

Methods in Molecular Biology™

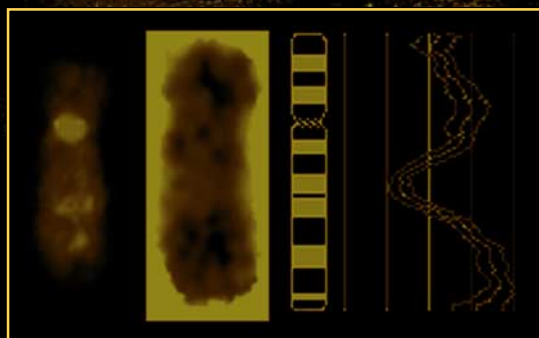
VOLUME 204

# Molecular Cytogenetics

*Protocols and Applications*

*Edited by*

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HUMANA PRESS

## Molecular Cytogenetics in Medicine

### *An Overview*

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### 1. Introduction

The word “chromosome” was introduced over a century ago from the Greek language meaning “colored body.” While cytogenetics refers to the study of chromosomes, the term molecular cytogenetics is used to describe the analysis of genomic alterations using mainly *in situ* hybridization based technology.

Fluorescent *in situ* hybridization (FISH) was initially developed in the late 1980s from radioactive hybridization procedures used for mapping human genes (1–4). Soon, this technology was utilized for the characterization of chromosomal rearrangements and marker chromosomes (5,6), the detection of microdeletions (7), and the prenatal diagnosis of common aneuploidies (8,9) in clinical cytogenetics laboratories. At the same time, numerous DNA probes have been commercialized, further promoting the wide-spread clinical applications of molecular cytogenetics. Many new FISH techniques have been developed, including primed *in situ* labeling (PRINS [10]), fiber FISH (11,12), comparative genomic hybridization (CGH) (13), chromosome microdissection (14,15), spectral karyotyping (SKY [16]), Multiple color FISH (M-FISH [17,18]), color banding (19), FISH with multiple subtelomeric probes (20), and array-based CGH (21,22). With the current FISH techniques, deletion or rearrangement of a single gene can be detected, cryptic chromosome translocations can be visualized, the copy number of oncogenes amplified in tumor cells can be assessed, and very complex rearrangements can be fully characterized. Using interphase FISH, genomic alterations can be studied in virtually all types of human tissues at any stage of cell division, without the need of cell culture and chromosome preparation. The development of FISH technology in the past two decades has brought cytogenetics into the molecular era, and made the “colored bodies” more colorful and brighter.

## **2. FISH Techniques**

### **2.1. DNA Probes**

All types of human DNA sequences have been used as probes for molecular cytogenetic studies. These include unique sequences, repetitive sequences such as  $\alpha$ -satellite and telomere DNA, locus specific DNA obtained by PCR amplification, large genomic DNA sequences cloned into cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs), yeast artificial chromosomes (YACs), chromosome band or arm specific sequences generated by microdissection, and DNA libraries established by chromosome flow sorting. To make the probes, DNA sequences are labeled directly with fluorescent dyes or indirectly with biotin or digoxigenin, which are then detected with immunofluorescent staining. DNA sequences can be labeled with a single color, dual colors, or multiple colors. Single or multiple probes can be used for each hybridization. In recent years, numerous fluorescent labeled DNA probes have been commercially available, making the FISH protocols much simpler, more cost-effective, and reliable for clinical applications.

### **2.2. FISH with Unique DNA Sequences**

FISH with unique DNA sequences represents the most basic molecular cytogenetic technique. The DNA segment used as a probe may represent a functional gene, or a particular chromosome region or locus. The basic steps of FISH procedure include labeling of DNA probes, preparing interphase or metaphase chromosome slides, *in situ* hybridization, and visualization with a fluorescence microscope (4). FISH with unique sequences is most commonly used for the diagnosis of microdeletion syndromes, and for the detection of gene fusion or rearrangements in cancer cells.

### **2.3. Chromosome Painting**

Chromosome painting refers to a FISH procedure using probes generated from specific chromosome libraries (23), Alu and L1 PCR (24) or chromosome microdissection (25). When the probe contains unique and repetitive sequences from an entire chromosome, whole chromosomes of a homologous pair in metaphases are illuminated with fluorescence (painted). The short or long arm of a particular chromosome can be painted with an arm-specific probe. Using a device containing a  $3 \times 8$  array, all 24 chromosomes can be painted simultaneously and detected sequentially on a single slide using a standard fluorescent microscope (26). Chromosome painting is usually performed as an adjunctive tool for identification and characterization of structural rearrangements.

### **2.4. Spectral Karyotyping (SKY)**

SKY is an automated chromosome painting procedure (16). The probe mixture is composed of chromosome specific libraries generated from flow-sorted human chromosomes. The probes are directly labeled with combinations of 5 fluorochromes: Rhodamine, Texas-red, Cy5, FITC, and Cy5.5. By the means of computer classification of the spectra, all 24 human chromosomes can be simultaneously visualized in different colors. SKY has been proven to be a powerful tool for the characterization of complex chromosomal rearrangements in cancer cells (27–29) and *de novo* constitu-

tional structural abnormalities (30,31). With a visual sensitivity to DNA alterations of 1–2 Mb in size, SKY is a useful tool for detecting cryptic translocations (32).

### **2.5. Multiple Color FISH (M-FISH)**

M-FISH has also been called multifluor FISH or multiplex FISH (17,18). Similar to SKY, the probes are labeled with the combinations of multiple fluorochromes. Different from spectral analysis, 24 chromosomes in unique colors are detected by a series of fluorochrome specific filters with the assistance of computer software. Whereas a wheel containing multiple filters can be installed onto a fluorescence microscope, the software designed for 24 color analysis can be added into an existing imaging system used for conventional karyotyping. Therefore, an additional imaging system is not required for M-FISH studies.

### **2.6. FISH Following Microdissection (MicroFISH)**

To perform MicroFISH (14,15) a whole chromosome, a marker, or a particular chromosome band is scraped from metaphase spreads using a micromanipulator. The scraped DNA is amplified by PCR, and labeled as probes. FISH of such probes with normal reference metaphase chromosomes reveals the composition of chromosomes or the chromosome regions in question. The term reverse *in situ* hybridization has been used to describe this procedure. This technique is useful for characterizing structural rearrangements and marker chromosomes. Microdissection has been used as a tool to produce commercial DNA probes for specific chromosomes, specific arms, or particular chromosome regions.

### **2.7. Comparative Genomic Hybridization (CGH)**

CGH gained its name from a FISH procedure that compares test DNA with normal reference DNA (13,33), and is also called reverse *in situ* hybridization. The test DNA is traditionally labeled with a green color and the normal reference DNA with a red color. The DNA mixture is then hybridized to normal metaphase chromosomes prepared from a blood culture. By measuring the ratio of green to red color, gains or losses of chromosomes or chromosomal regions in the test DNA can be detected. The size of DNA segments that CGH can detect is estimated to be in the range of 10–20 Mb. CGH is useful in the characterization of *de novo* unbalanced constitutional anomalies (34). Since the entire genome can be scanned for gains or losses without preparing metaphase chromosomes of the cells or tissues tested, CGH has been widely used in investigations of solid tumors (33).

### **2.8. Primed In Situ Labeling (PRINS)**

PRINS refers to a process of reannealing short oligonucleotide primers to target sequences *in situ*, followed by elongation of the sequences with a Taq polymerase and simultaneous labeling of the target sequences with a fluorochrome (10). The target sequences are examined under a fluorescence microscope. PRINS primarily targets short stretches of  $\alpha$ -satellite DNA unique to each chromosome. The reaction can be completed in less than 2 h. This technique has been used as an efficient alternative tool to detect aneuploidies (35,36).

## **2.9. Color Banding**

This technique is also called cross-species FISH (Rx FISH) because it utilizes DNA obtained from flow-sorted gibbon chromosomes by PCR amplification (19). The genome of gibbons (*Hylobates concolor* and *Hylobates syndactylus*) has a high degree of homology with human DNA but with extensively rearranged chromosomes. When hybridized with a set of gibbon DNA probes labeled with a combination of FITC, Cy3, and Cy5, human metaphase chromosomes show a distinctive color banding pattern. This technique has been used in cancer cytogenetics studies with commercially available probes (37).

## **2.10. FISH with Multiple Subtelomeric Probes (Multi-Telomere FISH)**

The technique of multi-telomere FISH utilizes a device containing 41 subtelomeric probes for all 24 different chromosomes (not including the short arms of acrocentrics [20]). Each of these probes is composed of unique sequences of 100–200 kb mapped in the subtelomeric regions (~300 kb from the chromosome end) of human chromosomes (38). The probes for the short arms and the long arms are dual labeled with a green and a red fluorochrome respectively. This allows detection of submicroscopic deletions or translocations in all subtelomeric regions with a single hybridization, and therefore can be used as screening a tool.

## **2.11. Fiber FISH**

Fiber FISH is a hybridization of DNA probes to extended chromatin fibers (i.e., free chromatin released from lysed cells) on a microscope slide (11). A modified method hybridizes probes to unfixed DNA fibers prepared from cells embedded in pulsed-field gel electrophoresis (PFGE) blocks (12). This technique has been used for high resolution gene mapping (11,12), and for direct visualization of gene duplication and chromosome breakpoints involved in translocations (39–41).

## **2.12. Combined Immunophenotyping and FISH**

The term FICTION, standing for fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms, was originally created to describe this technique (42). FICTION is a simultaneous analysis of cell surface immunologic markers and interphase FISH. The strategy of combining immunophenotyping with FISH enables correlation of chromosome aberrations of interest with cell lineage and differentiation stages of tumor cells, and therefore provides a useful tool for studies of leukemia and lymphomas (43,44).

## **2.13. Microarrays, Fluorescence Genotyping, and Other Molecular Approaches**

Many other molecular approaches have been developed for delineating chromosomal disorders and screening of submicroscopic genomic alterations. PCR-based microsatellite CA repeat analysis and methylation studies have been routinely used for detecting uniparental disomy and imprinting (45). Microsatellite markers have been utilized for a genome wide screening of chromosomal aberrations (46). A fluores-

cence-based genotyping technique has been recently developed for screening subtelomeric rearrangements (47). The techniques of tissue microarrays (48), array CGH (21,49), and c-DNA microarrays (22) are being rapidly developed and utilized in many areas of biomedical research, including molecular cytogenetic studies.

### 3. Applications

#### 3.1. FISH for Structural Abnormalities

The incidence of constitutional structural chromosome abnormalities which are visible at the level of 400 bands is approximately 1/200 at birth, including all balanced *de novo*, 1/1000; all balanced inherited, 1/400; all unbalanced *de novo*, 1/1000; and all unbalanced inherited, 1/2000 (50). While an inherited abnormality can usually be determined by its banding pattern and parental studies, FISH analysis is necessary for the identification and characterization of most unbalanced *de novo* structural rearrangements, including marker chromosomes. Numerous acquired aberrations which lead to gains or losses of chromosomal material have been described in leukemia, lymphomas, and solid tumors. It is important to know whether or not a particular chromosomal region or a particular gene is involved in a chromosomal aberration, so that a correct clinical diagnosis can be made and appropriate treatment initiated. In many cases, however, this may not be possible without FISH or other molecular studies.

Chromosome painting (51–53), SKY (27–31), M-FISH (54), CGH (33,34), and color banding (37) have all been utilized in the studies of structural abnormalities in conjunction with G-banding analysis. In many cases, however, FISH with unique sequences is necessary to determine the involvement of a particular gene in a structural abnormality. An example is the rearrangement of the *MLL* gene in a translocation with a breakpoint at 11q23 in acute myeloid leukemia (55,56). Nevertheless, a comprehensive molecular cytogenetic approach may be necessary depending on the nature of the disease and the particular aberration (57–59).

#### 3.2. FISH for Microdeletion Syndromes

More than thirty microdeletion syndromes have been described in the past two decades (7,60). Williams, Prader-Willi/Angelman, Smith-Magenis, 22q11.2 deletion, and 1p36 deletion, are the most common microdeletion syndromes. These syndromes are usually caused by a deletion of a 2–4 Mb DNA sequence, undetectable by standard chromosome analysis. The term “contiguous gene syndrome” is also used to describe these disorders because the deleted chromosome segment may contain a number of functional genes. The deletion of 22q11.2 is associated with a number of syndromes and psychiatric illnesses including DiGeorge, velocardiofacial, conotruncal anomaly face syndromes, and an increased risk of schizophrenia. Recently, these disorders have been considered to represent varying expression of the same genetic defect (61). The prevalence of 22q11.2 deletion alone was estimated to be 1/4500 in the general population (62,63). The deletion of 1p36 is believed to be the second most common microdeletion, with an estimated incidence of >1/10,000 newborns (64). Thus, the overall incidence of microdeletion syndromes is likely in the range of 1/1000–2000

newborns, or higher given that the number of microdeletion syndromes described is growing, and that clinical recognition is difficult for many of these syndromes. With an assumed incidence of 1/1000, the risk for one individual to be affected with two different microdeletion syndromes is 1/1,000,000 or 1/10<sup>6</sup>. Such a case has indeed been reported (65).

With the improvement of the quality of chromosome preparation, the deletion of 17p11.2 in Smith-Magenis syndrome can be detected by G-banding analysis without difficulties (66). For the majority of microdeletion syndromes however, a definitive diagnosis cannot be made without FISH analysis. Studies of microdeletion syndromes have created new concepts such as uniparental disomy and genomic imprinting, and opened exciting areas in human and medical genetics research. In return, the knowledge obtained from research has led to a combined diagnostic approach for Prader-Willi and Angelman syndromes, i.e., conventional karyotyping to exclude structural abnormalities, FISH to detect microdeletion, and DNA testing for uniparental disomy or gene mutation (45).

While the detection rate may vary in different laboratories, the data from our laboratory is shown here as an example of FISH utilization for the diagnosis of microdeletion syndromes. From 1996 to the end of 2000, a total of 550 blood samples were received for testing of common microdeletions. The diagnosis of a microdeletion syndrome was made in 59 of these cases (10.73%) by FISH, including 37/300 (12.33%) cases referred for 22q11.2 deletion, 10/167 (6%) for Prader-Willi/Angelman, 11/73 (15%) for Williams, and 1/10 (10%) for Miller-Dieker syndrome. FISH for Smith-Magenis syndrome is performed in our laboratory only when the chromosomal finding is inconsistent with the clinical indication. Although 1p36 deletion has been considered to be the second most common microdeletion syndrome, no case has been referred to our laboratory for testing of this deletion. This may reflect the difficulties in the clinical recognition of such a syndrome, or simply the level of awareness of this syndrome among local clinicians.

### **3.3. Detection of Subtelomeric Aberrations in Patients with Unexplained Mental Retardation**

Genomic alterations in the subtelomeric regions appear to be an important cause of developmental disabilities (67). It was suggested that subtelomeric anomalies may be second only to Down syndrome as the most common cause of mental retardation (68). Patients with unexplained mental retardation or developmental disabilities have been studied by FISH with multiple subtelomeric probes. In an earlier large study reported by Knight et al. (69), subtelomeric aberrations were detected in 7.4% of patients with moderate to severe idiopathic mental retardation. Our recent study (70) and two other large reports (71,72) have estimated that the frequency of clinically significant subtelomeric aberrations is 3–5% in the study population. FISH with multiple subtelomeric probes has been considered to be a valuable tool for a definitive diagnosis of patients with unexplained mental and developmental disabilities (70).

### **3.4. Interphase FISH for Prenatal Diagnosis of the Common Aneuploidies**

Aneuploidies of chromosomes 13, 18, 21, X, and Y account for about 95% of the chromosomal aberrations causing live-born birth defects. Extensive studies have been done in the past 15 yr to establish, refine, and assess the techniques of interphase FISH prenatal diagnosis of these common aneuploidies with uncultured amniocytes (8,9,73,74). DNA probes and FISH protocols were commercially standardized in the late 1990s. The currently used AneuVysion assay kit (Vysis, Downers Grove, IL) includes two sets of multicolor probe mixtures, one for chromosomes 13 and 21, and the other for chromosomes 18, X, and Y. The standardized probes and protocols have been proven to be accurate and very sensitive for prenatal diagnosis of the most common aneuploidies. This technique is particularly valuable for high risk pregnancies as indicated by ultrasonography or maternal serum screening (75).

Since 1998, our laboratory has routinely provided rapid interphase FISH prenatal diagnosis for patients with a high risk pregnancy noted at a late gestational age ( $\geq 20$  wk), i.e., either a positive maternal serum screening for trisomy 18 or 21, or an abnormal ultrasound indicating a probable chromosomal anomaly. FISH was performed using AneuVysion assay kits and the results were reported to the referring physicians. Among the 196 cases studied, aneuploidies were detected in 30 cases (15.3%), including 12 cases with trisomy 21, 10 cases with trisomy 18, 2 cases with trisomy 13, 2 cases with 45, X, and 2 cases with triploidy. All FISH results were confirmed by conventional G-banding analysis which showed 100% accuracy and zero false positive/false negative result. It is strongly believed that interphase FISH prenatal diagnosis is very beneficial to this group of patients and should be routinely provided as a standard diagnostic tool.

### **3.5. Prenatal Diagnosis of Chromosomal Disorders Using Maternal Blood**

Fetal nucleated red blood cells which pass into the maternal circulation during pregnancy provide a cell source for noninvasive prenatal genetic diagnosis. Cytogenetic analysis of fetal cells by FISH is a potentially useful method for prenatal diagnosis of chromosomal disorders, but requires relatively pure samples of fetal cells isolated from maternal blood (76). Many methods including density gradient centrifugation, magnetic activated cell sorting, fetal cell culture, and immunocytochemical staining have been developed for the isolation, enrichment and identification of fetal cells (77–80). Currently, noninvasive prenatal genetic diagnosis is still in the investigational phase. However, an approach of combined cell sorting, immunophenotyping, and FISH appears to improve the sensitivity and specificity of the methods, and thus offers new promise to the future of noninvasive prenatal genetic testing (81).

### **3.6. Preimplantation Diagnosis of the Common Aneuploidies**

Preimplantation diagnosis is a prepregnancy genetic test for in vitro fertilization (IVF) patients. A large proportion of patients undergoing IVF are at the age of  $\geq 35$  yr. It was

estimated in this group that about 50% of embryos are chromosomally abnormal with aneuploidy being the major contributor (82). Since most aneuploidies arise as the products of a maternal meiosis I non-disjunction, they can be detected by FISH analysis on the first and/or second polar bodies removed from oocytes following maturation and fertilization. DNA probes for chromosomes 13, 18, and 21 have been used most commonly for FISH studies on polar bodies (82–85). Many other chromosomes have been tested on single blastomeres biopsied from embryos at an early stage of development (d 3 [86–88]). Preimplantation diagnosis of aneuploidies has provided an accurate and reliable approach for the prevention of age-related aneuploidies in IVF patients with advanced maternal age (84,85). Selecting embryos with a normal chromosome complement can also improve the implantation rate in patients with advanced age or carriers of an altered karyotype (88).

### **3.7. Studies of Mosaicism and Its Effect on Early Human Development**

The recent molecular cytogenetic studies of chromosomal mosaicism and its effect on early human development represent a very interesting area of research. Constitutional mosaicism is the result of postfertilization mitotic error, i.e, a somatic event. Two types of mosaicism, meiotic and somatic, have been defined by molecular studies in determining the origin of the extra chromosome in the trisomic cell line. While meiotic mosaicism refers to the occurrence of a mitotic error producing a diploid cell line in a trisomic conception, somatic mosaicism means a trisomic cell line occurred in a conception which was initially diploid (89). Other terms, such as generalized vs confined mosaicism, are also commonly used. A generalized mosaicism involves all cell lineages of the conceptus, including both the placenta and the embryonic/fetal tissues. A mosaicism when occurred only in the placenta is called confined placenta mosaicism. Studies using FISH, CGH, and other molecular techniques have facilitated our understanding of the biological and clinical significance of chromosomal mosaicism in early embryonic/fetal development (89–92).

### **3.8. Detection of Specific Translocations and Gene Rearrangements in Human Cancer**

Over 100 recurrent chromosomal translocations in hematologic neoplasms, malignant lymphomas, and solid tumors have been identified, and rearrangement of a specific gene is known in most of these translocations (93). FISH has been a powerful tool in the characterization of these translocations. Clinically, the identification of specific chromosomal translocations and gene rearrangements is not only diagnostic, but also important for determining a therapy plan, monitoring treatment, and predicting prognosis. For patients with chronic myeloid leukemia, it has been shown that interphase FISH is highly sensitive in detecting the *BCR/ABL* fusion, and therefore is very useful for following patient's response to therapy (94–96). FISH is necessary to detect involvement of specific genes in many cases of acute leukemia, for example, the involvement of the *MLL* gene in an 11q23 rearrangement (55,56), and the *TEL/AML1* fusion in childhood acute lymphoblastic leukemia (97,98). FISH probes for several rearranged genes in non-Hodgkin lymphomas are now commercially available. The

technique of interphase FISH detection of the *BCL2* rearrangement in follicular lymphoma using breakpoint-flanking probes has shown advantages over the standard PCR method (99). Many specific chromosomal and gene rearrangements have been characterized in solid tumors. These rearrangements, for example, the translocation t(X;18)(p11.2;q11.2) in synovial sarcoma and the EWS/FLI1 fusion in Ewing sarcoma/peripheral primitive neuroectodermal tumor, can be detected by dual color interphase FISH in formalin-fixed, paraffin-embedded tumor tissues (100,101). It is apparent that interphase FISH detection of specific gene rearrangements in solid tumors has potential value for diagnosis and treatment, but an expanded variety of FISH probes needs to be made commercially available to clinical laboratories.

### **3.9. Analysis of Gains and Losses of Chromosomes or Chromosomal Regions in Tumors**

Conventional cytogenetic studies of solid tumors were hampered due to the difficulties of cell culture and chromosome preparation, as well as the complexity of their genomic alterations. With the interphase FISH approach, however, chromosomal aneuploidies can be detected without cell culturing in virtually any given tissue or cell source, such as touch preparations, sections of frozen tumor, and paraffin-embedded tissue (102,103). Similarly, almost all types of clinical specimens can be used for CGH studies of tumors (33). Many reports have shown gains or losses of individual chromosomes or chromosome regions correlating with particular tumors, different stages of the tumor, and the prognosis of patients (104–107). These studies have yielded extremely important information for our understanding of the biologic behavior of solid tumors.

### **3.10. Testing Deletion of Tumor Suppressor Genes and Amplification of Oncogenes**

Deletion of tumor suppressor genes, such as *p53* and *RB-1*, and amplification of oncogenes, such as *N-myc*, *C-myc*, and *HER-2/neu*, can be detected by FISH or CGH studies of tumor tissues. FISH has provided reliable estimates of *N-myc* amplification in neuroblastoma. FISH also has advantages over Southern blot analysis in terms of speed, technical simplicity, ability to discern heterogeneous gene amplification among tumor cells in the same specimen, and capacity to determine the source of the amplified *N-myc* signals (108). The interphase FISH method has been considered to be more accurate than the Southern blot method in detecting *N-myc* amplification when the number of cells with *N-myc* amplification is low, or when intra-tumor heterogeneity is present (109). In addition, FISH has also shown a strong correlation between 1p abnormalities and *N-myc* amplification (110). Amplification of *C-myc* has been detected by FISH in many different types of tumors, including medulloblastoma, malignant melanoma, lung cancer, nasopharyngeal carcinoma, ovarian cancer, and prostate cancer. Testing of the *HER-2/neu* (also known as *erbB-2*) gene in breast cancer has become very important for patient management owing to its association with more aggressive clinical and pathologic features. Amplification and/or overexpression of *HER-2/neu* can be assessed by either FISH, PCR, or immunohistochemistry. The

methods of immunohistochemistry and FISH have been compared in recent studies (*111–116*), and a combined approach has been suggested to make the test more sensitive and cost-effective (*114–116*). The status of many other oncogenes have been tested in human cancers, and amplifications of multiple oncogenes are known in some tumors. While the changes in copy number of multiple oncogenes can be simultaneously tested using CGH microarrays (*21,49*), a large number of tumor tissue specimens can be rapidly analyzed with FISH performed on consecutive tissue microarray sections (*48*).

### **3.11. Genome-wide Screening for Constitutional and Acquired Alterations**

As mentioned above, unbalanced constitutional or somatically acquired alterations can be screened by CGH in a single experiment, but the resolution is limited. The technique of array CGH has provided a high resolution screening method for DNA copy number changes (*21*). A cDNA microarray-based CGH method has also been developed to identify gene amplifications and deletions genome-wide (*22*). In a very recent report, the use of array CGH has achieved a resolution of approx 1 Mb in the analysis of the genome and demonstrated its sensitivity of detecting single copy gains and losses (*49*). Submicroscopic chromosomal aberrations can also be screened using genome-wide microsatellite markers (*46*). Automated fluorescent genotyping using microsatellites has been shown to be a very sensitive method for screening cryptic rearrangements of the telomeric regions (*47*).

## **4. Standards and Guidelines for Clinical Applications**

It is necessary that all cytogenetics laboratories have their protocols and standards established for the clinical applications of molecular cytogenetic techniques according to the guidelines provided by professional organizations. It is also important that the standards and guidelines be updated as new technologies evolve and the data on their clinical applications accumulates. In North America, the guiding principles of genetic testing have been provided by the American Society of Human Genetics (ASHG), the American College of Medical Genetics (ACMG), and the Canadian College of Medical Geneticists (CCMG).

In 1993, the ACMG recognized prenatal interphase FISH to be of diagnostic potential when the investigational nature of testing was stressed (*117*). The guidelines, including clinical indications, requirements of test validation, analysis standards, and reporting of FISH studies, were provided by the ACMG in 1996 (*118*), and were updated in 1999 (*119*). In 1997, the CCMG also provided guiding principles for clinical applications of FISH technology to Canadian cytogenetics laboratories (*120*), which were basically consistent with the ACMG policies. A Joint ASHG/ACMG Test and Technology Transfer Committee published a report on diagnostic testing for Prader-Willi and Angelman syndromes with recommended testing approaches including FISH in 1996 (*45*). Recently, the same committee published the first part of an ACMG/ASHG position statement on technical and clinical assessment of FISH (*121*). It was recommended in this statement that:

"FISH testing be considered a highly useful and accurate test for the diagnosis of microdeletions and for the identification of unknown material in the genome. In disorders in which FISH testing provides results not possible from standard cytogenetic testing, the testing is standalone and should be accepted as such."

## 5. Conclusion

As a research tool, molecular cytogenetics has contributed to the understanding of many aspects of human biology and has played an indispensable role in human genome mapping (not covered in this review). As a diagnostic tool, molecular cytogenetics has now become an essential component in many areas of medical practice, including medical genetics, maternal-fetal medicine, pediatrics, reproductive medicine, pathology, hematology, oncology, and psychiatrics.

We have experienced the exciting years of the Human Genome Project and the development of molecular cytogenetics. As the high-quality sequence of the human genome is achieved by 2003, our society will enter the postsequencing era. While we are speculating the possible future changes in medical practice resulting from the Human Genome Project, there is no doubt that the FISH techniques will be utilized more extensively in medical sciences and newer technologies will be developed. The colors of human chromosomes may further be enriched and the art of molecular cytogenetics will continue to flourish in the new century.

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## Labeling Fluorescence *In Situ* Hybridization Probes for Genomic Targets

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### 1. Introduction

Fluorescence *in situ* hybridization (FISH) requires nucleic acid probes, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or nucleic acid analogs, labeled directly with fluorophores, or capable of indirect association with fluorophores. The nucleic acid provides the FISH assay with its specificity through complementary pairing of the probe nucleotides with nucleotides of the target nucleic acid. The appended fluorophores provide the ability to visually detect the homologous regions within the cellular structure using a fluorescence microscope. Photographic or electronic cameras can also be used to provide permanent images of the fluorescence staining patterns, and the latter can be used to provide quantitative measurements of the probe fluorescence.

This chapter describes a variety of methods by which DNA can be coupled to fluorophores to form FISH probes directed toward genomic targets. Following a brief discussion of labeling methodologies, fluorophore selection, and sources of probe DNA, a number of detailed protocols are provided that describe both enzymatic and chemical labeling of FISH probes.

#### 1.1. Direct and Indirect Fluorophore Labeling

Fluorophores can be associated with nucleic acid probes by chemical conjugation to the nucleic acid, or by chemical conjugation of the nucleic acid with a nonfluorescent molecule that can bind fluorescent material after hybridization. The former method is called “direct labeling” and the latter method is called “indirect labeling.” In indirect labeling, the molecule directly attached to the nucleic acid probe is typically either biotin or a hapten, such as dinitrophenol (DNP) or digoxigenin. The *in situ* hybridization is performed with the hapten- or biotin-labeled probe, after which the specimen is incubated with fluorophore-labeled antibody or avidin. Because a number of

fluorophores can be attached to each antibody or avidin molecule, the indirect method allows for the association of multiple fluorophores with each directly attached binding moiety. Furthermore, additional rounds of antibody binding, sometimes referred to as “sandwiching,” can be utilized to further increase the number of bound fluorophores. For example, if goat IgG anti-DNP was used to bind to DNP-labeled probes, then fluorophore-labeled anti-goat IgG can be used to amplify the signal in a second round of indirect labeling.

In addition to binding multiple rounds of avidin and/or antibody secondary reagents, the amount of fluorescence staining can be increased using enzyme conjugates of avidin or antibodies. For enzyme conjugates to be effective in FISH, fluorescent products of the enzymatic reaction must remain localized near the site of probe binding. Two approaches to dye localization include the generation of a precipitating fluorescent product (ELF reagent, Molecular Probes, Inc., Eugene, OR) (1–3), and generation of highly reactive fluorescent compounds that covalently attach to neighboring cellular material (CARD/TSA system, NEN, Boston, MA) (4–6).

While indirect labeling has the potential for generating greater fluorescence signal, it also has the disadvantage of requiring additional incubation steps to bind the antibody and avidin reagents. The introduction of fluorescent antibodies also can increase the background fluorescence owing to nonspecific binding of the antibodies and avidin proteins to extraneous cellular material on the microscope slide, and the slide surface itself. Furthermore, when multicolor FISH is utilized to simultaneously identify several different genomic targets, a different, spectrally distinct fluorophore must be used to unambiguously identify each of the targets. For direct-labeled probes, this means finding  $N$  spectrally distinct fluorescent labels to identify  $N$  different targets. For indirect-labeled probes this means not only selecting  $N$  different labels, but also finding  $N$  different binding pairs (hapten-antibody or biotin-avidin pairs) for binding each of the  $N$  fluorescent labels. For very small genomic targets, for example, targets less than 70 kilobases (kb), indirect labeling may be required to achieve visually interpretable staining. However, larger targets are usually detectable using direct labeling alone. For research applications where probes or particular targets may be used infrequently or are under initial investigation, individual laboratories may opt for small target probes, such as plasmid or cosmid clones, for which indirect labeling may be a necessity. However, with the availability of bacterial artificial chromosome (BAC) libraries generated in connection with the Human Genome Project, large target probes can be easily generated and discerned with little difficulty when directly labeled.

## 1.2. Survey of Nucleic Acid Labeling Chemistry

For either direct- or indirect-labeling, the probe nucleic acid must be modified to attach a fluorophore, biotin, or hapten. Both chemical and enzymatic reactions have been used for this purpose. Early fluorescence *in situ* hybridization was performed with a chemically modified probe, using periodate oxidation of a 3'-terminal ribonucleotide to form the dialdehyde, coupled in turn with a hydrazine derivative of fluorescein (7). Biotin or a hapten could presumably be added by this same chemistry,

however, the chemistry is restricted to RNA probes or DNA probes to which a 3'-terminal ribonucleotide has been added, using terminal transferase, for example. Other chemical modifications reported for *in situ* hybridization probes include a reaction to introduce the hapten aminoacetylfluorene (AAF) (8–12), and mercuration (13). Mercurred probes are reacted post hybridization with a bifunctional molecule containing the detection moiety and a thiol group (14,15).

A convenient method of chemical labeling that is described in more detail below uses platinum complexes (16). In this method, the detection moiety is derivatized to form a coordinating ligand of a platinum complex. The labeled complex is further reacted with nucleic acid resulting in the formation of a coordinate covalent bond between the platinum and primarily guanine residues of the nucleic acid. Other chemistries employed in labeling hybridization probes include, bisulfite mediated transamination of cytosine (17,18), photochemical reaction with photobiotin (19), bromination of thymine, guanine, and cytosine with *N*-bromosuccinimide; followed by reaction with amine-containing detection moieties (20), and condensation of terminal phosphate groups with diamines, followed by coupling with amine reactive detection moieties (21,22).

Enzymatic reactions, especially those using polymerases to incorporate labeled nucleoside triphosphates, have been the most popular means of labeling nucleic acids by far. Of these, nick translation to incorporate biotinylated nucleoside triphosphates is the oldest and most frequently used method (23–25). Other haptens incorporated by this method include dinitrophenol (26), digoxigenin (27), and fluorescein (28). In addition to being used as an indirect label with anti-fluorescein antibodies, fluorescein incorporated by nick translation has been used for directly detected probes (26,28). A variety of fluorophores are now commercially available that can be incorporated by polymerases for directly detected FISH (e.g., from Molecular Probes Inc., Eugene, OR; New England Nuclear, Boston, MA; or Vysis, Inc., Downers Grove, IL).

In addition to nick translation, DNA polymerases have been used to incorporate labeled nucleoside triphosphates into FISH probes by PCR. This has included PCR with flanking primers that amplify DNA inserts within plasmids (29), as well as PCR with random and degenerate (30) primers. Examples of these important enzymatic labeling protocols are provided below. (See **Subheadings 3.1–3.3**.)

Note that *in situ* hybridization probes perform best when the probe lengths are <1 kb. Published procedures often call for probe lengths in the range of 200–600 base pairs (bp). Methods for fragmenting probes have included sonication, alkali treatment, heat, or enzymatic degradation. Smaller probe lengths can also be generated as a consequence of certain enzymatic labeling methods, such as the polymerase chain reaction (PCR).

### 1.3. Fluorophore Selection

A wide variety of fluorophores are available for labeling *in situ* hybridization probes, with emission extending from the ultraviolet end of the spectrum to the near infrared. The most frequently used fluorophores belong to several common chemical classes—the coumarins, fluoresceins, rhodamines, and cyanines. The structures of these compounds are shown in **Fig. 1**, together with two frequently used indirect-

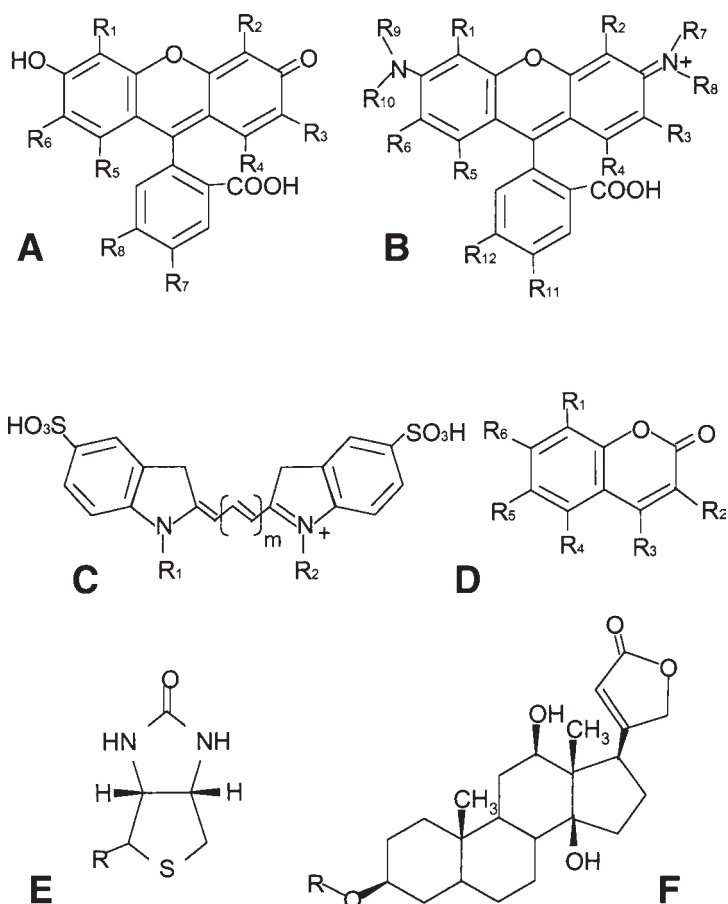


Fig. 1. Chemical structures of four common fluorophore classes (A–D) and two common indirect labels (E and F). **A.** fluoresceins, **B.** rhodamines, **C.** cyanines (Cy 3, Cy 5, and Cy 7 only), **D.** coumarins, **E.** biotin, **F.** digoxigenin. Specific compounds in each class differ by their chemical substituents, indicated as R's in the chemical structures.

labels. Changing the substituents ( $R_n$ ) on the basic structures modifies the chemical and spectral properties, including the extinction coefficient for absorption of light, the fluorescence quantum yield, the fluorescence lifetime, and the fluorescence excitation and emission spectra. For example, 7-amino-4-methylcoumarin-3-acetic acid (AMCA;  $R_6$  = amino,  $R_3$  = methyl,  $R_2$  = acetate, in Structure **D**, **Fig. 1**) has an excitation max at 354 nm and an emission max of 441 nm. Changing the substituents to form 7-diethylaminocoumarin-3-carboxylic acid ( $R_6$  = diethylamino,  $R_2$  = carboxylate, in structure **D**, **Fig. 1**) shifts the excitation max to 432 nm and the emission max to 472 nm. In the case of rhodamines, Rhodamine Green™ ( $R_7 = R_8 = R_9 = R_{10} =$  hydrogen,  $R_{11}$  or  $R_{12} =$  carboxylate, in Structure **B**, **Fig. 1**) has an excitation max at 504 nm and

an emission max of 532 nm, while tetramethylrhodamine isothiocyanate (TRITC;  $R_7 = R_8 = R_9 = R_{10} =$  methyl,  $R_{11}$  or  $R_{12} =$  isothiocyanate) has an excitation max at 544 nm and an emission max of 572 nm. The spectral characteristics of the cyanines are strongly affected by changing the number of carbons separating the two indole rings. For example, Cy 3 ( $m = 1$  in structure C, **Fig. 1**) has an excitation maximum at 550 nm and an emission maximum of 570 nm, while Cy 5 ( $m = 2$  in Structure C., **Fig. 1**) has an excitation max at 649 nm and an emission max of 670 nm. The excitation and emission maxima for a number of fluorescent labels used on *in situ* hybridization probes are listed in **Table 1**. Also included are two common nucleic acid counterstains, 4',6'-diamidino-2-phenylindole (DAPI) and propidium iodide (PI).

For *in situ* hybridization, the most desirable properties are high absorption extinction coefficient (preferably greater than 10,000/ $M \cdot \text{cm}$ ) and high fluorescence quantum yield (preferably  $>0.2$ ). The excitation and emission spectra are also very important and must be selected with regard to the spectral distribution of the excitation source, the microscope optical system, the fluorescence detector, and the filter sets available. In the case of multitarget hybridization, the spectral distributions of each fluorophore present in the assay must be chosen carefully to allow the fluorescence of each to be individually distinguished (for a review of multitarget hybridization, *see ref. 31*).

Spectral properties are not all that must be considered in label selection. In particular, the different chemical structures of the fluorescent labels lead to different levels of interaction with various cellular components, cellular debris, extracellular matrix, and the slide surface. Background staining of a specimen is highly dependent, therefore, upon the chemical structure of the fluorescent label. While highly hydrophobic labels can reduce probe solubility and increase adsorption to some cellular components, often the only way to determine how a label will perform in an *in situ* hybridization is by actually preparing the labeled probe and hybridizing it to the target tissue.

#### 1.4. Sources of Probe DNA

For most FISH applications, probes are prepared by culturing bacteria or yeast that contains the desired cloned sequence. The cells are harvested, lysed, and the clone DNA is purified from the host chromosomal DNA and cellular material. Bacterial cells containing the clone of interest are typically grown in media that selects for the clone by use of an antibiotic, or in the case of yeast, in media which lacks a particular nutrient. Bacterial clone DNA can be isolated by several common methods such as alkaline lysis or boiling (32).\*,\*\* Alternatively, extraction kits are available commercially from several suppliers, including Qiagen, Inc. (Chatsworth, CA), Stratagene (La Jolla, CA), and Genra Systems, Inc. (Minneapolis, MN). The preferred method for generating FISH probes from YACs involves amplification of the insert by Alu-PCR\*\*.

Vectors which maintain large inserts such as cosmids, PIs, PACs, or BACs, are best suited for generating FISH probes homologous to unique sequence DNA, as the

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\*See various volumes in the series *Current Protocols in Molecular Biology*, Janssen, K. (series editor), John Wiley and Sons, New York.

\*\*See various volumes in the series *Current Protocols in Human Genetics*, Boyle, A. L. (series editor), John Wiley and Sons, New York.

**Table 1**  
**Fluorescent Probe Labels and Counterstains**  
**Used in FISH (NHS = *N*-Hydroxysuccinimidyl Ester)**  
**Spectral Information is from Manufacturer Specification Sheets**

Fluorophore	Abs. Extinction Coeff.	Peak Wavelengths (nm)	
		Excitation	Emission
Labeling Compounds:			
Coumarins			
7-Amino-4-methylcoumarin-3-acetic acid, NHS	17,000	354	441
7-Diethylaminocoumarin-3-carboxylic acid, NHS	56,000	432	472
Pacific Blue™, NHS	36,000	416	451
Fluoresceins			
5- or 6-carboxyfluorescein, NHS	83,000	496	516
Fluorescein-5- and/or -6-isothiocyanate	80,000	494	519
Oregon Green® 488 isothiocyanate	78,000	493	520
Rhodamines			
Rhodamine Green™ carboxylic acid, NHS	78,000	504	532
Alexa Fluor® 488	71,000	494	519
Alexa Fluor® 532	81,000	530	555
Alexa Fluor® 546	104,000	554	570
Alexa Fluor® 568	91,000	577	602
Alexa Fluor® 594	85,000	590	615
Tetramethylrhodamine-5 and/or -6-isothiocyanate	84,000	544	572
Texas Red® sulfonyl chloride	85,000	587	602
Cyanines			
Cy2™	150,000	489	506
Cy3™	150,000	550	570
Cy3.5™	150,000	581	596
Cy5™	250,000	649	670
Cy5.5™	250,000	675	694
Cy7™	200,000	743	767
Commercial labeled probe preparations:			
SpectrumBlue™		400	450
SpectrumAqua™		433	480
SpectrumGreen™		497	524
SpectrumGold™		530	555
SpectrumOrange™		559	588
SpectrumRed™		592	612
SpectrumFRed™		655	675
DNA counterstains:			
4',6-Diamidino-2-phenylindole (DAPI)		367	452
Propidium iodide (PI)		543	614

probe should span at least 40 kb of contiguous sequence. Probes that represent highly repetitive sequences, such as that found near centromeres or telomeres, can be made from a single plasmid containing an insert of approximately 300–10,000 bp.

Two of the most common means for identifying a clone containing the desired target sequence are: (1) screening the appropriate clone libraries, or (2) searching online databases, such as Genbank at the National Center for Biotechnology Information, with a known sequence such as mRNA from a particular gene, or anonymous sequence from the end of a clone insert or a Sequence Tagged Site (STS).

Whole chromosome painting probes, which contain sequences spread across the breadth of a specific chromosome, were originally prepared from whole chromosome phage and bacterial libraries (33,34). These libraries were ultimately obtained from chromosomes that were isolated by flow sorting. More recently, whole chromosome probes are prepared from flow sorted chromosomes (35,36) or microdissected chromosomes (37) that are amplified by DOP-PCR, without the intervening steps required to make bacterial libraries (30,38).

## 2. Materials

### 2.1. Nick-Translation

1. Sample DNA, typically 1  $\mu\text{g}/50 \mu\text{L}$  labeling reaction.
2. Fluorophore labeled dUTP at a working concentration of 0.2 mM.
3. 0.3 mM dATP.
4. 0.3 mM dCTP.
5. 0.3 mM dGTP.
6. 0.3 mM dTTP.
7. Nick-translation enzyme: mix of DNA polymerase I (10,000 U/mL, DNase I (3 U/mL, such as Promega (Madison, WI) enzyme mix) .
8. 10X Nick-translation buffer: 500 mM Tris-HCl, pH 7.2, 100 mM  $\text{MgSO}_4$ , 1 mM dithiothreitol (DTT).
9. Nuclease-free water.
10. 15°C incubator.
11. Stop solution: 0.25 mM EDTA.
12. 3M sodium acetate, pH 5.5.
13. 70% ethanol.
14. 100% ethanol.
15. TE solution: 10 mM Tris-HCl, pH 7.5–8, 1 mM EDTA.
16. Sephadex G-50 type spin column (e.g., ProbeQuant™ G-50 spin columns, Amersham Pharmacia, Piscataway, NJ). Other methods can be used for removal of unincorporated nucleotides.
17. Microcentrifuge tubes.
18. Microcentrifuge.
19. Speed-vac lyophilizer.
20. Pipetors.
21. Pipet tips, preferably sterilized.
22. Tris-acetate buffer (TAE): 40 mM Tris-acetate, pH 7.5–7.8, 1 mM EDTA, or Tris-borate buffer (TBE): 45 mM Tris-borate, pH 8.0, 1 mM EDTA.
23. Agarose.

24. Hot plate or microwave oven.
25. 55°C water bath.
26. Mini horizontal gel electrophoresis apparatus with casting tray and combs, power supply.
27. DNA molecular weight markers, size range should cover 50–1000 bp.
28. 10 mg/mL ethidium bromide (EtBr).
29. 10X gel loading buffer: 50% (v/v) glycerol, 100 mM EDTA, 0.25% bromphenol blue.
30. UV transilluminator and UV protective visor.
31. Polaroid camera with Kodak Wratten red filter and type 667 Polaroid film.

## 2.2. Random Priming

1. Sample DNA, typically 10 ng to 3 µg/50 µL labeling reaction.
2. Fluorophore labeled dUTP at a working concentration of 1 mM.
3. 10 mM dATP.
4. 10 mM dCTP.
5. 10 mM dGTP.
6. 10 mM dTTP.
7. 40 U/mL Klenow fragment, (such as Life Technologies (Gaithersburg, MD) enzyme) .
8. 2.5X Random primer/buffer solution: 125 mM Tris-HCl, pH 6.8, 12.5 mM MgCl<sub>2</sub>, 25 mM 2-mercaptoethanol, 750 µg/mL random octamer primers (Life Technologies).
9. Nuclease-free water.
10. 37°C incubator.
11. Boiling water bath.
12. Ice.
13. Stop solution, 0.25 mM EDTA.
14. 3M sodium acetate, pH 5.5.
15. 70% ethanol.
16. 100% ethanol.
17. Sephadex G-50 type spin column (optional, depending on method preferred for removal of unincorporated nucleotides).
18. Microcentrifuge tubes.
19. Microcentrifuge.
20. Speed-vac lyophilizer.
21. Pipetors.
22. Pipet tips, preferably sterilized.

## 2.3. DOP-PCR

1. Flow sorted chromosomes (500–1000/PCR)
2. 10X PCR buffer I: Applied Biosystems (Foster City, CA).
3. PCR assay buffer: 10 µL of 10X PCR buffer I, 2 µL dNTPs (10 mM each), 1 µL of primer (100 ng), 1 µL Taq polymerase, nuclease-free water to 100 µL.
4. PCR labeling buffer: Same as PCR assay buffer, with addition of 1 µL of SpectrumOrange™ or SpectrumGreen™ dUTP, except with final vol of 99 µL.
5. 100 ng/µL primer.
6. 50 nmol SpectrumOrange™, SpectrumGreen™ 2'-deoxyuridine- 5'-triphosphate (Vysis, Inc., Downers Grove, IL) or other labeled nucleoside triphosphate—Reconstitute with 50-µL of nuclease-free water to give a 1 mM solution. Stable for up to 3 mo if stored at –20°C.
7. Taq polymerase: any commercial variety available can be used, but optimal concentration should be first determined by titrating with appropriate substrate.

8. Nuclease-free water.
9. Mineral oil (if necessary), light white (Sigma Chemical Co., St. Louis, MO).
10. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
11. 1X TBE buffer for gel electrophoresis (BioRad, Hercules, CA).
12. Primers: The primer used was designated 6 MW (30), with sequence 5' CGA CTC GAG NNN NNN ATG TGG 3'.

## 2.4. Labeling Probes with Aliphatic Amines

1. Amine-modified DNA to be labeled.
2. A suitable reaction buffer (50 mM sodium tetraborate at pH 9.3 for isothiocyanates and sulfonic acid chlorides, 0.2 M 3-[*N*-morpholino]-propanesulfonic acid (MOPS; Sigma Chemical Co.) at pH 7.4 or 50 mM sodium tetraborate at pH 8.5 for *N*-hydroxysuccinimidyl esters).
3. Amine-reactive derivative of the desired label.
4. Dimethyl sulfoxide, dimethyl formamide, or acetone (whichever is capable of dissolving the desired amine-reactive label in a 10–20 mM solution).
5. A prepacked Sephadex G-25 column (Amersham Pharmacia, Piscataway, NJ).
6. TE solution: 10 mM Tris-HCl, pH 7.5–8, 1 mM EDTA).

## 2.5. ULS Labeling

1. TE buffer 10 mM Tris-HCl, 0.3 mM EDTA, pH 8.0.
2. DNA for labeling.
3. DNase I (Roche Biochemicals, Nutley, New Jersey) cat. no. 104 159, approx 2000 Kunitz U/mg.
4. 5 mM sodium acetate, 1 mM CaCl<sub>2</sub>, 50% glycerol, pH 5.2
5. 10X nicking buffer: 50 mM Tris-HCl, pH 7.5, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>.
6. 10 M ammonium acetate.
7. 100% ethanol.
8. Kreatech kit components: Vial 1: code LK1101 (60 μL rhodamine-ULS<sup>®</sup>, 0.5 U/μL), or code LK1301 (60 μL dGreen-ULS<sup>®</sup>, 0.5 U/μL), Vial 2: code LK006, Labeling Solution, 2 mL.
9. Spin columns: e.g., ProbeQuant<sup>™</sup> G-50 spin columns (Amersham Pharmacia Biotech, Piscataway, NJ) or QIAquick<sup>™</sup> spin columns (Qiagen, Valencia, CA).
10. Ultrasonic disruptor (e.g., Branson Ultrasonics, Danbury, CT).

## 2.6. Labeling Proteins for Indirect Detection

1. *N*-hydroxysuccinimidyl derivative of desired fluorophore.
2. Dimethyl sulfoxide, dimethyl formamide, or acetone (whichever is capable of dissolving the desired fluorophore in a 20 mM solution).
3. A prepacked Sephadex G-25 column (Amersham Pharmacia).
4. 50 mM boric acid, pH 8.5 to 9.3.
5. TBS: 25 mM Tris-HCl, 140 mM NaCl, 51 mM KCl, pH 7.4.
6. PBS: 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 140 mM NaCl, 2.7 mM KCl, pH 7.4.

## 3. Methods

### 3.1. Nick-Translation

Nick translation is a method for incorporating labeled nucleotides into DNA such as an isolated fragment or an intact clone (39,40). The method uses a combination of

two enzymes, deoxyribonuclease I (DNase I) which nicks the DNA creating free 3' hydroxyls, and DNA polymerase I, which processively adds nucleotides to the 3' terminal hydroxyl. The 5' to 3' exonuclease activity of the DNA polymerase removes nucleotides from the 5' terminus of the nick as the polymerization proceeds. Both labeled and unlabeled nucleotides are substituted during the reaction and varying sized fragments are generated; however, there is no net synthesis of DNA. The resultant double-stranded fragments must be denatured prior to hybridization.

1. Prepare 0.1 mM dTTP by adding 100  $\mu$ L of 0.3 mM dTTP to 200  $\mu$ L nuclease-free water.
2. Prepare 0.1 mM dNTP mix by combining 100  $\mu$ L each of 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP. Excess nucleotide mixtures can be stored at  $-20^{\circ}$  or  $-80^{\circ}$ C.
3. For each labeling reaction, on ice, prepare a tube containing 1  $\mu$ g DNA, 2.5  $\mu$ L of 0.2 mM fluorophore-labeled dUTP, 5  $\mu$ L of 0.1 mM dTTP, 10  $\mu$ L of 0.1 mM dNTP mix, 5  $\mu$ L of 10X nick translation buffer and make up to a final vol of 40  $\mu$ L with nuclease-free water (*see Note 1*).
4. To each tube add 10  $\mu$ L nick-translation enzyme mix.
5. Mix and briefly centrifuge.
6. Incubate at  $15^{\circ}$ C for 8–16 h.
7. Add 5  $\mu$ L stop solution.
8. To remove unincorporated nucleotides, add 5  $\mu$ L 3 M sodium acetate, 125  $\mu$ L 100% ethanol; centrifuge at 12K for 20–30 min. Carefully pour off supernatant, or draw off with a pipettor. Add 100  $\mu$ L 70% ethanol per tube, briefly vortex pellet; centrifuge at 15,000g for 5 min. Carefully pour off supernatant, or draw off with pipettor (*see Note 2*).
9. Dry pellet under vacuum 10–20 min. Resuspend in 10  $\mu$ L TE, which yields an approx 100 ng/ $\mu$ L final concentration (*see Notes 3 and 4*). Alternatively, unincorporated nucleotides can be removed using a Sephadex G-50 type spin column according to the manufacturer's instructions. Labeled DNA will be in 50–100  $\mu$ L after the column and will need to be concentrated by ethanol precipitation as described in **step 8**.
10. To determine size of the labeled DNA fragments, add 0.5 g agarose to 50 mL of TAE buffer or TBE buffer, carefully heat to boiling in microwave, or on a hot plate. After all agarose is melted, cool to  $55^{\circ}$ C in a water bath.
11. Add 2.5  $\mu$ L EtBr to the agarose and mix. Pour molten agarose into casting tray using a 12 or 16 well comb.
12. For each labeled DNA, mix 2  $\mu$ L DNA (~200 ng) plus 7  $\mu$ L of water, or 1X TAE or TBE, plus 1  $\mu$ L loading buffer.
13. Run labeled DNAs plus molecular weight marker at 70–100 V until leading dye has migrated approx 5 cm into the gel.
14. View DNA with UV transilluminator and take a Polaroid picture. Majority of fragments should be in the range of 200–600 bp.

### 3.2. Random Priming

Random priming is a means of labeling DNA fragments whereby, a mixture of all possible combinations of hexamers, octamers, or nonamers are annealed to denatured DNA (**41,42**). These small oligonucleotides then act as primers that allow for synthesis of the complementary DNA strand by the Klenow enzyme and incorporation of both labeled and unlabeled nucleotides. The labeled material will be a combination of both double- and single stranded fragments that must be denatured prior to hybridization.

1. Prepare a 10X dNTP mixture such that final concentrations are 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.3 mM fluorophore-labeled dUTP, 0.7 mM dTTP. Excess mixture can be stored at  $-20^{\circ}$  or  $-80^{\circ}$ .
2. Dissolve DNA (400–500 ng preferred) in 20  $\mu$ L nuclease-free water (*see* **Notes 5** and **6**).
3. Add 20  $\mu$ L 2.5X random primer/buffer solution.
4. Heat denature for 5 min in boiling water; rapidly cool on ice.
5. On ice, add 5  $\mu$ L 10X dNTP mix and 4  $\mu$ L nuclease-free water to a final vol of 49  $\mu$ L.
6. Mix and briefly centrifuge.
7. Add 1  $\mu$ L Klenow enzyme and mix. Briefly centrifuge.
8. Incubate at  $37^{\circ}\text{C}$  for 1–6 h. Longer incubation times usually increase product yield (*see* **Note 7**).
9. Add 5  $\mu$ L stop solution.
10. Unincorporated nucleotides and primers can be removed by adding 5  $\mu$ L of 3 M sodium acetate, 125  $\mu$ L of 100% ethanol; centrifuge at 15,000g for 20–30 min. Carefully pour off supernatant, or draw off with a pipetor. Add 100  $\mu$ L of 70% ethanol per tube, briefly vortex pellet; centrifuge at 15,000g for 5 min. Carefully pour off supernatant, or draw off with pipetor.
11. Dry pellet under vacuum 10–20 min. Resuspend in 20  $\mu$ L TE, which yields an approx 100 ng/ $\mu$ L final concentration. Alternatively, unincorporated nucleotides can be removed using a Sephadex G-50 type spin column according to the manufacturer's instructions. Labeled DNA will be in 50–100  $\mu$ L after the column, and will need to be concentrated by ethanol precipitation as described in **step 10**.
12. Optimally, fragments should range from 200–600 bp, and can be visualized by gel electrophoresis. Refer to **steps 10–14** (*see* **Subheading 3.1.**) for electrophoresis protocol.

### 3.3. DOP-PCR

PCR generated whole chromosome DNA, either from microdissected or flow sorted chromosomes, is currently viewed as the preferred route to high quality probes suitable for whole chromosome staining of individual chromosomes. This is true for staining single chromosomes as well as for staining all 24 human chromosomes combinatorially in a multiplex FISH (M-FISH) (**43**) or spectral karyotyping (**44**) assay. The procedure provided below (**45**) is a modification of the original Degenerative-Oligonucleotide-Primed-PCR (DOP-PCR) protocol (**38**), and is optimized to amplify chromosome DNA in the presence of SpectrumOrange™ or SpectrumGreen™ dUTP (Vysis, Inc.). The protocol also should permit labeling with a variety of other labeled dUTP's, with little or no modification. The reaction involves the use of an oligonucleotide with an Xho-I restriction endonuclease site at its 5' end, a defined six-nucleotide sequence at the 3' end, and a set of degenerate nucleotides (a random mix of all 4 nucleotides) in between. Theoretical calculations indicate that the defined 3' sequence occurs every 4 kb along the genome. Under suitable conditions, an amplification using this primer could be primed off the specific 3' sequence, probably stabilized by annealing of one or more of the degenerate nucleotides (**38**). The specific 5' sequence permits the annealing of this primer at a higher temperature to previously amplified DNA. In practice, the initial cycles in the DOP-PCR protocol include a low temperature annealing step (low fidelity PCR), followed by multiple cycles at a higher annealing temperature (higher fidelity PCR), resulting in a population of randomly amplified DNA.

The flow sorted chromosomes are normally amplified through two rounds of DOP-PCR, and the resulting product is then labeled by a third round of amplification in the presence of fluorescently labeled nucleoside triphosphates. Although nick-translation protocols could be used to label probes for chromosomal analysis, this additional step is usually unnecessary to obtain high quality probes.

### 3.3.1. Amplification of DNA

Typically, flow sorted chromosomes are resuspended and amplified in PCR assay buffer, using the following conditions: 95°C for 5 min, followed by 9 cycles of 94°C for 1 min, 30°C for 1.5 min, and 72°C for 3 min, with a ramp time of 2 min, 5 s, followed by 35 cycles at 94°C for 15 s, 62°C for 15 s, and 72°C for 15 s (*see* **Notes 8–10**). This is followed by a single extension at 72°C for 10 min, and then holding the temperature at 4°C. Using the same procedure, 1 µL of the PCR product is then reamplified in a final vol of 100 µL. 10 µL of this reaction should then be electrophoresed on a 1% agarose gel, resulting in a smear, ranging in size from approx 300–1000 bp. The yield of DNA from this reaction varies from 1.5–3.0 µg (*see* **Note 11**).

### 3.3.2. Labeling of DNA

1 µL from the previous round of PCR is labeled in PCR labeling buffer, using the same conditions described for DNA amplification. 1–2 µL of the labeled DNA can then be used directly for hybridization without purification.

## 3.4. Labeling of DNA Probes with Aliphatic Amines

In addition to the one-step labeling of probes using polymerases to incorporate labeled nucleotides, a two-step labeling procedure can be utilized involving: (1) incorporating nucleoside triphosphates with aliphatic amine substituents into the probe DNA using the polymerase-based labeling protocols described above (*see* **Subheading 3.1–3.3.**), and (2) reacting the modified DNA with amine reactive fluorophores. The two-step procedure offers the advantage that any amine reactive fluorophore can be attached to the modified DNA, instead of only fluorophores that are commercially available already attached to nucleoside triphosphates. Additionally, all polymerase-based labeling reactions can use the same nucleoside triphosphate–allylamine–dUTP. Therefore, a single set of polymerase reaction conditions can be used for all fluorescent labels, instead of having to optimize for each different fluorescent nucleoside triphosphate. Also, incorporation of allylamine–dUTP may be more efficient than incorporation of other labeled nucleoside triphosphates. Labeling kits for two-step label incorporation via allylamine–dUTP are commercially available (ARES labeling kits, Molecular Probes, Inc., Eugene, OR).

Aliphatic amines introduced into DNA enzymatically, using allylamine–dUTP in place of labeled nucleoside triphosphates, can be conjugated with amine-reactive fluorophores, biotins, or haptens by the following procedure. The procedure can also be used to label probes containing aliphatic amines introduced by a number of other chemistries (**22,46**). This includes synthetic DNA, RNA, and PNA oligomers containing aliphatic amine modified bases, or 3'- or 5'-amine-modified termini, introduced via phosphoramidite chemistry.

1. Dissolve amine-modified DNA in a suitable reaction buffer at a concentration of 10 nmol aliphatic amines/0.6–1.0 mL reaction buffer.
2. Dissolve the amine reactive labeling compound in a suitable solvent to a final concentration of 10–20 mM.
3. Add a 50-fold (isothiocyanates) or 100- to 200-fold (*N*-hydroxysuccinimidyl esters or sulfonic acid chlorides) molar excess of the amine-reactive dye to the amine modified DNA (see **Note 12**).
4. Allow the reaction solution to stir at room temperature overnight.
5. Separate the probe from unconjugated label by gel permeation chromatography using a Sephadex G-25 column equilibrated and eluted with water or TE buffer. The labeled probe will elute in the excluded volume (see **Note 13**).
6. Store the labeled probe at 4°C or lower until ready for use.

### 3.5. ULS Labeling

The Universal Linkage System (ULS<sup>®</sup>; KREATECH Biotechnology BV, Amsterdam, The Netherlands) is a labeling methodology that uses a platinum dye complex to react with the N7 position of the guanine nucleotides. This reaction results in the formation of a stable bond between the nucleic acid and the platinum fluorophore complex. Depending on reaction conditions the ULS compound, to a lesser extent, will also form a complex with the adenine bases. This methodology has been used to label DNA (including plasmids, cosmids, molecular weight markers, DNA in low melting point agarose, BACs, PACs, YACs, whole chromosome libraries, DOP-PCR products and highly repetitive sequences such as satellite, centromeric, and telomeric DNA), RNA, PNA, oligonucleotides, and amplified nucleic acid products (**16,47**). ULS reagents and kits are also offered by Molecular Probes (Eugene, OR).

Prior to any labeling with the ULS compounds the template to be labeled must be of a size range <1000 bp. Template larger than 1000 bp will result in a substantial amount of spotted background. PCR products are usually <1000 bp and therefore can be labeled directly. The two methods of size reduction described will work with both the Kreatech and the Molecular Probes labeling protocols—sonication and enzymatic cleavage. Molecular Probes recommends an alternative DNase I protocol for use with the Alexa-ULS reagents. This protocol and the corresponding reagents are part of the ULS labeling kits provided by Molecular Probes.

#### 3.5.1. DNA Fragmentation

##### 3.5.1.1. SONICATION

1. Prepare a DNA solution at a concentration of 20 ng/μL in TE buffer. Using a minimum volume of 100 μL for sonication is best.
2. Sonicate the DNA solution in a small conical bottom plastic tube for 3 cycles of 1 min each, while keeping the sample on ice. Select ultrasonic disruptor power level and duty cycle to deliver the highest power possible while minimizing cavitation. Allow the DNA solution to cool on the ice for 1 min prior to the start of sonication, and after each cycle. In a microcentrifuge, centrifuge the DNA solution for 5 s at max speed before the second and third sonication steps to force all of the solution to the bottom of the tube (see **Note 14**).
3. Confirm adequate DNA size by electrophoresis on an aliquot of the DNA solution using a 1% agarose gel. Refer to **steps 10–14** of **Subheading 3.1.** for electrophoresis protocol.
4. Proceed to labeling protocols.

### 3.5.1.2. DNASE TREATMENT

1. Prepare a stock solution of DNase I by dissolving 1 mg of DNase I (Roche cat. no. 104 159, approx 2000 Kunitz U/mg) in 1 mL of 5 mM sodium acetate, 1 mM CaCl<sub>2</sub>, 50% glycerol, pH 5.2. Keep the buffer on ice prior to and during the addition of the lyophilized DNase. Invert this solution until completely mixed. Do not vortex. The stock solution should be stored at -20°C avoiding freeze thaw cycles.
2. Dilute the DNase I stock 1:5000 in 1X nicking buffer (*see Note 15*).
3. Add the following components to a microcentrifuge tube on ice: 1 µg template DNA, 2.5 µL 10X nicking buffer, 3–5 µL diluted DNase I, H<sub>2</sub>O to 25 µL.
4. Incubate this reaction at 37°C for 10 min.
5. Stop the reaction by placing on ice.
6. Precipitate the reaction mixture with 1/4 vol of 10 M ammonium acetate and 2.5 vol of 100% ethanol.
7. Resuspend the pellet in the appropriate amount of labeling solution (*see Subheading 3.5.2.*).
8. Confirm adequate DNA size by electrophoresis on an aliquot of the DNA solution using a 1% agarose gel.

### 3.5.2. Labeling

#### 3.5.2.1 PROTOCOL RECOMMENDED BY KREATECH

Optimal labeling efficiencies are achieved when the ULS reagent and nucleic acid are combined at a 1:1 ratio (i.e. 1 U ULS reagent:1 µg DNA). For labeling amounts of nucleic acid other than the standard 1 µg, the ratio of nucleic acid to ULS reagent should be kept at 1:1. When labeling small amounts, a minimum of 100 ng of nucleic acid in a 20 µL vol should be used. Alternatively, larger amounts should not exceed 10 µg of nucleic acid in a 20 µL vol. Labeling in a larger vol is possible as long as the template concentration is not lower than 5 ng/µL, and the amount of ULS reagent added is adjusted to the amount of input template. In the case of very dilute template, the labeling solution can be omitted.

1. Add 1 U (2 µL) ULS reagent to 1 µg of input template (*see Notes 16 and 17*).
2. Adjust volume with labeling solution to 20 µL and mix well.
3. Incubate for 15 min at 65°C.
4. Centrifuge briefly.
5. Purify probe on a spin column.

#### 3.5.2.2. PROTOCOL RECOMMENDED BY MOLECULAR PROBES

Molecular Probes provides a kit that utilizes the ULS labeling system coupled to a variety of their dyes. The kit contains reagents both for fragmentation of the DNA and labeling protocols. The labeling protocols and precautions are very similar with exception of the following:

1. Molecular Probes ULS dye reagents are dissolved in different buffers prior to labeling, depending upon the dye selected. A chart is provided with all specific information relating to each available dye.
2. Immediately prior to labeling, DNA is denatured at 95°C for 5 min, and then snap cooled on ice. It is noted that denaturation is not a necessity but can improve the labeling efficiency by 20–40%.

3. The labeling reaction total volume is 25  $\mu\text{L}$ .
4. Incubation is at 80°C for 15 min.
5. Molecular Probes states that the modifications to the previously stated Kreatech protocols have been made owing to the specifications of their own dyes that are coupled to the ULS reagents.

### 3.6. Labeling Proteins for Indirect Detection

Secondary detection reagents for indirectly labeled FISH probes are prepared by conjugating fluorophores to proteins such as avidin, streptavidin, or antibodies. A large variety of fluorophore labeled avidins and antibodies are commercially available (e.g., Accurate Chemical and Scientific Co., Westbury, NY; Calbiochem, San Diego, CA; Cappel Organon Teknika, Durham, NC; DAKO Corp., Carpinteria, CA; Jackson ImmunoResearch Laboratories, West Grove, PA; Kirkegaard and Perry Laboratories, West Grove, PA; Molecular Probes, Inc., Eugene, OR; Pierce Chemical Co., Rockford, IL; Sigma Chemical Co., St. Louis, MO). Labeled proteins can also be prepared fairly easily in the laboratory. Unlabeled avidins and antibodies can be obtained from the same suppliers listed above.

Streptavidin is often preferred over avidin as a secondary reagent because of its near-neutral pH isoelectric point, which is believed to reduce nonspecific binding. Similarly, while whole antibodies can be used in FISH, the more hydrophobic  $F_c$  region is often removed to reduce nonspecific binding. The  $F_c$  region can be cleaved from the antigen-binding regions of antibodies by digestion with papain, to produce Fab particles (single binding arms), or pepsin, to produce  $F(ab')_2$  with the two binding arms still connected. Fab' particles can be prepared from  $F(ab')_2$  preparations by selective reduction of disulfide bonds that joint the two binding arms. Methods for preparing  $F(ab')_2$ , Fab, and Fab' antibody fragments can be found in the literature (48,49).

1. Dissolve the protein to a final concentration of 1–10 mg/mL in 50 mM boric acid at pH 8.5–9.3.
2. Dissolve the *N*-hydroxysuccinimidyl derivative of the desired fluorophore in a solvent such as dimethyl sulfoxide, dimethyl formamide, or acetone, to a final concentration of 20 mM.
3. Add a sufficient volume of the fluorophore solution to the protein solution to provide a 1- to 20-fold molar excess of fluorophore to protein (see **Notes 18** and **19**).
4. Allow the reaction to proceed with gentle stirring at room temperature for 2 h.
5. Separate the protein from unconjugated fluorophore by gel permeation chromatography using a Sephadex G-25 column equilibrated and eluted with TBS or PBS. The labeled protein will elute in the excluded volume (see **Note 20**).
6. Store the labeled protein at 4°C until ready for use (see **Note 21**).

### 3.7. Characterization of Labeled Probes

To calculate accurate probe concentrations, enough DNA must be available to generate an absorbance value of at least approx 0.05 in the minimal volume of liquid required to fill the spectrophotometer cuvet. For conventional absorbance spectrophotometer cuvetts with 1 cm pathlengths, the minimal volumes are several hundred microliters (semimicro cuvetts). Spectrophotometers specifically designed for DNA work can utilize cuvetts with volumes near 10  $\mu\text{L}$ . The following equation can be used to

calculate the nucleic acid concentration, [nucl], from the measured absorbance at 260 nm,  $A_{260}$ :

$$[\text{nucl}] = [A_{260} - (\epsilon_{F,260}/\epsilon_{F,\text{MAX}})A_{F,\text{MAX}}]/\epsilon_{\text{nucl},260}$$

where  $\epsilon_{\text{nucl},260}$  is the absorbance extinction coefficient of the nucleic acid at 260 nm, and  $\epsilon_{F,260}/\epsilon_{F,\text{MAX}}$  is the ratio of the absorbance extinction coefficients of the label at 260 nm,  $\epsilon_{F,260}$ , and at the peak wavelength of the longest wavelength absorbance band,  $\epsilon_{F,\text{MAX}}$ . This ratio for the labeled probe is approximated by the ratio of absorbance values of the unconjugated label at these two wavelengths. For single-stranded DNA,  $\epsilon_{\text{nucl},260} = 10,000 M^{-1}\cdot\text{cm}^{-1}$ , or  $0.0286 (\mu\text{g}/\text{mL}) \text{ mL}\cdot\text{ng}^{-1}\cdot\text{cm}^{-1}$ . The former value gives the concentration in nucleotide molarity, while the latter value gives the concentration in micrograms nucleic acid per milliliter. The label concentration, [F], is calculated from the probe absorbance at the long wavelength absorbance maximum of the label:

$$[\text{F}] = A_{F,\text{MAX}}/\epsilon_{F,\text{MAX}}$$

at which the nucleic acid absorbance is assumed to be negligible. Values of  $\epsilon_{F,\text{MAX}}$  can be obtained from the suppliers of the labeling reagents. The percentage of nucleotides labeled is then equal to  $100[\text{F}]/[\text{nucl}]$ .

When the amount of probe is too small to obtain accurate absorbance measurements, or contains some contaminating RNA, fluorometry with bisbenzimidazole, commonly known as Hoescht 33258 dye, can be used to determine nucleic acid concentration. The Hoescht dye has little affinity for RNA but binds to the minor groove of double stranded DNA. Hoescht 33258 dye bound to DNA can be excited at 365 nm, and has peak emission at 458 nm. Fluorometry is very sensitive and can be used for DNA concentrations ranging from 0.01–5 mg/mL, with an optimum DNA concentration range of 0.05–0.3 mg/mL.

The relative fluorescence intensities are measured on the sample DNA solution,  $F_S$ , and the same solution minus the DNA (blank solution),  $F_B$ . In addition, the fluorescence intensity of a DNA standard solution,  $F_{\text{STD}}$ , and corresponding blank solution,  $F_{\text{STD},B}$  are also measured (typically the sample and standard blank solutions are the same). The DNA standard solution should have a DNA concentration,  $[\text{DNA}]_{\text{STD}}$ , close to that expected for the DNA sample solution. The sample DNA concentration,  $[\text{DNA}]_S$ , is then calculated as follows:

$$[\text{DNA}]_S = [\text{DNA}]_{\text{STD}}(F_S - F_B)/(F_{\text{STD}} - F_{\text{STD},B})$$

Accurate pipetting and thorough mixing of solutions is critical for reproducible results.

The concentrations and labeling percentages of proteins can be determined by absorbance spectroscopy using the same equations as for nucleic acid probes, except that measurements are recorded at 280 nm instead of 260 nm. The absorbance extinction coefficients for goat IgG and Fab' have been reported to be  $198,000/M^{-1}\cdot\text{cm}^{-1}$  and  $61,200/M^{-1}\cdot\text{cm}^{-1}$  (49), respectively, and the extinction coefficient for  $\text{F(ab')}_2$  should be twice that for Fab'.

#### 4. Notes

1. Fluorophores will photobleach if exposed to light for extended periods of time. Labeled DNAs and dUTPs can be handled for short periods of time in light but should not be exposed any longer than required to set up an experiment.

2. It is easier to assess range of fragment sizes if unincorporated nucleotides are removed prior to gel electrophoresis.
3. After ethanol precipitation, DNA pellets labeled with a red or orange fluorophore are usually readily seen by eye, whereas, those labeled with a green fluorophore may appear white, or very pale green.
4. 50–100 ng of a red or orange labeled unique sequence probe, and 200–400 ng of a green labeled probe per 10  $\mu$ L FISH mix typically yield sufficiently bright signals on the majority of sample types.
5. For optimal results, template DNA should be linear, preferably by digestion using a restriction enzyme with a six base pair recognition site and purified by phenol/chloroform extraction followed by ethanol precipitation.
6. Starting amounts of DNA can range from 10 ng to 3  $\mu$ g.
7. Labeling at 37°C can range from 1 h to overnight. Optimal conditions should be determined for individual applications.
8. Contamination is a very serious issue in PCR. To avoid contamination of PCR reactions, use the best quality reagents possible, and use them exclusively for PCR. It is best to routinely aliquot all solutions and store them frozen at  $-20^{\circ}$  C until ready for use. Also, use aerosol-resistant tips for pipetting to minimize cross-contamination. Designate one area in the lab exclusively for PCR work. When possible, prepare DNA samples in a separate room. In addition, it is absolutely essential to run suitable negative controls (i.e., without target sequence) each time an experimental PCR is performed.
9. Titrate the Taq polymerase with the specific target. Excess enzyme can result in nonspecific background.
10. Vary the number of cycles to find the appropriate number of cycles which gives the best signal to noise ratio.
11. The presence of high molecular weight DNA (visible during electrophoresis as a smear extending from the well to approx 2.0 kb size) indicates unacceptable labeled probe. Such probe results in very high nonspecific, noncellular background that can sometimes be reduced by sonication. As mentioned in **Note 9**, titrate the Taq polymerase, and find the appropriate number of PCR cycles.
12. The organic solvent: aqueous buffer ratio should not exceed 1:4 unless the DNA is known to be soluble at a higher ratio.
13. As an alternative to gel permeation chromatography, the unconjugated fluorophore can be separated from the protein by dialyzing in a storage buffer (e.g., TE), changing the buffer solution at several hour intervals until the unconjugated label is completely removed (2 or more buffer changes).
14. When sonicating, the volume of the sample, the type of vessel in which the sample is contained, the length of the sonication period, the number of sonication cycles, the ultrasonic disruptor power level, and the ultrasonic disruptor duty cycle, are all dependent upon the make and model of the ultrasonic disruptor instrument and the sonication probe used. Some experimentation may be required to obtain properly sized nucleic acid fragments.
15. When performing the DNase I digestion, all solutions should be kept on ice. Prepare solutions immediately before use.
16. DNA should be purified to remove proteins, RNA, and free nucleotides before labeling.
17. High tris(hydroxymethyl)aminomethane (Tris) concentrations ( $>40$  mM) or EDTA ( $>5$  mM), Mg acetate ( $>100$  mM), NaCl ( $>100$  mM), and restriction enzyme digestion buffers should be avoided because of their rate-limiting effect on the labeling reaction.
18. The molar excess of reactive fluorophore-to-protein, and the protein concentration will determine the extent of protein labeling. The labeling ratio required for optimal perfor-

mance of the labeled protein reagent will depend upon which fluorophore and protein are used, and will need to be determined experimentally. Too low a labeling ratio results in weak fluorescence signals, while too high a ratio can inhibit the specific protein binding reaction and increase nonspecific binding.

19. The amount of 20 mM fluorophore solution added to the protein should not result in the organic solvent concentration exceeding 20% of the total reaction volume, necessary to prevent denaturation of the protein.
20. As an alternative to gel permeation chromatography, the unconjugated fluorophore can be separated from the protein by dialyzing in TBS or PBS, changing the buffer solution at several hour intervals until the dye color is no longer imparted to the buffer.
21. Alternative protein labeling protocols abound in the literature and are available from suppliers of labeling reagents (e.g., see the "Amine-Reactive Probes" information sheet from Molecular Probes, Inc., or "Procedure for Labeling Proteins with Fluorochromes" by Research Organics, Cleveland, OH).

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## Labeling Fluorescence *In Situ* Hybridization Probes for RNA Targets

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### 1. Introduction

Ribonucleic acids (RNA) can be detected in cellular specimens by fluorescence *in situ* hybridization (FISH) using labeled RNA, deoxyribonucleic acid (DNA), or nucleic acids analogs. The RNA targets are often messenger RNA (mRNA) molecules that have been transcribed from the genomic DNA. Specific mRNA sequences can be selectively identified out of the many co-expressed mRNA molecules owing to the high specificity of nucleic acid base pairing. The analysis of selected mRNA sequences is an important measure of many cellular properties, including, for example, the identification of specific cell types, the identification of different stages of cell differentiation, and measuring abnormal mRNA expression, as often accompanies cancer development. RNA probes can be directly conjugated with fluorescent labels, or conjugated with labels that specifically associate with fluorescent compounds. Once hybridized, the presence and relative abundance of an RNA target can be qualitatively assessed visually based on the fluorescence intensity, or in a more quantitative fashion, by digital imaging and image analysis.

Much of what is stated about genomic FISH probes in the preceding chapter is also applicable to FISH probes for RNA targets, including statements related to direct and indirect labeling, amplified detection reactions, and fluorescent label selection. Since DNA probes are often used for RNA detection, DNA probe labeling methods described in the preceding chapter may also be employed in making DNA probes for mRNA detection.

In addition to the methods described in the preceding chapter, some enzymatic labeling reactions are unique to working with RNA. Reverse transcriptase can be used to incorporate labeled nucleoside triphosphates into DNA using an RNA template (1–4), and RNA polymerase can be used to incorporate labeled nucleoside triphosphates into RNA strands (5–8).

Synthetic oligonucleotides are more easily applied to RNA detection because many copies of an mRNA molecule are synthesized *in vivo*, in contrast to the diploid nature of the genomic DNA. Synthetic oligonucleotides, including DNA, RNA, and analogs such as peptide nucleic acids (PNA) can be synthesized relatively inexpensively and can have any of a number of different fluorophores incorporated terminally or attached to internal sites during the oligomer synthesis process.

The following protocols provide some common methods for labeling probes used to detect RNA targets via FISH. Owing to the ease of obtaining synthetic oligonucleotides, the methods presented here are entirely enzymatic, relying on reverse transcriptase, DNA polymerase I, and RNA polymerase.

### **1.1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

The first method described below generates fluorophore or hapten labeled DNA probes to RNA transcripts using the technique of RT-PCR (9,10). The process involves an initial reverse transcriptase reaction, using either random hexamer primers, or degenerate oligonucleotide primers (DOP), as described in the preceding chapter, resulting in single-stranded cDNA. Double stranded cDNA is generated using *E. coli* DNA polymerase I, followed by an amplification using Taq polymerase and either the same degenerate DOP primers, or transcript-specific PCR primers.

### **1.2. Single-Stranded Probes**

It is important to be able to generate single-strand-specific probes (antisense sequences) when using FISH to detect mRNA transcripts. Although double-stranded probes can, and are, used in many FISH applications for detection of transcripts in cells, there are two major reasons to prefer the single-stranded variety. The first reason is that although double-stranded probe can be denatured before hybridization, the solution-phase kinetics of reannealing is considerably faster than that of probe hybridizing to surface-immobilized target sequences. This results in a large portion of probe being titrated out of the hybridization mix, even before encountering the target. The second reason is that the use of the sense probe serves as an excellent negative control for the FISH assay, since its complementary sequence should not be present in the mRNA population to be studied.

There are a number of different methods for generating single-stranded probes for use in FISH. In **Subheading 1.2.1.** is described a method for generating single-stranded DNA probes, as well as, a method for generating antisense and sense RNA probes. Both of the methods make the assumption that the specific sequence of interest is available as a clone, or as part of a cDNA library.

#### **1.2.1. Single-Stranded DNA Probes—Asymmetric PCR**

The first method involves altering the conditions of PCR such that one of the strands is preferentially synthesized. This technique is referred to as asymmetric PCR (11,12), and involves performing the assay with one of the primers (complementary to the desired single strand) in vast excess to the other, “limiting” primer. Primer ratios normally range from 50:1 to 100:1. For the first few cycles (normally 10–15), double-stranded DNA is predominantly produced in an exponential manner. As the limiting

primer is depleted, an excess of one strand is produced, in a linear fashion. Consequently, about 40 cycles of PCR are required to generate adequate amounts of single-stranded probe. By switching the ratios of the primers, it is possible to generate single-stranded probe to each strand in separate reactions. A method is described below for the generation of single-stranded DNA probes to the human HLA-G mRNA sequence.

### 1.2.2. Single-Stranded RNA Probes

The second method involves attaching specific viral RNA polymerase promoter sequences to the 5' end of each sequence of interest. RNA polymerases are very sequence-specific. When the hybrid DNA molecule is transcribed *in vitro* in the presence of fluorescent ribonucleoside triphosphates, specific single-stranded cRNA molecules of high specific activity are generated. Different RNA polymerase promoter sequences (for example, T7 or SP6) can be attached to either end of the DNA molecule. By performing separate *in vitro* transcription reactions in the presence of the appropriate RNA polymerase, specific single-stranded transcripts to each strand (sense or anti-sense) can be generated. The *in vitro* transcription reaction typically results in a 50–100-fold linear amplification of the sequence of interest, in the presence of fluorescent ribonucleotides. A method is described below for the generation of single-stranded sense and antisense RNA probes to the human HLA-G mRNA sequence.

### 1.3. In Situ Reverse Transcriptase PCR (In Situ RT-PCR)

*In situ* hybridization is limited in sensitivity when low levels of nucleic acid are involved. *In situ* PCR (13–15) and *in situ* RT-PCR (16,17) can be used to localize DNA or RNA respectively, in appropriately prepared tissue samples. Below is described an *in situ* technique to detect HSV-1 RNA on selected sections from latently infected rat trigeminal ganglia or cortical neurons (16,17). Specific oligonucleotide primers to the HSV-1 glycoprotein B (gB) gene and digoxigenin-labeled nucleotides are used to produce amplified digoxigenin-labeled DNAs *in situ*, which are localized using an alkaline phosphatase-conjugated anti-digoxigenin antibody detected with the BCIP/NBT. Neurons in infected trigeminal ganglia as well as cortical neurons containing HSV-1 transcripts have been identified using this method (16,17).

## 2. Materials

### 2.1. RT PCR

1. 10X PCR buffer I (Applied Biosystems, Foster City, CA).
2. PCR assay buffer: 10  $\mu$ L of 10X PCR Buffer I, 2  $\mu$ L dNTPs (10 mM each), 1  $\mu$ L of primer (100 ng), 1  $\mu$ L Taq polymerase, and nuclease-free water to 100  $\mu$ L.
3. Deoxynucleoside triphosphates: 10 mM each (Promega Corp., Madison, WI).
4. PCR Labeling Buffer: same as PCR Assay Buffer, with addition of 1  $\mu$ L of SpectrumOrange™ or SpectrumGreen™ dUTP, except the final volume is 99  $\mu$ L.
5. 100 ng/ $\mu$ L primer (random hexamer (Promega Corp.) or DOP-PCR primer).
6. 50 nmol SpectrumOrange™/SpectrumGreen™ 2'-deoxyuridine-5'-triphosphate (Vysis, Inc., Downers Grove, IL) Reconstitute with 50  $\mu$ L of nuclease-free water to give a 1 mM solution. Stable for up to 3 mo if stored at  $-20^{\circ}\text{C}$ .

7. Taq polymerase: any commercial variety available can be used, but optimal concentration to be used must be first determined by titrating with appropriate substrate.
8. RNasin 40 U/ $\mu$ L (Promega Corp.).
9. Reverse Transcriptase: Superscript II at 200 U/ $\mu$ L (Gibco-BRL, Gaithersburg, MD).
10. 5X First strand buffer (Gibco-BRL).
11. *E. coli* DNA polymerase I—10 U/ $\mu$ L, (Gibco-BRL).
12. 0.1 M dithiothreitol (DTT).
13. RNase H—2 U/ $\mu$ L (Gibco-BRL).
14. 5 $\times$  Second strand buffer (Gibco-BRL).
15. Glycogen: molecular biology grade, 5 mg/mL (Promega Corp.).
16. 5 M Ammonium acetate, (Sigma Chemical Co., St. Louis, MO).
17. Isopropanol: absolute, (Aldrich Chemical Co., Milwaukee, WI).
18. 70% Ethyl alcohol.
19. Nuclease-free water.
20. Mineral oil (if necessary), light white (Sigma Chemical Co.).
21. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
22. 1X Tris-borate buffer (TBE): 45 mM Tris-borate, 1 mM EDTA, pH 8.0, for gel electrophoresis (BioRad, Hercules, CA).

## 2.2. Asymmetric PCR

### 2.2.1. Buffers and Enzymes

1. 10X PCR buffer I (Perkin-Elmer Applied Biosystems, Foster City, CA).
2. PCR assay buffer: 10  $\mu$ L of 10X PCR Buffer I, 1  $\mu$ L dNTPs (10 mM each), primer pair (concentration as below), 1  $\mu$ L of SpectrumOrange™ or SpectrumGreen™ dUTP (Vysis, Inc., Downers Grove, IL), 3 U of Taq polymerase, nuclease-free water to 99  $\mu$ L.
3. 50 pmol of “excess” primer.
4. 1 pmol of “limiting” primer.
5. SpectrumOrange™/SpectrumGreen™ 2'-deoxyuridine-5'-triphosphate (Vysis, Inc.): reconstitute with 50  $\mu$ L of nuclease-free water to give a 1 mM solution. Stable for up to 3 mo if stored at  $-20^{\circ}\text{C}$ .
6. Taq polymerase: any commercial variety available can be used.
7. Nuclease-free water.
8. Mineral oil (if necessary): light white (Sigma Chemical Co., St. Louis, MO).
9. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
10. 1X Tris-borate buffer (TBE): 45 mM Tris-borate, 1 mM EDTA, pH 8.0, for gel electrophoresis (Bio-Rad, Hercules, CA).

### 2.2.2. Primers

The primers used were designated:

1. HLA-A, sequence 5' TGG GAC TGA GTG GCA AGT.
2. HLA-B, sequence 5' TTC ATG GTG GCC TGA GCA.

## 2.3. Single-Stranded RNA Probes

### 2.3.1. Buffers and Enzymes

1. 10X PCR buffer I (Applied Biosystems, Foster City, CA).
2. PCR assay buffer: 10  $\mu$ L of 10X PCR buffer I, 2  $\mu$ L dNTPs (10 mM each), 1  $\mu$ L of each primer (100 ng), 1  $\mu$ L Taq polymerase, and nuclease-free water to 99  $\mu$ L.

3. SpectrumOrange/SpectrumGreen 2'-deoxyuridine- 5'-triphosphate (Vysis, Inc., Downers Grove, IL): reconstitute with 50  $\mu$ L of nuclease-free water to give a 1 mM solution. Stable for up to 3 mo if stored at  $-20^{\circ}\text{C}$ .
4. Taq polymerase: any commercial variety available can be used.
5. Mineral oil (if necessary), light white (Sigma Chemical Co., St. Louis, MO).
6. SP6 RNA polymerase, 10 units/ $\mu$ L (Promega Corp., Madison, WI).
7. 5X SP6 transcription buffer (Promega Corp.).
8. T7 RNA polymerase, 10 Units/ $\mu$ L (Promega Corp.).
9. 5X T7 transcription buffer (Promega Corp.).
10. Fluorescein RNA labeling mix, 10 $\times$  (Roche Biochemicals, Nutley, NJ).
11. Nuclease-free water.
12. In vitro transcription labeling buffer: 2  $\mu$ L of fluorescein RNA Labeling Mix, 4  $\mu$ L of 5X transcription buffer, 2  $\mu$ L of specific RNA polymerase, nuclease-free water to 18  $\mu$ L.
13. 0.5 M EDTA, pH 8.0.
14. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
15. 1X Tris-borate buffer (TBE): 45 mM Tris-borate, 1 mM EDTA, pH 8.0, for gel electrophoresis (BioRad, Hercules, CA).

### 2.3.2. Primers

The primers used were designated:

1. SP6HLA, 5' AGG GAT CCA TTT AGG TGA CAC TAT AGA ACC CGG GGC TGG GAC TGA GTG GCA AGT.
2. T7HLA, 5' GGA TCC TAA TAC GAC TCA CTA TAG GGA G TTC ATG GTG GCC TGA GCA.

### 2.4. In situ RT PCR

1. 100 ng/ $\mu$ L primers. The primer pairs used were (1) for gB: 5' primer ATT-CTC-CTC-CGA-CGC-CAT-ATC-CAC-CTT; 3' primer AGA-AAG-CCC-CCA-TTG-GCC-AGG-TAG-T. (2) For LAT: 5' primer GAC-AGC-AAA-AAT-CCC-GTC-AG; 3' primer ACG-AGG-GAA-AAC-AAT-AAG-GG (3) for glyceraldehyde phosphate dehydrogenase (GAPDH): 5' primer ATT-GGG-GGT-AGG-AAC-ACG-GAA; 3' primer ACC-CCT-TCA-TTG-ACC-TCA-ACT-A.
2. Graded ethanols: 100, 70, and 50% ethanol.
3. PBS: 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , 140 mM NaCl, 2.7 mM KCl, pH 7.5.
4. Xylene.
5. Taq polymerase: any commercial variety available can be used, but optimal concentration to be used must be first determined by titrating with appropriate substrate.
6. Reverse transcriptase: Superscript II at 200 U/ $\mu$ L (Gibco-BRL, Gaithersburg, MD).
7. DNase I, RNase free (Gibco-BRL).
8. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
9. *In situ* PCR Reaction mix: 1 $\times$  PCR Buffer II (Applied Biosystems, Foster City, CA), 1 mM  $\text{MgCl}_2$ , 1 $\times$  digoxigenin DNA labeling mix (Roche Molecular Biochemicals) 100 ng of primer mix, 50% glycerol, and 1  $\mu$ L of Taq polymerase.
10. RNasin 40 U/ $\mu$ L (Promega Corp.).
11. DNase digestion buffer: 1 mM NaCl, 10 mM Tris-HCl, pH 7.5.
12. *In situ* reverse transcriptase assay buffer: 10 U of SuperScript II (Gibco-BRL), 5 U of RNasin, 5  $\mu$ L of oligo(dT) primer (100 ng/mL), 10 mM DTT, 1 mM dNTP, in SuperScript II reaction buffer (Gibco-BRL) per 100  $\mu$ L.

13. Antidigoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals).
14. BCIP/NBT (Vector Lab.).

### 3. Methods

#### 3.1. RT-PCR

##### 3.1.1. Synthesis of cDNA

1. Denature RNA sample before assay, at 90°C, for 5 min.
2. Add 1 µg of poly (A)<sup>+</sup> RNA to 4 µL of 5× first strand buffer, 2 µL of 0.1 M DTT, 1 µL of 10 mM dNTP mix, 100 ng of primer and 1 µL of Superscript™ II reverse transcriptase (200 U/µL).
3. Incubate at 37°C for 60 min.
4. To the reaction mix add 92 µL of nuclease-free water, 30 µL of 5× second strand buffer, 3 µL of 10 mM dNTP mix, 4 µL of *E. coli* DNA polymerase I (10 U/µL) and 1 µL RNase H (2 U/µL), and incubate at 16°C for 2 h.
5. Add 2 µL of glycogen (5 mg/mL), 0.4 vol of 5 M ammonium acetate and twice the final volume of absolute isopropanol.
6. Microcentrifuge at max × *g* force value for 10 min at room temperature, and discard supernatant.
7. Wash pellet with 0.5 mL of ice-cold 70% ethanol and air dry.
8. Resuspend pellet in 10 µL of nuclease-free water.

##### 3.1.2. Amplification and Labeling of cDNA

Amplify and label double-stranded cDNA according to the procedure described in **Subheadings 3.3.1** and **3.3.2** of the preceding chapter. Alternately, use gene specific primers and appropriate PCR amplification/labeling conditions (*see* **Notes 1–5**).

#### 3.2. Asymmetric PCR

Amplify 1 µg of plasmid DNA (1 mg/mL) in PCR assay buffer, using the following conditions: 40 cycles at 94°C for 15 s, 55°C for 15 s, followed by a single extension at 72°C for 10 min, and then hold at 4°C. 10 µL of this reaction should then be electrophoresed on a 1% agarose gel. The yield of DNA from this reaction varies from 1.0–2.0 µg of labeled single-stranded DNA (*see* **Notes 6–8**).

#### 3.3. Single-Stranded RNA Probes

##### 3.3.1. Amplification of DNA

Amplify 1 µg of DNA (1 mg/mL) in PCR assay buffer using the following conditions: 30 cycles at 94°C for 15 s, 55°C for 15 s, followed by a single extension at 72°C for 10 min, and then hold at 4°C.

##### 3.3.2. In vitro Transcription of PCR Product

2 µL of cDNA is in vitro transcribed in in vitro transcription labeling buffer, for 2 h at 37°C. Stop the reaction by adding 2 µL of 0.5 M EDTA, pH 8.0, on ice. Make up the volume to 100 µL with nuclease-free water, and directly use for FISH (*see* **Notes 9–11**).

### 3.4. In Situ RT PCR

1. Perfuse animals with 4% paraformaldehyde, embed tissue in paraffin, and cut on a microtome.
2. Deparaffinize 6  $\mu\text{m}$  sections of tissue on glass slides in xylene ( $3 \times 2$  min).
3. Successively rehydrate tissue with graded ethanols in the following order: 100% ethanol ( $2 \times 2$  min); 70% ethanol (2 min), and 50% ethanol (2 min).
4. Wash sections in PBS (pH 7.5) for 5 min, twice.
5. Permeabilize sections with 1% HCl in PBS for 5 min, and then wash in PBS ( $3 \times 5$  min) (*see Note 12*).
6. Treat sections with 400 U of RNase-free DNase, and 10 U of RNasin in 200  $\mu\text{L}$  of DNase digestion buffer, at  $37^\circ\text{C}$  overnight (*see Note 12*).
7. Rinse samples with RNase-free PBS to remove excess DNase.
8. Samples are then reverse-transcribed in 100  $\mu\text{L}$  of *in situ* reverse transcriptase assay buffer, at  $37^\circ\text{C}$  for 60 min.
9. Rinse samples repeatedly with RNase-free PBS.
10. Rinse samples with PCR buffer II ( $3 \times 5$  min).
11. Layer 25  $\mu\text{L}$  of *in situ* PCR reaction mixture onto the sections, which are covered with a glass coverslip and sealed using nail polish, taking care to ensure that the polish does not seep into the reaction mix.
12. PCR amplification is carried out in a BioOven III Thermal Cycler (BioTherm Corp, St. John Associates, Inc., Beltsville, MD 20705) in two stages; first for 3 cycles at  $92^\circ\text{C}$  for 1 min,  $54^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 30 s, followed by 25 cycles at  $92^\circ\text{C}$  for 15 s,  $54^\circ\text{C}$  for 15 s, and  $72^\circ\text{C}$  for 15 s (*see Notes 13–15*).
13. After amplification, remove the coverslips and wash the sections successively with 1X SSC ( $2 \times 5$  min), 50% formamide in 1X SSC ( $3 \times 15$  min,  $56^\circ\text{C}$ ), and 1X SSC ( $2 \times 15$  min).
14. Following a rinse in Tris-buffered saline (pH 7.5) and 5% normal goat serum. Treat the digoxigenin-labeled amplified DNA with an antidigoxigenin antibody conjugated to alkaline phosphatase (1:250), and visualize with BCIP/NBT.
15. Monitor color development visually; reaction can be stopped typically after about 30 min, by washing with 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA.

### 4. Notes

1. To guard against nonspecific amplification resulting from contamination of PCR reagents, use the best quality of reagents possible, and use them exclusively for PCR. We routinely aliquot out all of our solutions and freeze them at  $-20^\circ\text{C}$ . We also use aerosol resistant tips for pipetting to minimize cross-contamination. Designate one area in the lab exclusively for PCR work.
2. When possible, prepare DNA and RNA samples in separate rooms. In addition, it is absolutely essential to run suitable negative controls (i.e., without target sequence) each time an experimental PCR is performed.
3. Titrate the Taq polymerase with the specific target. Too much enzyme can result in nonspecific background.
4. Titrate primers from 50 ng to 1  $\mu\text{g}$ .
5. Vary the number of cycles to find the appropriate number of cycles which gives the best signal-to-noise ratio.
6. For best results, the ratio of primers will have to be optimized by titration.

7. Since the reaction to generate single-stranded DNA by asymmetric PCR is not efficient, as many as 40 cycles will need to be run. At times, an aliquot may need to be removed after 40 cycles for a fresh round of asymmetric PCR.
8. Another method to enhance yield of single-stranded product is to double the amount of Taq polymerase used in a normal PCR reaction. A typical concentration is 3 *U* of enzyme per reaction.
9. As before, the quality of the labeled transcript can be monitored on a 1% agarose gel.
10. Do not stain gel with ethidium bromide, since fluorescein-labeled cRNA will auto-fluoresce on a UV-transilluminator.
11. Observe all precautions normally followed while handling RNA.
12. The most crucial steps in obtaining good results using this technique are determining the optimal time for tissue permeabilization and DNase treatment.
13. Include control samples in which the enzyme reverse transcriptase is not included; this should not produce any positive signals.
14. Include control samples that have been pretreated with RNase prior to reverse transcription; this should not produce any positive signals. A simple way to perform this step is to include RNase I (400 *U*) in the DNase digestion step, overnight.
15. Include control samples in which the *in situ* PCR is performed in the absence of specific primers; this should also not produce any positive signals.

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## Basic FISH Techniques and Troubleshooting

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### 1. Introduction

Fluorescence *in situ* hybridization (FISH) technology permits the detection of specific nucleic acid sequences in morphologically preserved chromosomes, cells, and tissue. The unambiguous detection of structural or copy number changes of whole chromosomes or chromosome specific regions is an important prognostic and predictive factor in human disease. The utility of FISH in chromosome analysis is divided into metaphase and interphase applications. This distinction is manifested primarily in the preparation of the cells prior to pretreatment and hybridization. The culturing of cells for metaphase analysis is identical to those used in standard karyotyping. FISH is particularly advantageous in samples of low cellularity, or containing a mixed population of cells. A wide variety of sample types are amendable to FISH making it particularly valuable in the study of archival material, such as formalin-fixed paraffin-embedded tissues. However, the success of probe hybridization is dependent on the optimal preparation of sample. In interphase applications, the efficiency of probe hybridization is greatly enhanced by treating the cell or tissue preparation with a variety of reagents that permeabilize the target while preserving important morphological features. Additionally, pretreatment will reduce the background autofluorescence of cells and tissue. A thorough understanding of the processes behind sample pretreatment will provide insight in troubleshooting efforts. Once the DNA in the sample is made available for hybridization, the conditions for optimal signal involves far fewer permutations. The purpose of this chapter is to review the salient features of FISH with respect to sample preparation and hybridization.

#### 1.1. Interphase vs Metaphase Analysis

Fluorescently labeled DNA probes can be hybridized to cells in both interphase and metaphase stages of the cell cycle. Interphase FISH is advantageous in the rapid screening of many nuclei without the need for cell culture and metaphase chromosome preparation. It is also beneficial in the study of samples with a low mitotic

index such as most solid tumors. The major limitation of interphase FISH is the inability to detect unknown structural chromosomal changes. Conversely, metaphase FISH analysis allows for a visual analysis of specific chromosome regions that contain structural changes, making it particularly useful in a variety of applications including chromosome painting probes, centromere specific, and locus identification probes. Structural changes in the genome such as translocations, inversions, and marker chromosomes are often readily identified by metaphase FISH. Methods combining banding techniques and FISH have been optimized for confirmation of aberrant findings.

Slide quality of interphase and metaphase preparations is a very significant factor in the success of FISH. A thorough understanding of the tissue of interest and of slide preparation techniques is the first step to obtaining high quality results. Each laboratory should validate a probe's performance on control specimens prior to use.

## **1.2. Slide Preparation**

### **1.2.1. From Culture**

The spreading of condensed chromosomes involves the precise application of the cell preparation across a slide under favorable drying conditions (*see Note 1*). Temperature and relative humidity are controlled to optimize chromosome spreading and morphology, and to minimize the presence of cytoplasm. A phase contrast microscope is essential for reviewing slide quality. The chromosomes in a metaphase should appear medium gray in color, have sharp borders, be well separated, and have little to no visible cytoplasm. Chromosomes should not appear refractile under phase microscopy. If the slides do not meet these specifications, modify slide preparation conditions by adjusting the temperature or humidity levels, and if necessary, by adjusting the density of the cell suspension. These measures are aimed at optimizing the rate of evaporation of the fixative. After drying, slides are aged overnight at ambient temperature. For long term storage, slides should be stored with desiccant in a  $-20^{\circ}\text{C}$  freezer.

### **1.2.2. From Fresh Specimens**

While slide preparation can be performed in either of the two formats listed below for cultured metaphase specimens, fresh specimens are usually less affected by controlled temperature and humidity. Nuclei in cells obtained from non-cultured sources such as amniocytes, squamous, and epithelial cell types contain significantly more cytoplasm than cultured cells which, in turn, prevents the swollen nuclei from spreading effectively in two dimensions across the slide. Consequently, fixed cells are simply dropped onto slides. With the aid of a microscope, additional sample can be applied to the slide until the desired cellularity is achieved. Cyto-centrifugation is also a popular method for depositing cells onto slides. If slides are to be hybridized the same day, they should be artificially aged in the same manner as cultured samples.

### **1.3. Slide Pretreatment**

#### **1.3.1. Fresh Cells**

The accessibility of DNA in metaphase chromosomes is optimized during slide dropping and usually does not require pretreatment. For interphase cells, following aging, cells are incubated in a protease solution to rid the cell of extracellular debris and further reduce the presence of cytoplasm. This step is particularly important in cell types that have a significant amount of cytoplasm, e.g., amniocytes. Following enzymatic treatment, cells are exposed to a 1% formaldehyde solution to stabilize nuclear morphology. Slides are incubated in a series of graded ethanol solutions to dehydrate the cells in preparation for denaturation. Pretreatment procedures are basically the same for each specimen regardless if the processing includes a cultured or uncultured format. The recommended protease concentration is lower for amniocytes than most other cell types due to their fragile nature. Many samples, particularly fresh cultured cells or lymphocytes do not require pretreatment. However, the use of a standard protocol may be warranted when hybridizing slides with excess cytoplasm or background debris. Pretreatment of uncultured bone marrow specimens is often beneficial and helps to minimize background noise while increasing specific signal intensity. Fixation in 1% formaldehyde prior to protease digestion may help maintain compact nuclear signals.

#### **1.3.2. Paraffin-Embedded Solid Tumor**

Freshly excised specimens should be fixed as soon as possible in 10% buffered formalin. Fixation times between 24–48 h are optimal for FISH applications may help delays in tissue fixation, as well as over- and under-fixation will result in weak or absent hybridization signals. Sections are typically cut at 4–6 microns and should be mounted on positively charged slides, such as amino-silane treated, then allowed to air dry. The slide can be used immediately or stored for an extended period of time at ambient temperature. Prior to use, the mounted slides are baked in an oven or on a slide warmer overnight at 56°C to improve tissue adherence. Formalin is a stronger fixative than acid or alcohols. Consequently formalin-fixed specimens require significantly more protease digestion for optimal probe hybridization.

To prepare paraffin-embedded samples for FISH, the paraffin must first be removed. There are commercial pretreatment kits available that are optimized to prepare tissue specimens for FISH. Samples are deparaffinized in a series of xylene or Hemo-D<sup>®</sup> solutions followed by dehydration in ethanol. The samples are then treated chemically with HCl, followed by sodium isothiocyanate at elevated temperatures prior to protease digestion.

### **1.4. Hybridization and Wash**

The process of specific and efficient hybrid formation of labeled DNA to partially digested chromatin involves denaturation of target and probe DNA, hybridization of labeled probe in the presence of unlabeled blocking DNA homologous to nonspecific repetitive sequences, and removal of unhybridized, or nonspecifically hybridized or

bound probe. Denaturation conditions for FISH are similar to those required for purified DNA, except that they are performed in the presence of formamide, a chaotrope, which lowers the melting point of duplexed nucleic acids (*I*). Hybridization of FISH probes made from large genomic DNA fragments, typically containing repetitive sequences, necessitates the use of excess blocking DNA. The suppression of probe hybridization to non-target sequences by blocking DNA constitutes the mechanism of FISH specificity. The amount of probe used in a hybridization is in the 10–900 ng range for a 10  $\mu$ L hybridization volume. Less probe is required if made from highly repetitive sequences near the centromere, which can span hundreds of thousands of base pairs. Depending on the complexity of the probe, hybridization buffers may also contain accelerators such as dextran sulfate.

## 1.5. Enumeration

Before a hybridized slide can be assessed, the operator needs to be aware of the quality characteristics of the probe in addition to the characteristics of the test sample. It is important to become familiar with the size, shape, and fluorescent intensity of each of the DNA probe signals in order to accurately evaluate the hybridization results. Signals should be bright, distinct, and easily enumerated. The background should be free of fluorescing particles and the probe should not cross-hybridize to other loci. The operator also needs to be familiar with the cell population of the test slide and recognize when to include or exclude cell types, or when to scan a population of cells for a specific malignancy. Certain probes are intended to target cells during interphase or metaphase stages of the cell cycle and the operator must adjust their focus accordingly.

## 2. Materials

### 2.1. Slide Preparation from Metaphase Cultures

1. Waterbath.
2. Humidifier.
3. Plastic wrap.
4. Hygrometer.
5. Test tube racks.
6. Carnoy's fixative (*see Note 2*). 3:1 Methanol:Glacial Acetic acid, cold ( $-20^{\circ}\text{C}$ ).
7. Slides.
8. 70% Ethanol.
9. Water.
10. Laboratory wipes, lint free.
11. Cell suspension(s).
12. Microscope, phase contrast.

### 2.2. Slide Preparation from Metaphase Culture Using a Controlled Cytogenetic Drying Chamber

1. Thermocon CDS-5 Cytogenetic Drying Chamber (Holland, MI).
2. Slides.
3. 70% Ethanol.

4. Laboratory wipes, lint free.
5. Cell suspension(s).
6. Microscope, phase contrast.

### **2.3. Slide Preparation from Primary Amniocytes**

1. Fixed cell suspension.
2. Microscope slides.
3. 70% Ethanol.
4. Laboratory wipes, lint free.
5. Microscope, phase contrast.

### **2.4. Slide Preparation from Uroepithelial Cells**

1. Fixed cell suspension.
2. 6 mm-12 well masked slides (Shandon Inc., Pittsburgh, PA).
3. 70% Ethanol.
4. Laboratory wipes, lint free.
5. Microscope, phase contrast.

### **2.5. Pretreatment of Fresh Cells**

1. 2X SSC.
2. Prewarmed pepsin working solution (0.5 mg/mL Pepsin/0.01 N HCl).
3. Phosphate buffered saline (PBS).
4. Post fixation solution: 0.95% formaldehyde: 1 mL of 37% formaldehyde, 0.18 g MgCl<sub>2</sub>, and 39 mL of PBS.
5. 100% Ethanol.
6. 85% Ethanol.
7. 70% Ethanol.

### **2.6. Pretreatment of Paraffin Pretreatment**

1. Paraffin slides baked overnight at 56°C.
2. Diamond-tipped scribe.
3. Slide warmer at 45–50°C.
4. Hemo-De clearing agent (Fisher Product No. 15-182-507A).
5. 100% Ethanol.
6. Hydrochloric acid, 0.2 N.
7. Purified water (distilled or deionized or Milli-Q).
8. Wash buffer: 2X SSC, pH 7.0.
9. Waterbath at 37 and 80°C.
10. Pretreatment solution: 1 M NaSCN.
11. Protease solution: 0.5 mg/mL pepsin (2500–3000 U/mg) in NaCl, pH 2.0.
12. Neutral buffered formalin (4% formaldehyde in PBS).

### **2.7. Hybridization and Wash**

1. Slides for hybridization.
2. DNA Probe.
3. Forceps.
4. Denaturing solution: 70% formamide / 2X SSC, pH 7.0–8.0.
5. 70, 85, and 100% Ethanol.

6. Waterbath at 73°C.
7. 22 × 22 mm Glass coverslips.
8. Rubber cement.
9. Humidified hybridization chamber (airtight container with a piece of damp blotting paper).
10. Incubator at 37°C.
11. Post-Hybridization wash buffer: 0.4X SSC/0.3% NP40 for nonformalin fixed samples or 2X SSC / 0.3% NP-40 for formalin fixed paraffin embedded.
12. DAPI Counterstain.

### 3. Method

#### 3.1. Slide Preparation from Metaphase Cultures

1. Place a waterbath and humidifier within a containment cabinet. Humidity should be approx 50%. Use a humidifier if the room hygrometer reading is below 45%.
2. Prewarm the waterbath to 67° ± 2°C. Place test tube racks in the center of the waterbath so that they do not touch the sides of the bath. Maintain the water level just below the top of the test tube rack throughout the procedure.
3. Optimize the cell pellet concentration with Carnoy's fixative so that the suspension is slightly cloudy (see **Note 3**).
4. Clean a microscope slide by flooding both sides of the slide with 70% ethanol. Wipe the slide dry with a laboratory wipe.
5. Dip the cleaned slide in a jar of water. Tilt the slide to evenly coat its upper surface.
6. Immediately hold the slide over the waterbath. Holding a Pasteur pipet 2–4 in above the slide, expel 3 to 4 drops of cell specimen suspension along the length of the slide.
7. Place the slide, specimen side up, on the top of the test tube racks in the waterbath. Let the slide dry for 5–10 min.
8. Remove the slide and view under a phase contrast microscope. Adjust slide dropping conditions as necessary.

#### 3.2. Slide Preparation from Metaphase Culture Using a Controlled Cytogenetic Drying Chamber (see **Note 4**)

1. Turn on the Thermotron and set the temperature to 22°C and the relative humidity to 44%. Allow the unit to equilibrate.
2. Optimize the cell pellet concentration with Carnoy's fixative so that the suspension is slightly cloudy (see **Note 5**).
3. Clean a microscope slide by flooding both sides of the slide with 70% ethanol. Wipe the slide dry with a laboratory wipe. Place the slide on the interior surface of the Thermotron.
4. Holding a Pasteur pipet 2–4 in above the slide, expel 3 to 4 drops of cell specimen suspension along the length of the slide.
5. Observe the drying pattern of the suspension. Optimal slides are often prepared from suspensions that dry within 45–60 s.
6. Review the slide under a phase contrast microscope and adjust slide-dropping conditions as necessary.

#### 3.3. Slide Preparation from Noncultured Samples (see **Note 6**)

1. Gently resuspend fixed cells.
2. Drop 15–20 µL of cell suspension per hybridization area (usually two areas per slide).
3. Air dry.

4. Check for cellularity under phase and repeat **steps 2–3** as required.
5. Age overnight or incubate in a 73°C, 2X SSC solution for two min (*see Note 7*).

### 3.4. Slide Preparation from Uroepithelial Cells

1. Gently resuspend fixed cells and aliquot 3  $\mu\text{L}$ , 10  $\mu\text{L}$ , and 30  $\mu\text{L}$  of the resuspended pellet onto 3 wells of the 6 mm masked slide.
2. Air dry.
3. Determine which sample contains the optimal cellularity where there are sufficient numbers of nonoverlapping cells.
4. Check for cellularity under phase and select appropriate well for hybridization.
5. Age overnight or incubate in a 73°C, 2X SSC solution for two min (*see Note 7*).

### 3.5. Fresh Cells Pretreatment

1. Place slides in 2X SSC at 73°C for 2 min.
  2. Place slides in pepsin working solution at 37°C for 10 min.
  3. Wash slides in PBS at room temperature for 5 min.
  4. Place slides in post fixation solution at room temperature for 5 min.
  5. Wash slides in PBS at room temperature for 5 min.
  6. Dry slides.
  7. Immerse slides in 70% ethanol at room temperature for 1 min.
  8. Immerse slides in 85% ethanol at room temperature for 1 min.
  9. Immerse slides in 100% ethanol at room temperature for 1 min.
- Proceed to slide denaturation.

### 3.6. Paraffin Pretreatment (*see Note 8*)

1. Mark the paraffin area to be hybridized with a diamond-tipped scribe.
2. Immerse slides in Hemo-De for 10 min at room temperature.
3. Repeat twice using new Hemo-De each time.
4. Dehydrate slides in 100% ethanol for 5 min at room temperature.
5. Repeat with new ethanol.
6. Air dry slides or place on a 45–50°C slide warmer for 2–5 min.
7. Immerse slides in 0.2 N HCl for 20 min (*see Note 9*).
8. Immerse slides in purified water for 3 min.
9. Immerse slides in wash buffer for 3 min.
10. Immerse slides in pretreatment solution at 80°C for 30 min.
11. Immerse slides in purified water for 1 min.
12. Immerse slides in wash buffer for 5 min.
13. Repeat wash buffer rinse.
14. Remove excess buffer by blotting edges of the slides on a paper towel.
15. Immerse slides in Protease Solution at 37°C for 10 min (*see Note 10*).
16. Immerse slides in wash buffer for 5 min.
17. Repeat wash buffer rinse.
18. Dry slides on a 45–50°C slide warmer for 2–5 min.
19. Immerse the specimen in neutral buffered formalin at room temperature for 10 min (*see Note 11*).
20. Immerse the slides in wash buffer for 5 min.
21. Repeat wash buffer rinse.
22. Dry slides on a 45–50°C slide warmer for 2–5 min.
23. Proceed with hybridization protocol.

### 3.7. Hybridization and Wash

The timing for preparing the probe solutions should be carefully coordinated with denaturing the specimen so that both will be ready for the hybridization step at the same time.

1. Immerse the slides in denaturing solution at 73°C for 5 min. Do not denature more than 4–6 slides at one time per Coplin jar (*see Note 12*).
2. Immerse slides in 70% ethanol at room temperature for 1 min.
3. Immerse slides in 85% ethanol at room temperature for 1 min.
4. Immerse slides in 100% ethanol at room temperature for 1 min.
5. Drain excess ethanol from the slide by blotting the edge of the slide to a towel and wipe the underside of the slide dry with a laboratory wipe.
6. Aliquot probe to a microfuge tube, cap tightly, and denature probe in a 73°C waterbath for 5 min.
7. Apply between 3–10  $\mu\text{L}$  (depending on specimen area) probe mixture to target area of slide (*see Note 13*).
8. Immediately place a 22  $\times$  22 mm or a 12-mm round (uroepithelial hybridizations **Subheading 3.4.**)glass coverslip over the probe and allow it to spread evenly under the coverslip.
9. Seal coverslip by applying rubber cement around the periphery of the coverslip.
10. Place slides in a humidified chamber prewarmed to 37°C and cover.
11. Incubate slides at 37°C overnight (14–18 h, *see Note 14*).
12. Remove the rubber cement seal by gently pulling up on the sealant with forceps.
13. Immerse slides in post-hybridization wash buffer at room temperature and float off the coverslip.
14. Remove excess liquid by wicking off the end of the slide.
15. Immerse slides in post-hybridization wash buffer at 73°C for 2 min. Do not wash more than 4–6 slides at one time per Coplin jar for nonparafinembedded samples, incubate in 2X SSC/0.1% NP40 at ambient temperature for 1 min. (*See Note 14*).
16. Remove slide and air dry in the dark in a vertical position.
17. Apply 10  $\mu\text{L}$  DAPI counterstain to the target area and apply a 22  $\times$  22 mm glass coverslip.
18. Store the hybridized slide in the dark. Place slides in –20°C storage when not in use.

### 3.8. Signal Enumeration in Interphase Cells

Evaluate slide adequacy using the following criteria (*see Notes 15–20*).

1. Probe signal intensity: The signal should be bright, distinct, and easily evaluated. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.
2. Background: The background should appear dark or black and relatively free of fluorescence particles or haziness.
3. Recognition of target signals: Use the prescribed filter. Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris).
4. Using a  $\times 40$  objective, scan several areas to assess any heterogeneity of cell type. Select an area of even nuclei distribution; avoid areas of the target where hybridization signals are weak.
5. Using a  $\times 63$  or  $\times 100$  objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each interpretable interphase cell according to the guidelines.
6. Focus up and down to find all of the signals present in the nucleus.

7. Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal.
8. Do not score nuclei with no signals or with signals of only one color when using two or more different fluorophores. Score only those nuclei with one or more FISH signals of each color.

## 4. Notes

### 4.1. Slide Preparation

1. The goal for optimal spreading of hypotonically swollen cells for metaphase or interphase analysis is the flattening of nucleus and cytoplasm. This flattening process is mediated by the combination of the evaporation and spreading rate of the fixative on the slide (2). This is particularly important in metaphase applications where over-spreading causes chromosomes from adjacent cells to overlap while under-spreading results in shorter chromosomes that are clustered on top of one another.
2. Optimal drying properties is a major reason that Carnoy's fixative is one of the most widely used fixatives in cytogenetics laboratories. The fixative is added slowly to cells to avoid rupture of the cell membrane that could lead to chromosome loss. Cold fixative is often preferred to accelerate fixation and maintain cell morphology. Carnoy's fixative should always be freshly prepared prior to use as the components lose their stability over time and fixative properties diminish.
3. Slides may be prepared directly from fixed cell pellet suspensions or the samples may be stored for extended periods of time at  $-20^{\circ}\text{C}$ . Prior to slide preparation, cell pellets are usually washed several times in additional fixative to rid the samples of debris that could interfere with slide production and FISH interpretation.
4. Reproducibility in the slide preparation process is critical to FISH success. Laboratories are very diverse and creative in their slide preparation techniques, and often have the need to change slide dropping variables with daily and seasonal weather changes. The basis of slide preparation and cell spreading is related to the drying rate of cells as they are applied to the slide. Temperature and relative humidity have a profound effect on this process and therefore laboratories often attempt to control these variables as much as possible. Many laboratories have incorporated the use of the Thermotron CDS-5 Cytogenetic Drying Chamber into their daily procedures. The Thermotron is a self-contained unit with controlled relative humidity and temperature settings used to achieve optimum chromosome spreading routinely and in a highly consistent manner (2). Self-sealing arm ports allow the user to prepare slides in a controlled environment inside the Thermotron. A great number of additional variables affect the slide preparation process.
5. The density of the cell suspension, type of slide, air flow, fixative ratio, and cell type, in addition to culturing and harvesting techniques, all contribute to the overall process. Consistency in protocol is often the best means of controlling the slide making process.
6. Many procedures such as cytocentrifugation involve the application of a specimen directly to the slide with subsequent fixation. Specimens on slides are allowed to air dry at room temperature and are promptly fixed in a solution compatible with FISH. Carnoy's fixative and 95% ethanol are routinely used. Slides are immersed in a Coplin jar of fixative solution for 10 min, or alternatively the slide can be flooded in a series of fixative washes. Following slide aging, slides can be used immediately in the FISH procedure or stored at  $-20^{\circ}\text{C}$ .
7. The aging process is thought to be an additional form of fixation. This step is of particular importance when running FISH assays the same day as slide preparation. Freshly pre-



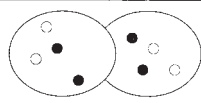


well understood  $T_m$  parameter, which is defined as the point at which 50% of the DNA is single stranded.  $T_m$  is dependent on GC content, monovalent cation concentration, and length of the duplex strands (7). The sample needs to be denatured well beyond the  $T_m$  where close to 100% of the DNA is single stranded. Typically, these conditions are 70–75°C in 2X SSC in 50–55% formamide. Often the probe and the sample are simultaneously or co-denatured on the slide. This results in more favorable kinetics for rapid heteroduplex formation. If denaturation conditions of the sample significantly exceed these conditions, a broader more speckled hybridization pattern can result, which is the result of the “uncoiling” of the compact DNA-histone structures. Adequate fixation can circumvent this to some extent. Conversely, if the DNA strands are not adequately denatured, little or no hybridization signal will result.

13. The nature of blocking DNA is different depending on the complexity of the probe. A DNA probe made from highly repetitive sequences such as centromeric or paracentromeric DNA is hybridized in the presence of 10–100 fold excess of total human genomic DNA. If the probe contains unique sequences to a specific region of a chromosome arm, hybridization is conducted in the presence of a similar mass excess of highly repetitive DNA, termed Cot-1 DNA. Cot-1 DNA is composed of DNA from highly repetitive alphoid sequences and intermediate repetitive sequences such as Alu family of repeats (8,9). It is these latter sequences which are present on all chromosomes as well as in virtually all genomic DNA fragments from which FISH probes are made.
14. Hybridization time will be dependent on the sequence complexity of the probe. Probes with a significant fraction of unique sequences will typically require an overnight incubation at 37°C, while probes containing centromeric or pericentromeric satellite sequences only require a few hours of hybridization at 37–42°C. Slight variations in hybridization temperature are employed to differentiate between closely related repetitive sequences. However, crosshybridization is more effectively eliminated through high-stringency wash. For paraffin embedded samples, use of a higher salt concentration in the wash buffer achieves a similar degree of specificity as does the higher stringency conditions for nonformalin fixed samples. This is because of a lower melting point for probe; target hybrids in the formalin fixed samples that could result from a much shorter length of target sequences as a result of degradation or could be owing to the persistence of protein in the hybridized region that causes hybrid destabilization.



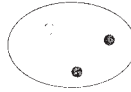
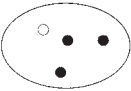

#### 4.4. Enumeration

15. Guideline:

● = orange probe      ○ = green probe

1.		Nuclei are overlapping and all areas of both of the nuclei are not visible but signals are not in overlapping area. Count as two orange and two green in each nucleus.
2.		Count as two orange signals and two green signals. One orange signal is diffuse.
3.		Don't count. Nuclei are overlapping, all areas of nuclei are not visible and some signals are in overlapping area.

*continued*

4.		Count as two orange signals and two green signals. One orange signal is split.
5.		Count as one orange signal and two green signals. One green signal is split and the orange signal is split.
6.		Count as two orange signals and one green signal.
7.		Count as three orange signals and one green signal.
8.		Count as four orange signals and two green signals.

16. It is extremely important to incorporate control slides with FISH testing to check for hybridization adequacy. Both negative and positive controls, ideally near the cut-off for diagnosis, should be included each day FISH tests are run. The control slides should be evaluated prior to any test slides. If the slides are found to exhibit the appropriate quality, the slides should be enumerated and compared to known values to determine counting accuracy. If control slides meet the acceptance criteria but the enumeration values are outside the specified range, the enumeration may not have been performed correctly and an independent, repeat assessment of the same slide may be appropriate. FISH test results should not be reported if assay controls fail, interpretation of the hybridization signal is difficult, or if there are insufficient cells for analysis.
17. For paraffin embedded tumor samples, use a  $\times 25$  objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and nonspecificity, or with noisy background.
18. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals. Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain on every 10th slide of the same tissue block. Identify these areas on the coverslip after the FISH assay is performed. Continue this process until 60 nuclei are enumerated and analyzed.
19. For single cell suspension samples, select an area where the specimen is distributed sparsely, few interphase nuclei or metaphase spreads are overlapping, and several interphase nuclei or metaphase spreads can be scanned within a viewing field. Avoid areas where the distribution of cells is dense, cells are overlapped, or the nuclear border of individual nuclei is unidentifiable. Identify the area of interest specific to the type of test being performed.

20. When more than one color fluorophore is involved, additional consideration is given to resolve signals that are randomly positioned in close proximity such that the colors blend. In some cases a color blend represents the confirmation of a translocation event. In other cases, the signals can be resolved with the use of filters specific to each fluorophore.

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## Chromosome Microdissection

Xin-Yuan Guan

### 1. Introduction

It has been known for decades that chromosome rearrangements exist in most if not all human tumors (1) and certain human hereditary diseases (2). Distinct chromosomal abnormalities in tumors lead to the activation of proto oncogene products, creation of tumor specific fusion proteins, or inactivation of tumor suppressor genes. Since chromosome banding techniques were developed, cytogenetic study of nonrandom chromosome abnormalities in malignant cells has become an integral part of the diagnostic and prognostic work up of many human cancers (3). However, not all cytogenetically visible chromosome rearrangements (e.g., complex chromosome rearrangements, small ring chromosomes, and unidentifiable *de novo* unbalanced translocation) can be determined by conventional cytogenetic banding analysis. This technique limitation prevents complete karyotypic analysis in many human cancers, particularly solid tumors. This technical limitation has been complemented by the combined use of chromosome microdissection and fluorescence *in situ* hybridization (FISH) techniques (4). Chromosome microdissection has been developed as a very powerful tool to proceed rapidly from cytogenetic observation to molecular analysis.

#### 1.1. The Development of Chromosome Microdissection

Chromosome microdissection was primarily developed to isolate DNA markers from a defined chromosomal region, which was first successfully applied to the *Drosophila* polytene chromosomes (5) and then mouse chromosome (6). In 1989, a significantly increased cloning efficiency was achieved when Lüdecke et al. (7) first developed an amplification strategy in which microdissected DNA was digested with *Rsa* I, ligated into a *Sma* I-cut pUC vector, and subsequently PCR amplified using vector sequences flanking the insertion site. This method was then modified by ligating the restriction digested DNA from microdissection to a 5' protruding linker-adaptor, which was constructed from oligonucleotides of 24- and 20-mer. The dissected DNA was then amplified by PCR with the 20-mer of the linker-adaptor as a

primer, and the amplified products were restriction digested and cloned into a plasmid vector (8–10). In these methods, microchemical manipulations are typically carried out in nanoliter-scale with the use of micropipettes controlled by microinjection. However, widespread application of these procedures was limited because of the extremely time consuming and labor intensive requirement for dissecting 20–80 copies of the selected regions, high risk of extraneous DNA contamination, as well as the difficulties in microchemical manipulations.

As an alternative, the method presented here applies a strategy for direct PCR amplification of microdissected DNA using a degenerate oligonucleotide primer (4,11). This method was further improved by treating microdissected DNA with Topoisomerase I prior to the PCR amplification (12). It significantly simplifies the procedure of microdissection and provides several advantages over previously described methods. This method reduces the time and effort necessary for microdissection by reducing the copies of microdissected DNA while also limiting the risk of contamination with extraneous DNA, during the manipulation. The procedure of this protocol is streamlined by eliminating the needs for microchemical manipulations and allows the routine utilization of microdissection in cytogenetic laboratories.

## **1.2. The Applications of Chromosome Microdissection**

Although it was originally designed for the isolation of DNA markers from defined regions, chromosome microdissection has been widely applied in cytogenetic research and molecular cloning once it has been combined with the FISH technique. After a decade of effort, this approach has been developed into a useful and reproducible approach for the following purposes to date.

### **1.2.1. Construction of DNA Clones for Regions of Interest**

The primary application of microdissection prior to the human genome project initiative was to isolate chromosomal region specific DNA markers to fill in gaps found in a physical map. Several region-specific libraries have been constructed for positional cloning projects including 8q24 for the Langer-Giedion syndrome (7), 6q16–q21 for melanoma (13), and 2q35–q37 for alveolar rhabdomyosarcoma (14). In addition, microdissection was applied to obtain highly polymorphic markers from 2p16, which led to the identification of *hMSH2* gene in hereditary nonpolyposis colorectal cancer (15).

### **1.2.2. Generation of FISH Probe**

Fluorescence *in situ* hybridization with different painting probes has been widely applied to detect previously unrecognizable chromosome abnormalities in tumors and hereditary diseases. To meet the increasing demand for high quality FISH painting probes, chromosome microdissection has been used to generate many different FISH probes, including human whole chromosome painting probes (WCP) (16), chromosome arm painting probes (Fig. 1A) (17), and band specific painting probes (Fig. 1B) (18).

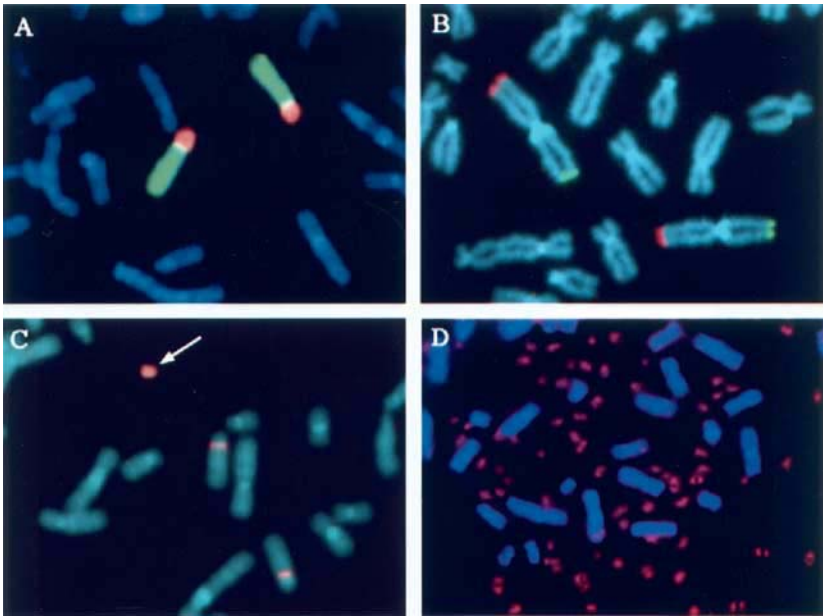


Fig. 1. (A) Microdissection was used to generate chromosome arm painting probes from 12p (red) and 12q (green). (B) An example of band-specific probes constructed by microdissection. Terminal band probes of 1p (red) and 1q (green) were shown. (C) A small ring chromosome was characterized by microdissection. The dissected DNA (red) was hybridized to the small ring chromosome (indicated by an arrow) and centromere region of chromosome 14. (D) Characterization of double minutes (DMs) by microdissection. The dissected DNA (red) was specifically hybridized to DMs.

### 1.2.3. Detection of Chromosome Rearrangement

One of the most appealing features of chromosome microdissection is its ability to directly detect the chromosomal constitution of virtually any cytogenetically visible chromosome rearrangement. This unique approach has been used to analyze different chromosome rearrangements including deletion, translocation, small ring chromosome (Fig. 1C), and amplification. Reinvestigation, using microdissection, of events which conventional banding analysis had cataloged as simple terminal deletions clearly demonstrated that the presumed terminal deletions were actually interstitial deletions or translocations (19–21). Application of microdissection to products resulting from gene amplification including homogeneously staining region (HSR) and double minutes (Fig. 1D) has revealed that the contents of these regions are usually composed of multiple chromosomal regions (22,23).

### 1.2.4. Selection of Region-Specific cDNA

The availability of microdissected DNA from HSR has prompted the development of techniques for isolating transcribed sequences contained within the microdissected

region. One strategy, microdissection combined with hybrid selection, has been applied to identify genes associated with 12q13–q15 HSR in an osteosarcoma cell line (24) and 20q11–q13.2 HSR in breast cancer (25). Briefly, PCR products of microdissected DNA were immobilized on a nylon membrane, then hybridized with random primed cDNAs prepared from a cancer cell line with the target HSR. After hybridization and a stringent wash, the specifically hybridized cDNA clones were eluted and recovered by PCR.

### **1.3. Strategy for Chromosome Microdissection**

The chromosome microdissection technique described here includes two major parts: microdissection of a target chromosomal region, and subsequent amplification of the dissected DNA fragments with a degenerate oligonucleotide primer by PCR.

#### **1.3.1. Chromosome Microdissection**

Metaphase chromosomes for microdissection are prepared from phytohemagglutinin (PHA)-stimulated normal peripheral blood lymphocytes. Metaphases are spread on clean coverslips (22 × 60 mm) and GTG-banded. Usually, 3–5 copies of a target chromosomal region are enough to obtain a high quality probe. The microdissection is performed with a glass-needle controlled by a micromanipulator attached to an inverted microscope. The microdissected chromosome fragments are transferred into 5  $\mu$ L collection solution containing Topoisomerase I (Topo I).

#### **1.3.2. Treatment of Dissected DNA with Topo I**

Before PCR amplification, the dissected DNA is treated with Topo I which can relaxes the very tight DNA coiling. This treatment has been shown to dramatically enhance the recovery of sequences from dissected DNA (12). After the desired number of dissected chromosomal region is collected, the dissected DNA is treated with Topo I by incubating at 37°C for 1 h.

#### **1.3.3. Amplification of Microdissected DNA**

The most difficult part of amplification of dissected DNA is effective initiation of the PCR reaction. Taq polymerase is not efficient for PCR initiation because its extension temperature is 72°C, whereas the optimal annealing temperature for the primer, a partially degenerated primer (26), is about 30°C. To solve this problem, T7 DNA polymerase (Sequenase) is introduced in the first 5–8 cycles of PCR (27). The enzyme is added during each cycle at the annealing temperature (30°C). Following this pre-amplification step, a conventional PCR reaction catalyzed by Taq polymerase is performed.

#### **1.3.4. Fluorescence in situ Hybridization**

FISH is a simple and rapid method to ensure the success of microdissection and to check the quality of the probe. PCR amplified dissected DNA is labeled with fluorochrome in a secondary PCR reaction. After labeling, the probe is purified and hybridized to normal metaphase chromosomes.

## 2. Materials

### 2.1. Preparation of Metaphase Chromosomes for Microdissection

1. Complete RPMI 1640 medium: RPMI 1640 medium, 15% fetal calf serum, 1% glutamine (200 mM), 1% Pen/Strep (10,000 U/mL).
2. Phytohemagglutinin (PHA) (Murex Biotech Ltd., Dartford, UK).
3. Colcemid (10  $\mu\text{g}/\text{mL}$ ) (Gibco-BRL, Gaithersburg, MD).
4. Hypotonic solution: 0.075 M KCl.
5. Carnoy's fixative: 3:1 methanol and glacial acetic acid.
6. 22  $\times$  60 mm glass coverslip.
7. Trypsin working solution: 0.005% trypsin (Gibco-BRL, Gaithersburg, MD) in Hank's balanced salt solution (HBSS) without calcium and magnesium (Gibco-BRL, Gaithersburg, MD).
8. Giemsa stain working solution: 1 mL Giemsa stock solution (BDH Chemicals Ltd., Poole, UK) in 50 mL 0.01 M sodium phosphate (pH 7.0).

### 2.2. Microdissection and Topo I Treatment

1. Glass needle: prepare glass needle by pipet puller using glass capillary tube (1.2 mm wide and 4 in in length) (WPI, Sarasota, FL).
2. Kopf needle/pipet puller, Model 750 (Kopf, Tujunga, CA).
3. Stratalinker 2400 (Stratagene, La Jolla, CA).
4. An inverted microscope.
5. A hydraulic micromanipulator, model MO-302 (Narishige, Japan).
6. Collection solution (final concentration): 4 mM Tris-HCl (pH 7.5), 2 mM  $\text{MgCl}_2$ , 5 mM NaCl, 0.2 mM each dNTP (Pharmacia LKB, Piscataway, NJ), 1  $\mu\text{M}$  UN1 (5' CCGACTCG AGNNNNNNATGTGG 3'), 1 U/50  $\mu\text{L}$  Topo I (Promega, Madison, WI).
7. Thermal cycler and thin-walled PCR tubes.

### 2.3. Amplification of Dissected DNA

1. Sequenase version 2 (13 U/ $\mu\text{L}$ , US Biochemicals, Cleveland, OH).
2. Enzyme dilution buffer (US Biochemicals, Cleveland, OH).
3. PCR reaction mixture (final concentration): 10 mM Tris-HCl (pH 8.4), 2 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.2 mM each dNTP, 1  $\mu\text{M}$  UN1 primer, 2 U/50  $\mu\text{L}$  AmpliTaq LD (Perkin Elmer, Norwalk, CT) (see Note 1).

### 2.4. Fluorescence In Situ Hybridization

1. Biotin-16-dUTP (Roche, Mannheim, Germany).
2. 3 M sodium acetate (pH 5.2).
3. TE buffer: 10 mM Tris-HCl, 1 mM EDTA.
4. BioSpin P6 column (BioRad, Hercules, CA).
5. Denaturing solution: 70% formamide, 2X SSC. Store the solution at 4°C and it can be repeatedly used for several months.
6. Hybridization buffer: 70% formamide, 2X SSC, 10% dextran sulfate.
7. Human Cot I-DNA (1 mg/mL, Gibco-BRL, Gaithersburg, MD).
8. 20X SSC: 175.3 g NaCl and 88.2 g sodium citrate, pH 7.0, in 1 L distilled water.
9. Washing solution I: 50% formamide, 2X SSC. Store the solution at 4°C and it can be repeatedly used for several months.

10. Washing solution II: 4X SSC, 0.1% NP40 (Calbiochem, La Jolla, CA).
11. Washing solution III: 4X SSC.
12. Block solution: 2% (w/v) BSA in 4X SSC, 0.05% Triton X-100. Store the solution at  $-20^{\circ}\text{C}$ .
13. Avidin and Anti-avidin (Vector Laboratories, Burlingame, CA).
14. PNM buffer: 95 mL 0.1 M sodium phosphate, 0.1 mL NP40, 20 mg sodium azide, and 5 g nonfat dry milk in 100 mL distilled water. Incubate the mixture in a  $37^{\circ}\text{C}$  waterbath for 2 h and shake several times during the period to allow all of the milk completely dissolved. Leave the solution at room temperature for 1 d, followed by spinning in a table top centrifuge to pellet the solids. Aliquot the supernatant and stored at  $4^{\circ}\text{C}$  until to be used.
15. Antifade solution: 100 mg *p*-phenylenediamine dihydrochloride in 10 mL PBS. Adjust to pH 8.0 and add 90 mL glycerol. Filter the solution with 0.22  $\mu\text{m}$  membrane to remove undissolved particles and store it at  $-20^{\circ}\text{C}$ .
16. 4', 6-Diamidino-2-phenylindole (DAPI).

### 3. Methods

#### 3.1. Preparation of Metaphase Chromosomes for Microdissection

1. Mix 0.5 mL peripheral blood with 4.5 mL Complete RPMI 1640 medium containing PHA and culture the cells for 64–68 h at  $37^{\circ}\text{C}$ .
2. The cells are harvested by adding 25  $\mu\text{L}$  colcemid (10  $\mu\text{g}/\text{mL}$ ) into the culture medium and incubated at  $37^{\circ}\text{C}$  for 20 min.
3. Transfer the cells to a 15 mL centrifuge tube and centrifuge at 250g for 5 min, then carefully discard the supernatant.
4. Resuspend the remaining cell pellet in 10 mL of hypotonic solution and incubate the cells in a waterbath at  $37^{\circ}\text{C}$  for 12–18 min. Centrifuge at 250g for 5 min and discard the supernatant.
5. Resuspend the remaining cell pellet thoroughly but gently in 8 mL freshly prepared Carnoy's fixative and place the tube on ice for a minimum of 2 h. Centrifuge at 250g for 5 min and then remove the supernatant carefully.
6. Wash the cells twice with 5 mL Carnoy's fixative by centrifuging at 250g for 5 min.
7. Resuspend the cell pellet in 0.5–1.5 mL Carnoy's fixative to obtain a lightly opaque cell suspension before preparing slides (*see Note 2*).
8. Drop 2–3 drops of cell suspension onto a clean coverslip held at  $45^{\circ}$  angle, allowing the cell suspension to run down the length of the coverslip. Do not blow the slide to avoid contamination.
9. Keep the prepared coverslips in a sterile container at  $37^{\circ}\text{C}$  for 3–5 d prior to G-banding (*see Note 3*).
10. Standard G-banding with trypsin-Giemsa (GTG) was performed prior to microdissection. Briefly, treat the slide with trypsin working solution at room temperature for 1–2 min. Transfer the slide to Giemsa working solution for 5 min at room temperature, then rinse the slide with distilled water and air dry.

(All steps above should be performed under sterile conditions.)

#### 3.2. Microdissection and Topo I Treatment

1. Find the target chromosome under an inverted microscope fitted with a stage that can be freely rotated to allow the target chromosome perpendicular to the axis along where the glass needle is moved. If possible, the microscope should reside on an anti-vibration table to dampen out vibration.

2. Dissect the target chromosomal region with glass needles controlled by a micromanipulator attached to the inverted microscope (*see Note 4*).
3. Pick up the dissected DNA fragment with the needle by positioning the tip of the needle directly above the DNA fragment and then moving the needle down and lightly touching the fragment. The DNA fragment will be held to the tip by electrostatic forces and then is transferred into a 5  $\mu\text{L}$  collection solution by touching the needle tip to the fluid.
4. After the desired number of dissected DNA fragments (5 copies) are collected, the collection solution is covered with a drop of mineral oil. Before the amplification of dissected DNA, the DNA is treated with Topo I at 37°C for 1 h to relax the very tight DNA coiling.

### 3.3. Amplification of Dissected DNA

1. An initial PCR (5–8 cycles at 94°C for 1 min, 30°C for 2 min, and 37°C for 2 min) is conducted by adding approx 0.3 U of Sequenase at 30°C each cycle [Sequenase (13 U/ $\mu\text{L}$ ) is diluted 1–8 in enzyme dilution buffer, and 0.2  $\mu\text{L}$  is added to 5  $\mu\text{L}$  reaction mixture].
2. Following this pre-amplification step, a conventional PCR reaction catalyzed by Taq DNA polymerase is performed in the same tube. Fifty  $\mu\text{L}$  PCR reaction mixture is added and a 30-cycle reaction at 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min is performed, with a final elongation time of 5 min at 72°C.
3. Add 2  $\mu\text{L}$  of first round PCR products into another 50  $\mu\text{L}$  PCR reaction mixture and perform 20 PCR cycles identical to that described in **step 2**.
4. The success of the process can be judged at this point by electrophoresis analysis. Load 5  $\mu\text{L}$  of the PCR products onto a 1% agarose gel. The size distribution of PCR products is 200–800 base pairs (*see Note 5*).

### 3.4. Fluorescence In Situ Hybridization

1. Prepare FISH probe by adding 2  $\mu\text{L}$  of second round PCR products into a 50  $\mu\text{L}$  PCR reaction mixture identical to that described in the above steps except for the addition of 20  $\mu\text{M}$  Biotin-16-dUTP for final concentration. The PCR reaction is continued for 20 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a 5 min final extension at 72°C.
2. Remove unincorporated Biotin-16-dUTP from the product by centrifuging on a Bio-gel P6 filtration column following the manufacturer's instructions.
3. Recover the probe by precipitation with 1/10 vol of 3 M sodium acetate and 2 volumes of ethanol for 20 min at 4°C and centrifugation at 10,000g for 15 min at 4°C. Resuspend the probe in 40  $\mu\text{L}$  TE buffer.
4. Hybridization of the FISH probes is based upon the procedure described previously (**12**). Briefly, for each hybridization, about 100 ng labeled probe is mixed in 10  $\mu\text{L}$  hybridization mixture (7  $\mu\text{L}$  hybridization buffer, 2  $\mu\text{L}$  probe, 1  $\mu\text{L}$  human Cot I DNA) which is denatured at 75°C for 5 min followed by a pre-hybridization at 37°C for 20 min.
5. Denature a slide bearing metaphase spreads for 2 min at 72°C in denaturing solution, then dehydrate the slide through a series of 70, 85, and 100% ethanol.
6. Place the hybridization mixture on the slide previously denatured and cover with a 22  $\times$  22 mm coverslip, seal with rubber cement, and incubate the slide at 37°C overnight in a humidified container.
7. After hybridization, the coverslip is removed and the slide is washed 3 $\times$  in washing solution I at 45°C for 5–10 min each.
8. Wash the slide 3 $\times$  in washing solution II and 1 $\times$  in washing solution III at room temperature for 2 min each.

9. Hybridization involving only directly labeled probes can be analyzed at this point. Hybridization involving biotin labeled probe is then treated with 40  $\mu\text{L}$  FITC-conjugated avidin (5  $\mu\text{g}/\text{mL}$ ) in PNM buffer for 20 min at room temperature. The slide is then washed as described at **step 8**.
10. The fluorescence signal is then amplified by treating the slide with 40  $\mu\text{L}$  anti-avidin antibody (5  $\mu\text{g}/\text{mL}$ ) in PNM buffer for 20 min at room temperature. The slide is then washed as described in **step 8**.
11. The slide is usually treated with one more layer of FITC-conjugated avidin as the identical to that described in **step 9**.
12. The slide is dehydrated through a series of 70, 85, and 100% ethanol washes and air dried. Counterstain the slide with 40  $\mu\text{L}$  antifade solution containing DAPI (1  $\mu\text{g}/\text{mL}$ ). The slide is then coverslipped and examined with a fluorescence microscope equipped with appropriate filters.

#### 4. Notes

1. To diminish the impact of contamination, all glassware for the preparation of reagents should be washed and autoclaved twice before use. Water with highest purified grade should be filtered through a 0.22 micron filtration unit, then autoclaved twice for 40 min before use. Likewise, salts solutions are prepared from reagents of the highest available purity, and are filtered and autoclaved in the same fashion as the water.
2. Fix cells with Carnoy's fixative solution (3:1 methanol and glacial acetic acid is acceptable). High quality FISH probe can be obtained using cells fixed in Carnoy's fixative within one year.
3. The age of coverslip with metaphase chromosomes for microdissection should be between 3–14 d. Exposure of coverslips to high temperature ( $>60^\circ\text{C}$ ) or prolonged the storage time may reduce the quality of the product from microdissection.
4. Glass needles are prepared on a spring-loaded, automated pipet puller. Needle diameters at the point of breakage should be the width of a chromosome. Needles are placed on plasticine strips in culture dishes for contamination-free storage. Once prepared, the needles are irradiated with UV light for 5 min in an ultraviolet cross linker to ensure that they are free of DNA contamination.
5. DNA contamination is a critical problem in the amplification of dissected DNA. Since the initial amount of dissected material is exceedingly small (in the range of  $10^{-13}$  to  $10^{-14}$  g/fragment), even minute amounts of contaminating DNA can overwhelm the dissected DNA and lead to useless amplification products. The contaminated DNA may derive from a glass needles which touches DNA other than the target DNA, could be introduced into the collection drop from the air when the tube is repeatedly opened and closed, or even from one of the reagents which has been contaminated with extraneous DNA before microdissection. Therefore, all reagents used for microdissection should be tested for contamination prior to microdissection experiment. The test is carried out by regular PCR reaction with 0.1 ng total human genomic DNA or without DNA. After 40 cycles at  $94^\circ\text{C}$  for 1 min,  $56^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min, the purity of the tested PCR reagents can be checked by electrophoresis analysis. If the positive control produces extensive products in the size range of 200–800 base pairs, whereas the negative control produces little or no visible product, the test reagent is suitable for microdissection. Otherwise, it is necessary systematically to replace each of the reagents until a negative blank is obtained. After all PCR reagents are confirmed suitable for microdissection, Topo I and Sequenase should be also tested.

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## Primed *In Situ* Labeling

Jørn Koch

### 1. Introduction

PRINS (Primed *in situ* labeling) produces the same type of result as FISH; namely a hybridization dependant staining of specific DNA sequences in cell and tissue preparations. However, the staining is obtained differently, as the probe used in PRINS is unlabeled and staining results from the synthesis of labeled DNA at sites binding the probe. To obtain this site specific DNA synthesis, the probe is hybridized in the presence of a primer-dependant DNA polymerase that can synthesize DNA from the 3'-end of the probe (primer) as it hybridizes, as well as labeled nucleotides serving as substrates for the DNA synthesis. The main advantages of the PRINS design are faster and cheaper detection of target sequences with probes that penetrate well (*see Note 1*), probes are readily available from any oligonucleotide synthesis facility, and can be designed to suit the purpose. The disadvantages are that PRINS requires a better quality of the specimen and better incubators than the more rough FISH technique (*see Notes 2–4*), fewer optimized probes are commercially available, and multiple single copy targets can not be detected simultaneously. An international PRINS working group has been established, and there is a website at [www.icg-prins.org](http://www.icg-prins.org). A list of tested primer sequences will be published on that site. However, with the lack of commercial interest in the PRINS technique, these are not likely to be taken through official governmental approval procedures, which may limit the diagnostic use in some parts of the world.

### 1.1. Applications of the PRINS Technique

#### 1.1.1. Sensitive, High Discrimination Detection of Tandem-Repeat DNA

The technique was first designed to enable the discrimination between closely related tandem-repeat sequences (*1*), and detection of tandemly repeated target sequences in chromosomes and nuclei (*in situ*) by oligonucleotide probes is still the primary use of the technique (*2*). With a simple short procedure, such sequences can be efficiently

detected down to small target sizes and with single nucleotide discrimination (3,4). Recently, this application was further enhanced by the development of the dideoxy-PRINS (ddPRINS) approach. In ddPRINS, the absence of one or more nucleotides from certain repeat sequences is employed to further improve discrimination and signal/noise ratios in PRINS. The basis for ddPRINS is that such DNA can be efficiently synthesized also when the missing nucleotide is included as a dideoxy-analogue, whereas nonspecific synthesis of DNA is blocked. Telomeric repeats e.g., contain a G-rich strand missing cytosine, and a complementary C-rich strand missing guanine. Consequently, the G-rich strand can be produced in a ddPRINS including ddCTP, and the C-rich strand in a dd-PRINS including dGTP. Relative to a standard PRINS, the ddPRINS improve the signal/noise ratio of telomere staining by about an order of magnitude, increases the efficiency to virtually 100%, and lowers the detection limit to a few hundred bases of target DNA (presumably corresponding to 2–3 priming events per target site) (5–8). Furthermore, since one strand of the target is flanked at its 5'-end by one sequence, and the other strand by another, it is possible to also determine the orientation of the target. It has been possible to determine the relative orientation of various telomeric repeat variants with respect to each other by this method (6–8).

### 1.1.2. Quantitative Detection of DNA Sequences (Q-PRINS)

As the PRINS design allows for high concentrations of small probes that penetrate the specimen well, targets can be effectively saturated, and the reaction made quantitative. This possibility has been used in particular for measuring the size of telomeric repeat domains at individual chromosome ends, based on the ddPRINS reaction (5). A similar approach can be applied to the sizing of other simple repeat domains that vary in size, such as trinucleotide repeats. Q-PRINS reactions can be evaluated in either of two ways. The first is simply to measure the staining frequency (how many out of 100 potential targets are actually stained). The higher the staining frequency, the larger the target (9). With the dd-PRINS, this often becomes impractical as the staining efficiency in many cases is close to 100%. In normal blood lymphocytes, the main telomeric repeat is stained with an average efficiency of 99.8% (see Fig. 1). In such cases, it is preferable to measure the light intensities of the PRINS signals instead. We have described a procedure for this, employing the pre-existing image analysis program QUIPS from VYSIS for the purpose (5). Another analysis program has become available from DAKO. This program seems equally suited for signal quantification. Expanded trinucleotide repeats are generally stained with a low enough efficiency to be evaluated for relative size by determining the percentage of cells labeled.

### 1.1.3. Detection of Single Copy Targets

The detection of such targets has traditionally been the Achilles heel of the PRINS technique. However, it was recently reported that single copy sequences could be detected at high sensitivity, if multiple primers are used and signals are enhanced with the Tyramide amplification system (10,11). Apart from these two modifications, the single copy target detection procedure does not differ from previous PRINS protocols.

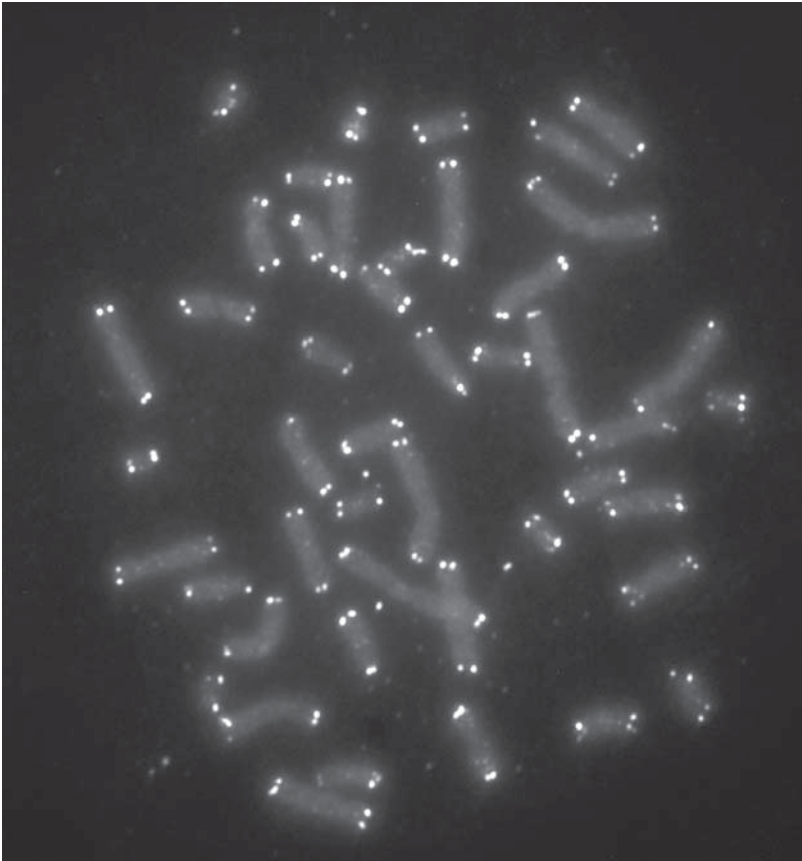


Fig. 1. ddPRINS on a spread of human chromosomes. Each chromosome is made of two chromatids, and each chromatid has two ends, so the number of targets/signals/cell is in principle 184. However, signals on neighboring chromatids may appear fused. The outline of the chromosomes is also visible, mainly owing to autofluorescence from the chromatin.

#### 1.1.4. Self-Primed In Situ Labeling (SPRINS)

In the absence of added primer, primed *in situ* labeling from endogenous priming sites (self-labeling) can be detected. In normal chromosome spreads it is seen as a very faint background staining (absent in ddPRINS). In spreads of low quality, the background staining is increased (*see Note 2*). However, most notably, the phenomenon can be employed to study the *in vitro* and *in vivo* activity of DNA modifying enzymes. Digestion of chromosome spreads with nucleases or restriction enzymes produce nicked chromosomes where the density of nicks (and thus sites of enzyme activity) can be displayed by SPRINS (**Fig. 2**). Similarly, if DNA is nicked *in vivo* as a consequence of cell processes, the resulting nicks can subsequently be detected by SPRINS (e.g., detection of apoptosis or topoisomerase activity) (*12*).

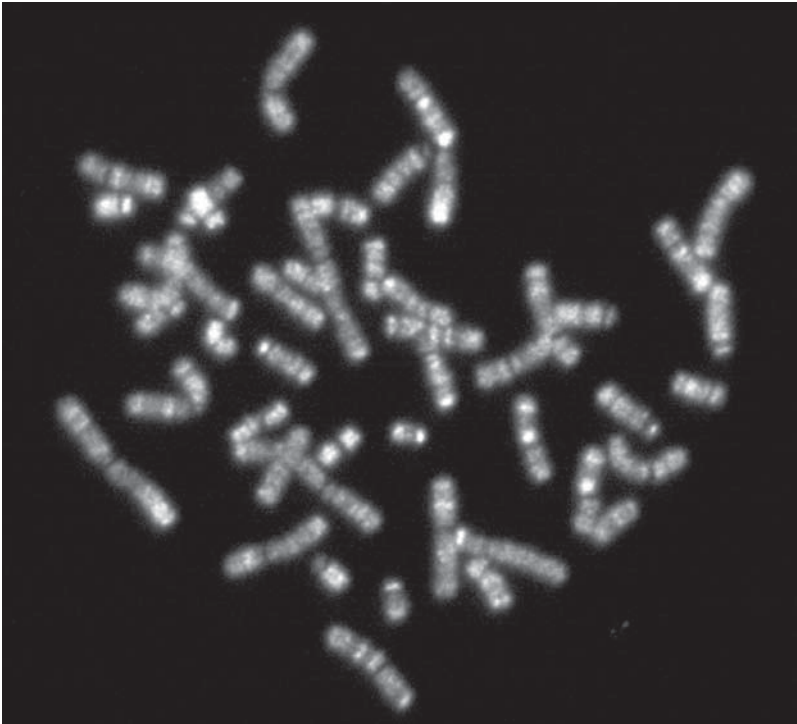


Fig. 2. SPRINS on a spread of human metaphase chromosomes digested with the restriction enzyme Msp I. The chromosomes appear with a chromosome specific banding pattern.

### 1.1.5. Combinatorial Labeling

PRINS can be combined with PRINS, FISH, or immunostaining for the simultaneous detection of multiple targets (13–15). Doing a PRINS reaction ahead of a FISH reaction often eliminates the need for other pretreatments (e.g., proteinase treatment). PRINS is less destructive to the quality of the specimen than FISH, and also less destructive to the quality of a preceding immunostaining reaction. Thus, if the antibody has been “secured” at the site by fixation in paraformaldehyde, very little loss of antibody staining occurs as a result of the following PRINS reaction.

## 2. Materials

1. Chromosome spreads (*see Note 3*).
2. Cover slips.
3. Coplin jars, or other suitable containers for washing the slides.
4. Standard nucleoside triphosphates, dideoxy nucleotides for ddPRINS (Lithium salt, Roche). Hapten or fluorochrome labeled nucleotides (digoxigenin-dUTP, biotin-dUTP, fluorescein-dUTP, rhodamine-dUTP [Roche]).
5. Tth or Taq DNA polymerase and 10× polymerase buffer (enzyme and buffer are supplied together by Roche).

6. 5 M NaCl.
7. 500 mM EDTA.
8. 20X SSC.
9. 20% Tween 20.
10. Ice cold ( $-20^{\circ}\text{C}$ ) ethanol series (70, 90, 99% (vol/vol)) for PRINS on predenatured slides.
11. Nonfat dry milk (powder).
12. Fluorochrome labeled anti-digoxigenin for detection of digoxigenin labeled PRINS product (Fab fragment, Roche). Fluorochrome labeled Streptavidin for detection of biotin labeled PRINS product (Roche, Vector Laboratories).
13. Antifade solution: Vectashield (Vector Laboratories) or *p*-phenylenediaminedihydrochloride (Sigma).
14. Counterstain: either propidium iodide (Sigma) or DAPI (Sigma).
15. 10X dNTP for basic PRINS (1.0 mM each of dATP, dCTP, and dGTP, 0.1 mM of labeled dUTP, mix in 50% glycerol). Can be stored at  $-20^{\circ}\text{C}$  for at least 1 yr. The glycerol keeps the liquid from solidifying at  $20^{\circ}\text{C}$  so it is possible to aliquot from a stock without cycles of thawing and freezing.
16. 10X dNTP for dideoxy-PRINS (with ddGTP) (1.0 mM each of dATP, dCTP, 1.0 mM ddGTP, 0.1 mM of labeled dUTP, mix in 50% glycerol). Can be stored at  $-20^{\circ}\text{C}$  for 1 yr.
17. Reaction mixture (for basic PRINS in a 50  $\mu\text{L}$  volume): 38  $\mu\text{L}$  ddH<sub>2</sub>O, 5  $\mu\text{L}$  10X polymerase buffer, 5  $\mu\text{L}$  10X dNTP, 1  $\mu\text{L}$  DNA probe (from 500 ng and up), 1  $\mu\text{L}$  DNA polymerase (1 U), mix gently by tapping a finger on the Eppendorf tube, and use immediately after inclusion of the polymerase.). Reaction mixture without polymerase may be stored at  $-20^{\circ}\text{C}$  for months.
18. Stop buffer: 50 mM NaCl, 50 mM EDTA, pH 8.0.
19. Wash buffer: 4X SSC, pH 7.0 (1X SSC: 150 mM NaCl, 15 mM sodium citrate), 0.05% Tween 20.
20. Blocking solution: 5% (w/vol) nonfat dry milk dissolved in washing buffer. Centrifuge for 2 min in an Eppendorf centrifuge and use supernatant). Can be stored at  $-20^{\circ}\text{C}$  for years. When in use, it is preferable to store the blocking solution at  $20^{\circ}\text{C}$  and do not use it for more than 1 wk (the milk turns sour with time).
21. Either two incubators (thermoblocks, waterbaths) where the slide can be put on a metal surface to ensure good heat transfer, one with a surface temperature of  $94^{\circ}\text{C}$  and the other with a surface temperature of  $55\text{--}65^{\circ}\text{C}$ . The temperature of the  $94^{\circ}\text{C}$  incubator should be measured very carefully, as it should not deviate more than  $1\text{--}2^{\circ}\text{C}$  from the desired value. To ensure this it may be helpful to cover the slide and the hot plate with an insulating lid (see **Note 3**), or a special PRINS/*in situ* PCR machine (from Hybaid, MJ-Research or Techne) preset (simulated slide function) to incubate the slide at  $91\text{--}94^{\circ}\text{C}$  for 2–4 min and at  $55\text{--}65^{\circ}\text{C}$  for 5–60 min (depending on the oligonucleotide used). The reason for the slightly lower setting during denaturation in this case stems from the build-in compensation for the  $1.5^{\circ}\text{C}$  temperature difference between the hotplate and the interior of the slide on a metal surface covered with a lid (see **Note 4**).

### 3. Methods

#### 3.1. Standard One-Step Reaction

The PRINS protocol comes in two variants. Either the slide is denatured in hot formamide, as for FISH, and immediately quenched in an ice cold ethanol series as described in **Subheading 3.4.**, or the slide is denatured by heating the slide with the reaction mixture added to a denaturing temperature as follows:

1. Decide how large a region of the slide should be analyzed, and choose a cover slip and an amount of reaction mixture that fits the area. Use 1  $\mu\text{L}$  reaction mixture for each mm of cover slip length. To cover a standard slide completely, prepare 60  $\mu\text{L}$  reaction mixture and cover with a 24  $\times$  60 mm cover slip. To cover a smaller area, prepare less reaction mixture (and use a smaller coverslip).
2. As soon as the reaction mixture has been spread with the cover slip, the slide is denatured by placing it on a hotplate covered with a lid for 4 min at 94°C (*see Note 4*).
3. Transfer the slide quickly to 55–65°C and incubate for 5–60 min for probe annealing and chain elongation. Probes for simple repeats anneal rapidly, more complex probes more slowly, and chain elongation is virtually instantaneous, so the annealing time determines the incubation time.
4. Place the slide in preheated stop buffer at the temperature used in **step 3** for 1 min to terminate the PRINS reaction.
5. Transfer the slide to 50 mL of wash buffer and wash for approx 3 min at room temperature (the reaction can be paused at this step, and the slide stored overnight in wash buffer at 4°C).
- 6a. If fluorochrome labeled nucleotides have been used, the slide can now be counterstained, mounted and evaluated under the microscope.
- 6b. If digoxigenin labeled nucleotides have been used, they need to be visualized with anti-digoxigenin antibody as described in **Subheading 3.6**.

### 3.2. Dideoxy-PRINS

In the PRINS procedure, simply replace the nucleotide(s) not needed for DNA synthesis from correctly hybridized probes with the corresponding dideoxy-nucleotides (e.g., replace dGTP when synthesizing the CCCTAA element of the human telomere).

### 3.3. Combinatorial Labeling

There are two basically different strategies to combinatorial labeling, either the PRINS is done first or the PRINS is done last. In PRINS-FISH, PRINS is done first to avoid PRINS reactions from the FISH probe(s), and the denatured FISH probe is added to the slide after the latter has been quenched in ice-cold ethanol. It is important that the slide is not (re)denatured after the PRINS, as this may cause the PRINS signal to disappear. In PRINS-immunostaining the PRINS is done last, and after the immunostaining the slide is fixed in 2% paraformaldehyde for 2 min at room temperature and dehydrated through an (70, 90, and 99%) ethanol series prior to the PRINS to enhance the survival of the immunostaining.

### 3.4. PRINS on Already Denatured Slides

If it is preferred to use a slide which has been denatured in formamide, e.g., if the incubator is not sufficiently reliable for robust heat denaturation, the slide is denatured in 70% formamide, 70°C, for 2 min, or according to your standard protocol for FISH denaturation. The slide may have also been previously denatured if a previous PRINS reaction has been conducted. In both cases it is important that the slide is immediately quenched in a –20°C ethanol series to fix the target DNA in the denatured configuration.

1. Dehydrate the slide in a –20°C ethanol series (70, 90, and 99%, 3 min each) as soon as possible after denaturation.

2. Remove the slide from the 99% ethanol, drain and air-dry.
3. Prepare a reaction mixture as above steps. If this is a second PRINS reaction, make sure to use a different label on the dNTP this time.
4. Annealing and chain elongation: the slide is preheated to 55–65°C for 1 min. Then, the likewise preheated reaction mixture is put on the slide and spread with a coverslip.
5. Incubate, stop and wash as above. The slide is now ready for antibody staining and counterstaining/mounting.
6. If a third PRINS reaction is desired, repeat steps 1–5.

### 3.5. Self-Primed In Situ Labeling (SPRINS)

SPRINS can be done according to the above protocol, **Steps 2–5** (the slide should not be denatured at any point, so there is no need for **Step 1**).

### 3.6. Visualization of Digoxigenin or Biotin Labeled PRINS Product

1. Apply 50  $\mu\text{L}$  of fluorochrome conjugated anti-digoxigenin (or fluorochrome conjugated Streptavidin) in blocking solution (2 ng/ $\mu\text{L}$ ) to the slide. Incubate under a coverslip for 30–60 min in the dark.
2. Wash 2  $\times$  5 min in 50 mL of washing buffer. The slide is now ready for counterstaining and mounting.

### 3.7. Counterstaining and Mounting

1. Blue counterstaining of DNA is obtained by including 0.4  $\mu\text{M}$  DAPI in the antifade solution.
2. Red counterstaining of DNA is obtained by including propidium iodide in the antifade solution. Mount the slide in 20  $\mu\text{L}$  of antifade solution containing 0.5  $\mu\text{g}/\text{mL}$  propidium iodide.

## 4. Notes

1. An advantage of the PRINS technique is that the probes are very small, and thus penetrate easily to the target, even when this is in a tight conformation. This is exploited by Franck Pellestor's group for efficient staining of target sequences in sperm cells (16).
2. An occasional problem in PRINS is "self-labeling" of certain regions of satellite DNA. In human chromosomes, satellite III on chromosome 9, and more rarely, satellite II on chromosomes 1 and 16 may self-label. This self-labeling is somewhat primer dependent, but may occur with any primer. Some primers always induce the self-labeling, but with most primers it is only seen in low quality chromosome spreads, and the solution to the problem therefore is to make better spreads.
3. The PRINS technique relies strongly on the intact nature of the target sequences. Nicked or broken DNA is not only a poor template for chain elongation, *in situ*, through endogenous chain elongation *in situ* nicks in the chromosomal DNA may also raise the otherwise low background staining associated with PRINS. As accessibility of the target is generally not a problem in PRINS on standard chromosome spreads, freshly prepared slides are the preferred starting point for an optimal reaction. If optimal slides can not be prepared, accessibility can be increased by treatment with a proteinase (DNAse-free!), vulnerable chromatin can be stabilized by fixation with paraformaldehyde, and damaged DNA can be repaired with a DNA ligase. The chromatin in "aged" slides may be so hardened that it requires long incubations to denature it by heat, e.g., 10 min for a slide aged one month at room temperature.
4. If heat denaturation of slides is used, a strict temperature control is needed to provide optimal denaturation of target sequences without destroying the polymerase. Suitable

computerized incubators are available from a range of companies (we use incubators from Hybaid, and from MJ-Research). If specialized incubators are not available, stable waterbaths or hot-plates can be used. It is important that any such equipment is covered with an insulating lid to ensure the right and reproducible incubation temperature.

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## Spectral Karyotyping

Jane Bayani and Jeremy A. Squire

### 1. Introduction

Historically in clinical cytogenetics, G-banding has been the gold standard for detecting gross chromosomal abnormalities, ranging from simple numerical changes to the identification of complex structural rearrangements in clinical samples. The designation “marker chromosome” or “derivative chromosome” has been used to indicate that G-banding has been unable to provide a definitive identification of the aberration. This is often because the complexity of the rearrangement has resulted in the lack of a coherent and recognizable banding pattern. The advent of the various multicolor fluorescence *in situ* hybridization (FISH) chromosomal painting techniques (1,2) has greatly improved our ability to identify all marker chromosomes, but these techniques still need some careful planning in rapidly achieving the goal of identifying complex chromosomal rearrangements.

Spectral karyotyping (SKY) involves the use of 24-color, whole chromosome-painting permitting visualization of each chromosome in one experiment. This technology is based on the principles of spectral imaging (3) and Fourier spectroscopy (4). Flow sorted chromosomes are PCR-labeled (5), either directly or indirectly, with fluorochromes or haptens. Five pure dyes that are spectrally distinct are used in combination to create the unique chromosome cocktail of probes. This probe cocktail is hybridized to metaphase preparations and detected using sophisticated image analytical methodologies. Image acquisition (Fig. 1) is accomplished by conventional fluorescence microscopy and the use of a specially designed triple filter (SKY CUBE™, Applied Spectral Imaging). In this design, light passes through a Sagnac interferometer focussed on a charged-coupled device (CCD) detector. Spectral images are acquired and analyzed with the commercially available SD 200 Spectral Bio-Imaging System (ASI Ltd., MigdalHaemek, Israel). The generation of a spectral image is achieved by acquiring ~100 frames of the same image that differ from each other only by the optical path difference (OPD). The collected images are Fourier Transformed and the data sorted in the software (SKYVIEW™). Each chromosome has a unique

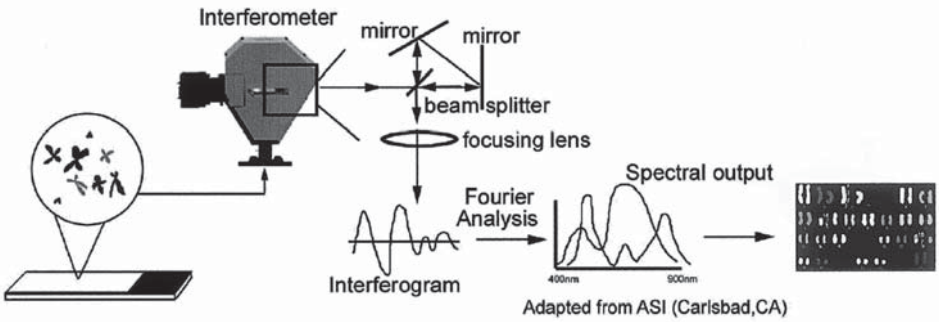


Fig. 1. Schematic representation of image acquisition using an interferometer housed in the optical head (Applied Spectral Imaging, Carlsbad, CA). Images from the hybridized specimen are collected, Fourier transformed and processed.

spectral “signature”, generated by the specific combination of one or more of the five pure dyes. Once a spectral image is acquired, the SKYVIEW™ software compares the acquired spectral image against the combinatorial library containing the fluoro-chrome combinations for each chromosome and generates a “classified” image. The classified image pseudo-colors the chromosomes to aid in the delineation of specific structural aberrations where the RGB (Red-Green-Blue) display image, which displays the fluorescent colors of the chromosomes may appear quite similar (**Fig. 1**). For every chromosomal region, identity is determined by measuring the spectral emission pixel by pixel. Regions where sites for rearrangement or translocation between different chromosomes occur are visualized by a change in the display color at the point of transition (**Fig. 2A**).

One of the most obvious limitations of SKY is its inability to readily detect deletions or other intrachromosomal structural changes such as inversions. However, there are some compelling reasons for the systematic use of SKY in the analysis of abnormal chromosome preparations of the type encountered in cancer cells. The ease of interpretation of the clearly assigned color patterns means that it is not essential to have a highly experienced metaphase analyst to perform the microscopy. In addition the acquired images can be analyzed objectively so that subtle translocations as small as ~2 megabases of DNA can be detected (**6**). Such a small chromosomal region would be difficult to detect with certainty by conventional banding analysis. SKY therefore provides a method for rapid high-resolution screening of the cancer karyotype and has applications both in the research and clinical cytogenetics laboratories.

To date, spectral karyotyping is the most widely used method for 24-color FISH analysis. It has been used to examine a variety of neoplasms including hematopoietic malignancies (**7–13**), carcinomas (**14–20**), sarcomas (**21–27**), and brain tumors (**28,29**). Its contributions to clinical genetics is evident in the number of publications examining various syndromes (**30–32**) and it has been applied to examining assisted reproductive technologies (**33–37**). SKY has also been successfully applied to murine systems to address diverse research questions (**Fig. 2B**) often associated with mouse

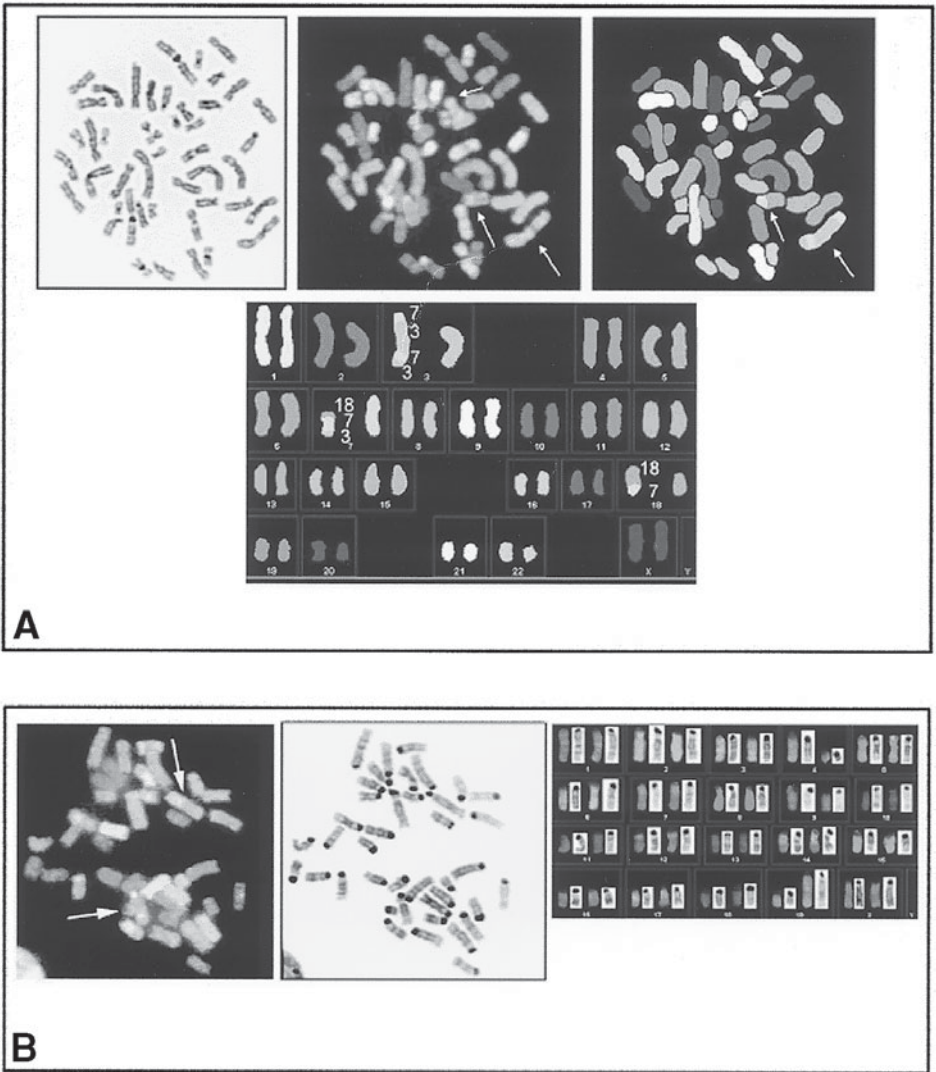


Fig. 2. SKY analysis of cancer metaphase preparations. **(A)** SKY Analysis of a human soft tissue sarcoma. The (top left panel) illustrates the inverted DAPI banding of a metaphase which permits identification of chromosomal regions in using patterns similar to G-banding. The central (top panel) shows the display of the same metaphase with arrows indicating the rearranged chromosomes. The (top right panel) shows the aberrant chromosomes as detected by SKY analysis. The (central panel) shows the SKY karyotype using classified in which structural alterations of chromosomes 3, 7, and 18 can be clearly seen. **(B)** SKY analysis of a murine fibrosarcoma. The (bottom right panel) shows the display of a murine metaphase with arrows indicating the rearranged chromosomes. The (bottom central panel) shows inverted DAPI banding of the same metaphase. The (bottom right panel) shows the SKY karyotype in which structural alterations can be clearly seen.

models of human cancer (38–50). The increased interest in SKY over the last 4–5 yr stems from the convenience and interpretative ease of this powerful molecular cytogenetic screening technique.

### **1.1. Probe Labeling**

The SKY probes are available commercially and are created from flow-sorted chromosomes that are usually amplified and labeled using DOP-PCR (5). The 24-chromosome probe cocktail is generated by the combination of 5 pure dyes. This allows  $2^{n-1}$  or 31 combinations. The color separation for each chromosome is based on individual spectral properties of each fluorochrome as detected by a specially created triple filter (SKYCube™) during a single acquisition step. The creation of customized probes is possible using flow cytometry (2) to sort each chromosome. Thereafter, it is necessary to use the correct spectral settings and fluorochrome combinations for data acquisition and analysis in the preference file of the supplied SKY software.

### **1.2. Chromosome Preparation from Tissues**

The utility of SKY in both clinical and research molecular cytogenetics laboratories lies in the ability to hybridize and detect all the chromosomes in the genome. Thus, metaphase chromosomes must be present in cytogenetic preparations. Whether the sample to be tested is derived from hematopoietic sources or solid tissues, the cells used must be mitotically active so that adequate good quality metaphases can be generated after short-term tissue culture. Agents such as colcemid prevent spindle formation and keep cells at metaphase. Finding the proper colcemid times and concentration is a matter of experience and practice. Cell fixation also involves hypotonically swelling the cells, then slowly introducing a methanol:acetic acid fixative. Careful attention to the hypotonic treatment and first methanol:acetic acid fixation can yield cell suspensions that are free from cellular and cytoplasmic debris. Most experienced cytogeneticists acknowledge that the quality of chromosomal preparations for both banding and all FISH procedures can be greatly influenced by the drying environment during slide making and by the age of the slide. Humidity and temperature will significantly influence the rate of fixative evaporation during slide making. The drying time should be well controlled so that when the overall humidity is less than 30% slide making is performed near a steam source or humidifier. In recent years a controlled environmental evaporation chamber developed by Thermotron Inc. has made for more reproducible slide making and has gained in popularity in clinical laboratories. In view of the cost of performing SKY procedures, it is advisable to ensure that the target slides are consistently of optimal quality and age.

### **1.3. Slide Pretreatment and Denaturation**

Once metaphase preparations have been made, the slides must be assessed to determine the necessary slide pre-treatment. All slides should be assessed by phase contrast microscopy before SKY analysis. The presence of cellular and cytoplasmic debris that may contribute to the overall background is sometimes unavoidable, and can be removed by protease treatments. Once a protease treatment has been applied, the DNA must be denatured to a single stranded state to hybridize to the labeled probe. This is

achieved through heat denaturation using a 70% formamide solution. Formamide is used because it lowers the melting temperature of DNA, thus a 75°C solution of formamide is sufficient to denature DNA into single strands. Once the denaturation of the target DNA has been achieved, the slides must immediately be placed in 70% ethanol followed by an increasing alcohol series to keep the DNA target in its denatured state and to remove any residual moisture. The denaturation of the target DNA is the most critical step in any FISH-based assay. Too little denaturation causes poor hybridization efficiency and accessibility of the probe to the target DNA. Too much denaturation destroys the DNA also resulting in poor hybridization efficiency.

#### **1.4. Probe Denaturation**

The SKY probe, like the target DNA, must also be denatured into its single stranded state in order for hybridization to occur. The probe comes already resuspended in a hybridization solution usually containing formamide and dextran sulfate. As described previously, the formamide allows the DNA to melt at lower temperatures. The use of unlabeled COT-1 DNA in FISH probes serves to suppress repetitive sequence hybridization that is widely dispersed throughout the human genome. Thus, the probe must first be heat denatured at 75°C, then allowed to pre-anneal at 37°C such that the COT-1 DNA can hybridize to and block background signals from these highly repetitive sequences. Because COT-1 DNA represents the fraction of DNA sequences, which have the fastest annealing properties, the 1 h incubation at 37°C will not cause the re-annealing of the labeled DNA.

#### **1.5. Hybridization**

Once both the target DNA on the slides and the DNA probe has been denatured, the probe is applied to the slides and allowed to hybridize together at 37°C for a minimum of 48 h. The signal strength following hybridization will depend primarily on the completeness of the denaturation process of the target DNA. In addition, the presence of cellular and cytoplasmic debris surrounding the chromosomes, can greatly impair the accessibility of the probe to the target chromosomes.

#### **1.6. Post-Hybridization Washes and Detection**

The post hybridization washes and detections take the bulk of the laboratory time. To remove any unbound probes, a lower concentration of formamide at 45°C is used followed by a treatment with 1X SSC also at 45°C. Like many hybridization assays, a blocking step is performed to reduce any background or noise present during the immunological detection steps to follow. The blocking solutions are usually made of some combination of bovine serum albumin and a detergent in an isotonic buffer. The commercial SKY probes are combinations of specific paints in which some chromosomal paints have been directly labeled with a fluorochrome, and others have been indirectly labeled with a hapten. The detection reagents provided in the kit contain antibodies to indirectly detect labeled paints. Between incubations with the antibody reagents, a detergent wash is carried out to remove any unbound residual antibodies that could contribute to background. Gentle agitation during these washes helps to reduce the background signals that may appear due to the presence of cellular and

cytoplasmic debris. The final step is the counterstaining of the DNA using DAPI, mixed with an antifading mounting medium. The slides can then be stored at  $-20^{\circ}\text{C}$  where the signals can last for several months.

### **1.7. Image Acquisition**

For the greatest success in applying SKY analysis to cytogenetic cases, it is not only important to have the proper slide preparations, slide pre-treatment, DNA denaturation, and stringency in post hybridization washes, but to use the image acquisition and analysis software to its fullest potential. As discussed above, the differentially labeled whole chromosome paints are distinguished from each other by their unique spectral properties. Using a fluorescent microscope equipped with the SKYCUBE, light passes through a Sagnac interferometer focussed on a charged-coupled device (CCD) detector. The generation of a spectral image is achieved by acquiring  $\sim 100$  frames of the same image that differ from each other only by the optical path difference (OPD). These collected images are Fourier transformed and the data sorted in the software (SKYVIEW<sup>TM</sup>). The spectral properties for each pixel can then be compared to the spectral or combinatorial library that drives the SKYVIEW<sup>TM</sup> software. Acquisition times are generally dependent on the overall fluorescence intensity, although it is *not* necessarily the signal intensity that influences the accuracy of SKY analysis. Weak signal strength can give equally reliable results provided that the spectral properties of each dye is maintained. Thus, the fidelity of the fluorescence of the probe cocktail and subsequent immunochemical detections is important.

### **1.8. Hybridization of SKY probes to Previously G-banded Slides: Slide Pretreatment**

In some situations it may be necessary to perform SKY analysis on previously G-banded material. This procedure can be especially important for verifying an inconclusive chromosomal aberration on a specific metaphase cell. In this situation an image of the G-banded metaphase cell of interest should be captured, the microscope coordinates noted, and promptly prepared for SKY analysis as soon as possible (i.e., within 2 wk). For older samples, slide storage variables such as humidity and ambient temperature will greatly influence success. It is thus very important to find out how the sample was prepared and stored. If there are a limited number of slides remaining from the patient sample of interest, it is sometimes helpful to use a superfluous slide from another sample prepared the same week to derive the best conditions with respect to aging and storage.

## **2. Materials**

### **2.1. Probes**

Vial 1 in kit from Applied Spectral Imaging (MigdalHaemek, Israel).

### **2.2. Chromosome Preparation from Tissues**

1. Sample Tissue.
2. Tissue culture media appropriate for short term culturing.
3. Blades and scalpels.

4. Sterile Petri dishes.
5. Vented tissue culture flasks and sterile pipets.
6. 37°C CO<sub>2</sub> Incubator.
7. Colcemid (10 mg/mL Gibco-BRL).
8. 1X Trypsin (Gibco-BRL) prewarmed to 37°C.
9. Citrate saline at room temperature.
10. 0.075 M KCl prewarmed to 37°C.
11. 37°C Oven or waterbath.
12. 15-mL conical tubes.
13. Tissue culture centrifuge.
14. 3:1 (v/v) methanol:acetic acid.
15. Glass slides (Fisher).
16. 200- $\mu$ L pipet and tips.
17. Slide storage box.

### **2.3. Slide Pretreatment and Denaturation**

1. Metaphase slides aged for 2–3 d.
2. Ethanol series: 70, 80, 95% in plastic or glass Coplin jars cold.
3. Small forceps.
4. 75°C Waterbath.
5. Deionized, distilled high grade formamide.
6. 20X SSC.
7. 10% (w/v) Pepsin.
8. 0.01 M HCl prewarmed to 37°C in an oven or waterbath.
9. 1X PBS at room temperature.
10. 1X PBS/50 mM MgCl<sub>2</sub> at room temperature.
11. 1% Formalin/1X PBS/MgCl<sub>2</sub> at room temperature.
12. Timer.

### **2.4. Probe Denaturation**

1. Aliquoted labeled probe.
2. 75°C Waterbath or PCR machine.
3. 37°C Waterbath or PCR machine.

### **2.5. Hybridization**

1. Denatured metaphase slides from **Subheading 2.3**.
2. Denatured probe from **Subheading 2.4**.
3. Glass or plastic coverslips (22  $\times$  20 mm).
4. 10- $\mu$ L or 20- $\mu$ L pipeter and tips.
5. Rubber cement.
6. Hybridization box.
7. Plastic bag.
8. 37°C Oven.

### **2.6. Post-Hybridization Washes and Detection**

1. Forceps.
2. 45°C Waterbath.
3. Three glass or plastic Coplin jars containing 50% formamide/2X SSC at 45°C.

4. Three glass or plastic Coplin jars containing 1X SSC at 45°C.
5. Seven glass or plastic Coplin jars containing 0.01% Tween-20/4X SSC at 45°C.
6. One glass or plastic Coplin jar containing 2X SSC at room temperature.
7. Commercially available kit from Applied Spectral Imaging containing: Vial 2: Blocking reagent; Vial 3: Detection reagent; Vial 4: Detection reagent; Vial 5: DAPI/Antifade solution.
8. Plastic slide box for storage at -20°C.
9. 22 × 30-mm glass coverslips.

## 2.7. Image Acquisition

1. Fluorescent microscope attached to Applied Spectral Imaging Optical Head.
2. Spectral Imaging Acquisition software (Applied Spectral Imaging).
3. SKYVIEW analysis software (Applied Spectral Imaging).

## 2.8. Hybridization of SKY Probes to Previously G-banded Slides: Slide Pretreatment

1. 2 Coplin jars of xylene.
2. 2 Coplin jars of methanol.
3. Ethanol series: 70, 80, 95% in plastic or glass Coplin jars stored at a cold temperature.

## 3. Methods

### 3.1. Probes

Commercially available probes are pre-labeled in combinatorial fashion and suspended in a hybridization solution.

### 3.2. Chromosome Preparation from Tissues

1. Using aseptic techniques in a tissue culture hood, finely mince the tissue using the blades in a sterile Petri dish.
2. Transfer the finely minced tissues into a vented-tissue culture flask containing sufficient media to cover the surface of the flask when placed down (usually 10–15 mL is sufficient).
3. Place the flask in the CO<sub>2</sub> incubator and leave undisturbed for 24 h.
4. Check for growth using a light microscope.
5. When the desired growth or culture time has been achieved, add colcemid to the flask to a final concentration of 10 µg/mL for 2–3 h (*see Note 1*) in the CO<sub>2</sub> incubator.
6. Transfer the media and any floating tissues or cells to a 15-mL conical tube (*see Note 2*). For non adherent cells, proceed to **Step 10**.
7. Add 10 mL of citrate saline to the flask and rinse the attached cells. Discard the citrate saline.
8. Add 10 mL of 1X trypsin to the flask. Monitor the release of adherent cells from the flask surface. Gently pipet the cells off the flask surface and transfer to a 15-mL conical tube.
9. Add 5 mL of fresh media to the 15 mL conical containing the detached cells (*see Note 3*).
10. Centrifuge the collected cells at 1500g for 10 min.
11. Pour or aspirate off the supernatant leaving approx 0.5–1 mL of solution. Gently resuspend the pelleted cells well by tapping the conical tube with fingers.
12. Add the prewarmed 0.075 M KCl solution in a drop-wise fashion for the first 1 mL, with mixing between drops (*see Note 4*). After the first mL has been added, add the remaining 14 mL of solution and invert gently. Place at 37°C for 10 min.

13. Add 5 drops of 3:1 methanol:acetic acid solution and invert (*see Note 5*).
14. Centrifuge at 1500g for 10 min.
15. Pour or aspirate off the supernatant and resuspend the pellet (*see Note 6*). Add fresh 3:1 methanol:acetic acid fixative with the first 1 mL added dropwise as before. Add the remaining 14 mL of fixative and invert gently.
16. Centrifuge at 1500g for 10 min.
17. Repeat the fixation **Step 15**. However, the first 1 mL does not need to be added in a dropwise fashion. Centrifuge and repeat for a total of 3 fixations.
18. The cell suspension may be stored in its pellet state under fixative at  $-20^{\circ}\text{C}$ .
19. To prepare cytogenetic preparations, pour off the fixative and resuspend the pellet. Add sufficient fresh fixative so that the suspension is slightly translucent.
20. Using a 200- $\mu\text{L}$  pipet, add 100  $\mu\text{L}$  of suspension to clean glass slides (*see Note 7*). Allow the slides to dry and store at room temperature. Allow the slides to age for at least 2 d naturally, or place the slides at  $37^{\circ}\text{C}$  overnight.

### 3.3. Slide Pretreatment and Denaturation

1. Metaphase slides should be aged for at least 2 d at room temperature or placed at  $37^{\circ}\text{C}$  overnight before use.
2. Dehydrate the slides in an ethanol series (70, 80, 95%) for 5 min each. Allow the slides to air dry.
3. Add 15–20  $\mu\text{L}$  of a 10% pepsin stock (*see Note 8*) to 50 mL of prewarmed 0.01 M HCl in a Coplin jar. Place the slides in the jar for 5 min (*see Note 9*) at  $37^{\circ}\text{C}$ . Meanwhile, prepare a 70% formamide/2X SSC solution in a Coplin jar and place at  $75^{\circ}\text{C}$ .
4. Remove the slides and place them in 1X PBS at room temperature for 5 min.
5. Incubate the slides in 1X PBS/MgCl<sub>2</sub> at room temperature for 5 min.
6. To 50 mL of 1X PBS/MgCl<sub>2</sub>, add 1.5 mL of 37% formalin. Incubate the slides for 10 min in the formalin solution (*see Note 10*) at room temperature.
7. Incubate the slides in 1X PBS for 5 min at room temperature.
8. Pass the slides in a dehydration series as in **Step 2**.
9. When the slides have dried, check that the temperature of the formamide solution has reached  $75^{\circ}\text{C}$  (*see Note 11*). Denature the slides in the formamide solution for 2 min.
10. Immediately place the slides in 70% ethanol and proceed through the last dehydration series (*see Note 12*). Allow the slides to air dry.

### 3.4. Probe Denaturation

1. For each slide, use 10  $\mu\text{L}$  of probe to cover a 22  $\times$  22-mm coverslip. If the area to be probed is larger than this, more probe must be used. Place the appropriate amount of probe into an eppendorf tube.
2. Heat denature the probe at  $75^{\circ}\text{C}$  for 10 min either in a waterbath or PCR machine.
3. Place the probe at  $37^{\circ}\text{C}$  to preanneal for 1 h.

### 3.5. Hybridization

1. Take the preannealed probe from  $37^{\circ}\text{C}$  and carefully add to the denatured metaphase slide preparations. Since the probes are both directly and nondirectly labeled, work quickly and avoid extended exposure to light.
2. Carefully place a coverslip on the probe, avoiding bubbles. If bubbles do appear, gently push them to the slide by pressing down on the coverslip.
3. Ring the perimeter of the coverslip with rubber cement.

4. Place in a hybridization box (*see Note 13*) and place in a plastic bag (optional). Allow the slides to hybridize at 37°C for 48 h.

### **3.6. Post-Hybridization Washes and Detection**

1. Preheat all the solutions in a 45°C waterbath.
2. Remove the slides from the hybridization box and carefully peel off the rubber cement. The coverslip may come away with the rubber cement or remain on the slide. Should the coverslip remain on the slide, proceed to place the slide in the first wash solution.
3. Wash the slides in 50% formamide/2X SSC, 3× for 5 min each. The coverslip should slide off in the first wash. Gently agitate the Coplin jar for a few seconds (*see Note 14*).
4. Wash the slides in 1X SSC, three times for 5 min each with gentle agitation.
5. Dip the slides in 0.01% Tween-20/4X SSC. Remove the slides and allow the excess solution to dip off, but do not allow the slides to dry.
6. Using Vial 2 from the SKY Kit provided by Applied Spectral Imaging, add 30 µL to 40 µL of blocking solution to the slides and coverslip.
7. Place the slides back in the hybridization box and incubate for 30 min at 37°C.
8. Carefully remove the coverslips and add 30 µL to 40 µL of detection reagent from vial 3 from the SKY Kit provided by Applied Spectral Imaging and coverslip.
9. Place the slides back in the hybridization box and incubate for approx 30 min at 37°C.
10. Carefully remove the coverslips and wash the slides in three washes of 0.01% Tween-20/4X SSC for 5 min each. Agitate gently.
11. Allow the excess solution to run off the slide and add 30 µL to 40 µL of detection reagent from vial 4 in the SKY Kit provided by Applied Spectral Imaging, and coverslip.
12. Place the slides back in the hybridization box and incubate for approx 30 min at 37°C.
13. Carefully remove the coverslips and wash the slides in three washes of 0.01% Tween-20/4X SSC for 5 min each. Agitate gently.
14. Rinse the slides in 2X SSC at room temperature.
15. Using 15 µL to 20 µL of the DAPI/Antifade mounting medium provided in vial 5, counterstain the sides and coverslip making sure that there are no bubbles.
16. The slides can now be viewed. The slides should be stored at -20°C.

### **3.7. Image Acquisition**

1. Image acquisition and analysis should be carried out as soon as possible. Although the fluorescence can last for several months if stored at -20°C and exposed to as little light as possible, the signal intensity will diminish over time (*see Notes 17–20*).
2. Training for use of the system's acquisition and analysis is carried out at the time of installation of the system in the laboratory.

### **3.8. Hybridization of SKY Probes to Previously G-banded Slides: Slide Pretreatment**

1. Remove any residual immersion oil from previously G-banded slides by washing in 2 washes of xylene for 5 min each at room temperature (*see Note 15*).
2. Destain the slides by soaking slides in methanol for 10–15 min, or until the slide has been thoroughly destained.
3. Once the slides have been destained, pass the slides through a rehydration series: 95, 80, and 70% ethanol for 5 min each. Allow the slides to air dry.
4. Continue the protocol outlined in **Subheading 3.3**, starting at **Step 5**.
5. In the case of previously banded slides, the denaturation time in 70% formamide/2X SSC

**Table 1**  
**Suggested G-Banding Steps Yielding Both**  
**Good Banding and Subsequent SKY Hybridization**

Banding steps	Slide 3 d-1 mo	Slide >1 mo	<i>In situ</i> slide preparation 3 d-1 mo	<i>In situ</i> slide preparation >1 mo
Trypsin (5% w/v stock) diluted 1/100 times	10-20 s with agitation	20-30 s with agitation	40-60 s with agitation	60-90 s with agitation
Saline	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Saline	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Stain (Giemsa)	50 s	1+ min	50 s	1+ min
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Denaturing Time	20-30 s	45 s-1 min	30-45 s	1 min-?

should be adjusted to reflect the properties of the slide (*see Note 16, Table 1*). A conservative denaturation time is 40 s.

- All other steps, probe denaturation, hybridization and post-hybridization washes and detections are the same as outlined above.

#### 4. Notes

(Also *see Table 2* for General Troubleshooting Tips)

- If there is sufficient material, try different colcemid times to produce metaphase spreads that are a good length for cytogenetic analysis. Often times, different tissues and tumor types will behave differently with respect to their growth patterns. For slow growing cultures, an overnight treatment with colcemid may yield more metaphase spreads. This will require the colcemid concentration to be considerably reduced. Whereas for cultures which appear to be growing quickly, a shorter period of colcemid treatment, perhaps only 30 min, is sufficient to arrest enough cells in metaphase to produce spreads. In general it is advisable to give the culture a "half feed" one day before harvesting the cells, by removing half of the media from the culture and replacing it with fresh media. The sensitivity of the cells to colcemid is also a factor in obtaining good size chromosomes for analysis. It has been our experience that some tumors will have a very low tolerance for colcemid treatment, producing very tight and short chromosomes. This can be alleviated somewhat by reducing the final concentration of colcemid and increasing the incubation time, but this could sacrifice the number of cells arrested in metaphase.
- Some cultures grow both as adherent and as nonadherent cells. To ensure that the entire tumor has been represented in the cytogenetic preparation, it is advisable to collect the cells that may be floating in the media. Once collected, they can be added to the rest of the cells which have been lifted off the culture flask using trypsin.
- The use of fresh culturing media containing fetal calf serum when washing the collected cells helps to inactivate the trypsin. Since trypsin is a protease, prolonged exposure of the cells to the trypsin could rupture membranes and damage the DNA.

**Table 2**  
**Commonly Encountered SKY Difficulties**

	Slide	Probe	Technical
<i>Weak Signal</i>	<p><i>Slide Age:</i> optimal results have been obtained from slides not older than 2 mo in age. As the slides age, they become harder to denature. Conversely, very old preparations often have degraded DNA not adequate for FISH. Previously G-banded slides will have an even shorter lifespan and should be processed within 2 wk.</p> <p><i>Denaturation Time:</i> As the slide ages, the chromosomes become harder to separate into single strands. Slides used within 1–2 wk of preparation should be denatured for 1.5–2 min. Slides that are older may require times that range from 2–3 min.</p> <p><i>Cytoplasmic Debris:</i> The presence of cytoplasm may inhibit binding and contribute to background. A more aggressive protein pretreatment may be required.</p> <p><i>Excessive Slide Pretreatment:</i> Excessive enzymatic treatment may damage the target DNA making it less efficient for hybridization with the probe.</p>	<p><i>Commercial Probes:</i> Usually the company has properly processed the product with the necessary quality controls. Check that the probes were properly stored and used within the expiration date.</p> <p><i>Probe Concentration:</i> The probe is usually in excess of the target DNA, however, make sure that sufficient probe has been added to adequately cover the area of interest on the slide.</p>	<p><i>Sealing of coverslip:</i> It is critical that the coverslip is adequately sealed, preventing any moisture from entering the hybridized area and diluting the probe.</p> <p><i>Proper Temperature:</i> Check that the temperature in the oven or hotplate unit is correct for hybridization or incubations.</p> <p><i>Incubation Times:</i> Avoid taking shortcuts during incubation times with blocking reagents or detection reagents. Also be sure that the reagents do not dry up on the slide making washing more difficult.</p>

<i>Background</i>	<p><i>Cytoplasmic Debris:</i> This is the most common culprit causing background. Increase the incubation time during the protein digestion or maintain the same time but change the concentration.</p> <p><i>Bacterial/Yeast Contamination:</i> Microorganisms that have been cultured along with the specimen may have also made its way onto the slide. Unfortunately, this may be unavoidable.</p> <p><i>Coverslips:</i> If cells are to be grown <i>in situ</i>, use only glass coverslips. Plastic coverslips will cause autofluorescence.</p> <p><i>Residual Oils:</i> Slides that have been previously visualized using immersion oil should be cleaned with xylene. Residual oils will prevent hybridization and cause background problems.</p>	<p><i>Commercial Probes:</i> This is usually not the cause for background, However, note the lot number for future reference.</p>	<p><i>Post-Hybridization Washes:</i> Make sure that the correct temperature has been reached for the washes and incubations. Agitation during the washes can help to remove unbound probe and antibodies. Increasing the stringency of the washes by either increasing the temperature or altering the amount of SSC in the washes can also help. Avoid drying of the slide with any of the blocking or detection reagents.</p>
<i>Fading Signals</i>	NA	<p><i>Commercial Probes:</i> Although these probes are supposed to be quality controlled, these too are under the same limitations of In-House probes. Take note of the lot number and expiry date of commercially made probes.</p>	<p><i>Antifade:</i> Check that the DAPI/ Antifade solution is within its expiry date. Normally, the antifade is clear with a slight pink tinge. Antifade which has gone “off” will turn increasingly amber in colour. Expired antifade medium will cause rapid signal degradation and display a red glow when viewed under the microscope.</p>

(continued)

**Table 2 (continued)**

	Slide	Probe	Technical
<i>Poor Chromosome Morphology</i>	<i>Over-denaturation:</i> The cause of poor chromosome morphology is usually caused by over-denaturation causing the DNA to be destroyed. This can be sample specific and/or slide age related.	NA	<i>Denaturation Temperature:</i> Check that the temperature for denaturation is accurate. The final internal temperature of the Coplin jar should be 72°C. Add 1 degree for each slide that will be denatured in that jar. Avoid denaturing more than 5 slides in one jar at one time. Plastic Coplin jars will have a greater differential temperature between the bath and the internal temperature, as compared to a glass Coplin jar.
<i>Previously G-Banded Slides</i>	<i>Over-Processing:</i> Over trypsinization for optimal banding can cause the DNA to become sensitive to subsequent denaturation. For this reason, denaturation times are greatly reduced. This will also have an affect on the quality of hybridization and signal strength.		

4. KCl has been traditionally used as the hypotonic solution used to swell the cells. Adding the first mL in a dropwise fashion to the cells prevents the sudden rush of water into the cells and bursting the contents into the supernatant. Gentle mixing between drops helps to ensure that the hypotonic solution is incorporated into the cell suspension. A gentle heat treatment at 37°C assists in swelling the cells more rapidly. If there is not a 37°C incubation, 30 min at room temperature will also adequately swell the cells. If the cells do not swell (*see Note 5*), increase the hypotonic incubation time in 5 min increments for subsequent harvesting of the same culture.
5. After the hypotonic swelling, the cells are very sensitive. Doing a “prefixation” with a few drops of methanol:acetic acid fixative helps to harden the cell membranes for subsequent centrifugation and fixations. The addition of the fixative also lyses any red blood cells that may be present. After the centrifugation, the pellet size should have doubled. This is an indication that the hypotonic treatment was successful.
6. Once the cells have been pelleted, the supernatant can be removed. This contains any ruptured membranes and cytoplasmic material. The pelleted cells should be gently resuspended with gentle flicking of the bottom of the conical tube. The addition of fixative in a dropwise solution helps to slowly introduce the fixative to the still swollen and sensitive cells.
7. There are many ways to prepare metaphase slides. Some laboratories use disposable 1-mL bulb pipets, glass pipets or pipetmans for adding the fixed cells to the slide. The use of a 200  $\mu$ L pipetman can help to control the amount of suspension used, particularly if there are few cells. The cells are generally added to a slide that is tilted to an approx 45° angle. The slide may be dry or wet with either water or fixative. These different methods of slide making are based on trial and error. The relative humidity in the room as well as the season will influence the success of slide making. If the air is too dry, the use of a humidifier or steam generated by a boiling kettle or water on a hotplate can help to increase the relative humidity. If the surrounding air is too humid, a few seconds on a hotplate, which has been warmed to the touch, can facilitate proper drying. The slides should always be assessed by phase contrast microscopy to determine whether the current slide making technique is appropriate. To ensure successful SKY, the slides should have minimal cytoplasmic debris around the metaphases and chromosomes that are not too long or are overlapping. Adjust the cell density as required. In general, the chromosomes should appear dark grey. The banding patterns should be slightly noticeable. The slides can be stored at room temperature in a slide box. They should be used within the first 2 wk, but can be used as early as the next day. As the slides begin to age, they will first become more difficult to denature (*see Note 9*) then proceed to degrade making the DNA useless.
8. Pepsin is mildest of the proteases used for slide pretreatments. A 10% (w/v) pepsin stock should be made and aliquoted. They can be stored at -20°C almost indefinitely. The aliquoted pepsin should be a one use aliquot. Because each new batch of pepsin is different, the amount used to achieve the cleanest slides may vary.
9. A conservative approach to controlling the extent of protein digestion is to use the same amount of pepsin but increase the incubation time. In contrast, the increase in amount of pepsin used requires more strict timing, making the former method more flexible and forgiving. Gentle agitation can also facilitate the loosening of cytoplasmic debris. The extent of digestion can be viewed by phase contrast microscopy after the treatment, but before the formalin fixation step (**Subheading 3.3., Step 6**).
10. The use of formalin helps to maintain the chromosome morphology. This also makes the DNA a little bit more resistant to denaturation. Although the denaturation time of 2 min is sufficient to denature the DNA to single strands, the formalin treatment can be reduced in

time for slides that are much older (>2 mo). If the slides are old, reduce the formalin treatment to 5 min. For previously G-banded slides (**Subheading 3.8.**), the formalin fixation also helps to maintain the chromosome morphology which may have been harshly treated by the banding steps including trypsin digestion and heat treatment for artificial aging.

11. Formamide is used in the denaturing solution because it has the ability to reduce the melting temperature of DNA. Ensure that the formamide solution has reached the proper internal temperature by measuring it with a thermometer. The solution inside plastic Coplin jars tend to be 3–4°C lower than the waterbath temperature, thus the temperature of the bath should be increased. Glass Coplin jars tend to conduct the heat much better from the water to the solution inside the jar. As a rule of thumb, the temperature of the bath should be increased 1°C for each slide that will be placed for denaturation, since the temperature will drop with addition of each slide. Thus for a glass Coplin jar, where 5 slides will be added for denaturation, the waterbath temperature should be set at about 78–79°C so that the temperature of the solution is about 74–75°C. Two minutes is a standard time for slide denaturation. However, adjustments in the time should be made based on the quality and age of the slide. For much fresher slide preparations (i.e., 1–3 d old), the DNA may not have had enough time to be properly aged. Although the formalin fixation helps to achieve this, the DNA may still be delicate. Reducing the time to 90 s may be sufficient. One can determine whether the slides were over-denatured upon visual inspection after hybridization. The chromosomes will appear puffy with a visible “skeleton” running down the axis of each chromosome or chromatid, or ghost-like chromosomes with defined borders, but pale staining interiors. Over-denatured chromosomes will not pick up the DAPI counterstain as well as well denatured slides, often showing very brightly staining centromeres only. There may also be very little to no hybridization of the probe to the target DNA because the over-denaturation has damaged the DNA. Target slides that have been under-denatured will show more intact chromosomes with patchy hybridization. The chromosomes will pick up the DAPI staining well indicating that the DNA has not been over-processed and damaged.
12. Denaturation of the slides is the most critical step when carrying out FISH based assays. Once the slides have been denatured, they must immediately be placed in 70% ethanol to keep them in their denatured state. The ethanol can be cold or at room temperature. While it is not required to have the ethanol replaced after each step, the 70% ethanol should be replaced after each experiment. The other ethanols (80 and 95%) can be topped up and replenished after every other experiment. If they are not stored at –20°C, they can be stored on the benchtop at room temperature, provided that they are tightly sealed to prevent evaporation.
13. Any light proof container can be used as a hybridization box. A black video cassette box is a good container for hybridization. Although the box does not need to be dampened, it has been our experience that the hybridization kinetics seems to be better when there is a damp (not soaking wet) piece of paper towel or gauze lining the bottom of the box. Be sure to use clean distilled water since any bacteria that may be present can make its way onto the slide and pick up the antibodies, causing unnecessary background. The hybridization container can also be wrapped in a plastic bag to prevent the box from drying out. This is optional.
14. All post-hybridization washes should be carried out with some gentle agitation. This helps to loosen any unbound probes or antibodies from the slide making it a much cleaner preparation. Ensure that all the reagents are free from bacteria, which can pick up the

antibodies. Should there be high background upon UV microscopy, one should consider more stringent washing conditions: either adding an extra wash for each step with more vigorous agitation, or adjusting the solutions. The concentration of Tween-20 for example could be increased from 0.01% to 0.2%. Background also appears when the slides have been allowed to dry during the washing and antibody incubation steps.

15. Previously G-banded slides are often the only source of material left in clinical cytogenetics laboratories. Their slide pre-treatment and storage also make them difficult to be used for subsequent FISH-based analysis. However, a few steps can be done to increase the chances of success when using previously banded slides. **Table 1** also outlines some of the guidelines for preparing G-banded slides, which will be used for SKY analysis. After G-banding analysis, the slides should be treated with xylene or some other agent that removes any residual emersion oil. The oil can quickly degrade the DNA. If the slides were coverslipped and sealed, the mounting media may also have degraded the DNA. This may be unavoidable, but try to avoid further DNA damage by adjusting protease treatments (which may be eliminated entirely since G-banding already requires a trypsin treatment), denaturing times and formalin fixations as described above.
16. Adjust the denaturation time when denaturing previously G-banded slides. The DNA has already undergone some harsh treatment during the banding process, including a trypsin treatment to remove cytoplasm and enhance banding by stripping away some of the DNA associated proteins, and high heat treatment to allow for better contrast of the banding with the stain. Together with exposure to emersion oil, the target DNA is more likely to be in a more fragile state. To compensate for this, decrease the denaturing time to 40 s. It might also be useful to test out a slide from another source that was made and processed in the same fashion, before using the actual test slide. This will give an idea as to how well the assay will work and how much more processing the slide can handle.
17. Try to image metaphase spreads that do not include a nucleus. While the chromosomes often fluoresce as brightly or more brightly than surrounding nuclei, smaller micronuclei tend to have very intense fluorescence that will change the intensity of the metaphase of interest by automatically adjusting the contrast during image acquisition.
18. Try to avoid “bright spots” during image acquisition. For the same reasons described above, extraneous bright spots from conjugated antibodies that failed to be washed off can also reduce the intensity of the metaphase of interest.
19. If possible, capture metaphases with few overlaps. This may be unavoidable when there are few metaphases, or the entire suspension consists of long chromosomes. The overlapping of chromosomes causes the false detection of “translocations” owing to the combinations of fluorochromes at the site of crossing which may correspond to one of the combinations in the spectral library.
20. To save space on the hard drive as well as reducing the acquisition time, only select the metaphase of interest to be captured rather than including a lot of “dead space” surrounding the metaphase.

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## Multicolor FISH

Syed M. Jalal and Mark E. Law

### 1. Introduction

Chromosome “painting” by fluorescent labeled probes for each human chromosome has been available since the late 1980s. The DNA or RNA radioactive isotopes have been in use since 1969 as nucleic acid probes (1,2). The introduction of fluorescent labeled probes by Bauman et al. (1980) presented an attractive alternative for chromosomal *in situ* hybridization (3). Based on unique DNA sequences specific to individual chromosomes, painting probes were generated for each human chromosome (4,5). Pinkel et al. (1988) used *in situ* hybridization of 120 fluorescent DNA probes (FISH) along the entire length of chromosome 4 to generate a specific painting probe for this chromosome (6). By the early 1990s, whole chromosome painting probes (wcp) were commercially available for each human chromosome in two colors (green and orange). Jalal et al. presented the characteristics of the direct labeled wcps for every chromosome and their utility in clinical practice (7). The ability to resolve chromosomal anomalies was greatly enhanced when wcps became available as a complement to chromosome analysis by banding techniques.

It was indeed exciting that by 1996, it became possible to “paint” the entire human genome simultaneously so that each chromosome fluoresced in a unique and distinct color (8). As is customary for wcps and multicolor FISH (M-FISH) methods, the highly repetitive DNA sequences (COT-1 DNA) are suppressed by excess unlabeled complementary DNA. The combinatorial labeling of five fluorophores makes it possible to assign a specific fluor combination and thus, a unique spectral signature to each human chromosome. The chromosomal regions that are rich in repetitive DNA sequences (constitutive heterochromatin) and acrocentric chromosome short arms are not labeled by these strategies. Schröck et al. (8) point out that for spectral karyotyping, all the information from emitted light is used by Fourier spectroscopy and charged coupled device imaging. The spectral signature for each chromosome is then converted to display as classification colors with the help of imaging software (8). Multicolor

spectral karyotyping (SKY) has been used as a diagnostic or research tool for over 300 cases (9).

During 1996 also, another option to SKY was proposed to monitor each human chromosome in a unique and specific fluorescent color (10). This method also used combinatorial labeling based on five fluorophores (spectral intervals of 350–770 nm) to generate a unique label for each chromosome with a background staining of DAPI (4'-6-diamidino-2-phenylindole). Rather than spectroscopy, M-FISH utilizes a series of filters with defined emission spectra (see Note 1). Coupled with imaging software, the procedure generates unique and distinct fluorescent images for each chromosome. M-FISH has been called multicolor FISH, multicolor FISH, or multiplex FISH, but we prefer the term multicolor FISH. An advantage of M-FISH is that the standard FISH imaging systems can be relatively easily adapted for M-FISH analysis. More than 70 US laboratories have some type of computer image analysis system (11). Eils et al. have proposed strategies for optimization of probe labels, filter sets, and image analysis for M-FISH (12). Law and Jalal have also provided the practical guidelines for set up and analysis by M-FISH (13).

M-FISH has been successfully utilized for identification of markers and derivative chromosomes for congenital disorders (14–16). It has also been used in clinical practice for identification of multiple chromosomal abnormalities in complex karyotypes more recently. M-FISH has been useful diagnostically for the detection of critical chromosome rearrangements in hematologic disorders (17).

### **1.1. Clinical Application of M-FISH**

M-FISH has been successfully utilized both for constitutional abnormalities in congenital disorders and acquired chromosome anomalies in hematologic diseases (see Note 2). In particular, M-FISH can often identify unknown markers, derivative chromosomes, and can resolve complex karyotypes. M-FISH can also play a key role in the detection of critical (primary) chromosome anomalies in hematologic diseases, especially when the karyotype is complex or when the chromosome morphology is of poor quality. However, such an effort involving M-FISH often needs to be complemented with use of locus specific fluorescent DNA probes for detection of subtle anomalies. Another important question is whether M-FISH analysis of karyotypically normal samples of hematologic disorders can be worthwhile in the detection of structural abnormalities. Our experience and that of the Eastern Cooperative Oncology Group (personal communication—Dr. Dewald) is that it is generally not helpful. The only exceptions are where the anomaly is too subtle to be picked up by banded chromosome analysis [e.g., t(12;21)(p13;q21)] or when the chromosome morphology is of such poor quality that even relatively major structural anomalies cannot be ruled out.

#### **1.1.1. Congenital Disorders**

One of the more labor intensive and challenging efforts in clinical cytogenetics is to provide a comprehensive identification of markers, derivative chromosomes, or to resolve a complex karyotype. It has been estimated that *de novo* structural rearrangements occur with a frequency from 0.70/1000 rising to 2.4/1000 among mentally retarded individuals (18). The prenatal frequency of *de novo* supernumerary markers

(including chromosome 15 bisatellited marker) is around 1/2500 (19). Deletions associated with defined phenotypic anomalies have been well documented for almost all major bands (20). Balanced reciprocal translocations occur with a frequency of about 1 in 600. The risk for unbalanced chromosome composition in the progeny of such balanced carriers vary widely from 50% to <1% (21). Unbalanced chromosome complements are widely associated with phenotypic abnormalities. It is speculated that Robertsonian translocations, occurring with a frequency of 1.2/1000, are the most common chromosome abnormality in human populations. Gravholt et al. have provided evidence that banded chromosomes are produced most commonly from the acrocentrics by break and fusion in satellite III DNA of the short arm near the centromere (22). Although the balanced carriers are phenotypically normal, they are at risk for unbalanced combinations as well as uniparental disomy in the offspring (23). Complex chromosome rearrangements (CCR) are rare abnormalities involving more than two chromosomes with three or more breakpoints. As many as 10 breakpoints have been reported in some cases with CCR (24,25). A summary of structural and numeric chromosomal anomalies for each chromosome and their associated dysmorphic features are well documented (26,27).

Banded chromosome analysis is labor intensive and the detailed characterization of markers, derivative chromosomes, and complex karyotypes are limited. Utility of *in situ* hybridization and fluorescent labeled DNA probes (locus specific, centromere specific, and chromosome specific) ushered in the new era of molecular cytogenetics. This has allowed description of markers, derivative chromosomes, and complex karyotypes in much greater detail for prenatal as well as postnatal tissues, based on both metaphase and interphase cell analysis (7,28–34). The specific characterization of the anomaly and its origin is important not only for fully assessing the clinical significance, but also for genotype-phenotype correlations, gene mapping, and genetic counseling.

Complementation of FISH based probes for the centromeres, specific loci, and whole chromosome paint probes to banded chromosome analysis has provided an unprecedented progress in detailed characterization of chromosomal anomalies. However, even this approach is cumbersome and inadequate in certain situations. When an extra marker chromosome of unknown origin or a *de novo* derivative chromosome is encountered, the question is what type of FISH probe should be used. Paint probes have been available commercially for every chromosome since early 1990s. The centromere specific probes based on the diversity of alpha satellite sequences have also been available for every chromosome except 13/21, 14/22, and 5/19 due to cross hybridization (5). In addition, a limited number of locus specific probes are commercially available. In practice, the selection of probes in such a situation was a matter of trial and error that could often be very time consuming and expensive. Since 1996, the introduction of SKY and M-FISH have largely resolved this dilemma since these techniques simultaneously paint each chromosome in a unique color (8,10). The utility of M-FISH (see Notes 3 and 4) in clinical practice has been of particular help in specific situations as illustrated by the following representative examples:

*Case 1.* An amniotic fluid based chromosome analysis was conducted due to intra-uterine growth retardation. The interphase FISH analysis based on chromosomes 13, 18,

21, X and Y (35) from uncultured cells for aneuploidy was normal. Chromosome analysis from 20 GTL (G band by trypsin using Leishman Giemsa) banded metaphases from five colonies and four primary cultures showed a karyotype of 46,XX,add(15)(q26.1). M-FISH analysis readily identified the *de novo* translocated segment of chromosome 15 to be from chromosome 9. This was confirmed by use of wcp9 and wcp15 (**Fig. 1A, B**).

*Case 2.* An amniotic fluid based chromosome analysis was performed owing to advanced maternal age. Analysis of 20 GTL-banded metaphases from 15 colonies and 3 primary cultures indicated a karyotype of 47, XX, +r. The *de novo* ring was identified to be from chromosome 19 by M-FISH analysis and confirmed by use of wcp19 (**Fig. 1C, D**).

*Case 3.* A fibroblast culture was established from products of conception (POC) of a 25 yr old at about 7.5 wk of gestation for chromosome analysis. Twenty GTL-banded metaphases indicated a karyotype of 46,XY,add(4)(p12). M-FISH analysis from a previously G-banded cell (**Fig. 1E, F**) indicated that a segment from chromosome 2 was translocated to the abnormal chromosome 4 (*see Note 3*). This was confirmed by use of wcp2. The father was unavailable for chromosome analysis, and the mother was chromosomally normal.

These three cases have been published in *Genetics in Medicine* (14). Although the M-FISH findings were confirmed by use of whole chromosome paint probes for the first 25 cases analyzed by our laboratory, this practice has been stopped, and we feel that it is no longer necessary. There are cases where simultaneous fluorescent labeling of all chromosomes provided by M-FISH was necessary for efficiency and details. There are also circumstances when M-FISH is very efficient, although just confirmatory to painstaking high resolution banded chromosome analysis. A few examples as illustration are provided as Cases 4, 5, and 6.

*Cases 4 and 5.* Two brothers with multiple anomalies and cognitive impairment inherited unbalanced derivative chromosomes from a familial balanced translocation. One had 46,XY,der(4)t(4;8)(p16.1;p23.1) and the other had 46,XY,der(8)t(4;8)(p16.1;p23.1). It required a great deal of effort from high resolution chromosome analysis (550–850 band stage) to determine the relatively subtle breakpoints in the two derivative chromosomes. M-FISH detected the abnormality of der(4), but the translocated segment of chromosome 4 to chromosome 8 in der(8) could not be readily detected by M-FISH or wcp4. Use of Wolf-Hirschhorn critical region specific probe was helpful to demonstrate the anomaly (**Fig. 2A, B**). The parents were not available for chromosome analysis.

*Case 6.* A female infant with multiple dysmorphic features had a karyotype of 46,XX,der(7)t(7;12)(q34;p13.1)mat. The abnormal chromosome 7 was deficient for 7q34-qter and duplicate for 12p13.1-pter. This was readily resolved by M-FISH analysis complemented by the use of locus specific probes (**Fig. 2C**).

Cases 4, 5, and 6 have been published by us in *Mayo Clinic Proceedings* (16). In all these cases, the most rewarding experience was to rapidly identify the segments of the translocated chromosomes. However, M-FISH has to be complemented with the use of locus specific probes for the detection and characterization of the anomaly, especially if it is subtle.

A case was published where an insertional translocation from 8q24.1 region to 3p21 could not be detected by SKY, but the translocation was detectable by the use of the

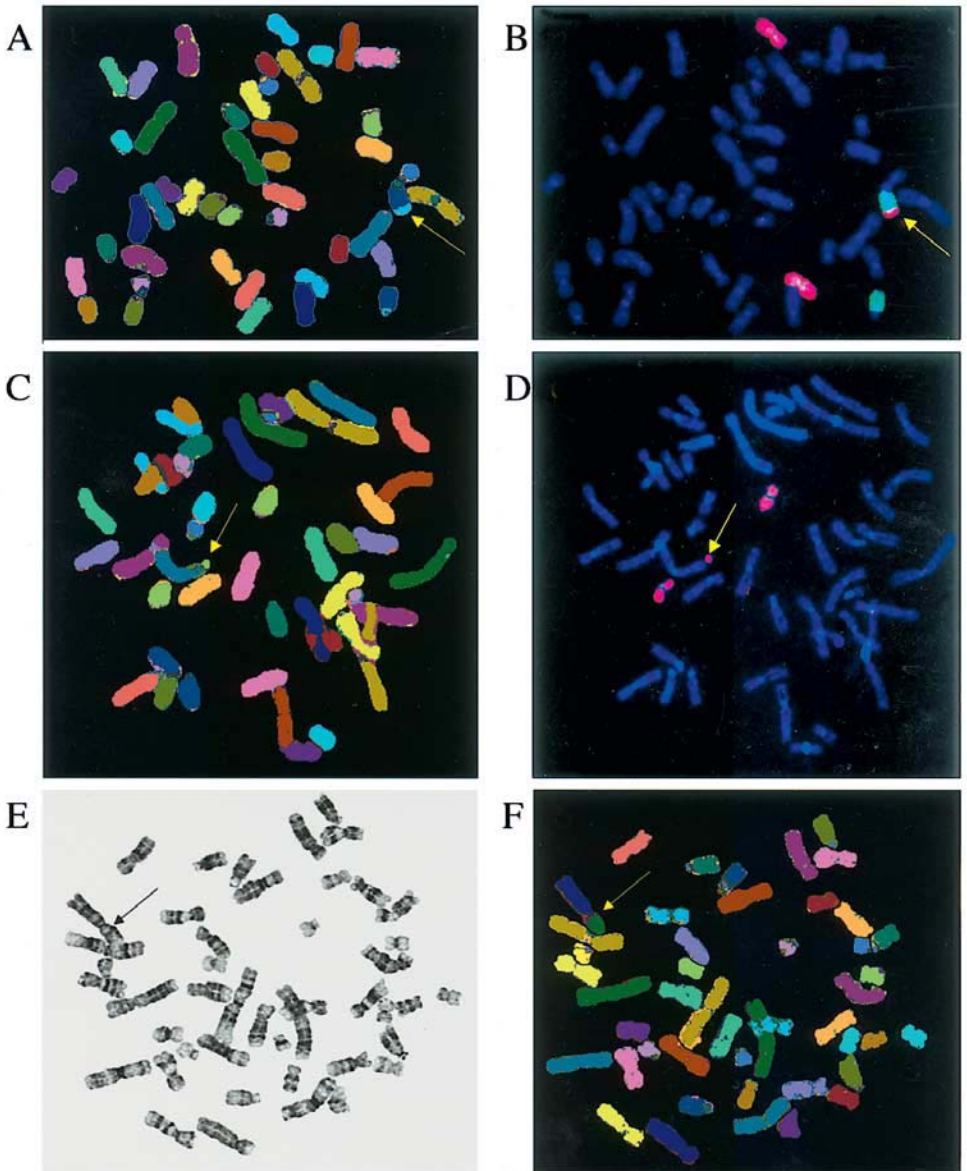


Fig. 1. (A) M-FISH analysis from an amniocyte culture where the segment translocated to an abnormal chromosome 15 was identified to be from chromosome 9. (B) Confirmation of the M-FISH result was obtained by use of wcp9. (C) M-FISH analysis from an amniocyte culture determined a small ring to be from chromosome 19. (D) The M-FISH finding was confirmed by use of wcp19. (E, F) Sequential M-FISH analysis of G banded fibroblast culture from a spontaneous abortion identified the segment translocated from chromosome 2 onto the abnormal chromosome 4.

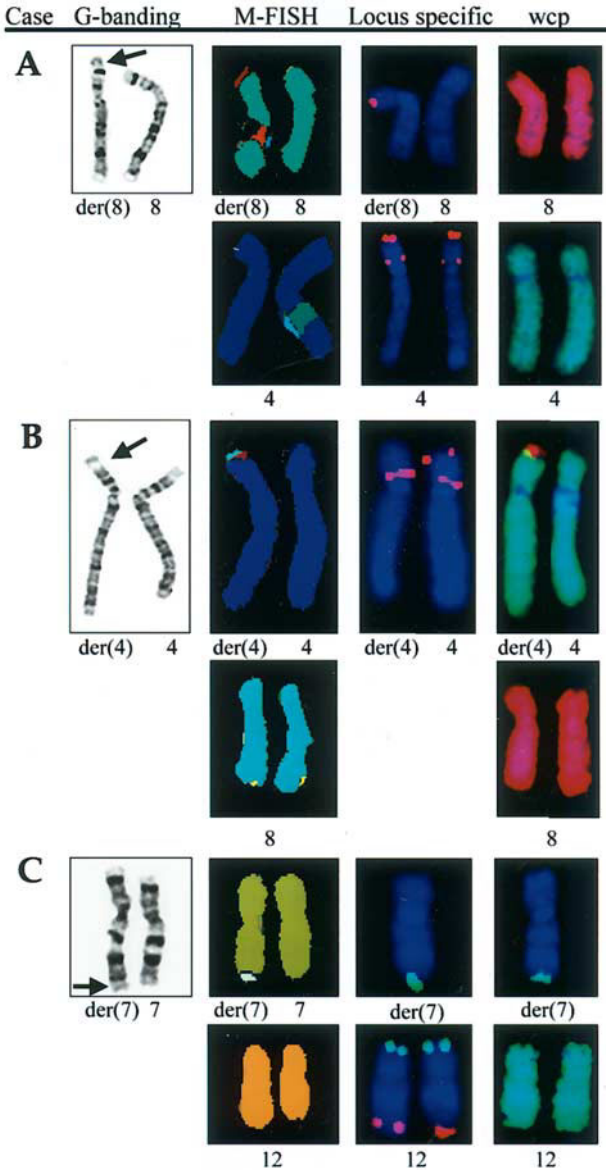


Fig. 2. M-FISH analysis from whole blood cultures of two subtle derivative chromosomes segregating from a  $t(4;8)(p16.1;p23.1)$  in two brothers. (A) The translocated segment in der(8) was from chromosome 4 that was confirmed by use of wcp4 and presence of 4p16.3 locus specific probe. (B) The translocated segment in der(4) was from chromosome 8, confirmed by use of wcp8 and absence of locus specific probe for 4p16.3. (C) M-FISH analysis of the subtle translocated segment in an  $add(7)(q34)$  from whole blood culture was identified to be from chromosome 12 and confirmed by the use of wcp12 and 12-p telomere specific probe.

c-myc probe (36). This insertional translocation was also undetectable by M-FISH (our unpublished result). Uhrig et al. analyzed 26 prenatal and postnatal cases by M-FISH involving markers, derivative chromosomes, and reciprocal translocations (15). They also conclude that most of these abnormalities could not be identified by banding but were detected by M-FISH. However, in some of the cases of deletion or duplication, it was necessary to use comparative genomic hybridization or chromosome region based specific multicolor bar codes to resolve the abnormality.

### 1.1.2. Hematologic Disorders

In the Western world, the incidence of leukemia is about one in 25,000. Chronic myelogenous leukemia (CML), a stem cell defect characterized by elevated granulocyte counts, accounts for approximately a quarter of all leukemia cases. CML provides an excellent model for the onset and progression of a disease with well established genetic events. The primary cytogenetic aberration is the t(9;22)(q34;q11.2) that translocates the oncogene C-ABL to the breakpoint cluster region (bcr) on chromosome 22 (Philadelphia chromosome, Ph). The chimeric protein that results from this fusion appears to be responsible for the onset of CML. The chronic phase, if untreated, lasts for about three years. The disease then enters an accelerated phase and terminates with a blast crisis. During the more malignant phase, secondary chromosome abnormalities commonly encountered include +8,i(17q) and an extra Ph (translocated 22) chromosomes. It is noteworthy that the critical genetic-cytogenetic event is the occurrence of t(9;22)(q34;q11.2). This can be identified by standard metaphase based cytogenetic studies, especially if the frequency of the neoplastic clone with the translocation is significant. The translocation can also be detected in interphase cells by FISH with a much higher sensitivity, especially to monitor residual disease after bone marrow transplantation or chemotherapy. The translocation is detectable by single fusion or double fusion FISH strategies involving DNA fluorescent probes and *in situ* hybridization primarily in interphase cells to monitor the residual disease (37–39).

Nonrandom and recurrent chromosome abnormalities have been documented for essentially all human neoplasms (40). Over 150 most commonly occurring translocations, deletions, duplications, inversions, isochromosomes, and aneuploidy in hematologic malignancies have been documented (41). Mitelman et al. have enlisted 215 balanced and 1,588 unbalanced recurrent aberrations among 75 neoplastic disorders (42). In this compilation, aneuploidy was excluded because of the uncertainty of their role as a primary chromosomal anomaly. M-FISH or SKY analysis can resolve the major chromosomal abnormalities in complex karyotypes from hematologic disorders (9,14). Of particular interest is the question if M-FISH can help identify the primary (critical) anomaly from a complex karyotype in hematologic disorders. Several cases (Figs. 3 and 4) are illustrated here as examples from our publication in British Journal of Hematology (17).

*Case 1.* A one-year-old girl diagnosed with acute myeloid leukemia had a karyotype of: 46,X,t(X;1)(q24;q25),t(6;8)(q25;q24.1), -11, -13, add(15)(q26)+2 mar[19]/46,XX[1] based on G-banding. M-FISH analysis confirmed the t(X;1) and t(6;8) and resolved the markers to be der(13)t(11;13) and the add(15) as der(15)t(13;15). The

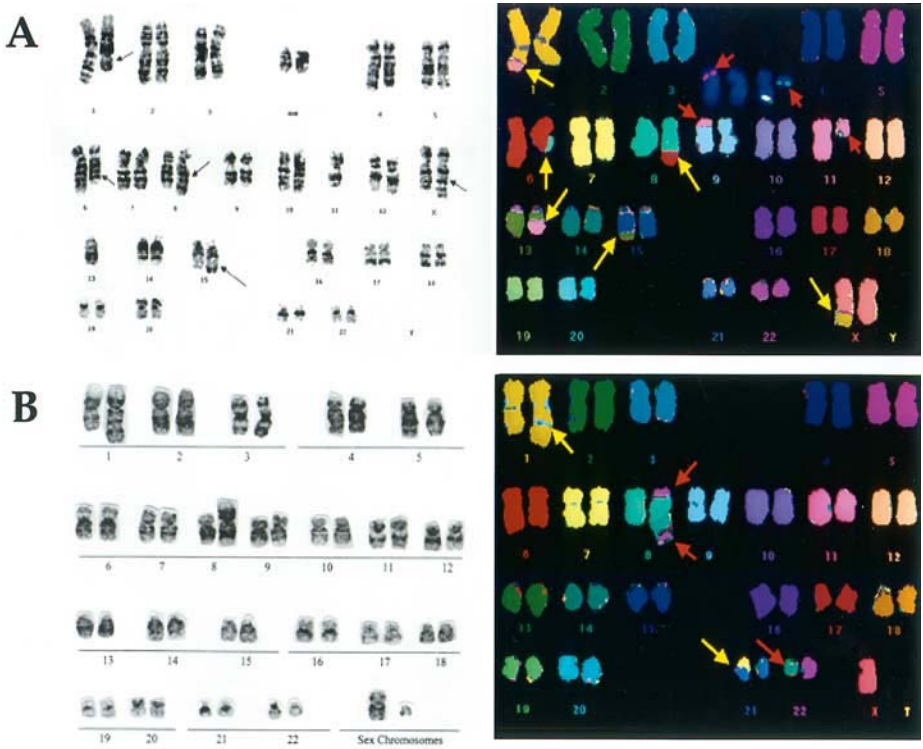


Fig. 3. M-FISH analysis was conducted to detect critical chromosome rearrangements (red arrows) and other chromosomal abnormalities (yellow arrows) in complex karyotypes, often of poor chromosome morphology, from bone marrow samples of hematological disorders. (A) The missed  $t(9;11)$  in *Case 1* was identified and confirmed by MLL locus rearrangement. The  $t(X;1)$  and  $t(6;8)$  were confirmed and the two markers were identified as  $der(13)t(11;13)$  and  $der(15)t(13;15)$ . (B) The critical rearrangement involving  $add(8)$  of *Case 2* was determined to be  $ider(8q)(q10)t(8;22)(q24;q11)$ . In addition,  $add(1)(q24)$  was identified to be  $dup(1)$  and  $add(14)$  was determined to be  $der(14)t(3;14)$ .

critical rearrangement of  $t(9;11)(p21;q23)$  was also identified and was confirmed by MLL locus rearrangement by FISH (Fig. 3A). The patient died seven months after diagnosis from transplant-related complications.

*Case 2.* A 38-yr-old male was diagnosed with acute lymphoblastic leukemia. The karyotype based on G-banding was  $46,XY,add(1)(q42),add(8),add(14)$ . M-FISH analysis clarified that  $add(1)$  was  $dup(1)$ , and  $add(14)$  to be  $der(14)t(3;14)$ . The  $add(8)$  turned out to be  $ider(8q)(q10)t(8;22)(q24;q11)$  (Fig. 3B). The identification of  $t(8;22)$  was critical to the diagnosis of variant Burkitt's lymphoma involving the translocation of oncogene *c-myc* adjacent to the Immunoglobulin lambda light chain locus.

*Case 3.* Bone marrow specimen from a 9-yr-old male was received for chromosome analysis for an unspecified hematologic disorder. The G-banded karyotype was:

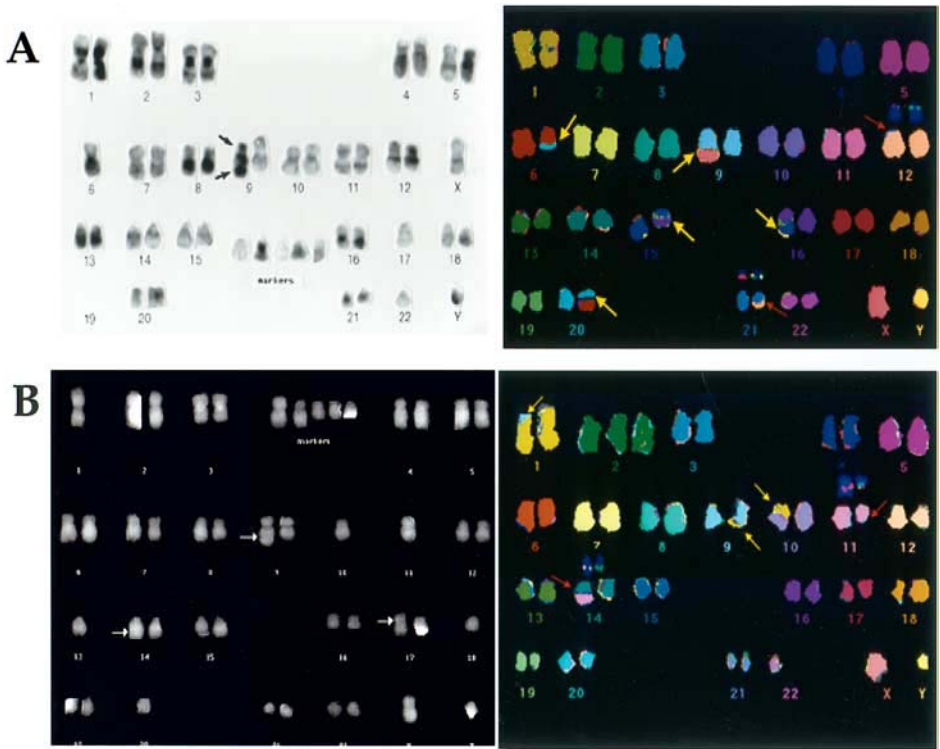


Fig. 4. M-FISH analysis was conducted to detect critical chromosome rearrangements (red arrows) and other chromosome abnormalities (yellow arrows) in complex karyotypes, often of poor chromosome morphology, from bone marrow samples of hematological disorders. **(A)** The missed rearrangement in *Case 3* of  $t(12;21)(p13;q21)$  was identified and confirmed by TEL-AML1 fusion. The  $der(6), add(9), -17, -19, -19, -22 +5mar$  were resolved. **(B)** The missed and critical rearrangement  $t(11;14)(q13;q32)$  was detected in *Case 4* and confirmed by cyclin D1 and IgH fusion. The other markers were identified as  $t(1;9)$  and  $der(10)t(1;10)$ .

45–47, XY, -6,  $der(9)add(9)(p13) add(9)(q34), -17, -19, -19, -22, +5 mar[cp19]/46,XY[1]$  with a poor morphology. M-FISH analysis detected the cryptic translocation of  $t(12;21)(p13;q21)$  that was confirmed by FISH as TEL-AML1 fusion (**Fig. 4A**). The  $t(12;21)$  is so subtle that it is often missed by standard cytogenetic analysis in children with ALL. M-FISH also identified all the remaining markers as  $der(6), der(9), der(15), der(20)$ , and two normal chromosomes 19.

*Case 4.* An 80-yr-old male with an unclassified malignant lymphoma was referred for chromosome analysis. The chromosome morphology was poor and based on Q-banding, the karyotype was described as  $44–46, XY, -1, add(9)(q34), -10, -11, -13, add(14)(q32), add(17)(p11.2), -18, -20, +5 mar[cp7]/46,XY[3]$ . Analysis by M-FISH revealed a translocation  $t(11;14)(q13;q32)$  which provided a definitive diagnosis for mantle cell lymphoma. The  $t(11;14)$  was confirmed by

cyclin D1 and IgH locus fusion. The other markers were identified as t(1;19) and der(10)t(1;10) (**Fig. 4B**).

## 2. Materials

1. DAPI counterstain (Vysis, Downers Grove, IL): Store at  $-20^{\circ}$ . See product label for expiration dates.
2. DAPI 10% solution: Mix 1 part DAPI in 9 parts Vectashield. Store at  $-20^{\circ}\text{C}$ . Use within 6 mo.
3. Ethanol (70%): Add 300 mL of distilled  $\text{H}_2\text{O}$  to 700 mL of 100% ethanol. Mix well and store at room temperature. Use within 1 yr.
4. Ethanol (85%): Add 150 mL of distilled  $\text{H}_2\text{O}$  to 850 mL of 100% ethanol. Mix well and store at room temperature. Use within 1 yr.
5. Ethanol (100%). Pure grain alcohol. Store 1000 mL aliquot at room temperature. Use within 1 yr.
6. 0.4X SSC (pH 7.0): Mix 10 mL of 20X SSC with 490 mL of distilled water. Store at room temperature. Use within 6 mo.
7. 2X SSC (pH 7.0): 17.53 g sodium chloride, 8.82 g sodium citrate, and bring to 1 L with distilled water or 100 mL 20X SSC (pH 6.3) with 900 mL distilled water. Store at  $4^{\circ}\text{C}$ . Use within 6 mo.
8. 2X SSC/0.1% NP40: Add 1 mL Nonidet P-40 to 1 L of 2X SSC. Store at room temperature. Use within 6 mo.
9. 20X SSC (pH 6.3): 175.3 g sodium chloride, 88.2 g sodium citrate, bring to 900 mL with deionized water; or add 132 g 20X SSC powder (Vysis) to final volume of 500 mL distilled water, pH to 6.3 with concentrated HCl. Store at room temperature. Use within 6 mo.
10. 20X SSC powder (Vysis): 500 g lots. Store at room temperature. See product label for expiration date.
11. Hydrochloric acid (1 N). (Ricca Chemical Company): 32 oz lots. Store at  $4^{\circ}\text{C}$ . See product label for expiration date.
12. 0.01 N HCl: Add 1 mL of hydrochloric acid (1 N) to 100 mL of distilled water. Store at  $4^{\circ}\text{C}$ . Use within 1 yr.
13. Nonidet P-40 (Vysis): 2 mL lots. Store at room temperature. See product label for expiration date.
14. Pepsin (Sigma): 2100 units/mg solid. 5 gram lots. Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
15. 10% Pepsin solution: Dissolve 1 gram of pepsin into 10 mL of distilled water. Store at  $4^{\circ}\text{C}$ . Use within 3 mo.
16. Pepsin working solution: Add 25  $\mu\text{L}$  of 10% pepsin solution to 40 mL of 0.01 N HCl. Use within 2 h.
17. Sodium chloride (Sigma): 500 g lots. Use within 3 yr.
18. Sodium citrate (Sigma): 12 kg lots. Store at room temperature. Use within 2 yr.
19. Spectra Vysion probe (Vysis): 50  $\mu\text{L}$  lots. Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
20. Vectashield (Vector Laboratories): Store at  $4^{\circ}\text{C}$ . See product label for expiration date.
21. M-FISH probes (*see Note 1*).

## 3. Methods

Based on our experience (**13**) and the manufacturer's guidelines, we present the following M-FISH method (*see Notes 2–4*), precautions, and possible solutions to common problems (*see Notes 5–10*) encountered with this technique.

### 3.1. Slide Preparation

1. Prepare slides according to the standard procedure for the kind of specimen submitted (blood, amniotic fluid, fibroblast culture, or bone marrow).
2. Find suitable metaphases using phase microscopy and etch area to be hybridized.
3. Allow slides to stand for 2 min in a Coplin jar containing 40 mL of pepsin working solution at 37°C.
4. Artificially age the slides on a HYBrite (Vysis) set on hold at 90°C for 2 min.
5. Flood the slide while still on the HYBrite with 2X SSC for 2 min.
6. Dehydrate the cells by placing the slides for 1 min each in a series of Coplin jars containing 40 mL of 70, 85, and 100% ethanol at room temperature. Jet air dry the slides.

### 3.2. Hybridization and Wash

1. Apply the M-FISH probe mix (Vysis) to the marked hybridization area of the slides.
2. Place a coverslip on each slide at the hybridization area and seal the border with rubber cement.
3. Set slides in a humidified HYBrite (Vysis) with the following settings: denaturing temperature at 80°C, denaturing time of 3 min, hybridization temperature of 37°C, hybridization time of 16 h minimum.
4. Wash the slides for 1–2 min in a Coplin jar containing 40 mL of 0.4X SSC at 70°C. Rinse for 2 s under running deionized water.
5. Transfer the slides to a Coplin jar containing 40 mL of fresh 2X SSC/0.1% NP40 solution at room temperature for 10 s.
6. Apply DAPI 10% counterstain to each hybridization area and place a coverslip over the area. Do not seal the coverslip onto the slide.

### 3.3. Analysis

1. Use a high-quality microscope equipped with the following filter sets (*see Note 1*) for fluorescent microscopy: 1) Triple pass FITC/Texas Red/DAPI filter to capture red, green, and counterstain planes. 2) Single pass Gold filter to capture gold plane. 3) Single pass Cy-5 filter to capture far red plane.
2. The analysis is initiated with a  $\times 40$  objective lens and gold filter set for scanning. The choice of gold for scanning is two fold. It is bright and is least likely to photo bleach. When a metaphase is selected, the image is carefully focused with a  $\times 100$  objective lens. Multiple images are then captured using the manufacturer's instructions. When capture software is initiated, the user is asked to move the barrier filter to the correct setting for the first fluorophores plane. After the first exposure, the barrier filter is moved to the next fluorophore and exposed. This process is repeated until all five fluorophores and the DAPI counterstain have been exposed separately. Excitation filters are located in a filter wheel and controlled by the computer software. Any image plane that appears to be out of focus may be recaptured. The computer software then merges all the images to a single image. Each chromosome is individually classified according to the assigned fluorescence signature (provided by the manufacturer). Images can be immediately analyzed and karyotyped or saved for later analysis. We typically capture and analyze five metaphases and karyotype two for each case and/or a clone (**Fig. 5A, B**). Individual chromosome pairs can be pseudocolored differently when necessary to highlight their fluorescence pattern. The pseudocolor of each chromosome pair can be changed. This feature is especially useful to accentuate chromosome rearrangements between two chromosomes where the default pseudocolors are similar.

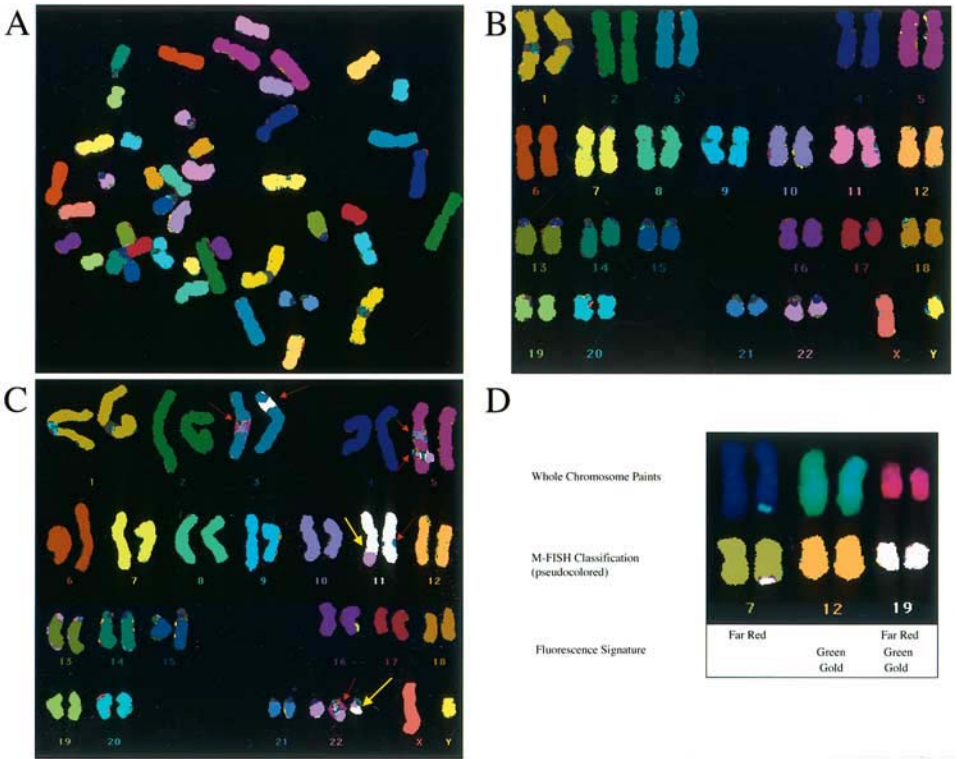


Fig. 5. (A, B) M-FISH analysis of a PHA-stimulated whole blood culture, showing a metaphase and a normal male karyotype. (C) M-FISH karyotype from a fibroblast culture showing a t(11;22) and extra chromosome 22. The yellow arrows identify the translocation, and the red arrows indicate areas of chromosome overlaps. (D) M-FISH analysis from a whole blood culture involving an unbalanced 7;12 translocation. The translocated chromatin on chromosome 7 appears to be from chromosome 19 owing to overlapping signals. Individual chromosome 12 painting probe was used to correctly identify the source of translocated material to chromosome 7 to be chromosome 12.

#### 4. Notes

1. The M-FISH probes, filter set, and computer software are commercially available as accessories to fluorescent image analysis systems from several companies. The M-FISH system that we use (Vysis, Downers Grove, IL) has the following fluor combinations and their respective absorption and emission peaks (nm): Far Red (655/675), Red (592/612), Gold (530/555), Green (497/524), and Aqua (433/480).
2. Cells can be harvested using standard cytogenetic techniques, both for congenital (PHA stimulated blood, amniotic fluid, fibroblast, chorionic villus or abortus tissue) or oncologic (bone marrow, pleural effusions, or solid tumors) tissues. For consistency of chromosome spreading and morphology, we use a Thermantron drying chamber for harvesting. The optimum setting of the chamber for blood harvest is 25°C and 50% relative humidity.

At these settings, a single drop of fixed cell suspension is placed on a dry microscope slide in the drying chamber. The settings are different for each tissue and may vary somewhat depending on the ambient temperature and humidity. It is desirable to check the first slide under phase microscopy for inadequate or over spreading and adjust the temperature and humidity settings accordingly.

3. We have successfully used sequential G-banding followed by M-FISH essentially as it is done for whole chromosome paint probes for banded chromosomes (**14**). The sequential staining requires greater care for optimum results. Immersion oil is removed with xylene (we do not use cover slips) and the G-banded slide is dried in a ventilated hood. Our G-banding procedure includes artificial aging for 1 h at 90°C, trypsinization and staining with Leishman's Giemsa stain. Following removal of immersion oil, slides are destained in 3:1 methanol and glacial acetic acid fixative for 10–15 s and jet air dried. The slide is then treated with 2X SSC for 5–15 min at 37°C followed by 1% formaldehyde (1% formaldehyde/phosphate balanced saline, pH 7.4) for 5 min in room temperature, PBS (phosphate balanced saline, pH 7.4) for 5 min at room temperature, and dehydrated in ethanol series (70, 85, and 100%) for 1 min in each solution. The hybridization procedure and posthybridization washes are the same as outlined.
4. For freshly prepared slides as well as for sequential staining with M-FISH, the M-FISH probe (Vysis, Downers Grove, IL) is placed on the hybridization site, cover slipped, the cover slip is sealed with rubber cement and placed in a humidified HYBrite with the following settings of melting temperature 80°C for 3 min and hybridization temperature 37°C for at least 16 h. The M-FISH results are usually much better from fresh preparations because of the artificial aging necessary for G-banding.
5. One of the most important considerations when using the M-FISH assay is the contrast between targeted hybridizations and nonspecific binding. If the contrast is poor, the chromosome classification colors will also not be sharp. The contrast is especially critical for the far red fluorophores. Increasing the time of exposure to pepsin during the pretreatment procedures can help to improve the contrast. Exposure to pepsin for too long, however, can cause a loss of chromosomal DNA and a loss of signal that can cause a dull appearance. Low humidity in the hybridization chamber can cause the hybridization reagents to dehydrate and increase the concentration of the solution. This in turn could cause probe and chromosomal DNA to hybridize somewhat randomly (loosely) affecting the contrast negatively.
6. Another important factor for optimum results is chromosome morphology. Poor morphology can be a problem because it makes DAPI bands, when converted to gray scale, difficult to interpret. It can also cause “bleeding” of colors from one chromosome to another. The most frequent cause of poor morphology is inadequate aging. Artificially aging or treating the slide with hot 2X SSC for 2 min may improve the morphology. Frequently, rinsing the slide briefly in deionized water at room temperature then counterstaining with DAPI will also improve morphology but may reduce the intensity of hybridization. Increased aging and/or prolonged 2X SSC pretreatment can also reduce hybridization intensity. Poor morphology may also result from over denaturation. Good morphology and poor hybridization is usually indicative of too much aging, excessive exposure to 2X SSC, or under denaturation. A “dirty” preparation with a lot of background debris can also compromise results. Brightly fluorescent debris, especially prevalent in Texas red, can decrease the exposure duration and thus cause problems with chromosome classification. This problem can be corrected by exposing the Texas red plane with debris for longer periods of time by use of a manual setting.

7. Sometimes, despite best efforts, an optimum result may be elusive when there is nothing wrong with the reagents or the procedure. It is then necessary to examine each image plane individually and compare them with the table provided by the manufacturer. For instance, chromosome 22 (aqua, red, and green) is sometimes misclassified as chromosome 3 (aqua). One can examine the green and red planes individually to make sure that these spectra are present to confirm the identity for chromosome 22.
8. At the junctions of break and fusion points of interchromosomal rearrangements and chromosomal overlaps, chromosome classification may sometimes indicate the involvement of a "third chromosome" or be "grayed out" owing to overlapping fluorophores (**Fig. 5C**). This is particularly critical when translocated segments are small. All possible chromosomes should be considered for the color classification observed. For instance, a derivative chromosome 7 with a small translocated segment of chromosome 12 may actually be classified as a chromatin from chromosome 19 (**Fig. 5D**). This is because the label for chromosome 7 is far red, that of chromosome 12 is gold and green, and the combination of the three (gold, far red, and green) represents the fluorophore signature for chromosome 19. It is therefore imperative that to resolve the problem, the sample must be analyzed by wcp12 probe. If positive for wcp12, the segment is concluded to be from chromosome 12 and if the result is negative, then it must be from chromosome 19. Gray areas also appear when a combination of fluors occur that is outside of the range of the classification system. For instance, if an overlap occurs between chromosomes 20 (far red, aqua, and red) and 21 (gold and aqua) resulting in four colors (far red, aqua, red, and gold), a gray area will be encountered since no chromosome is assigned more than three colors. The illustrations in **Fig. 5** are from our publication in the *Journal of Association of Genetic Technologists* (**13**).
9. Small translocations, especially insertions, can be misread especially if the derivative chromosome's fluorophore signature contains all the fluorophores of the translocated chromosome. An example would be a small segment of chromosome 1 (gold) translocated to chromosome 5 (gold and far red). The entire length may appear to be chromosome 5. Again, this can be resolved by use of individual chromosome paint probes for chromosome 1.
10. Constitutive heterochromatin including segments rich in alpha satellite and acrocentric p-arms rich in repetitive DNA sequences are either blocked out by use of unlabeled Cot 1 DNA or do not have complementary DNA sequences in the probes. These segments are also "grayed out" or randomly assigned a pseudocolor and classified (**Fig. 5C**). This is especially important to bear in mind when analyzing markers or derivative chromosomes. Other limitations of M-FISH include the inability to detect most intra-chromosomal abnormalities such as inversions and inter-chromosomal anomalies especially if they are 3 Mb or less.

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# Characterization of Constitutional Chromosome Abnormalities by Comparative Genomic Hybridization

Brynn Levy and Kurt Hirschhorn

## 1. Introduction

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that can characterize excess and missing cytogenetic material often unrecognizable by G-banding, in a one step global screening procedure. The advantage of CGH over conventional fluorescence *in situ* hybridization (FISH) with whole chromosome paints (wcp) and multicolor FISH is its ability to identify not only the chromosome from which the additional unknown material was derived but also to map the region involved to specific bands on the source chromosome. Defining the origin of additional cytogenetic material by FISH with various probes is expensive and laborious as numerous wcp may be required until the source chromosome is identified. In addition, the number of available region specific probes is limited and covers only a fraction of the genome.

To date, more than 1100 articles have been published on CGH with approximately 90% reporting the utility of CGH to delineate cytogenetic changes in cancer specimens. About 6% of CGH papers have dealt with technical aspects and only a limited number have described the application of CGH in a clinical cytogenetics setting. CGH has been particularly useful in clinical cytogenetics by facilitating the identification and characterization of intrachromosomal duplications, deletions, unbalanced translocations and marker chromosomes, including neocentric marker chromosomes (1-4). In addition to identifying the origin of extra/missing chromosomal material, CGH also maps the chromosomal position of the lost or gained material to specific chromosomal G-bands. CGH analysis software is now readily available as an optional addition to many FISH imaging systems and does not require additional specialized equipment. With many comprehensive cytogenetic laboratories now acquiring image analysis equipment for routine cytogenetic and FISH analysis, the utilization of CGH as a tool in clinical cytogenetics is likely to increase. In addition to being able to identify excess and/or missing chromosomal material not resolvable by G-banding, CGH could also

From: *Methods in Molecular Biology, Vol. 204: Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

be used as a backup method for aneuploidy analysis of specimens that have failed to grow in cell culture. This would be particularly useful in the analysis of nonviable fetal tissue derived from products of conception which are estimated to have a chromosome abnormality (mainly aneuploidy) approx 50% of the time (5).

CGH was developed in 1992 by Kallioniemi et al. (6) who utilized it to identify 16 different previously unknown regions of amplification in tumor cell lines and primary bladder tumors. CGH effectively reveals any DNA sequence copy number changes (i.e., gains, amplifications, or losses) in a particular specimen and maps these changes on normal chromosomes (6–9). CGH can detect changes that are present in as little as 30–50% or more of the specimen cells (9). It does not reveal balanced translocations, inversions, and other aberrations that do not change copy number. CGH is accomplished by simultaneous *in situ* hybridization of differentially labeled total genomic specimen DNA and normal reference DNA to normal human metaphase chromosome spreads (6–9). Hybridized specimen and reference DNA can be distinguished by their different fluorescent colors. The relative amounts of specimen and reference DNA hybridized at a particular chromosome position are contingent on the relative excess of those sequences in the two DNA samples and can be quantified by calculation of the ratio of their different fluorescent colors (6–9). Specimen DNA is traditionally labeled with a green fluorochrome such as fluorescein isothiocyanate (FITC) and the normal reference DNA with a red fluorochrome like Texas Red. A gain of chromosomal material in a specimen can be detected by an elevated green to red ratio, while deletions or chromosomal losses produce a reduced green to red ratio (6–9).

### **1.1. The Sensitivity of CGH in Clinical Cytogenetics**

The sensitivity of CGH is an issue that has received much attention. The theoretical detection limit of deletions by CGH has been estimated to be about 2 Mb (Piper, 1995 [10]). We have been able to identify deletions and duplication and marker chromosomes as small as 3–6 Mb using our standard CGH protocol. Conventional cytogenetic analysis detects imbalances in the range of 5–10 Mb at the 400-band level and 2–3 Mb at the 1000 band level. It would therefore appear that the detection limits of CGH correlates with what is observed in standard G-banded chromosome preparations, i.e. if chromosomal imbalance is not evident on routine cytogenetic analysis, it is unlikely that CGH analysis will provide additional “karyotypic” information.

#### **1.1.1. CGH Workup of Suspected Inversion Cases**

CGH together with FISH may be useful in resolving the question of whether an unusual banding pattern observed on a particular derivative chromosome is the result of a small duplication or a subtle inversion. In such cases, a whole chromosome paint is necessary to confirm that the abnormal chromosome does not contain material from other chromosomes. If the derivative chromosome paints completely and uniformly, then a normal CGH result would imply that the derivative chromosome is most likely an inversion. In some cases, the breakpoints could be ascertained by visual inspection of the GTG image. However, further molecular cytogenetic analysis would be required to define the exact breakpoints. Obviously, a gain in the CGH ratio of that chromosome would confirm the presence of an duplication.

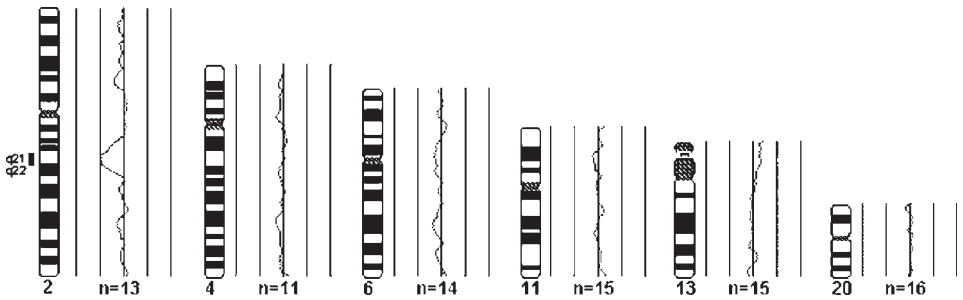


Fig. 1. CGH analysis on a patient with an apparently balanced translocation reveals a small interstitial deletion of 2q21–q22.

### 1.1.2. CGH Workup of Clinically Abnormal Cases with Apparently Balanced Translocations

In balanced translocation cases where the clinical picture suggests a chromosomal etiology, CGH analysis has the potential to reveal subtle imbalances at the site of the breakpoints (**Fig. 1**). When the CGH results indicate normal ratio profiles for all chromosomes, the translocation would appear to be truly balanced. It is still possible that the patient's clinical phenotype may have resulted from a microdeletion or from a disruption of critical genes at the breakpoint regions.

### 1.1.3. CGH Workup of Clinically Abnormal Cases with Normal Karyotypes

As with unbalanced translocations, CGH workup of cases with normal karyotypes is only recommended when the clinical presentation of the patient is strongly suggestive of a chromosome abnormality. CGH analysis would only be useful in those rare cases when an unequal exchange of chromatin with similar banding patterns has occurred, giving the false impression of a normal karyotype. When both the GTG pattern and the CGH results are normal, the etiology of the patient's phenotype may be attributed to other genetic and/or environmental factors. A normal CGH result, in such cases, also has to be interpreted within the boundaries of the test's limitations i.e., a normal CGH result does NOT rule out balanced cryptic rearrangements nor does it rule out submicroscopic imbalances such as microdeletions.

## 1.2. Working with the GTG Karyotype

While the CGH results add valuable information to the karyotype, the GTG image remains an important factor when revising the karyotype. The identification of a triplicated region in **Fig. 2** could not have been made by CGH alone and required correlating the over-represented regions with the GTG image. In some cases where CGH has delineated the specific regions comprising marker chromosomes and duplications, the orientation of the additional material can be assessed by visual inspection of the GTG image without requiring further molecular cytogenetic investigation.

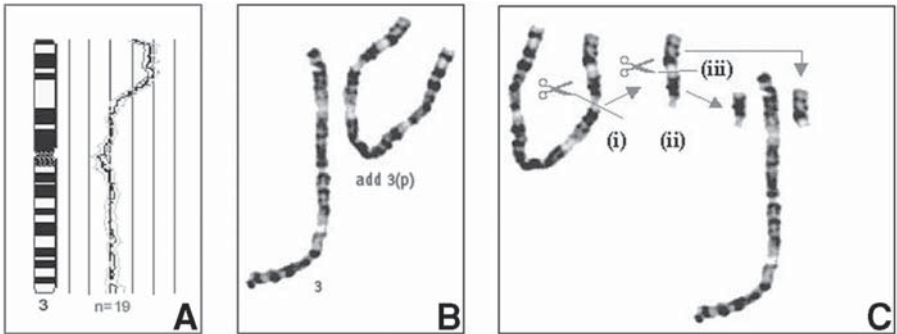


Fig. 2. Identification of a triplicated region by correlating the CGH result with the GTG image. (A) Average CGH ratio profile of chromosome 3 showing significant over-representation of regions 3p25.3 to 3p21.33. The average ratio is illustrated between the (upper) and (lower) 95% confidence intervals. (B) Partial G-banded karyotype showing the normal chromosome 3 and the add 3p chromosome (C) (1) Digital excision of the additional chromosomal material from the derivative chromosome. (2) and (3) Alignment of portions of the additional material (digitally separated) with the 3p25.3–3p21.33 region of the normal chromosome 3.

### 1.3. Overview of CGH

The major steps in CGH involve:

1. Preparation of karyotypically normal 46, XY metaphase spreads.
2. Isolation of high molecular weight DNA from patient (specimen) and normal (reference) samples.
3. Labeling of patient and reference DNA with different fluorochromes by nick translation.
4. In situ hybridization of equal amounts of labeled specimen and reference DNAs together with unlabeled Cot1-DNA to normal metaphase spreads.
5. Washing off unbound DNA.
6. Counterstaining metaphase spreads with DAPI for chromosome identification.
7. Fluorescence microscopy utilizing fluorochrome specific filters to visualize and capture color ratio differences along the chromosomes.
8. Chromosome identification by the inverted DAPI banding pattern.
9. Quantitation of copy number differences by generating a ratio profile of the specimen to reference DNA fluorescence intensities along each chromosome.
10. Combining the profiles from several metaphase spreads to improve the significance of the result.

## 2. Materials

### 2.1. Normal Male Peripheral Blood Culture

1. Fresh blood (~10 mL) from a karyotypically normal male should be drawn into sodium heparin tubes and kept at room temperature; (the blood should not be frozen). The blood may also be stored overnight at 4°C if necessary (*see Note 1*).
2. Freshly made fixative (3:1 methanol:glacial acetic acid).
3. KaryoMAX colcemid (10 µg/mL) (Gibco-BRL; Life Technologies, Grand Island NY).

4. Colcemid solution: Dilute KaryoMAX colcemid 1:1 with sterile water (store at 4°C).
5. RPMI 1640 medium with L-glutamine 1X (Cellgro; Mediatech Inc., Herndon VA).
6. Fetal bovine serum (Irvine Scientific, Santa Ana CA).
7. Penicillin (10,000 U/mL) Streptomycin (10,000 µg/mL) (Gibco-BRL; Life Technologies, Grand Island NY).
8. L-glutamine (200 mM) (Gibco-BRL; Life Technologies, Grand Island NY).
9. Phytohemagglutinin (45 mg) (Irvine Scientific, Santa Ana CA). Reconstitute in 4.5 mL of sterile distilled H<sub>2</sub>O to give a stock solution of 10 mg/mL (store at 4°C).
10. HEPES buffer solution (1 M) (Gibco-BRL; Life Technologies, Grand Island NY).
11. KCl (0.075 M). Store at room temperature and prewarm at 37°C before use.
12. 1X Dulbecco's phosphate buffered saline (PBS) (Cellgro; Mediatech Inc., Herndon VA).
13. Thymidine solution (15 mg/mL) made up in PBS (aliquot and store at -20°C).
14. Starting medium (100 mL): 86 mL RPMI 1640 medium with L-glutamine, 10 mL fetal bovine serum, 1 mL penicillin-streptomycin, 1 mL L-glutamine, 2 mL phytohemagglutinin.
15. Continuing medium (100 mL): 87 mL RPMI 1640 medium with L-glutamine, 10 mL fetal bovine serum, 1 mL HEPES buffer, 1 mL penicillin-streptomycin, 1 mL L-glutamine.
16. Pasteur pipets.

## **2.2. Preparation of Normal Male Target Metaphase Spreads**

1. Precleaned glass microscope slides.
2. Freshly made fixative (3:1 methanol:glacial acetic acid).
3. Coplin jar.
4. 1X Dulbecco's phosphate buffered saline (PBS) (Cellgro; Mediatech Inc., Herndon VA).
5. 1% Formaldehyde post fixation solution: 1.35 mL of 37% formaldehyde and 48.65 mL of PBS. Store at 4°C and use within 1 mo.
6. Ethanol series: 70, 85, and 100% ethanol. Store at room temperature. May be reused for 1 wk if used on a daily basis. May be reused for up to 4 wk if used only once or twice a week.

## **2.3. Isolation of High Molecular Weight DNA from Patient and Normal Samples**

1. Blood (≥3 mL) from the patient should be drawn into EDTA or sodium heparin tubes and kept at room temperature (*see Note 2*).
2. Blood (≥10 mL) from a sex-matched normal reference control should be drawn into EDTA or sodium heparin tubes and kept at room temperature (*see Note 3*).
3. PureGene DNA Purification Kit (Gentra Systems, Minneapolis, MN).

## **2.4. Probe Labeling by Nick-Translation**

1. Concentrated DNA (~0.2–1.5 µg/µL). Label 1 µg of DNA at a time.
2. Ice bucket or 15°C waterbath.
3. Waterbath set to 72°C.
4. Fluorescein-12-dUTP (1 mM) (Perkin Elmer Life Sciences NEN, Boston, MA). Store at -20°C.
5. Texas Red<sup>®</sup>-5-dUTP (1 mM) (Perkin Elmer Life Sciences NEN, Boston, MA). Store at -20°C.
6. dNTP set (dATP, dCTP, dGTP, dTTP each at 100 mM) (Life Technologies, Grand Island NY).
7. 14.3 M 2-Mercaptoethanol (Sigma, St. Louis MO).
8. Bovine serum albumin (20 mg/mL) (Sigma, St. Louis MO).
9. dNTP mix: 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.03 mM dTTP, 500 mM Tris pH 7.5, 50 mM MgCl<sub>2</sub>, 100 mM 2-mercaptoethanol, 100 µg/mL bovine serum albumin. Aliquot and store at -20°C.

10. RNase-Free DNase (1 U/ $\mu$ L) (Promega, Madison, WI). Store at  $-20^{\circ}\text{C}$ .
11. RNase-Free DNase (various concentrations: 0.01–0.1 U/ $\mu$ L) (Promega, Madison WI). Dilute DNase to appropriate concentrations using chilled distilled  $\text{H}_2\text{O}$ . Prepare fresh and discard after use (*see Note 4*).
12. *E. Coli* DNA Polymerase I (10 U/ $\mu$ L) (New England Biolabs, Inc., Beverly MA). Store at  $-20^{\circ}\text{C}$ .
13. 10X reaction buffer mix (supplied with *E. Coli* DNA Polymerase I): 100 mM Tris-HCl, 50 mM  $\text{MgCl}_2$ , 75 mM dithiothreitol. Store at  $-20^{\circ}\text{C}$ .
14. 50X Tris-acetate (TAE): 242g Tris-base, 57.1 mL glacial acetic acid, 100 mL 0.5 mL EDTA (pH 8.0),  $\text{H}_2\text{O}$  to 1 L. Store at room temperature.
15. 1X TAE buffer: 10 mL 50X TAE, 490 mL  $\text{H}_2\text{O}$ . Store at room temperature.
16. Ethidium bromide (1% solution) (Fisher Scientific, Fair Lawn NJ).
17. Electrophoresis buffer with ethidium bromide: 10 mL 50X TAE, 490 mL  $\text{H}_2\text{O}$ , 25  $\mu$ L of 1% ethidium bromide (EtBr) solution. EtBr is carcinogenic and mutagenic. Use gloves when preparing solution and discard as hazardous waste. Store at room temperature and protect from light by covering container with aluminum foil. May recycle used buffer for future use. Use recycled buffer up to 6–8 $\times$ .
18. 1% agarose gel (1 g agarose melted in 100 mL 1X TAE buffer). Prepare fresh.
19. Minigel apparatus and power supply.
20. UV transilluminator, UV protective visor, and goggles.
21. Reaction can be scaled up for labeling of  $>1$   $\mu\text{g}$  of DNA (*see Note 5*).

## 2.5. Preparation of CGH Probes

1. Labeled patient DNA (200 ng) and labeled reference DNA (200 ng) from the nick translation reaction.
2. Human Cot-1 DNA (1  $\mu\text{g}/\mu\text{L}$ ) (Gibco-BRL; Life Technologies, Grand Island NY).
3. 3 M Sodium acetate: Dissolve 408.1 g of sodium acetate $\cdot$ 3  $\text{H}_2\text{O}$  in 800 mL of  $\text{H}_2\text{O}$ . Adjust the pH to 5.2 with glacial acetic acid.  $\text{H}_2\text{O}$  to 1 L.
4. 100% Chilled ethanol. Store at  $-20^{\circ}\text{C}$  and bring out just prior to use.
5. Cotton tipped applicators (Citmed, Citronelle AL).
6. Slide warmer set to  $\sim 42^{\circ}\text{C}$ .
7. Metal block or PCR block that holds 1.5 mL microfuge tubes. Place block on slide warmer so that the block warms to approx  $42^{\circ}\text{C}$ .
8. Hybrisol VII hybridization mix (50% formamide, 2X sodium saline citrate) (Ventana Medical Systems, Inc., Tucson AZ).

## 2.6. Denaturation of Target Metaphase Slides

1. Coplin jars
2. 20X sodium saline citrate (SSC) (Q-Biogene, Carlsbad CA). Store at room temperature.
3. 2X SSC solution: 50 mL 20X SSC, 450 mL distilled water. Store at room temperature.
4. Deionized formamide (Q-Biogene, Carlsbad CA). Store at  $4^{\circ}\text{C}$ .
5. Denaturation solution (pH 7): 70% formamide, 2X SSC. Can be made in large quantities (500 mL and 1 L) and stored at  $4^{\circ}\text{C}$ . Denaturation solution may also be reused up to 4 or 5 $\times$  and stored at  $4^{\circ}\text{C}$  between use.
6. Waterbath set to  $75 \pm 1^{\circ}\text{C}$ .
7. Slide warmer set to  $40 \pm 2^{\circ}\text{C}$ .
8. Ethanol series (same as in **Subheading 2.2., step 6**): 70, 85, and 100% ethanol. Store at room temperature. May be reused for 1 wk if used on a daily basis. May be reused for up to 4 wk if used only once or twice a week.

9. Chilled ethanol series: 70%, 85% and 100% ethanol. Store at  $-20^{\circ}\text{C}$ . May be reused for 1 week if used on a daily basis. May be reused for up to 4 weeks if used only 1 $\times$  or 2 $\times$  a wk.

## **2.7. Probe Denaturation**

1. Waterbath set to  $75 \pm 1^{\circ}\text{C}$ .

## **2.8. Hybridization**

1. Slide warmer set to  $40 \pm 2^{\circ}\text{C}$ .
2. Incubator set to  $37^{\circ}\text{C}$ .
3. 22  $\times$  22 mm glass coverslips.
4. Rubber cement.
5. Syringe: (1, 3, 5, or 10cc; chose size according to the number of slides you will be preparing) carefully discard needle.
6. Humid chamber: Most empty pipet containers may be utilized by placing a paper towel in the lower section under the rack and saturating the paper towel with water.

## **2.9. Post-Hybridization Wash**

1. 20X Sodium saline citrate (SSC) (Q-Biogene, Carlsbad CA). Store at room temperature.
2. 4X SSC Solution: 100 mL 20X SSC, 400 mL distilled water. Store at room temperature.
3. 4X SSC/0.1% Triton X-100 solution: 100 mL 20X SSC, 0.5 mL Triton X-100, 399.5 mL distilled water. Store at room temperature.
4. 2X SSC Solution: 50 mL 20X SSC, 450 mL distilled water. Store at room temperature.
5. Distilled water.
6. Waterbath set to  $75 \pm 1^{\circ}\text{C}$ .
7. Waterbath set to  $39^{\circ}\text{C}$ .
8. Coplin jars.
9. Antifade Mounting Medium (Vectashield, H-1000) (Vector Laboratories, Burlingame, CA).
10. DAPI stock solution (1.5  $\mu\text{g}/\text{mL}$ ) (Vectashield with DAPI, H-1200) (Vector Laboratories, Burlingame, CA).
11. DAPI counterstain (0.5  $\mu\text{g}/\text{mL}$ ): Dilute DAPI stock solution with the Vectashield antifade mounting medium as follows: 400  $\mu\text{L}$  DAPI stock solution; 800  $\mu\text{L}$  anti-fade mounting medium.
12. 24  $\times$  60 mm glass coverslips.

## **3. Methods**

### **3.1. Normal Male Peripheral Blood Culture**

1. Add 500–650  $\mu\text{L}$  of whole fresh blood from a karyotypically normal male to 10 mL of starting medium.
2. Incubate at  $37^{\circ}\text{C}$  for  $\sim 72$  h (3 d).
3. Add 200  $\mu\text{L}$  of thymidine solution and gently mix.
4. Incubate at  $37^{\circ}\text{C}$  for 14–18 h (Overnight).
5. Centrifuge at  $\sim 155g$  for 10 min.
6. Discard supernatant and add 10 mL 1X PBS. Invert to mix.
7. Centrifuge at  $\sim 155g$  for 10 min.
8. Discard supernatant and add 10 mL of continuing medium. Invert gently to mix.
9. Incubate at  $37^{\circ}\text{C}$  for 4–5 h.
10. Add 6–7 drops of colcemid solution. Invert to mix.

11. Incubate in a 37°C water bath for 20 min.
12. Centrifuge at ~170g for 10 min.
13. Remove supernatant, leaving about 0.5 mL. Tap each tube several times to resuspend cells. Do this thoroughly to avoid clumping. Add 0.5 mL 75 mM KCl (which has been prewarmed to 37°C) quickly and tap tube to mix. Add 4.5 mL more KCl.
14. Incubate in a 37°C waterbath for 15 min.
15. Slowly add 1 mL of fresh fixative to each tube from the bottom up with a pasteur pipet. Invert gently to mix.
16. Centrifuge at ~170g for 10 min.
17. Remove supernatant except for about 0.5 mL.
18. Add fresh fixative drop-wise while mixing the tube. Add a total of 5 mL fixative.
19. Incubate at room temperature for 10 min.
20. Centrifuge at ~170g for 10 min.
21. Remove all of the supernatant except for about 0.5 mL. Flick pellet.
22. Add fresh fixative drop-wise while mixing the tube. Add a total of 5 mL fixative.
23. Repeat **steps 20–22** two–three more times as necessary.
24. Remove supernatant.
25. Dilute pellet with appropriate amount of fix. The solution should be turbid and slightly cloudy (i.e., if a large pellet is obtained, add ~ 1–1.5 mL of fix. Add < 1 mL of fix if smaller pellet is obtained (*see Note 6*).
26. Drop slides as detailed in **Subheading 3.2**.

### **3.2. Preparation of Normal Male Target Metaphase Spreads**

1. Prepare a closed environment with a relative humidity of approximately 55% (can range from 50–60% humidity) and a temperature of about 24–26.5°C.
2. Hold a Pasteur pipet about 2–4 in above the glass slide and let a single drop of the cell suspension fall onto the left hand side of the slide. Repeat quickly and let the second single drop fall on the right hand side of the slide (*see Note 7*).
3. Before all the fixative evaporates, immerse the slide into a Coplin jar containing fresh fixative for 1–3 s. Remove the slide from the Coplin jar and allow it to air dry in the closed humid/temperature environment described in **step 1** above.
4. Check quality of the slides under light microscope (*see Note 8*).
5. Leave slides overnight at room temperature and then perform a further postfixation step by immersing the slides in a 1% formaldehyde solution for 5–10 min at 4°C.
6. Dehydrate slides by running them through an ethanol series, 2 min in each of 70, 85, and 100% ethanol.
7. Allow slides to air dry at room temperature or gently blow air (from laboratory air flow) over slides to dry quickly.
8. Store slides at room temperature for 2–4 wk.
9. Perform a test CGH analysis on one of the slides using either a normal reference vs normal specimen or using a specimen with a known chromosomal imbalance (*see Note 9*).

### **3.3. Isolation of High Molecular Weight DNA from Patient and Normal Samples**

DNA should be extracted according to the manufacturer's instructions. Alternatively, DNA may be extracted using standard methods (**10**) or any reliable DNA extraction kit.

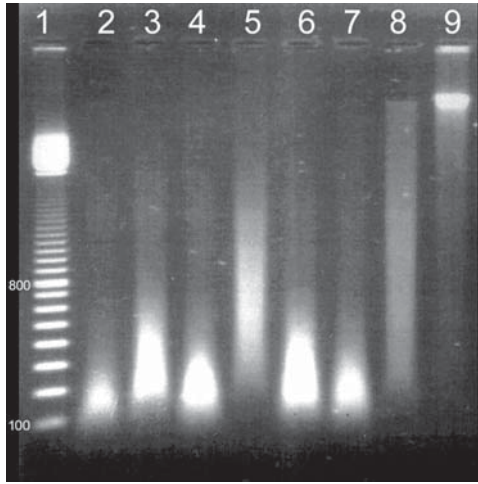


Fig. 3. Electrophoresis gel (1% agarose) of Nick-translation products which have been titrated using varying concentrations of DNase. (Lane 1): 100 bp DNA ladder. (Lanes 5 and 8) show fragment sizes that would be suitable for preparation of CGH probes.

### 3.4. Probe Labeling by Nick Translation

1. Fluorescein-12-dUTP is the fluorochrome used to label patient DNA, and Texas Red<sup>®</sup>-5-dUTP is the fluorochrome used to label the reference DNA.
2. Prepare a 15°C waterbath, or add small amounts of ice to an ice bucket filled with tap water until the temperature reaches 15°C.
3. Set another waterbath to 72°C.
4. Add the following to a 1.5-mL microfuge tube (keep all reagents chilled on ice): 1 µg patient or reference DNA; 5 µL 10X reaction buffer mix; 5 µL dNTP mix; 1 µL fluorochrome-dUTP (1 mM); 1 µL DNase (0.01–0.1 U/µL); 2.5 µL DNA Pol I (10 U/µL).
5. Total volume made up to 50 µL with H<sub>2</sub>O.
6. Spin the tubes briefly to collect the reagents at the bottom.
7. Incubate for 60 min in 15°C waterbath.
8. Stop reaction with a 10 min incubation at 72°C.
9. Run a 1% agarose gel to check the fragment sizes. The optimal fragment sizes for CGH are from 300–2000 bp (**Fig. 3**). If the fragment sizes are too long, further incubation at 15°C with DNase I can be done.
10. Reaction can be scaled up for nick translation of larger samples.

### 3.5. Preparation of CGH Probes

Add the following to a 1.5 mL microfuge tube:

1. 200 ng of labeled normal (reference) DNA from nick-translation reaction (10 µL) (*see Note 10*).
2. 200 ng of labeled patient DNA from nick translation reaction (10 µL).
3. 20–30 µg of Cot-1 DNA (20–30 µL) (*see Note 11*).
4. Add 1/10th vol of 3 M sodium acetate (4–5 µL).

5. Add 150  $\mu\text{L}$  ( $>2$  vol) of 100% ice cold ethanol.
6. Place at  $-80^{\circ}\text{C}$  for 40–60 min.
7. Centrifuge for 40 min at 29,000g (or highest speed on centrifuge) at  $4^{\circ}\text{C}$ .
8. Remove ethanol by gently pouring off.
9. Wipe remaining ethanol with a sterile cotton tipped applicator (do not touch pellet).
10. Place microfuge tube on metal block which is on the slide warmer (set to  $42^{\circ}\text{C}$ ) to aid the evaporation of remaining ethanol. This should take approx 2–3 min.
11. Resuspend pellet in 10  $\mu\text{L}$  of Hybrisol VII hybridization mix by pipeting up and down and vortexing for about 1 min.
12. The CGH probe can now be used immediately or may be stored at  $-20^{\circ}\text{C}$  protected from light until needed.

### **3.6. Denaturation of Target Metaphase Slides**

1. Pour approx 50 mL of the denaturation solution into a Coplin jar and place Coplin jar in a waterbath set to  $75\text{--}76^{\circ}\text{C}$ . Prewarm the denaturation solution to a temperature of  $\sim 73 \pm 2^{\circ}\text{C}$  (see **Note 12**).
2. Take required number of target metaphase slides out from the  $-20^{\circ}\text{C}$  freezer and run the slides through the room temperature ethanol series (70, 85, and 100%), 2 min in each.
3. Allow slides to dry (5 min) or gently blow air (from laboratory air flow) over slides to dry.
4. Prewarm slides on a  $37\text{--}40^{\circ}\text{C}$  slide warmer for  $\sim 30$  s.
5. Denature slides by immersing them in the denaturation solution (at  $73 \pm 1^{\circ}\text{C}$ ) for 2.5–5 min (see **Note 13**).
6. Quickly place slides in ice cold 70% alcohol for 3 min and then continue dehydrating the slides in the 85 and 100% ethanol series for 3 min each.
7. Allow slides to dry (5 min) or gently blow air (from laboratory air flow) over slides to dry.

### **3.7. Probe Denaturation**

1. While running the target metaphase slides through the ethanol series, place the microfuge tube with the CGH probe in a water bath set to  $75^{\circ}\text{C} \pm 1$  and denature the probe for 10 min.

### **3.8. Hybridization**

1. Fill a syringe (needle already discarded) with rubber cement.
2. Prewarm slides at about  $37\text{--}42^{\circ}\text{C}$  for  $\sim 1$  min on slide warmer.
3. While slides are on the slide warmer, place each denatured CGH probe onto a single hybridization area (see **Note 10**) of the denatured slide and cover each area with a  $22 \times 22$  mm glass coverslip.
4. Seal the glass coverslip by squeezing rubber cement from the syringe along the edges of the coverslip.
5. Incubate the slides at  $37^{\circ}\text{C}$  in a humid chamber for 2–3 d.

### **3.9. Post-Hybridization Washing**

1. Pour approx 50 mL of the 2X SSC solution into a Coplin jar and prewarm the solution to  $72 \pm 2^{\circ}\text{C}$  (waterbath should be set to about  $75^{\circ}\text{C}$ ).
2. Pour approx 50 mL of the 4X SSC solution in to a Coplin jar and prewarm the solution to  $37^{\circ}\text{C}$  (waterbath should be set to about  $39^{\circ}\text{C}$ ).
3. Pour approx 50 mL of the 4X SSC/0.1% TritonX solution into a Coplin jar and prewarm the solution to  $37^{\circ}\text{C}$  (waterbath should be set to about  $39^{\circ}\text{C}$ ).
4. Pour approx 50 mL of the 2X SSC solution into a Coplin jar and leave at room temperature.
5. Pour approx 50 mL of distilled  $\text{H}_2\text{O}$  into a Coplin jar and leave at room temperature.

6. Remove rubber cement from glass slide.
7. Gently remove the coverslip (without scratching slides).
8. Place the slides in the 2X SSC solution at  $72 \pm 2^\circ\text{C}$ , agitate the slides for ~ 2 and then leave in the solution for 5 min.
9. Transfer the slides to the 4X SSC solution at  $37^\circ\text{C}$  and leave for 5 min.
10. Transfer the slides to the 4X SSC/0.1% Triton X solution at  $37^\circ\text{C}$  and leave for 5 min.
11. Transfer the slides back to the 4X SSC solution at  $37^\circ\text{C}$  and leave for 5 min. (Can use the same solution as in **step 9**).
12. Transfer the slides to the 2X SSC solution at room temperature for 5 min.
13. Transfer the slides to the  $\text{H}_2\text{O}$  at room temperature and leave for 5 min.
14. Remove the slides from the  $\text{H}_2\text{O}$  and allow slides to air dry at room temperature in the dark or gently blow air (from laboratory air flow) over slides to dry quickly.
15. Apply 10  $\mu\text{L}$  DAPI counterstain to the target area of the slide.
16. Apply coverslip.
17. Leave slides in dark for 5–10 min and then proceed with CGH capturing and analysis.
18. Capture at least 10 of each autosome and 7 of each sex chromosome.

#### 4. Notes

1. A male reference is chosen so that both the X and Y chromosome are represented on the normal target metaphase spreads.
2. If the initial cytogenetic workup of the patient was performed by another laboratory, then blood from the patient should be drawn into a sodium heparin tube so that DNA can be extracted and a peripheral blood culture can also be set up for FISH confirmation if required. In such cases, obtaining more than 3 mL of blood would be optimum. When the patient is an infant, < 3 mL of blood is often obtained and in these cases, you may only have enough blood for DNA extraction (CGH analysis).
3. Large quantities of male and female reference DNA may be extracted and stored at  $-20^\circ\text{C}$  until needed.
4. With each fresh batch of DNase, try a range of concentrations from 0.0–0.1 U/ $\mu\text{L}$  and see which gives the best fragment sizes on an agarose gel. This concentration generally remains optimum.
5. A stock of labeled reference DNA may be generated by labeling more than 1  $\mu\text{g}$  of DNA at a time. Store stock solution at  $-20^\circ\text{C}$ .
6. A test slide should be prepared to examine the mitotic index and assess if the suspension needs to be diluted with fixative or concentrated by centrifugation and resuspension in a lower volume of fixative.
7. Dropping in two areas on the slide allows for two independent CGH hybridizations to occur on a single slide. Make sure that both drops are well separated.
8. When preparing the target metaphase slides, the goals are: (1) obtain metaphases that are evenly spread, relatively straight and have minimal overlaps, (2) minimize the amount of cytoplasmic debris on the slide and over the chromosomes, and (3) obtain chromosomes that appear greyish (not too black and/or “shiny” nor too light or “ghostly”) under phase microscopy.
9. The quality of the target metaphase slides appears to be a key factor in the CGH procedure. Each batch of slides should be tested and suitable batches should be stored at  $-20^\circ\text{C}$ . Begin test CGH analysis at about 2 wk after dropping slides and once satisfactory results are obtained, store slides at  $-20^\circ\text{C}$ . Generally, the quality tends to hold true for the entire batch, i.e., if a particular batch does not give satisfactory result either keep these slides for other purposes such as FISH or discard. Laboratories that are proficient in making

metaphase slides should first try their regular protocol for preparation of metaphase slides. In many cases, their protocol will provide metaphases suitable for CGH analysis if they fit the criteria mentioned in **Note 8**.

10. When preparing CGH probes, consider preparing two separate probes, one where the reference is sex matched to the patient and the other where the reference is sex mismatched to the patient. The sex matched probe is important as it will reveal imbalances involving the sex chromosomes. If each probe is used on each of the two hybridization areas of a single slide (*see Note 7*), then the sex mismatched probe will serve as an internal control as color ratio differences are expected along the entire length of the sex chromosomes.
11. Use ~ 20 µg Cot-1 DNA for marker cases and 30 µg for all other cases. As most marker chromosomes contain centromeric material, in some cases, using less Cot-1 DNA improves the detection.
12. Denaturation solution should be prewarmed for at least 30 min. Solution can be microwaved at full power for about 20 s to speed up the prewarming period.
13. Determine the optimal denaturation time for each batch of slides. Suitable slides will show both good hybridization and good inverse DAPI bands across a wider range of denaturation times. Try an initial denaturation time of 3.5–4 min.

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## Color Banding

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### 1. Introduction

#### 1.1. History

##### 1.1.1. Banding Techniques

In the early 1970s, a number of chromosome banding techniques were developed which produced reproducible transverse bands of different lengths along human chromosomes. These techniques included Q-banding, a fluorescent banding technique discovered by Caspersson in 1969; G-banding, proposed in 1971 by both Sumner and Seabright; and R-banding, introduced by Dutrillaux and Lejeune in 1971 (1-4). Today conventional G-banding induced by either pretreatment with hot salt solutions or proteolytic enzymes has remained the gold standard in chromosome identification.

While G-banding has remained the gold standard in clinical cytogenetics, morphologic and technical difficulties can prevent chromosome identification when the chromosomes are too short to detect small aberrations, when the chromosome morphology is poor, when karyotypes contain marker chromosomes, or chromosomes that have arisen from complex multiple rearrangements.

##### 1.1.2. Fluorescent In Situ Hybridization

The fluorescent in situ hybridization (FISH) technique, developed in 1986, in which colored DNA probes hybridize to various chromosome centromeres, was the beginning of the development of a number of subsequent FISH techniques that attempt to delineate chromosome abnormalities unresolved by conventional cytogenetics (5). In 1988, DNA painting probes were developed by fluorescence activated bivariate chromosome flow sorting (FACS). From the flow sorted chromosomes, DNA libraries from single chromosomes were created via degenerate oligonucleotide primed PCR (DOP-PCR). These whole chromosome painting probes have proven useful in verifying chromosome translocations that were suspected by G-banding (6,7).

### 1.1.3. Multiplex FISH

By the end of the 1980s, multiple color FISH was being developed by using either combinatorial or ratio labeling of DNA probes with different fluorochromes to create multiple unique color combinations (8–10). In the early 1990s, up to 7 different color combinations were being used to simultaneously color individual whole chromosomes. This soon led to the idea that subregional probes for different regions of chromosomes could be used to create a type of chromosome bar code in multiple colors to allow simultaneous analysis of multiple chromosome regions in a single hybridization experiment (11–13). In 1996, two different research groups discovered that combinatorial labeling with 5 different fluorochromes (Cy3, Cy3.5, Cy5, Cy5.5, and FITC) could produce enough color combinations to color all 24 human chromosomes a uniquely different color (14,15).

### 1.1.4. Color Banding Using Human DNA Probes

In 1997, a group from Cambridge University in England successfully introduced a color banding technique that uniquely labeled all of the human chromosomes (16). A banding resolution of 110 distinct color bands per haploid set was achieved by labeling 2 sets of subregional DNA probes with either red or green fluorescence. These probes were then hybridized to human metaphase spreads prepared by conventional cytogenetic airdried technique and counterstained with DAPI (4,6 diamidino-2-phenylino-2-phenylindole). This division of the DNA probes into two different color labeled sets allowed a four color banding pattern to be discerned along the length of the chromosomes. Red and green for unique DNA fragments found in only one set but not the other, yellow, when DNA fragments existed in both sets in equal amounts and blue when no DNA probe existed in either set. The subregional DNA probes were created from Alu/polymerase chain reaction products produced from fragment somatic cell hybrids (human/rodent) that contained over 300 regions of the human genome (17). The Alu-PCR in this experiment caused specific amplification of human DNA but failed to amplify rodent DNA owing to the fact that Alu repeats occur only in primate DNA.

### 1.1.5. Color Banding Using Primate DNA Probes

This same research team from Cambridge, England developed color banding using cross-species comparative genomic hybridization. This team, which included Malcolm A. Ferguson-Smith, Johannes Wienberg, and Stefan Muller, termed this technique “cross species color banding” because they utilized DNA probes derived from bivariate fluorescence activated chromosome flow sorted (FACS) gibbon chromosomes (*Hylobates concolor* and *Hylobates syndactylus*) to produce color banding on human chromosomes (18). These gibbon DNA probes were uniquely labeled with FITC, Cy3 and Cy5 using a combinatorial labeling technique that resulted in 7 different color bands. Gibbons belong to the family of great apes. Gibbon DNA and human DNA have ~98% sequence homology. Color banding is achieved owing to this extensive sequence homology and the evolutionary chromosomal rearrangements that occurred during the phyletic evolution of humans from great apes. These evolutionary rearrangements provide unique color bands on different chromosomes when hybridized to

human DNA. In this paper, approx 100 bands were identified. Each chromosome, with the exception of chromosomes 15, 18, 21, 22, and the sex chromosomes, showed at least two and as many as six different colored subregions.

Prior to this discovery, human chromosome painting probes were being used to study phyletic evolution in great apes, gibbons and Old World monkeys (19–24). This was the first time that higher primate DNA was hybridized to human chromosomes to produce a unique and specific color banding pattern. Utilizing the multiplex FISH techniques, these investigators were able to produce this color cross-species banding in a single hybridization experiment utilizing a fluorescence microscope with a quad band pass filter and a digital imaging system.

## **1.2. Applications of Color Banding**

### **1.2.1. Cancer Cytogenetics**

In cancer cytogenetics, G-banding is still the gold standard. It is simple and robust. GTG Banding is the technique of choice for initial screening of karyotypic abnormalities. Chromosomes in cancer cells however, often present with very complex karyotypes or chromosomes with less than optimal morphology.

Characterization of chromosomal breakpoints is emerging as an important area for cancer diagnosis and prognosis. Cross species color banding can identify marker chromosomes and redefine interpretations of ISCN bands involved in complex rearrangements. To date, 25 hematological malignancies and 13 solid tumors have been analyzed with the commercially available probe kit RxFISH (Rainbow cross-species FISH) (25–31). In all cases, color banding either confirmed or identified complex chromosome changes previously unidentifiable by conventional cytogenetics. Utilization of cross-species color banding can directly impact both the rate at which cytogenetic data is accumulated, as well as impact the accuracy of the breakpoints reported in the literature.

Common chromosome breakpoints have been associated with the location of tumor suppressor genes, oncogenes, and other genes that play a role in cell growth, drug resistance, and apoptotic pathways. These common chromosome breakpoints, when well characterized, can play a role in patient diagnosis and prognosis and therefore have the potential to impact patient treatment.

### **1.2.2. Constitutional and Prenatal Cytogenetics**

There have been two reports in the literature of RxFISH being used to study constitutional chromosomal rearrangements. Four cases total were analyzed with color banding technique, including a case of partial duplication of 4q and a deletion of 4p in a 32 wk-old fetus (32). GAG banding was performed on fetal cord blood and an unbalanced karyotype was observed. The karyotype showed additional material of unknown origin on 4p. With color banding it could be determined that this was a case of partial trisomy 4q26-qter and monosomy 4p16. Deletion of the Wolf-Hirschhorn critical region was confirmed by use of a LSI Wolf-Hirschhorn probe (Vysis). Muller et al. utilized the following constitutional abnormalities: a translocation  $t(1;2)(q37;q42.3)$ , a pericentric inversion  $inv(2)(p23q13)$ , and a pericentric inversion  $inv(6)(p25q22)$  to illustrate the utility of their cross species color banding technique (18).

### **1.3. Strategies in Color Banding—Benefits and Limitations**

#### **1.3.1. Benefits**

Cross species color banding has been shown to be a rapid and easy way to detect chromosomal aberrations that can not be identified by G-banding alone (25–32). This technique is especially useful when GTG banding does not provide sufficient clues to enable followup by use of single whole chromosome painting probes. In addition, color banding can detect intrachromosomal abnormalities such as inversions, duplication, and small deletions that can not be detected by other multiplex FISH techniques such as MFISH and SKY. All referenced authors extol the benefits of this technique when used in combination with G-banding and other FISH detection systems.

#### **1.3.2. Limitations**

Color banding resolution is only approx 100 bands or less. This number of bands provides some differentiation of chromosomes but is inadequate for small translocations, deletions or inversions. In addition, translocations are nondetectable by this technique if they involve regions painted a similar color.

## **2. Materials**

1. Slides prepared by conventional cytogenetic methods.
2. 10  $\mu$ L RxFISH probe (Applied Imaging, Santa Clara, CA), store at  $-20^{\circ}\text{C}$ .
3. Formamide, molecular biology grade, 50 and 70% in 2X SSC, pH 7.5.
4. Ethanol, denatured HPLC grade, 70, 85, and 100%.
5. Methanol, absolute.
6. Glacial acetic acid.
7. 20X SSC (Saline sodium citrate), pH 7.0.
8. 2X SSC, pH 7.0.
9. HCl (2 N).
10. Tween-20.
11. Rabbit antiFITC antibodies (DAKO, V0403).
12. FITC labeled goat anti-rabbit IgG antibodies (Vector, FI-1000).
13. DAPI (4,6-Diamidino-2-phenylindole), (Vysis, 32-804831).
14. Glass coverslips (22  $\times$  22 mm).
15. Glass Coplin jars (50 mL).
16. Microscope slides.
17. Humidified chamber at  $37^{\circ}\text{C}$ .
18. Purified water (distilled, deionized).
19. Rubber cement.
20. Phase microscope.
21. Waterbath set at 37, 45, 65, and  $72^{\circ}\text{C}$ .
22. Oven set at  $37^{\circ}\text{C}$ .
23. Oven set at  $60\text{--}65^{\circ}\text{C}$  with metal slide tray.
24. Micropipetors and tips.
25. pH meter.
26. Microcentrifuge and microcentrifuge tubes.
27. Freezer,  $-20^{\circ}\text{C}$ .
28. RxFISH CytoVision System (Applied Imaging, Santa Clara, CA).

29. Fluorescence microscope with automated filter wheel with a Quad filter block.
30. Cooled charge-coupled device video camera.

### 3. Methods

#### 3.1. Slide Preparation and Aging

1. Prepare cytogenetic cell suspensions using the methanol/acetic acid fixation method.
2. Prepare microscope slides by placing a drop of cell suspension onto the slide and allowing the slide to dry at the proper temperature (25°C) and humidity (45–50%) required for chromosome spreading.
3. Using a phase microscope, monitor quality of slides and mark area of slide containing well spread metaphases, or those metaphases of interest.
4. Age slides prepared the same day in 60°C oven for 2 h or age 1-d-old slides stored overnight at room temperature in a 60°C oven for 1 h. Following aging, bring the slide to room temperature before proceeding with the next step of the RxFISH procedure.
5. Dehydrate slides in a 70, 85, and 100% ethanol series for 2 min each at room temperature and allow to air dry.

#### 3.2. Preparation of Solutions for Denaturation and Hybridization Steps

1. Prepare 70% ethanol and place in –20°C freezer.
2. Prepare 50 mL of 70% formamide/2X SSC, pH 7.5 and place in 72°C waterbath.
3. Prepare 70, 85, and 100% ethanol and store at room temperature.
4. Prepare humidified chamber and prewarm in 37°C oven.

#### 3.3. Probe Denaturation

1. Warm tube containing RxFISH probe in a 37°C waterbath for 5 min.
2. Gently mix and then briefly centrifuge the tube.
3. Using a sterile pipet tip, transfer 10 µL of probe solution for each slide to be hybridized to a 0.5 mL microcentrifuge tube and cap the tube.
4. Return the unused portion of the RxFISH probe to –20°C.
5. Denature the aliquoted RxFISH probe by incubating in a 65°C waterbath for 10 min.
6. Following denaturation store probe in a 37°C waterbath for a minimum of 10 min and no longer than 2 h before being used.

#### 3.4. Slide Denaturation

1. Confirm that the internal temperature of the 70% formamide/2X SSC solution is at the desired temperature of 72°C (*see Notes 1 and 2*).
2. Denature no more than 4 slides by incubation in the 70% formamide/2X SSC solution for 1.5 min (*see Note 3*).
3. Immediately quench the denatured slide in –20°C 70% ethanol (*see Note 4*).
4. Dehydrate slides in a 70, 85, and 100% ethanol series for 2 min each at room temperature and allow to air dry.

#### 3.5. Hybridization Procedure

1. Reduce ambient lighting in order not to photo bleach RxFISH probe (*see Note 5*).
2. Pipet 10 µL of the denatured probe mixture onto the area of the slide that was marked for hybridization because it contains well spread metaphases or those metaphases of interest.
3. Slowly add 25 × 25 mm coverslip to avoid air bubbles.

4. Allow the probe solution to spread to the edges of the coverslip. Tap gently to remove any air bubbles avoiding any movement of the coverslip.
5. Seal the coverslip to the slide with rubber cement.
6. Incubate the slide in a humidified chamber for 48 h at 37°C (*see Note 6*).

### **3.6. Preparation of Solutions for Posthybridization Steps**

1. Prepare 150 mL 2X SSC, pH 7.0. Dispense into three Coplin jars and place in waterbath set at 45°C (*see Note 7*).
2. Prepare 100 mL of 50% formamide/0.5X SSC, pH 7.0, dispense into two Coplin jars and place in 45°C waterbath.
3. Dispense 50 mL of 4X Tween solution, pH 7.5 (500 mL 4X SSC and 0.25 mL Tween 20 stock) in a Coplin jar and place in 45°C waterbath.

### **3.7. Posthybridization Wash**

1. Carefully remove the rubber cement from coverslipped slide(s) with forceps and float the coverslip off by incubating the slide in the first Coplin jar of 2X SSC at 45°C for 5 min (*see Notes 8–10*).
2. Wash the slide(s) for 5 min at 45°C in a Coplin jar containing 50% formamide/0.5X SSC. Transfer slide to second Coplin jar containing this same solution and repeat wash for another 5 min.
3. Wash slide(s) for 5 min at 45°C in a Coplin jar containing 2X SSC. Repeat this wash in the third Coplin jar containing this solution for an additional 5 min.
4. Transfer the slide to the Coplin jar containing the 4X Tween solution and incubate at 45°C for 10 min.

### **3.8. Antibody Detection Steps (Optional)**

1. During the second formamide wash step in **Subheading 3.7.**, centrifuge the two antibody tubes for FITC detection at 14,000g for 10 min at room temperature. Use only the supernatants from these tubes in the following steps.
2. To microcentrifuge tube add 1 µL of rabbit antiFITC antibody and 199 µL of 4X Tween for each slide to be processed. Gently mix the dilution. Incubate in the dark at room temperature for 10 min.
3. Drain excess fluid from the slide(s).
4. Add 200 µL rabbit antiFITC antibody, 1:200 dilution to each slide and incubate for 30 min in a humid chamber kept at 37°C.
5. Lower the temperature of the waterbath from 45 to 42°C. Prewarm a 50 mL Coplin jar of 4X Tween. Also prewarm an additional 250 mL of 45X Tween for a total of 6 additional washes.
6. Check that the waterbath and 4X Tween solutions are at 42°C. Remove coverslip from slide(s). Wash the slide for 5 min. Decant the solution and refill with fresh prewarmed 4X Tween, wash again for 5 min. Repeat this step once.
7. During these wash steps, in another microcentrifuge tube add 2 µL of FITC-labeled goat anti-rabbit IgG antibody and 198 µL of 4X Tween for each slide to be processed. Gently mix the dilution. Incubate in the dark at room temperature for 10 min.
8. Drain excess fluid from the slide.
9. Add 200 µL FITC-labeled goat anti-rabbit IgG antibody, 1:100 dilution to each slide and incubate for 30 min in a humid chamber kept at 37°C.
10. Check that the waterbath and 4X Tween solutions are at 42°C. Wash 3× at 42°C for 5 min each.

### 3.9. DAPI Counterstain

1. Mount the slide with 10  $\mu\text{L}$  of the DAPI counterstain and antifade solution using a 22  $\times$  22 mm coverslip (*see Note 11*).

### 3.10. Image Capture and Analysis

1. Turn on computer, select RxFISH software under the research menu. When activated, select the capture screen. Set the filter wheel in the proper order before beginning image capture. The probe is captured in layers of raw images that are combined for a final image. Rx-Cy5 is captured first followed by Rx-Cy3, Rx-FITC, and lastly Rx-DAPI.
2. Scan for metaphases using FITC. FITC has a less tendency to fade the signal, which should stay bright throughout all analysis. When you have found a metaphase, focus the metaphase using a  $\times 100$  oil objective. Select Rx-Cy5.
3. Rx-Cy5 is undetectable by the naked eye. Rely on the computer image for detail. When the exposure setting is 5 s a faint white image on a black background should be detectable. Focus slowly and in increments, as there is a time delay between the camera and the software. Adjust the bright and black until you have a good contrast ( $\sim 90\%$  accuracy). These settings will be slightly different for each individual image.
4. Hit the “Live” button and the filter wheel will automatically turn to the next fluorochrome on the list. For the other fluorochromes an exposure of 2 s seems to work best most of the time. Once a good image with a clear banding pattern has been achieved, click on the “Capture” button.
5. After completing captures for all four fluorochromes, select the box marked RxFISH Image Capture. Within the box select, batch complete. Keep all images; never delete them even when prompted. These images are often useful during analysis.
6. Activate the analysis menu. Choose the cell you wish to analyze (highlight the probe cell image). Select “Load cell” and multicolored banded chromosomes can be observed (*see Note 12*). In the Analysis Customize menu, the RxFISH image can also be displayed as an inverted DAPI image, which can help in chromosome identification. The Analysis Profile tool is used to display individual intensity profile of the chromosomes.
7. The chromosome must be separated using the Analysis commands. Once separation has been completed the Classifier and Auto commands are used to generate the karyotype of the cell.

## 4. Notes

1. The denaturation step is very critical to the procedure. Temperature and times are dependent upon the type and age of the sample. Each user should determine the appropriate temperature and time based on their particular need. Acceptable results can be obtained using a temperature range of 70–75°C and a time range of 1.5–2 min.
2. The internal temperature of the solutions in each Coplin jar throughout the procedure must be checked prior to each use. Often the temperature of the solutions in the Coplin jar will be one or more degrees below that of the water in the waterbath itself.
3. Each room temperature slide that is added to the 72°C formamide lowers the temperature of the 70% formamide/2X SSC by 0.5–1°C. If more than 4 slides are added per run, the temperature of the formamide/2X SSC will be insufficient for denaturation to occur.
4. If more than 4 slides are to be processed, both the denaturation temperature of the 70% formamide/2X SSC and the first Coplin jar of 70% ethanol must be allowed to return to their initial temperature, 72°C and  $-20^\circ\text{C}$  respectively.

5. The Cy5 fluorochrome approaches the infrared wavelength range, which is very sensitive to being excited by white light. Accordingly, during image capture, this is the first fluorochrome to be exposed to light since it is the first to fade.
6. Slides may be hybridized for 12–96 h in a moist chamber at 37°C. Never hybridize probe for less than 12 h as signal intensity is greatly diminished. Forty-eight hour hybridization times provide slides with consistently strong signals. This is essential for successful image capture. Reduction in hybridization times should be based on each laboratory's experience.
7. To ensure quality results, discard solutions made at the end of each day. The solutions used in the denaturation steps should not be saved for use during the post-hybridization wash steps.
8. Posthybridization wash steps should be done in dim light to prevent photo bleaching of direct labeled fluorochromes (Cy5 and Cy3).
9. The slide should not be allowed to dry completely during any post-hybridization step.
10. If the cover slip fails to come off the slide, place the slide back into the 2X SSC for an additional 5 min and then check the coverslip again.
11. Store slides a -20°C and in the dark until they are ready to be imaged.
12. If the colors are pastel and not bright, recapture images on a darker setting (less exposure). When focusing images before capture, keep the exposure times lower as there will be less of a delay. There is a chip in the camera that gathers light for the exposure time and then sends the information to the computer, this is why there is a delay. The DAPI image should not be too dark. The DAPI image provides the background for the other colors. If the DAPI image is not bright enough, the chromosomes will look ragged. For this reason as well, it is essential that the DAPI image be in good focus.

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## Multicolor Fiber FISH

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### 1. Introduction

Various genetic abnormalities can be observed in many human diseases. They vary from numerical chromosomal aberrations, deletions, and amplification of specific regions to chromosomal translocations and insertions. In the past decade, numerous fluorescence *in situ* hybridization (FISH) methods have been developed for the detection of these abnormalities. FISH to interphase nuclei is one of the methods that is widely used to detect numerical chromosomal abnormalities, DNA amplification, and structural abnormalities ranging in size from approximately 10–1000 kilobases. Interphase cytogenetics requires *a priori* knowledge regarding the DNA sequences involved in the aberration. Particularly for the diagnostic detection of structural abnormalities such as chromosomal translocation breakpoints, the interphase method is highly dependent on the availability of appropriate breakpoint flanking probes.

More recently, a set of high resolution FISH methods have been developed that collectively are referred to as Fiber FISH. The basis of Fiber FISH is to release DNA-molecules from the nucleus and to fix the stretched DNA in a (near) linear fashion onto a glass slide. The DNA fibers are hybridized with a set of probes labeled with different fluorochromes that cover the entire region of interest resulting in a characteristic string of FISH signals that have the attributes of a multicolor barcode (**Fig. 1A, B**). Barcodes up to 1000 kb can be generated, but a length of approx 300 kb is used in practice. When larger regions are targeted, artificial breakage of the target DNA may complicate interpretation of results.

With its high and wide ranging resolution (1–1000 kb) as well as its color barcoding capacity, Fiber FISH has proven to be well suited as an adjunct physical mapping tool and for analysis of length polymorphisms as well as a valuable tool in molecular pathological research to pinpoint regions of chromosome (DNA) rearrangements at a much larger genomic scope than PCR, Southern blotting, and interphase-FISH.

From: *Methods in Molecular Biology, Vol. 204: Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

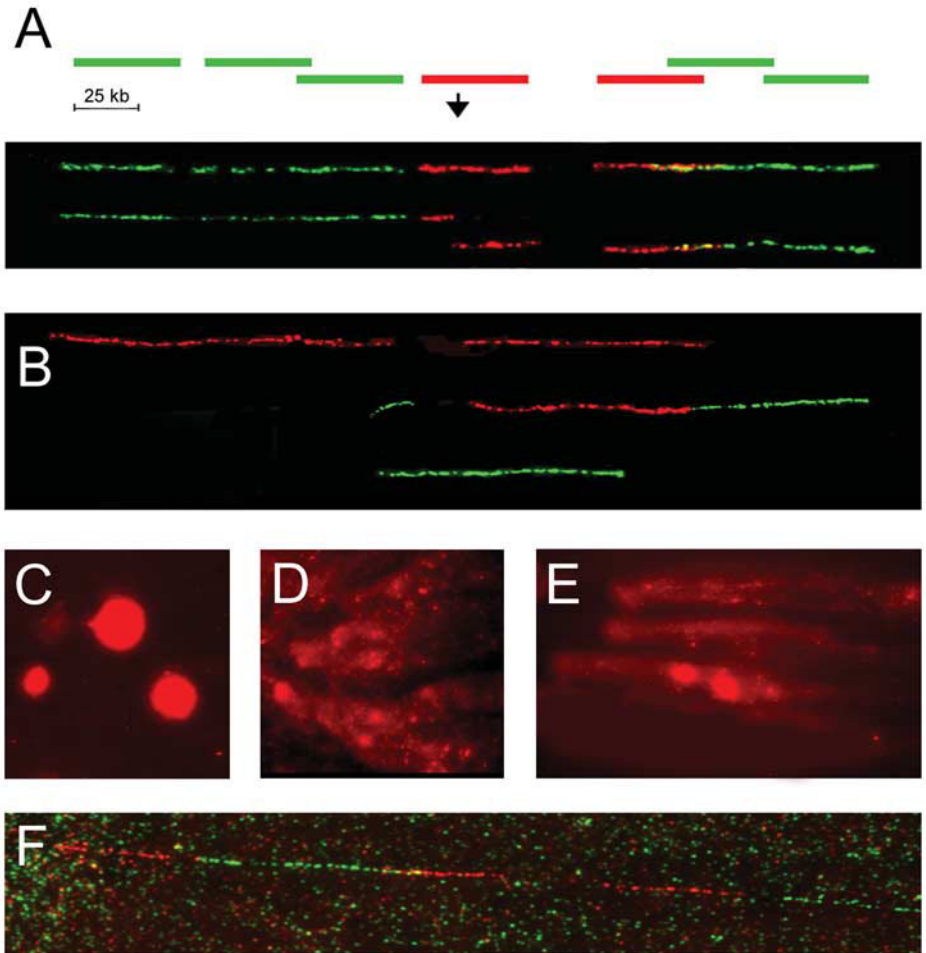


Fig. 1. (A) Fiber FISH to map a breakpoint in the *ETV6* (12p13) gene, present in a  $t(9;12)(q34;p13)$ . The normal 300 kb barcode consists of seven cosmid probes as illustrated in the superimposed cosmid map. The upper fiber, representing the normal allele, shows hybridization of the normal barcode pattern. The second and third fiber show the complementary patterns representing the breakpoint products. The breakpoint in these leukemic cells was mapped within the middle cosmid (arrow). (B) Simultaneous hybridization of two specific barcodes for the involved genes in a 200 kb insertion. The upper fiber shows the normal barcode of one gene (*MLL* on 11q23), whereas the bottom fiber shows the normal barcode of the partner gene (*AF4* on 4q21). The junction of both loci in the tumor DNA is visualized in the middle fiber. In this case, a part of the barcode of the *MLL* gene is inserted into the barcode of the *AF4* gene (20). Note that a simple single color barcode may also enable exact mapping of breakpoints. (C) Fiber preparation stained with propidium iodide. If nuclei are present on the slide as red bright balls, as shown in this picture, the cell lysis has been insufficient. (D) Fiber preparation stained with propidium iodide. If the slide looks cloudy and bright crystals are present, as shown in this picture, the amount of cells is too high. (E) Fiber preparation stained

In this chapter, we will briefly summarize the development of Fiber FISH, describe some of its applications and present a detailed Fiber FISH protocol we have used over the last few years in our Lab.

### 1.1. The Development of Fiber FISH

To prepare linear stretches of DNA released from the nucleus, various methods have been described (reviewed in *1*) and published under different names including high resolution FISH, molecular combing, DNA fiber FISH, halo FISH and DIRVISH (*2–7*). All are based on the release of most of the DNA contents of cells on microscope object slides and linearizing DNA. In most methods, nuclei are prepared from fresh cells, frozen cells or frozen tissues and put on glass slides before release of the DNA (*1*). Other methods used isolated genomic DNA prepared in agarose blocks or in solution (*2*). For linearization of genomic DNA, most methods rely on mechanical or gravitational force. Wiegant et al. (*4*) used the halo method to release the DNA from nucleus. This method was originally used in the early sixties to visualize DNA strands with electromicroscopy. Using this approach, the DNA was released and combined with a histone extraction step, using a concentrated salt solution, and a DNA unwinding step, involving induction of single strand breaks by a combination of the DNA intercalator propidium iodide and UV radiation. The Fiber FISH method described in this chapter is based on this DNA halo technique.

Following stretching and immobilization of (nuclear) DNA fibers, they are used as targets of multiple probes for the region of interest labeled with different haptens/fluorochromes. It is estimated that DNA fragments in size up to approx 2–4 kb will hybridize as a single signal. Genomic probes cloned in large insert vectors as cosmids, PACs, and BACs in general have a complexity of 30–150 kb, and thus a single probe will be visible as numerous beads-on-a-string (**Fig. 1A, B, F**). The total length of the signals (i.e., the collection of beads-on-a-string) corresponds well with the real size (or complexity) of the probe (within the limits of the standard deviation of approx 1–2 kb) so that the genomic distance approximates the physical distance (3 kb = 1  $\mu$ m). Because of the typical linear beads-on-a-string signal of the probe, this pattern can easily be discriminated from all other single background signals in the preparation that are due to aspecific hybridization (**Fig. 1F**). This enables the use of low stringency hybridization and washing conditions in Fiber FISH and is an important advantage over interphase-FISH especially if different probes are combined in a single experiment. Moreover, by using a set of different probes that cover a certain region and that are alternatively fluorescently labeled (red or green), probe contigs appear as multicolored, barcode like patterns in red, green, yellow (the overlap of clones labeled in green and red) and small gaps remaining dark when hybridized on linear DNA molecules

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(*continued*) with propidium iodide. In this picture, the cells are sufficiently lysed and the cells are evenly distributed along the slide. DNA halos are present as comet like structures. (**F**) Fiber FISH preparation hybridized with a 250 kb two color (green and red) cosmid barcode. Observed with a double or triple filter, it is very easy to distinguish between the beads-on-a-string fiber signals and single background signals.

(Fig. 1A, B, F). Each contig, i.e., each barcode, will be unique for a certain region. The availability of such multicolored barcodes allows a rapid visual identification for physical mapping of new probes and for the analysis of DNA rearrangements in patient specimens.

## 1.2. Some Applications for Fiber FISH

Fiber FISH finds application in physical mapping of DNA clones. Novel clones can be rapidly analyzed for size, position, and orientation when compared to already fully characterized clones in the region of interest (5,8). Such an approach has been used in genome projects. Fiber FISH was also used to map the genomic structure of a gene by hybridization of a cDNA clone in combination with genomic probes that covers all presumed exons and introns (9). This analysis illustrated that fragments as small as 500 bp can be detected. With a Fiber FISH barcode, various genetic abnormalities such as deletions, insertions, length polymorphisms, and translocations can be mapped physically in clinical specimens conveniently if they occur within the barcode. For instance, Florijn et al., (5) were the first to map two Duchenne dystrophy deletion breakpoints with Fiber FISH with an accuracy of 1–2 kb. We have applied our Fiber FISH protocol on a subset of B-cell lymphomas that had down-regulated expression of certain HLA-genes on chromosome 6p21.3 (10). Apart from confirming several length polymorphisms, this analysis revealed homozygous deletions in size ranging from 50–150 kb. This study enabled us to rapidly narrow down the size of the deleted region. With a set of probes that covers the constant region of the immunoglobulin heavy chain locus, numerous polymorphisms as well as somatic class switch recombination events were observed (11). In cases of DNA amplification, Fiber FISH permitted the determination of the number of amplicons, the orientation of each amplified fragment within the cluster, the size of each amplicon, the variation among different cells and also copy numbers of moderately repeated sequences (12). Fiber FISH has also been used to investigate viral insertions in the mammalian genome (13). Using Fiber FISH with contigs that cover the breakpoint regions in mantle cell lymphomas with t(11;14), follicular lymphomas with t(14;18) and ALL/AML with MLL-breaks on 11q23, the location of the breakpoints could be determined with an accuracy of few kilobases (14–16).

At this stage, Fiber FISH is not used as a routine diagnostic assay to detect genetic aberrations in clinical specimens, because it is relatively time consuming and generally can only be applied to fresh or frozen cells or tissues. Also, the success rate (i.e., the number of cases from which sufficient fibers are obtained to draw reliable conclusions) is not 100% and depends on many experimental conditions. This implies that some experiments have to be repeated or that the conditions have to be adapted to the specific cellular material. Nevertheless, in our experience, Fiber FISH as a research tool, has provided essential contributions to the design of optimal probe-sets to detect all abnormalities by interphase-FISH (reviewed in ref. 17). The Fiber FISH analysis of the t(11;14) and t(14;18) translocations characteristic for mantle cell and follicular lymphoma respectively may serve as illustrative examples. The mapping of many breakpoints by Fiber FISH showed that they are scattered over large 350–500 kb regions, a size that can not be accommodated by conventional methods such as PCR and Southern blotting. Fiber FISH mapping clearly defined the maximum size of the

breakpoint regions. The Fiber FISH analysis also revealed a considerable number of cases (approx 20%) with uncommon complex, mono-allelic breakpoints. These included mono-allelic double breakpoints 3' and 5' of cyclin D1 at 11q13 or BCL2 at 18q21, either or not in combination with an insertion into the IgH-locus (**18**) or a small deletion in the UTR of cyclin D1 (**19**). Based on these data, we designed probe sets flanking all possible breakpoints and use these sets in our routine diagnostic setting for the detection of translocations using interphase FISH (**14,15**).

## 2. Materials

### 2.1. Preparation of DNA Fibers Using the Halo Method

1. Cultured cell suspension or 45  $\mu$ M sections of frozen soft tissues/tumors or isolated PBL.
2. PBS: 8 g NaCl, 0.29 g KCl, 1.449 g  $\text{Na}_2\text{HPO}_4$ , 0.249 g  $\text{KH}_2\text{PO}_4/\text{L}$ .
3. Microfuge.
4. Halo-solutions:
  - a. Halo 1: 0.5% NP-40, 25 mM Tris-HCl, pH 8.0, 10 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CaCl}_2$ .
  - b. Halo 2: 25 mM Tris-HCl pH 8.0, 0.2 mM  $\text{MgCl}_2$ , 2 M NaCl.
  - c. Halo 3: as Halo 2, with 4 mg/100 mL propidium iodide.
  - d. Halo 4: 25 mM Tris-HCl pH8.0, 0.2 mM  $\text{MgCl}_2$ , 0.2 M NaCl.
  - e. Halo 5: 25 mM Tris-HCl pH8.0, 0.2 mM  $\text{MgCl}_2$ .
5. 20% bovine serum albumine (A9647 from Sigma, St. Louis, MO) in PBS; dissolve 2 g of BSA in 10 mL of sterile PBS, store in small portions at  $-20^\circ\text{C}$ .
6. Camag UV Cabinet II, low pressure mercury tube (8 W), 256 nm UV lamp.
7. 10% SDS in ultrapure water; dilute to 0.05% (or 0.1%) in ultrapure water.
8. Ethanol/ether 1:1.
9. Glass microscope slides ca  $76 \times 26$  mm with frosted edge, cleaned by dipping in a mixture of ethanol/diethylether 1:1 and wiped with a non-woven tissue (*see Note 1*).
10. Glass plate, thickness 5 mm, on crunched ice.
11. Fetal calf serum (FCS).
12. Fluorescence microscope equipped with a PL Fluotar  $\times 16$  objective and a PL Fluotar  $\times 100$ , objective and filters for viewing Texas Red/PI and fluorescein isothiocyanate (FITC).

### 2.2. Probe Labeling

1. Probe DNA (*see Note 2*).
2. 10X Nicking buffer: 0.5 M Tris-HCl pH 7.8, 50 mM  $\text{MgCl}_2$ , 0.5 mg BSA/mL.
3. Nucleotide mixture (0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dCTP, 0.1 mM dTTP).
4. 100 mM Bio-16-dUTP (Roche Diagnostics, Basel) or 100 mM Dig-11-dUTP (Roche Diagnostics).
5. 100 mM DTT.
6. Sterile ultrapure water.
7. DNase I grade II, Roche Diagnostics 104159, 1 mg/mL.
8. DNA-polymerase I (M502A, 10 U/ $\mu$ L from Promega, Madison, WI).
9. Yeast RNA (10  $\mu$ g/ $\mu$ L Sigma R 6750).
10. SS-DNA (salmon sperm DNA, 10  $\mu$ g/ $\mu$ L Sigma D7290).
11. 3 M NaAc pH 5.3–5.9.
12. Ethanol absolute.
13. Waterbath at  $16^\circ\text{C}$ .
14. Centrifuge 13000 rpm at  $4^\circ\text{C}$ .

15. Hybridization mixture, for 1 mL: 300  $\mu$ L deionized formamide, 100  $\mu$ L 0.5 M sodium phosphate buffer pH 7.0, 100  $\mu$ L 20X SSC, 500  $\mu$ L ultrapure water, 100 mg dextran-sulphate; dissolve for 3 h at 70°C.

### **2.3. Hybridization**

1. Slide warmer.
2. Labeled cosmid/PAC/P1/BAC/YAC probe.
3. Cot-1 DNA (Life Technologies, Gaithersburg, MD), ethanol precipitated and dissolved in hybridization mixture in a concentration of 3  $\mu$ g/mL.
4. Denaturing mixture, for 1 mL: 700  $\mu$ L deionized formamide, 100  $\mu$ L 0.5 M sodium phosphate buffer pH 7.0, 100  $\mu$ L 20X SSC, 100  $\mu$ L ultrapure water.
5. Hybridization mixture.
6. Ethanol absolute.
7. 24  $\times$  60 mm glass coverslips.
8. 24  $\times$  24 mm glass coverslips.

### **2.4. Posthybridization and Immunodetection**

1. 2X SSC.
2. Waterbath at 37°C.
3. Coplin jar 100 mL.
4. TNT: 0.1 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20.
5. TNB: 0.5% Boehringer blocking reagent in 0.1 mM Tris-HCl, pH 7.5, 0.15 M NaCl.
6. Texas Red-labeled streptavidin (Vector Laboratories, Burlingame, CA) 1.0 mg/mL.
7. Mouse anti-digoxigenin (Sigma) 4.0 mg/mL.
8. Goat biotinylated anti-streptavidin (Vector) 0.5 mg/mL.
9. Rabbit anti-mouse-Ig-FITC (Sigma) 15.3 mg/mL.
10. Goat anti-rabbit-FITC (Vector) 1.5 mg/mL.
11. Antifade mounting medium (Vectashield, Vector).
12. 24  $\times$  60 mm coverslips.

## **3. Methods**

### **3.1. Preparing Slides with DNA Fibers**

Most types of cells are suitable for fiber FISH, as long as the cells have not been fixated or dried and the nuclei are intact. Frozen tissue samples or pelleted cells can be used as long as the tissue can be suspended to a cell suspension. Cells which have a lot of cytoplasm often need more rigorous treatment to lyse. Always start with the most gentle method and increase SDS-treatment if lysis is insufficient.

Prior to beginning, place on ice:

1. Each Halo-solution and 0.05% SDS in ultrapure water in 100-mL beakers and two 100-mL beakers containing ultrapure water.
2. A tube containing 20% BSA and a tube containing PBS.
3. A glass plate (5 mm thickness).

#### **3.1.1. Preparing a Cell Suspension**

From cultured cells, peripheral blood cells, or other cell suspensions:

1. Centrifuge 1 mL of cell suspension or trypsinized cells (approx 10,000–100,000 cells) in an Eppendorf tube for a few seconds in a microcentrifuge.

2. Remove supernatant.
3. Resuspend cells in 200  $\mu\text{L}$  PBS (4°C).

From frozen tissues:

1. Cut one or a few frozen sections of 45  $\mu\text{m}$  (*see Note 3*).
2. Place in an eppendorf tube on dry ice.
3. Keep on dry ice until use.
4. Thaw section for  $\pm 30$  s.
5. Add  $\pm 500$   $\mu\text{L}$  PBS (4°C).
6. Suspend section by pipetting up and down vigorously.

### 3.1.2. Preparing DNA Fiber Slides

1. Pipet 20  $\mu\text{L}$  of cells in a tube, adjust the volume with PBS (4°C) to 47.5  $\mu\text{L}$  and add 2.5  $\mu\text{L}$  20% BSA/PBS and mix the suspension well.
2. Pipet the suspension onto a slide and evenly spread over the whole slide using a pipet tip.
3. Place the slide on an ice cold glass plate for 2 min.
4. Drain excess of fluid by placing the slide vertically (frosted edge upwards) onto a tissue for 40 s.
5. Blowdry very gently using compressed air until the edges are starting to dry (*see Note 4*). (For easily lysing cells like peripheral blood cells or cultured cells **steps 6–9** can be omitted).
6. Dip slide gently vertically in Halo 1 for 30 s.
7. Remove slide slowly from solution in 3 s, drain excess of fluid on a tissue for 7 s (*see step 4*).
8. Dip slide gently vertically in Halo 2 for 30 s.
9. Remove slide slowly from solution in 3 s, drain excess of fluid on a tissue for 7 s (*see step 4*).
10. Dip slide gently vertically in Halo 3 for 45 s.
11. Remove slide slowly from solution in 3 s, drain excess of fluid on a tissue for 7 s (*see step 4*).
12. Clean the backside of the slide with a piece of tissue.
13. Place the slide horizontally on the ice cold glass plate.
14. Place the still wet slide under the UV lamp at a distance of 1–10 cm (*see Note 5*).
15. Irradiate immediately for 1–10 min at 254 nm (*see Note 5*).
16. Place slide vertically onto a tissue for 10 s (*see step 4*).
17. Dip the slide gently vertically for 30 s in Halo 4.
18. Remove slide slowly from solution in 3 s, drain excess of fluid on a tissue for 7 s (*see step 4*).
19. Dip the slide gently vertically for 30 s in Halo 5.
20. Remove slide slowly from solution in 3 s, drain excess of fluid onto a tissue for 7 s (*see step 4*).
21. Repeat for 2 washes in ultrapure water.
22. Leave slide to dry vertically in a rack at room temperature for few minutes.
23. Examine the slide under a fluorescent microscope with  $\times 16$  objective and TRITC-filter (*see Note 6*) and examine whether (a) the cells are properly lysed (**Fig. 1C**) (*see Note 7*); (b) the amount of cells is sufficient (**Fig. 1D**) (*see Note 8*); (c) the number of comet like structures (representative for fibers) on the slide is sufficient (**Fig. 1E**) (*see Note 8*).
24. Leave slides to dry overnight at room temperature before proceeding with hybridization; long term storage of slides is possible at  $-20^\circ\text{C}$  in a closed container.

### 3.2. Probe Labeling

1. Mix in a tube: 5  $\mu\text{L}$  10X nicking buffer, 5  $\mu\text{L}$  0.1 M DTT, 4  $\mu\text{L}$  nucleotide mixture, 0.5  $\mu\text{L}$  of labeled nucleotide, 1  $\mu\text{g}$  of DNA and adjust volume with ultrapure water to 44  $\mu\text{L}$ .
2. Place on ice and add 1  $\mu\text{L}$  DNA-polymerase.

3. Dilute DNase I stock solution 1:500 /1:1000/ 1:2000 in ice cold ultrapure water.
4. Add 5  $\mu\text{L}$  of diluted DNase I.
5. Incubate for 2 h at 16°C.
6. Place on melting ice and take an aliquot of 5  $\mu\text{L}$ , test fragment length on a 2% agarose gel (*see Note 9*).
7. Add 20  $\mu\text{g}$  ss-DNA and 20  $\mu\text{g}$  yeast RNA.
8. Precipitate with 0.1 vol 3 M NaAc and 2.5 volume ethanol absolute  $-20^\circ\text{C}$ .
9. Leave for 30 min on melting ice.
10. Spin in a microcentrifuge at full speed for 30 min, remove ethanol and air dry pellet.
11. Resuspend probe in hybridization mixture in a concentration of 30–60 ng/ $\mu\text{L}$ , dissolve for 30 min at 37°C, store in the dark (at 4°C or  $-20^\circ\text{C}$ ).

### 3.3. Hybridization

1. Bake the slides with fibers on a slide warmer of 80°C for 2 h (*see Note 10*).
2. Prepare probe mixture: 3 ng/ $\mu\text{L}$  of labeled cosmid/PAC/P1 probe of each probe, cot-1 DNA in a 50-fold excess per probe, add up to 10  $\mu\text{L}$  with hybridization mixture.
3. Denature probe mixture for 8 min at 80°C.
4. Quench on melting ice for 2 min.
5. Mix, spin down briefly in a microcentrifuge.
6. Pre-anneal probe mixture for 30 min in a 37°C waterbath.
7. Apply 120  $\mu\text{L}$  of Denaturing mixture (70% deionized formamide/2X SSC/50 mM sodiumphosphate) on a 24  $\times$  60 mm coverslip.
8. Invert the slide with the fiber side onto the cover slip allowing denaturing mixture to spread.
9. Turn the glass slide back with coverslip up and put the slide on a slide warmer of 80°C for exactly 3 min.
10. Remove coverslips gently by tilting the glass slide and let the coverslip slide off.
11. Wash for 2 min in 2X SSC on ice (4°C).
12. Wash for 5 min in 70% ethanol ( $-20^\circ\text{C}$ ).
13. Dehydrate slide in 90 and 100% ethanol at RT.
14. Leave to air dry vertically in a rack.
15. Put 10  $\mu\text{L}$  probe mixture on slide.
16. Cover with a 24  $\times$  24 mm coverslip.
17. Hybridize upside down overnight at 37°C in a moist chamber.

### 3.4. Posthybridization and Immunodetection

Prewarm 400 mL 2X SSC to 37°C in waterbath:

1. Soak coverslip off in 2X SSC for 5 min at 37°C in a Coplin jar (*see Note 11*).
2. Wash 3 $\times$  for 5 min in 2X SSC at 37°C.
3. Wash for 5 min in TNT.
4. Add 120  $\mu\text{L}$  of TNB onto a coverslip 24  $\times$  60 mm and invert slide onto it.
5. Incubate upside down for 20 min at 37°C in a moist chamber.
6. Prepare a 1:1000 mouse antidigoxigenin antibody and 1:100 Texas Red-labeled streptavidin mixture in TNB; 120  $\mu\text{L}$  per slide.
7. Soak coverslip off in TNT.
8. Apply antibody mixture to a 24  $\times$  60 mm coverslip, invert slide onto it.
9. Incubate upside down for 30 min at 37°C in a moist chamber.
10. Soak coverslip off in TNT.
11. Wash 3 $\times$  5 min in TNT.

12. Prepare a rabbit 1:1000 antimouse-Ig-FITC and 1:100 goat biotinylated-antistreptavidin antibody mixture in TNB; 120  $\mu$ L per slide.
13. Apply antibody mixture to a 24  $\times$  60 mm coverslip, invert slide onto it.
14. Incubate upside down for 30 min at 37°C in a moist chamber.
15. Prepare a 1:100 goat antirabbit FITC antibody and 1:200 Texas Red-labeled streptavidin mixture in TNB; 120  $\mu$ L per slide.
16. Soak coverslip off in TNT.
17. Wash 3  $\times$  5 min in TNT.
18. Apply antibody mixture to a 24  $\times$  60 mm coverslip, invert slide onto it.
19. Incubate upside down for 30 min at 37°C in a moist chamber.
20. Soak coverslip off in TNT.
21. Wash 3  $\times$  5 min in TNT.
22. Dehydrate in a series of 70–90–100% ethanol.
23. Allow to air dry.
24. Apply 10  $\mu$ L antifade medium to slide and cover with 24  $\times$  60 mm coverslip.

### 3.5. Signal Viewing and Interpretation

The amount of background on the slide is very high due to the large probe fragments. However, the background is very easy to distinguish from the beads-on-a-string fibers (**Fig. 1E**). FISH signals are analyzed according to Vaandrager et al. (**11**). Known distances between probes and/or probe lengths (e.g., obtained by restriction mapping or in case of large probes by pulsed field gel electrophoresis) provide internal length references (*see* e.g. in **ref. 14**). Differences in degree of DNA condensation on individual glass slides can be eliminated by computer-aided stretching or compressing of the digital images and normalization to the total length of the barcode. In case of the presence of abnormal barcodes, e.g., derived from breaks, the hybridization efficiency can be verified by the presence of normal barcode signals from normal alleles. The total amount of fibers that is needed to map a breakpoint depends on the amount of tumor cells in the initial cell suspension. However, in general, measurement of five of each normal and abnormal barcode residues will be sufficient for accurate mapping of breakpoints (**14**).

### 4. Notes

1. Although many slide surfaces have appeared to be suitable for Fiber FISH, in our experience, a special coating is not required.
2. It is advised to use column cleaned probe DNA for nick translation since DNase I is very sensitive to contamination of small amounts of phenol, salt or RNA.
3. It is possible to cut several sections and store them in  $-70^{\circ}\text{C}$  for up to two months. How many sections are required for a single experiment depends on the specific conditions used to lyse the cells, as more rigorous lysing will detach more cells from the slide.
4. Keep slide horizontal and rainbow like patterns will occur.
5. The UV radiation nicks the DNA. Too much irradiation will result in small DNA-fragments that will detach, whereas, too little irradiation will give an inefficient hybridization with just a few signals. It is therefore important to initially verify the proper UV dose frequently and each time the lamps are replaced by applying a series of irradiation times varying from 1–10 min and by varying the distance from the slide to the UV-lamp between 1–10 cm. A Strata-Linker might also be used. Be aware to use appropriate eye- and skin-protection at all times against UV irradiation.

6. Do not cover slide and do not use oil.
7. There should be comet like structures present (**Fig. 1E**). If nuclei are still present as red bright balls, the lysis is insufficient (**Fig. 1C**). In that case one should perform the following protocol: dip slide gently vertically in 0.05% SDS for 10–30 s, drain excess of fluid, dip slide in Halo 1 for 10 s, proceed with **step 10**. However, use of SDS usually decreases the amount of cells that remain on the slide. In that case, one should also apply more cells or try to dry the slide more. The drier the slide, the more difficult lysis will be, so be careful not to dry too much.
8. The amount of fibers is sufficient when you see comet like structures all over the field, and when they are neatly arranged (**Fig. 1E**). If it is a cloudy view and you see bright crystals, the amount of cells is too high and should be decreased (**Fig. 1D**). If the slide is completely black (except for dirt), check the cell suspension after **step 3** for presence of cells. If there are easily visible cells in the suspension, they probably were removed during the lysis step. Skip **step 5** and blow dry the slide in **step 6** drier, i.e., dry the upper part completely but leave the lower part still quite wet and proceed with Halo 3. If lysis after the above steps is still insufficient, the last resort is to spin down the cell suspension, remove supernatant, resuspend cells in 50–100  $\mu$ L FCS and freeze overnight at  $-70^{\circ}\text{C}$ . Thaw rapidly and start with **Subheading 3.1.1**.
9. Probes are labeled with biotin and/or digoxigenin. Ideal sizes for probe fragments are in between 400–800 bp. This is achieved by changing the amount of DNase I from standard nick translation protocols. Check fragment lengths on a 2% agarose-gel, and test on germline fibers. After labeling, probes are precipitated and dissolved in hybridization mixture, thus allowing more probes to be mixed without altering hybridization conditions.
10. Slides can be used after overnight drying at room temperature, or after storage at  $-20^{\circ}\text{C}$ .
11. Do not remove coverslip at any time by any force; soak coverslip off in a Coplin jar.

## Acknowledgment

We are grateful to Berna Beverloo for providing the contig of cosmid probes for the EVT6 region and the cells with the ETV6-breakpoint. The work was in part financially supported by grants of the Dutch Cancer Society NKB-UL-99-1997 and NKB-KUN-99-1912 and of the IRS/J.A. Cohen Institute IRS6.3.4.

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## Multi-Telomere FISH

Samantha J. L. Knight and Jonathan Flint

### 1. Introduction

The standard investigation for suspected chromosomal rearrangements in patients is cytogenetic analysis at a 400–550 band resolution, yet this cannot routinely detect rearrangements smaller than 5 Megabases (Mb), and much larger abnormalities escape notice if they occur in regions where the banding pattern is not distinctive. In the future, this problem will largely be solved by the use of high resolution micro-arrays that will allow the entire genome to be investigated for submicroscopic chromosomal rearrangements. However, until this technology becomes routine, the only way of achieving increased reliability and resolution is to focus on specific chromosomal regions such as the ends of chromosomes (telomeres).

Screening telomeres for rearrangements has a number of attractions. First, the majority of translocations involve chromosome ends and therefore an assay that targets telomeres will detect these with 100% sensitivity regardless of size. Second, the regions adjacent to telomeres are gene-rich, so rearrangements involving such regions are more likely to have phenotypic consequences than rearrangements in many other parts of the genome (*1*). Finally, rearrangements involving telomeres are emerging as an important cause of human genetic diseases (*2–7*). For example, Wolf-Hirschhorn syndrome (chromosome 4p), Cri du Chat syndrome (chromosome 5p), Miller-Dieker syndrome (chromosome 17p), and alpha-thalassaemia with mental retardation (ATR-16, chromosome 16p) are all known to be caused by the unbalanced products of subtelomeric translocations.

Until recently, testing for rearrangements involving telomeres has proved problematical both owing to the complexity of the sequence structure of chromosome ends (*8–13*) and the technical problem of designing a highly sensitive and specific telomere assay which would also be financially acceptable. Both difficulties have now been addressed and multi-telomere FISH assays that use telomere specific probes for the detection of cryptic rearrangements are available. The assay outlined in this chapter is the Chromoprobe Multiprobe<sup>®</sup>-T System (Cytocell Ltd) and involves the use of

a microscope slide that is divided into 24 squares and a Multiprobe<sup>®</sup> device, which is essentially a coverslip with 24 raised square bosses (14). This arrangement allows up to 24 dual hybridizations (48 in total) to be performed, so all telomeres can be investigated on a single microscope slide. An overview of the procedure is shown in Fig. 1. In brief, each assay involves adding a drop of fixed chromosome suspension from the patient sample onto each square of the slide. To each square of the device, the appropriately labeled telomere probes are added. The slide and the device are then sandwiched together. The probe and chromosomal DNA are denatured simultaneously on a hotplate and hybridization allowed to proceed overnight. Following hybridization, a series of wash steps are performed and the resulting slides are analyzed by fluorescence microscopy. The numbers of signals specific to each telomere probe are counted and any anomalies indicating possible rearrangements are investigated. Further investigation may be achieved either by using individual telomere specific clones, which are commercially available ready labeled, or alternatively, by acquiring a glycerol set of telomere-specific clones and preparing and labeling the DNA "in-house."

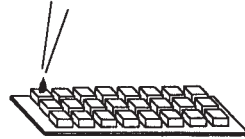
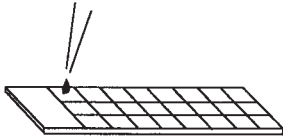
The Chromoprobe Multiprobe<sup>®</sup>-T System is widely used for the detection of cryptic subtelomeric rearrangements in individuals with idiopathic mental retardation. The probes within the current system are mainly PACs from the 2<sup>nd</sup> Generation set of telomere clones (see Table 1[b]). However, the original Multiprobe<sup>®</sup> system used the 1<sup>st</sup> Generation set of telomere clones that consisted mainly of cosmids with a few PACs and P1s (see Table 1[a]). It was using these 1<sup>st</sup> Generation clones that Knight et al. showed that subtelomeric rearrangements are an important cause of mental retardation, occurring in 7.4% individuals with moderate to severe developmental delay and dysmorphic features, and in about half of those presenting with a positive family history (25). These findings have since been confirmed in numerous case reports (26–34) and in larger studies where researchers have been able to divide their patients according to the degree of mental disability (35–43). There are other large studies which present frequencies of subtelomeric anomalies varying from 2.2–9%, but in these cases the selection criteria were more diverse (44–48).

In an attempt to identify a more refined subset of individuals in whom subtelomeric rearrangements might be occurring, de Vries et al. assessed clinical variables for a number of developmentally delayed individuals and normal controls whose samples had been tested for telomeric integrity (49). The results indicated that prenatal onset of growth retardation and a positive family history for developmental delay are good indicators for the presence of subtelomeric anomalies. These clinical criteria, in addition to features suggestive of a chromosomal phenotype, were included in a five item clinical checklist that de Vries et al. predicted would improve the diagnostic pick up rate of subtelomeric rearrangements among the developmentally delayed (49).

Although the Chromoprobe Multiprobe<sup>®</sup>-T System was originally used in the study of developmental delay, providing new diagnoses in affected individuals, it is now also proving invaluable for other clinical applications (50). For example, there are an increasing number of reports where the study of telomere integrity has allowed the full resolution of patients' karyotypes (51–54). In such cases, diagnostic laboratories had previously discovered an abnormal karyotype, but further submicroscopic rearrangements could not be ruled

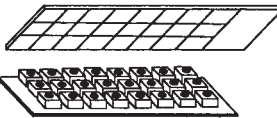
Add 2µl fixed chromosome suspension to each square of cleaned template slide.

Add 2µl pre-warmed hybridization solution to each boss of Multiprobe device.

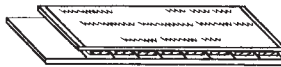


Wash slide in 2XSSC and dehydrate through ethanol series. Pre-warm slide on 37°C hotblock.

Pre-warm Multiprobe device on 37°C hotblock.



Carefully align template slide with Multiprobe device and bring into contact to form sandwich. Invert so that slide is beneath Multiprobe device.



Denature at 75°C on heating block and hybridize overnight in sealed plastic box floating in 37°C waterbath.

Remove Multiprobe device and wash template slide:

0.4XSSC (pH7.0) 72°C.

2XSSC, 0.05% Tween 20 at room temperature.



Mount slide and view by fluorescence microscopy.

Fig. 1. Overview of the Chromprobe Multiprobe®-T Assay.

**Table 1(a)**  
**1<sup>st</sup> Generation Telomere Clones**

Telomere	Clone ID	Availability <sup>a</sup>	Clone Type <sup>b</sup>
1p	CEB108 <sup>d</sup>	ATCC	Cosmid ( <b>16</b> )
1p	GS-13-P11	Incyte	PAC
1q	112N326Q6	ICRF	Cosmid
1q	2123.2a1	ATCC	Cosmid ( <b>14</b> )
2p	GS-68-13	Incyte	PAC
2p	2052f6	ATCC	Cosmid
2q	210-E14	DuPont/Incyte	PI
2q	2112b2	ATCC	Cosmid
3p	B47a2,AT-B47b2	ATCC	Cosmid ( <b>14</b> )
3q	B35c1	ATCC	Cosmid
4p	B31	ATCC	Cosmid ( <b>17</b> )
4q	cT55	ATCC	Cosmid ( <b>18</b> )
5p	84c11	ATCC	Cosmid
5q	B22a4	ATCC	Cosmid
6p	GS-36-12	Incyte	PAC
6q	2158e3	ATCC	Cosmid
7p	109a6 <sup>4</sup>	ATCC	Cosmid
7q	3	OA	Cosmid ( <b>19</b> )
7q	2000a5 <sup>d</sup>	ATCC	Cosmid
8p	GS-63-M14	Incyte	PAC
8q	2205a2	ATCC	Cosmid
8q	129A5	LANL	Cosmid
8q	2053b3	ATCC	Cosmid
9p	34-H2	Incyte	PAC
9q	2241c1	ATCC	Cosmid ( <b>14</b> )
10p	2189b6	ATCC	Cosmid
10q	2136a1	ATCC	Cosmid ( <b>14</b> )
11p	2209a2	ATCC	Cosmid
11p	146C2	LLNL	Cosmid
11q	161D7	LLNL	Cosmid
11q	2072c1	ATCC	Cosmid
12p	GS-90-15	Incyte	PAC
12q	GS-221-K18	Incyte	PAC
12q	2196b2	ATCC	Cosmid
13q	85-A10	DuPont	PI
13q	2002e1	ATCC	Cosmid
14q	60-H4	DuPont	PI
14q	2006a1	ATCC	Cosmid
15q	GS-154-PI	Incyte	PAC
16p	GS-119-L6	Incyte	PAC
16p	cGG4	ATCC	Cosmid
16q	D3b1	ATCC	Cosmid
17p	2111b1	ATCC	Cosmid ( <b>14</b> )

(continued)

Table 1(a) (continued)

Telomere	Clone ID	Availability <sup>a</sup>	Clone Type <sup>b</sup>
17q	111D9	LYNL	Cosmid
17q	B37c1	ATCC	Cosmid
18p	GS-52-M11	Incyte	PI
18q	127B6	LYNL	Cosmid
18q	2050a6	ATCC	Cosmid
19p	F20643	LYNL	Cosmid
19q	F21283 <sup>d</sup>	LYNL	Cosmid
20p	THY7	OA	Cosmid
20p	2005a4	ATCC	Cosmid
20q	GS-204-A16	Incyte	PAC (14)
21q	2H14	DuPont/Incyte	PI
21q	C9a1	ATCC	Cosmid
22q	106G1220	ICRF	Cosmid (20)
22q	N85A3	ATCC/ICRF	Cosmid (21)
XpYp	CY29	ATCC	Cosmid (22)
XqYq	C8.2/1 <sup>d</sup>	ATCC	Cosmid (23)

<sup>a</sup>Clones are available from ATCC (American Type Culture Collection), Incyte (<http://www.incyte.com>), ICRF (Imperial Cancer Research Fund MPI for Molecular Genetics reference library database), Du Pont (Du Pont Merck Pharmaceutical Company Human Foreskin Fibroblast PI Library 1), LANL (Los Alamos National Library), LLNL (Lawrence Livermore National Library), RGI (Research Genetics Inc), Bacpac website (<http://bacpac.med.buffalo.edu/human/overview.html>), from the originating author ('OA') or, for research purposes and for registered users only, from the Human Genome Mapping Project Resource Centre (<http://www.hgmp.mrc.ac.uk>).

<sup>b</sup>All clones except those referenced separately are attributed to the National Institutes of Health and Institute of Molecular Medicine Collaboration (15)

<sup>c</sup>All clones are attributed to Knight and Lese et al. (24)

<sup>d</sup>Clones used in Cytocell Ltd's Chromoprobe Multiprobe®-T System.

out because of the insufficient resolution of standard karyotyping. Multi-telomere FISH is also being used in this way for the characterization of the often complex karyotypes found in hematological malignancies (55–58). A further application is in providing new diagnoses in spontaneous recurrent miscarriages and infertility (26,59). Telomere specific probes also find use in the investigation of rearrangements when there are already clues regarding the possible involvement of particular telomeric regions. For example, in selected cases, individual telomere specific probes have proved informative for testing cleavage stage embryos for preimplantation diagnosis (60–62).

A major aim of the Multiprobe® telomere assay is to improve diagnostic capabilities so that families can be better informed, genetic counselling made as accurate as possible and therapeutic and preventative strategies advanced. Currently, there at least five other strategies that may also be employed for the study of telomere integrity (1) the telomere chip (63), (2) a comparative genome hybridization approach (64), (3) scanning short tandem repeat polymorphisms (65–67), (4) locus copy number measurement by hybridization with amplifiable probes (68,69) and (5) multiplex or multicolor

**Table 1(b)**  
**2<sup>nd</sup> Generation Telomere Clones**

Telomere	Clone ID	Availability <sup>a</sup>	Clone Type <sup>c</sup>
1p	GS-62-L8	Incyte	PAC
1p	GS-232-B23 <sup>d</sup>	Incyte	BAC
1q	GS-160-H23	Incyte	PAC
1q	GS-167-K11	Incyte	BAC
2p	GS-892-G20 <sup>d</sup>	Incyte	PAC
2p	GS-8-L3	Incyte	BAC
2q	GS-1011-O17 <sup>d</sup>	Incyte	PAC
2q	RG-172-113	RGI	BAC
3p	GS-1186-B18 <sup>d</sup>	Incyte	PAC
3p	RG-228-K22	RGI	BAC
3q	GS-196-F4 <sup>d</sup>	Incyte	PAC
3q	GS-56-H22	Incyte	BAC
4p	GS-36-P21 <sup>d</sup>	Incyte	PAC
4p	GS-118-B13	Incyte	BAC
4q	GS-963-K6 <sup>d</sup>	Incyte	PAC
4q	GS-31-J3	Incyte	BAC
5p	GS-189-N21 <sup>d</sup>	Incyte	PAC
5p	GS-24-H17	Incyte	PAC
5q	GS-240-G13 <sup>d</sup>	Incyte	PAC
6p	GS-62-I11 <sup>d</sup>	Incyte	PAC
6p	GS-196-I5	Incyte	BAC
6q	GS-57-H24 <sup>d</sup>	Incyte	PAC
7p	GS-164-D18	Incyte	PAC
7q	GS-3K-23	Incyte	PAC
8p	GS-580-L5 <sup>d</sup>	Incyte	PAC
8p	GS-77-L23	Incyte	BAC
8q	GS-489-D14 <sup>d</sup>	Incyte	PAC
8q	GS-261-I1	Incyte	BAC
9p	GS-43-N6 <sup>d</sup>	Incyte	PAC
9p	RG-41-L13	Incyte	BAC
9q	GS-112-N13 <sup>d</sup>	Incyte	PAC
9q	GS-135-I17	Incyte	BAC
10p	GS-306-F7 <sup>d</sup>	Incyte	PAC
10p	GS-23-B11	Incyte	BAC
10q	GS-137-E24 <sup>d</sup>	Incyte	PAC
10q	GS-261-B16	Incyte	BAC
11p	GS-908-H22 <sup>d</sup>	Incyte	PAC
11p	GS-44-H16	Incyte	PAC
11q	GS-770-G7 <sup>d</sup>	Incyte	PAC
11q	GS-26-N8	Incyte	PAC
12p	GS-496-A11 <sup>d</sup>	Incyte	PAC
12p	GS-8-M16	Incyte	BAC
12q	GS-124-K20	Incyte	BAC

(continued)

**Table 1(b) (continued)**

Telomere	Clone ID	Availability <sup>a</sup>	Clone Type <sup>c</sup>
12q	GS-221-K18 <sup>d</sup>	Incyte	PAC
13q	GS-163-C9 <sup>d</sup>	Incyte	PAC
13q	GS-1-L16	Incyte	PAC
14q	GS-820-M16 <sup>d</sup>	Incyte	PAC
14q	GS-200-D12	Incyte	BAC
15q	GS-124-05 <sup>d</sup>	Incyte	PAC
15q	GS-154-P1 <sup>d</sup>	Incyte	PAC
16p	GS-121-I4 <sup>d</sup>	Incyte	PAC
16p	RG-191-K2	RGI	BAC
16q	GS-240-G10 <sup>d</sup>	Incyte	PAC
16q	GS-191-P24	Incyte	PAC
17p	GS-202-L17 <sup>d</sup>	Incyte	PAC
17p	GS-68-F18	Incyte	BAC
17q	GS-362-K4 <sup>d</sup>	Incyte	PAC
17q	GS-50-C4	Incyte	BAC
18p	GS-52-M11	Incyte	PI
18p	GS-74-G18 <sup>d</sup>	Incyte	BAC
18q	GS-964-M9 <sup>d</sup>	Incyte	PAC
18q	GS-75-F20	Incyte	BAC
19p	GS-546-C1 <sup>d</sup>	Incyte	PAC
19p	RG-129-F16	RGI	BAC
19q	GS-48-O23	Incyte	PAC
19q	GS-325-I23	Incyte	BAC
20p	GS-1061-L1 <sup>d</sup>	Incyte	PAC
20p	GS-82-O2	Incyte	PAC
20q	GS-81-F12 <sup>d</sup>	Incyte	PAC
21q	GS-63-H24 <sup>d</sup>	Incyte	PI
21q	GS-2-H14b	Incyte	PAC
22q	GS-99-K24 <sup>d</sup>	Incyte	PAC
22q	CTD-3018-K24 <sup>d</sup>	Incyte	BAC
XpYp	GS-98-C4	Incyte	PAC
XpYp	RPCI-11-839-D20 <sup>d</sup>	BacPac	BAC
XqYq	GS-225-F6	Incyte	BAC
Xq	GS-202-M24	Incyte	PAC

FISH (M-TEL) (70–72). Two approaches for assessing all possible human telomeres are commercially available – the Chromoprobe Multiprobe<sup>®</sup>-T System of Cytocell Ltd and the ToTel Vysion Multicolor FISH Probe Panel of Vysis Inc. Of these, the Chromoprobe Multiprobe<sup>®</sup>-T System is used widely in clinical diagnostic laboratories. In this chapter we give a detailed account of the methodology involved in this assay and troubleshoot problems that are commonly encountered. We also present the materials and methodology necessary to prepare and use a complete set of indirectly-labeled telomere FISH probes “in house.”

## 2. Materials

### 2.1. Preparing Fixed Chromosome Suspensions

1. 1–5 mL peripheral blood sample or 25 mL healthy, dividing lymphoblastoid cell line (*see Note 1*).
2. Access to class II tissue culture hood (e.g., BioMAT-2, Envair Ltd., Rossendale, UK).
3. 25 cm<sup>3</sup> and 75 cm<sup>3</sup> tissue culture flasks (Corning Inc., New York, USA).
4. Nunclutubes (Gibco-BRL, Paisley, UK).
5. RPMI 1640 Media: with NaHCO<sub>3</sub>, without Glutamine (Sigma, Poole, UK).
6. Penicillin (500 IU/mL)/Streptomycin (–5000 UG/mL) (Gibco-BRL, Paisley, UK).
7. L-glutamine : 200 mM (Gibco-BRL, Paisley, UK).
8. Foetal calf serum (FCS) (GlobePharm).
9. M form phytohaemagglutinin (PHA) (Gibco-BRL, Paisley, UK).
10. Thymidine (Sigma, Poole, UK).
11. KaryoMax colcemid<sup>®</sup> solution 10 mg/mL (Gibco-BRL, Paisley, UK).
12. Potassium chloride (KCl) (Sigma, Poole, UK).
13. Methanol, AnaLaR Grade (BDH Laboratory Supplies, Poole, UK).
14. Glacial acetic acid (BDH Laboratory Supplies, Poole, UK).

### 2.2. Hybridization and Post-Hybridization Processing Using Chromoprobe Multiprobe<sup>®</sup>-T System (Cytocell Ltd.)

1. Phase contrast microscope (e.g., Olympus BX40, Olympus Optical Co. Ltd., UK).
2. Plastic or glass Coplin jars (Raymond Lamb Inc., Eastbourne, UK).
3. Two heating blocks with flat surface (e.g., Cytocell Hotplate, Cytocell Ltd., Adderbury, UK).
4. CCD microscope equipped with Pinkel filter wheel containing DAPI, Texas Red, FITC, dual and triple bandpass filters (e.g., Olympus BX60, Olympus Optical Co. Ltd., UK).
5. Fluorescence grade microscope lens immersion oil (Carl Zeiss Ltd., Welwyn Garden City, UK).
6. Fixed chromosome suspension
7. Super premium microscope slides (BDH Laboratory Supplies, Poole, UK).
8. Chromoprobe Multiprobe<sup>®</sup>-T System Kit (Cytocell Ltd).
9. Fixative (3:1 methanol(AnalaR): glacial acetic acid).
10. 20X SSC: 3 M sodium chloride (Sigma, Poole, UK), 0.3 M tri-sodium citrate (Sigma, Poole, UK).
11. Wash solution I: 0.4X SSC
12. Wash solution II: 2X SSC + 0.05% Tween 20 (BDH Laboratory Supplies, Poole, UK).
13. Nail varnish (available from many high street stores).

### 2.3. Extraction of Telomere Clone DNA

1. Orbital shaking incubator (e.g., G24, New Brunswick Scientific Co. Inc., Edison, NJ).
2. Vacuum microcentrifuge (e.g., DNA Speed Vac DNA120, Savant Instruments Inc., Farmingdale, USA).
3. Glycerol stocks or stabs of telomere clones (*see Table 1* for availability).
4. Ampicillin (Sigma, Poole, UK).
5. Kanamycin (Sigma, Poole, UK).
6. Chloramphenicol (Sigma, Poole, UK).
7. LB agar plates: 1% w/v NaCl (Sigma, Poole, UK), 1% w/v Bactotryptone (Difco Labs, Michigan, USA), 0.5% w/v Bactoyeast extract (Difco Labs, Michigan, USA), 1.5% w/v

- Bactoagar (Difco Labs, Michigan, USA) and appropriate antibiotic (ampicillin at 50 µg/mL, chloramphenicol at 12.5 µg/mL or kanamycin at 35 µg/mL).
- 2X TY Broth: 1.6% w/v Bactotryptone (Difco Labs, Michigan, USA), 1% w/v Bactoyeast extract (Difco Labs, Michigan, USA), 0.5% w/v NaCl (Sigma, Poole, UK).
  - Glycerol (Sigma, Poole, UK).
  - 250 mL centrifuge tubes with orange screw caps (Corning Incorporated, New York, USA).
  - Cell resuspension solution: 1% glucose (filter-sterilized), 10 mM EDTA, 25 mM Tris-HCl, pH 7.4.
  - Cell lysis solution: 0.2M NaOH (Sigma, Poole, UK), 1% sodium dodecyl sulphate (SDS) (USB).
  - Neutralizing solution: 3M potassium acetate (Sigma, Poole, UK), 11.5% v/v glacial acetic acid, AnalaR Grade (BDH Laboratory Supplies, Poole, UK).
  - Plastic funnels (BDH Laboratory Supplies, Poole, UK).
  - Mesh (Hybaid Ltd., Ashford, UK).
  - Isopropanol, AnaLar Grade (BDH Laboratory Supplies, Poole, UK).
  - Ethanol, AnaLar Grade (BDH Laboratory Supplies, Poole, UK).
  - TE Buffer 10 mM Tris-HCl, pH 8.0 (Sigma, Poole, UK), 1 mM EDTA (Sigma, Poole, UK).

#### **2.4. Indirect Labeling of Telomere Clone DNAs by Nick-Translation**

- 1 mM Biotin-16-dUTP (Roche, Lewes, UK).
- 1 mM Digoxigenin-11-dUTP (Roche, Lewes, UK).
- 10X dNTP mix: 0.5 mM each of dATP, dGTP, dCTP; 0.1 mM dTTP (100 mM stocks from Roche, Lewes, UK).
- 10X nick translation buffer: 0.5M Tris-HCl, pH 7.5, 50 mM MgCl<sub>2</sub>, 0.5 mg/mL nuclease-free bovine serum albumin (BSA), fraction V (Sigma, Poole, UK).
- 100 mM dithiothrietol (Sigma, Poole, UK).
- 2.5 ng/µL DNase I (Amersham Life Sciences Ltd., Little Chalfont, UK).
- 10,000 U/mL DNA polymerase I (New England Biolabs, Hitchin, UK).
- SELECT™-B spin columns packed with Sephadex G50 (5 Prime→3 Prime Inc., Arapahoe Boulder, USA).
- Salmon sperm DNA (2 mg/mL stock sonicated to 200–500 bp).
- 1 mg/mL human COT-1 DNA (GibcoBRL, Paisley, UK).
- Formamide (Fluka).
- Hybridization solution: 65% formamide, 1X SSC, 10% dextran sulfate MW 500,000 (BDH Laboratory Supplies, Poole, UK).

#### **2.5. Hybridization of Indirectly Labeled Probes and Post- Hybridization Processing**

- Super premium microscope slides (BDH Laboratory Supplies, Poole, UK).
- 22 mm × 22 mm glass coverslips (BDH Laboratory Supplies, Poole, UK).
- Light proof plastic slide box (Raymond Lamb Inc., Eastbourne, UK).
- 22 mm × 64 mm coverslips manually cut from Parafilm (American National Cam. USA).
- Wash solution I: 0.4X SSC
- Wash solution II: 2X SSC + 0.05% Tween-20 (BDH Laboratory Supplies, Poole, UK).
- ST buffer: 4X SSC +0.05% Tween-20 (BDH Laboratory Supplies, Poole, UK).
- BSA fraction V (Sigma, Poole, UK).
- Blocking solution: 3% w/v BSA in ST buffer, filter-sterilized through 0.45 µm filter (*see Note 2*).

10. Antibody suspension 1 for dual detection: 1 mL blocking solution + 18  $\mu$ L mouse anti-digoxin fluorescein isothiocyanate (FITC) (Sigma, Poole, UK) + 0.65  $\mu$ L avidin Texas Red (Vector Laboratories, Peterborough, UK) (*see Note 3*).
11. Antibody suspension 1 for digoxigenin detection only: 1 mL blocking solution + 18  $\mu$ L Mouse anti-digoxin fluorescein isothiocyanate (FITC) (Sigma, Poole, UK).
12. Antibody suspension 1 for biotin detection only: 1 mL blocking solution + 0.65  $\mu$ L avidin Texas Red (Vector Laboratories, Peterborough, UK).
13. Antibody suspension 2 for dual detection: 1 mL blocking solution + 1.7  $\mu$ L rabbit antimouse FITC (Sigma, Poole, UK) + 7.5  $\mu$ L biotin anti-avidin (Vector Laboratories, Peterborough, UK).
14. Antibody suspension 2 for digoxigenin detection only: 1 mL blocking solution + 1.7  $\mu$ L rabbit anti-mouse FITC (Sigma, Poole, UK).
15. Antibody suspension 2 for biotin detection only: 1 mL blocking solution + 7.5  $\mu$ L biotin anti-avidin (Vector Laboratories, Peterborough, UK).
16. Antibody suspension 3 for dual detection: 1 mL blocking solution + 1.7  $\mu$ L goat anti-rabbit FITC (Sigma, Poole, UK) + 0.65  $\mu$ L avidin Texas Red (Vector Laboratories, Peterborough, UK).
17. Antibody suspension 3 for digoxigenin detection only: 1 mL blocking solution + 1.7  $\mu$ L goat anti-rabbit FITC (Sigma, Poole, UK).
18. Antibody suspension 3 for biotin detection only: 1 mL blocking solution + 0.65  $\mu$ L avidin Texas Red (Vector Laboratories, Peterborough, UK).
19. Vectashield mounting medium: (Vector Laboratories, Peterborough, UK) containing 1.5  $\mu$ g/mL 4',6-diamidino-2-phenylindole (DAPI).
20. Nail varnish (available from many high street stores).

### 3. Method

#### 3.1. Preparing Fixed Metaphase Chromosome Suspensions

##### 3.1.1. From Peripheral Blood

1. Equilibrate RPMI-1640, FCS, L-glutamine and penicillin/streptomycin to 37°C in a waterbath (*see Note 4*).
2. In a Class II tissue culture hood, use sterile technique and make complete medium by adding 100 mL FCS, 5 mL L-glutamine and 5 mL penicillin/streptomycin to the 500 mL bottle of RPMI-1640.
3. To duplicate Nunc tubes, add 0.25 mL peripheral blood, 5 mL complete medium, and add PHA to a final concentration of 2%.
4. Place in a 37°C incubator for 72 h.
5. Add 100  $\mu$ L of 15 mg/mL thymidine to each culture and mix gently.
6. Place in a 37°C incubator for 16–18 h.
7. Centrifuge cultures at 180g for 5 min.
8. Pour off supernatant and resuspend cell pellet in 5 mL complete medium.
9. Centrifuge at 180g for 5 min.
10. Pour off supernatant and resuspend cell pellet in 5 mL complete medium.
11. Place in a 37°C incubator for 5 h.
12. Add 100  $\mu$ L of 10  $\mu$ g/mL KaryoMax colcemid
13. Place in a 37°C incubator for 10 min.
14. Centrifuge at 180g for 5 min.
15. Pour off the supernatant and resuspend the cell pellet in 7 mL 0.075M KCl prewarmed to 37°C.

16. Incubate at 37°C for 15 min.
17. Centrifuge at 180g for 5 min.
18. Discard supernatant.
19. Rest tube on a vortex set at medium speed and fix the cells by the careful, dropwise addition of precooled 3:1 methanol: acetic acid (fixative). When the solution turns brown, the fixative may be added more rapidly until a total of 5 mL have been added.
20. Centrifuge at 180g for 5 min and discard supernatant.
21. Add 5 mL cold fixative.
22. Centrifuge at 180g for 5 min and discard supernatant.
23. Add 5 mL cold fixative.
24. Centrifuge at 180g for 5 min and discard supernatant
25. Resuspend pellet in 3 mL fixative.
26. Store at -20°C until ready to use.

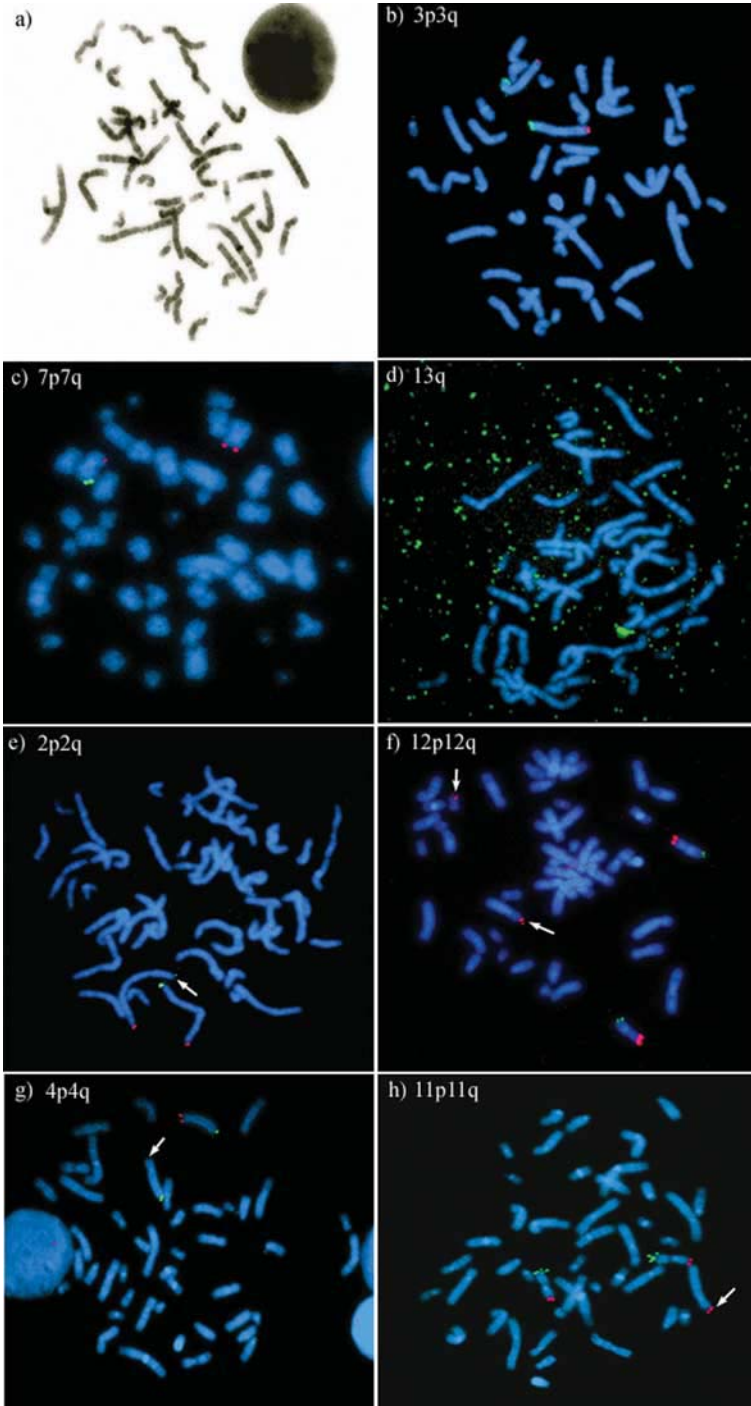
### **3.1.2. From Lymphoblastoid Cell Lines**

1. Grow cells until they are proliferating well in a vol of 50 mL complete medium.
2. 18–24 h prior to harvesting, remove old medium and replace with fresh medium.
3. When ready to harvest, transfer 20 mL of healthy proliferating cells to a 50 mL Falcon tube.
4. Add 200  $\mu$ L of 10  $\mu$ g/mL KaryoMax colcemid and mix gently.
5. Place in a 37°C incubator for 50–60 min (*see Note 5*).
6. Centrifuge at 180g for 5 min.
7. Pour off the supernatant and resuspend the cell pellet in 7 mL 0.075M KCl prewarmed to 37°C.
8. Incubate at 37°C for 20 min (*see Note 6*).
9. Centrifuge at 180g for 5 min and discard supernatant.
10. Rest the tube on vortex set at medium speed and fix cells by the dropwise addition of 2 mL precooled fixative.
11. Add a further 13 mL cold fixative.
12. Centrifuge at 180g for 5 min and discard supernatant.
13. Add 15 mL cold fixative.
14. Centrifuge at 180g for 5 min and discard supernatant.
15. Add 15 mL cold fixative.
16. Centrifuge at 180g for 5 min and discard supernatant
17. Resuspend pellet in 1 mL fixative.
18. Store at -20°C until ready to use.

## **3.2. Hybridization and Post-Hybridization Processing Using Cytocell Ltd Chromoprobe Multiprobe<sup>®</sup>-T System (*see Note 7*)**

### **3.2.1. Optimize Concentration of Fixed Chromosome Suspension**

1. Gently apply 2  $\mu$ L of the suspension to the surface of a clean, Super Premium Microscope slide.
2. Allow the drop to dry and view by phase-contrast microscopy using a  $\times 10$  objective.
3. Ideally there should be a mitotic index of  $\geq 5\%$  (*see Note 8*) and individual metaphases should be well defined with minimal overlapping chromosomes (*see Fig. 2a*).
4. If the cell density is too high, dilute the suspension with cold fresh fixative until the desired concentration is achieved.
5. If the concentration is too low, pellet the cells at 160g for 5 min. Pour off the supernatant and resuspend the pellet with increasing volumes of fresh fixative until the desired concentration is achieved.



### 3.2.2. Prepare the Multiprobe<sup>®</sup>-T Microscope Slide

1. Soak the slide in 100% methanol for 2 min.
2. Polish dry with a clean soft tissue
3. Pipet 2  $\mu$ L of optimized fixed chromosome suspension onto each square of the Multiprobe<sup>®</sup>-T slide in four discrete sets of pipetting following the order: (A) Square 1, 19, 5, X, 17, 3, 21, and 7. Allow to dry. (B) Square 2, 20, 6, Y, 18, 4, 22, and 8. Allow to dry. (C) Square 9, 11, 13, and 15. Allow to dry. (D) Square 10, 12, 14, and 16. Allow to dry.
4. Examine each spot under phase-contrast to ensure that no squares have been missed and that all squares have an acceptable number of metaphases for analysis (*see Note 9*).

### 3.2.3. Hybridization Procedure

1. Preheat one heating block to 37°C and one to 75°C (*see Note 10*).
2. Place the Chromoprobe Multiprobe<sup>®</sup> Hybridization Chamber in a 37°C waterbath to equilibrate.
3. Mix the hybridization solution (provided in kit) by repeated pipetting.
4. Transfer 60  $\mu$ L to a fresh Eppendorf tube and prewarm to 37°C in a heating block, incubator or waterbath.
5. Prewarm the Multiprobe<sup>®</sup> device, probe side up (i.e., raised bossed surfaces up and labeled surface down). Do not touch the bossed surfaces.
6. Prepare one Coplin jar containing 2X SSC and three Coplin jars containing the series 70, 85, and 100% Ethanol respectively.
7. Wash preprepared, spotted template slide for 2 min in 2X SSC and then dehydrate for 2 min in each of 70, 85, and 100% Ethanol.
8. Air-dry the slide and place spotted side up on a 37°C heating block.
9. Meanwhile, use a P10 micropipet to add 2  $\mu$ L of prewarmed hybridization solution to each raised square/boss of the Multiprobe<sup>®</sup> device (keep on 37°C heating block).
10. Carefully invert the template slide (so that spots are now facing downwards) and bring into contact with the Multiprobe<sup>®</sup> device with the appropriate squares matching i.e., so that square 1 is located over the top right hand square of the Multiprobe<sup>®</sup> device, which is marked with a yellow spot.
11. Gently lift the slide/Multiprobe<sup>®</sup> device and invert so that the slide is now beneath the device (*see Note 11*).
12. Place on a 37°C heating block for 10 min.
13. Keeping the slide/Multiprobe<sup>®</sup> device arrangement flat, carefully transfer to the surface of a 75°C heating block.
14. Denature for 1–3 min depending on the age of the fixed chromosome suspension (*see Note 12*).

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Fig. 2. **(a)** An example of a metaphase spread that is of acceptable quality for hybridization. **(b)** A normal hybridization pattern shown by the 3p3q telomere specific PACs. **(c)** The bloated appearance of chromosomes which have been overdenatured. In this case, the del 7p result remains clear. **(d)** The speckled appearance is typical of excess antibody – a similar appearance may arise through insufficient washing. **(e)** The 2p2q polymorphism – in this case the arrowed 2q signal is barely visible. **(f)** The crosshybridization of the 12p cosmid to the subtelomeric regions of 6p and 20q. **(g)** and **(h)** hybridization patterns on squares 4 and 11 of the same microscope slide revealing a clinically significant unbalanced translocation (4p monosomy and 11p trisomy) in an individual with developmental delay. In all hybridization examples, the p-arm signals are shown in red and the q-arm signals in green.

15. Immediately transfer the slide/Multiprobe<sup>®</sup> device to the prewarmed Chromoprobe Multiprobe<sup>®</sup> Hybridization Chamber (or light-proof plastic slide box), replace the lid and leave overnight with the lid of the waterbath off.

### 3.2.4. Post-Hybridization Washing and Mounting

1. Add wash solution I to a clean Coplin jar and equilibrate in a 72°C waterbath. Then, adjust to pH 7.0.
2. Add wash solution II to a clean Coplin jar and stand at room temperature (20–25°C).
3. Retrieve the slide/Multiprobe<sup>®</sup> device from the hybridization chamber.
4. Carefully remove the Multiprobe<sup>®</sup> device from the template slide and immediately place the slide in solution I for 2 min. Using forceps, agitate the slide gently up and down.
5. Transfer to solution II for 30 s.
6. Drain excess wash solution by holding the long edge of the slide against tissue for 10–20 s. Do not allow the slide surface to dry.
7. Add 20 µL DAPI-Antifade solution (provided in kit) to both ends of a large (24 × 60 mm) coverslip (also provided in kit) and gently lower the hybridized area of the slide onto the prepared coverslip.
8. Inspect the mounted area and coax out any air bubbles by gentle pressure with blunt ended forceps.
9. Place a paper towel over the slide and coverslip and apply gentle pressure to remove any excess mounting medium.
10. Seal by brushing a thin layer of nail varnish around the rim of the coverslip.
11. Leave in a dark box or cupboard for at least 10 min prior to viewing.

### 3.2.5. Viewing the Hybridized Slide by Fluorescence Microscopy

1. Place the slide on the microscope stage (*see Note 13*).
2. Using the ×10 objective and DAPI filter wheel, focus on Square 1 and identify metaphase chromosomes of a single cell.
3. Add a drop of mineral oil to the square and switch to the oil immersion objective (×100).
4. Identify the selected metaphase chromosomes and switch to the triple band pass filter wheel (DAPI, FITC, and Texas Red).
5. Score the p-arm (green) and q-arm (red) telomere signals and note the chromosomal location. A normal result for Square 1 is two green signals and two red signals on the p-arm and q-arm telomeres of chromosome 1 (*see Note 14*).
6. Confirm the results using FITC and Texas Red filters individually.
7. Repeat **steps 2–6** until 5 unambiguous results from 5 different metaphases on the square have been scored.
8. Repeat **steps 2–7** for every square of the slide.
9. Note the results. Any abnormal results or unsatisfactory hybridizations for certain telomere probes indicate those probes for which repeat hybridizations should be carried out on the sample (*see Note 15*).
10. Confirm abnormalities on a fresh sample from the test patient and where possible, also analyze samples from each parent. This will determine whether the abnormality is a true anomaly (and if so, whether it is *de novo* or familial in origin), or whether it might be a benign polymorphic variant (*see Note 16*).

### 3.3. Extraction of Telomere Clone DNA (*see Note 17*)

1. Make LB plates containing the correct antibiotic (ampicillin at 50 µg/mL, kanamycin at 30 µg/mL or chloramphenicol at 12.5 µg/mL).

2. Remove required glycerols from  $-70^{\circ}\text{C}$  freezer and place in a container of dry ice (*see Note 18*).
3. Streak desired glycerols onto plates and place in a  $37^{\circ}\text{C}$  bacterial incubator overnight.
4. Add 100 mL 2X TY media to a 250 mL centrifuge tube. Add appropriate antibiotic as outlined in **step 1**.
5. Inoculate the medium with a single colony from the plate and grow overnight at  $37^{\circ}\text{C}$  in a shaking incubator.
6. Make glycerol stocks by removing 700  $\mu\text{L}$  from the overnight culture and adding 300  $\mu\text{L}$  100% glycerol. Mix well, split between two Eppendorf tubes, label and store at  $-70^{\circ}\text{C}$ .
7. Pellet the remaining bacteria by centrifugation at 350g for 15 min.
8. Pour off supernatant and invert tubes on paper towels to allow excess supernatant to drain.
9. Resuspend the pellet in 30 mL cold Cell Resuspension Solution. Mix vigorously and pipet up and down to ensure that all lumps are removed.
10. Add 30 mL of cell lysis solution. Gently invert tubes once to mix.
11. Leave standing at room temperature for 5 min (no longer)
12. Immediately add 30 mL of cold Neutralizing solution. Invert gently to mix.
13. Place on ice for 15 min—invert gently every 5 min.
14. Centrifuge at 4000g at  $4^{\circ}\text{C}$  for 40 min.
15. Label a fresh 250 mL-tube and place a plastic funnel in the top.
16. Cut a circle of hybridization mesh, cut a slit to the center and fit into funnel.
17. Carefully pour centrifuged sample through the mesh and funnel into the 250-mL tube. Ensure that no flocculent material passes through the mesh.
18. Remove the funnel and mesh and wash with water and ethanol for subsequent use.
19. Precipitate the DNA by adding 0.7 vol 100% isopropanol.
20. Mix well and centrifuge at 4000g for 40 min
21. Pour off supernatant and add 25 mL 70% ethanol.
22. Vortex and centrifuge at 4000g for 10 min.
23. Pour off supernatant and drain excess by inversion on paper towel.
24. Resuspend pellet in 1 mL TE buffer (pH 8.0) (*see Note 19*).
25. Check the extracted DNA by running 5  $\mu\text{L}$  on a 0.9% minigel alongside a  $\lambda\text{HindIII}$  marker. Intact, cosmid DNA should remain in a high molecular weight position.
26. Measure the concentration of the extracted DNA (e.g., using fluorometer).
27. Store the extracted DNA at  $-20^{\circ}\text{C}$

### 3.4. Indirect Labeling of Telomere Clone DNAs by Nick-Translation (*see Notes 20 and 21*)

1. Place an Eppendorf tube on ice and add: 1.0  $\mu\text{g}$  probe DNA, 1.2  $\mu\text{L}$  biotin-16-dUTP (e.g., for p-arm probes) or digoxigenin-11-dUTP (e.g., for q-arm probes), 5.0  $\mu\text{L}$  10X dNTP mix, 5.0  $\mu\text{L}$  10X nick translation buffer, 5.0  $\mu\text{L}$  DTT (100 mM), 4.0  $\mu\text{L}$  DNase (2.5 ng/ $\mu\text{L}$ ) (*see Note 22*), 1.0  $\mu\text{L}$  DNA Polymerase I (10,000 U/ $\mu\text{L}$ ), x  $\mu\text{L}$  dH<sub>2</sub>O to give total reaction vol of 50  $\mu\text{L}$ .
2. Vortex briefly and microfuge on “touch” setting to eliminate air bubbles.
3. Transfer to  $15^{\circ}\text{C}$  waterbath for 90 min.
4. Pause the reaction by placing the tube on ice.
5. Remove 5  $\mu\text{L}$  and run on 2% w/v agarose gel with a PhiX174 size standard.
6. A smear of fragments from 50–500 bp is optimal for hybridization. If larger than this, add a further 1  $\mu\text{L}$  DNase to the reaction and incubate at  $15^{\circ}\text{C}$  for an additional 30 min.

7. When the probe is the correct size, remove unincorporated nucleotides by passing the through a SELECT™-B spin column prepacked with Sephadex™ G-50.
8. Add 25  $\mu\text{L}$  salmon sperm (scale up accordingly if more than 1  $\mu\text{g}$  DNA was labeled).
9. Add  $\text{dH}_2\text{O}$  to give a final probe concentration of 10  $\text{ng}/\mu\text{L}$  (take into account the amount of DNA removed for checking).
10. Note the volume of the 10  $\text{ng}/\mu\text{L}$  probe mix.
11. Add the appropriate amount of COT-1 DNA per 1  $\mu\text{L}$  of probe mix (calculate the amount to be added based on the probe and its original insert size [see **Note 23**]).
12. Calculate the volume of probe/COT-1 mix containing 120  $\text{ng}$  probe and aliquot this volume into Eppendorf tubes.
13. Dry down the aliquots by centrifugation in a speedyvac (do not overdry).
14. To create chromosome specific p-arm and q-arm telomere hybridization mixes, resuspend each pellet in 38  $\mu\text{L}$  hybridization solution and mix solutions together to give a total vol of 76  $\mu\text{L}$ .
15. To create individual telomere-specific mixes (i.e., p-arm or q-arm probes only), resuspend each pellet in 76  $\mu\text{L}$  hybridization solution.
16. Mix well and store at  $-20^\circ\text{C}$ .

### **3.5. Hybridization of Indirect Labeled Probes and Post-Hybridization Processing (see Note 20)**

#### **3.5.1. Hybridization**

1. Switch on heating block and preheat to  $75^\circ\text{C}$ .
2. Transfer 18  $\mu\text{L}$  probe hybridization solution to a clean Eppendorf tube and prewarm to  $37^\circ\text{C}$  in a waterbath or heating block.
3. Optimize concentration of test fixed cell suspension as described in **Subheading 3.2.1**.
4. Soak super premium microscope slide in 100% methanol for 2 min.
5. Polish dry with clean soft tissue.
6. Drop  $\sim 10$ – $20$   $\mu\text{L}$  of optimized fixed chromosome suspension onto the center of the slide.
7. Allow to dry and check quality by phase-contrast microscopy using  $\times 10$  objective.
8. Label the frosted end of the slide with the experiment details and mark boundaries of the chromosome spot on the reverse side of the slide using a diamond cutter.
9. Prepare one Coplin jar containing 2X SSC and three Coplin jars containing the ethanol series 70, 85, and 100% respectively.
10. Wash preprepared template slide (spotted with test fixed cell suspension) for 2 min in 2X SSC and then dehydrate for 2 min in each of 70, 85, and 100% ethanol.
11. Air dry slide and place chromosome spotted side up on a  $37^\circ\text{C}$  heating block.
12. Mix the prewarmed hybridization solution by gentle repeated pipeting (avoid bubbles).
13. Pipet hybridization solution onto the central region of the slide demarcated by the diamond cutter.
14. Carefully lower a  $22 \times 22$  mm coverslip over the hybridization solution. Coax out any air bubbles by gentle pressure with blunt ended forceps.
15. Carefully transfer the slide (coverslip uppermost) to surface of the  $75^\circ\text{C}$  heating block.
16. Allow to denature for 1–3 min depending on the age of the fixed chromosome suspension (see **Notes 12** and **24**).
17. Immediately transfer to an empty light-proof plastic slide container, replace the lid and place in a  $37^\circ\text{C}$  waterbath with the lid off overnight.

### 3.5.2. Post-Hybridization Washing, Antibody Detection, and Mounting

1. Prepare blocking solution and antibody suspensions 1–3 (*see Note 25*).
2. Vortex antibody suspensions well and microcentrifuge at top speed for 10 min. Keep in the dark and if necessary, equilibrate to room temperature prior to use.
3. Add wash solution I to a clean Coplin jar and equilibrate in a 72°C waterbath. Then, adjust the pH to pH 7.0.
4. Add wash solution II to a clean Coplin jar and stand at room temperature (20–25°C).
5. Meanwhile, place a piece of tissue moistened with water in the bottom of one end of a light proof plastic box (e.g., an empty slide box).
6. Using scissors, cut 4 pieces of ~22 × 64 mm parafilm for each slide being processed.
7. Remove the slide from light-proof box in the waterbath and jerk sharply over tissue paper to remove the coverslip.
8. Immediately place the slide in solution I for 2 min. Using forceps, agitate slide gently up and down.
9. Transfer to solution II for 30 s.
10. Drain excess wash solution by holding the long edge of the slide against tissue for 10–20 s. Do not allow the slide surface to dry.
11. Place the slide in the prepared light-proof plastic box in a raised position (e.g., with each end resting on a cocktail stick or on the inside platforms of the box). Quickly pipet 300 µL blocking solution over the slide and cover with a parafilm coverslip. Avoid air bubbles.
12. Put the lid on the box and place in a 37°C oven for 40–50 min.
13. During the incubation period, prewarm 1.5 L of ST solution to 37°C.
14. Following incubation, remove the parafilm coverslip from the slide and place the slide in a glass slide holder containing prewarmed ST solution.
15. Place on a shaking platform for 3 min. Keep in the dark by placing an inverted light-proof container (e.g., a cardboard box) over the top.
16. Remove the slide and drain the excess wash solution as in **step 10**.
17. Place the slide in the light-proof plastic box as before and immediately add ~80 µL antibody suspension 1 to the hybridized area.
18. Avoiding air bubbles, carefully overlay parafilm coverslip.
19. Put the lid on the box and place in a 37°C incubator for 10 min.
20. Remove the parafilm coverslip and immediately transfer the slide to a glass slide holder containing prewarmed ST solution.
21. Agitate on shaking platform as before for 3 min.
22. Pour off the solution and replace with fresh prewarmed ST solution.
23. Repeat **steps 21** and **22** a further 2×.
24. After the third wash, remove the slide and drain the excess wash solution as described in **step 10**.
25. Place the slide in the light-proof plastic box as before and immediately add ~80 µL appropriate antibody suspension 2 to the hybridized area
26. Avoiding air bubbles, carefully overlay a parafilm coverslip.
27. Put the lid on the box and place in 37°C incubator for 10 min.
28. Remove the parafilm and wash the slide 3× each for 3 min in fresh prewarmed ST solution.
29. Remove the slide and drain excess wash solution as in **step 10**.
30. Place the slide in the light-proof plastic box as before and immediately add ~80 µL appropriate antibody suspension 3 to the hybridized area.
31. Put the lid on the box and place in a 37°C incubator for 10 min.

32. Remove the parafilm coverslip and perform a final 3 washes each for 3 min in fresh prewarmed ST solution.
33. During the final wash, add ~25  $\mu\text{L}$  DAPI in Vectashield to a  $22 \times 22$  mm coverslip resting on a paper towel.
34. Remove the slide and drain the excess wash solution as in **step 10**.
35. Gently lower the hybridized area of the slide onto the prepared coverslip.
36. Place a paper towel over the slide and coverslip and apply gentle pressure to remove any excess mounting medium.
37. Inspect the mounted area and coax out any air bubbles by gentle pressure with blunt ended forceps.
38. Seal by brushing a thin layer of nail varnish around the rim of the coverslip.
39. Place in the dark and allow to dry before viewing by fluorescence microscopy as described in **Subheading 3.2.5**.

#### 4. Notes

1. For optimal chromosome preparations, peripheral blood samples should be collected in lithium-heparin tubes, the samples should be transported at room temperature and cultured as soon as possible. The quality of the fixed cell suspensions deteriorates markedly with time.
2. Blocking solution can be made in bulk and stored in filter-sterilized aliquots at  $4^\circ\text{C}$  for up to 6 mo.
3. 1-mL aliquots of antibody suspensions can be stored in light-proof containers at  $4^\circ\text{C}$  for up to 1 mo.
4. To avoid contamination, tissue culture media always should be placed in a designated waterbath reserved for tissue culture work.
5. Colcemid prevents spindle formation and so arrests the chromosomes in the metaphase stage of the cell cycle. The amount of time the cells are left in the colcemid solution affects the morphology of the chromosomes. If they are left for too short a period of time, the chromosomes may be long, but the resulting MI may not be sufficient. However, if the cells are left for too long, they appear shorter and sub-optimal for analysis.
6. Under-incubation of cells in hypotonic solution will fail to burst open the cell whereas, over-incubation will lead to excessive bursting.
7. Although the Chromoprobe Multiprobe<sup>®</sup> System Kit contains probes and reagents which have been quality-controlled by Cytocell Ltd., it is advisable to confirm optimal performance of the kit by carrying out an initial test hybridization using a good quality normal control chromosome suspension prior to proceeding with valuable study samples.
8. Although the ideal MI is  $\geq 5\%$ , this may not be achievable owing to problems, such as the age of the sample or its origin (e.g., leukaemic samples often have a suboptimal MI). In these circumstances, one should aim for approx 5 metaphases in the central region of the chromosome spread. In particularly difficult cases where the MI is very low and there is limited sample, it may be more feasible to opt for a multicolor FISH technique such as M-TEL (71).
9. If there are not enough metaphases on only a few squares then it is possible to respot some suspension over the original spot on these squares.
10. For the  $37^\circ\text{C}$  heating block it is sufficient to invert a standard tube-holding heating block so that the flat surface is uppermost. However, the  $75^\circ\text{C}$  hotplate must be a solid block with even surface temperature.
11. The best technique is to first lift the slide/Multiprobe arrangement upwards holding only the etched end of the slide between the forefinger and thumb of one hand. Then, gently

hold the arrangement between the thumb and forefinger of the other hand (do not squeeze) and invert. The arrangement can then be transported safely held flat in the palm of the hand or a tray.

12. It is important to get the denaturing step right. If the chromosomes are denatured for too long, then the chromosomes become bloated and there may be tracking of the probe signal along the chromosome arm (*see Fig. 2c*). Conversely, if the denaturation time is not enough, then the morphology will be retained, but there will be little if any hybridization. In general, the longer the cell suspension has been kept at  $-20^{\circ}\text{C}$ , the longer the chromosomes require to become denatured. For example, a cell suspension prepared and hybridized on the same day may need only 45 s to denature whereas, a sample over a year old may need more than 3 min. On average, samples that are 1–3 mo old require about 1 min 15 s denaturing time at  $75^{\circ}\text{C}$ .
13. Some microscopes have stages that make it difficult to view the extreme ends of the slides. In these situations, turning the slide around  $180^{\circ}$  should alleviate the problem.
14. Before attempting to score any results it is important to be familiar with certain characteristics associated with some of the telomere clones so that misinterpretation can be avoided. It is entirely normal for some of the clones to detect polymorphisms – these clones hybridize only to their cognate telomeres, but if a polymorphism is present, they hybridize only partially or not at all to one of the chromosome homologues. It is also characteristic for some clones to exhibit crosshybridization – these clones hybridize to other telomeres in addition to their cognate telomeres (**24**). Examples of a normal 3p3q hybridization pattern, the 2q polymorphism, the crosshybridization of 12p and a clinically significant unbalanced telomeric rearrangement are shown in **Fig. 2**. A list of known polymorphisms and crosshybridization patterns associated with the 1st and 2nd Generation telomere clones are listed in **Table 2**.
15. When repeat hybridizations are necessary one must decide whether the use of a whole new Multiprobe<sup>®</sup> slide and device is justified. The alternative to this is either to (1) purchase ready labeled individual telomere probes (e.g., from Cytocell Ltd., Appligene Oncor or Vysis Inc.) or (2) to obtain a set of telomere clone glycerols and prepare one's own stocks of DNA and labeled probes for hybridization. The latter can be very cost-effective: once the initial preparation of the clones has been achieved, bulk stores of labeled, optimized probes will be stable at  $-20^{\circ}\text{C}$  at least 2 yr. A list of the 1<sup>st</sup> and 2<sup>nd</sup> generation telomere-specific clones and where glycerol stocks or stabs may be obtained is given in **Table 1**. The telomere clones in the Chromoprobe Multiprobe<sup>®</sup>-T System Kit are indicated.
16. Unusual results may reflect true subtelomeric rearrangements or benign polymorphisms and misinterpretation must be avoided. *See Table 2* for a list of polymorphisms and crosshybridization patterns known to be associated with the 1<sup>st</sup> and 2<sup>nd</sup> Generation telomere specific clones and **Fig. 2** for FISH examples.
17. It is not necessary to prepare DNA from telomere clones if the Chromoprobe Multiprobe<sup>®</sup>-T System Kit (Cytocell Ltd., UK) or any other commercially available kit (e.g., ToTel Vysion Multicolor FISH Probe Panel from Vysis Inc.) is being used. However, it may be desirable to obtain and process a complete set of telomere clones, especially when small numbers of repeat hybridizations are required. A list of the 1<sup>st</sup> and 2<sup>nd</sup> generation telomere-specific clones and where glycerol stocks or stabs may be obtained is given in **Table 1**. The telomere clones in the Chromoprobe Multiprobe<sup>®</sup>-T System Kit are indicated.
18. If dry ice is not available, glycerols can be kept on ice for a limited period, but repeated access in this manner will reduce the viability of the glycerol stock.
19. If the pellet does not resuspend quickly, it may be left at  $4^{\circ}\text{C}$  overnight.

**Table 2**  
**Cross Hybridization and Polymorphisms of 1<sup>st</sup> and 2<sup>nd</sup> Generation Telomere Clones<sup>a</sup>**

	1 <sup>st</sup> Generation Clones		2 <sup>nd</sup> Generation Clones	
	Telomere	Clone	Telomere	Clone
Polymorphisms:	2q XpYp	210-E14 CY29	2q 9p	GS-1011-O17 GS-43-N6
Cross-hybridizations:	5p with acrocentric p-arms 5q with acroncentric centromeres 8p with 1p 11p with 17p 12p with 6p and 20q (both faint)	84c11 GS-90-I5 2205a2 2209a2 GS-90-I5	8p with 1p and 3q (both faint) 9q with 109p and 16p, 18p, XqYq (faint) 11p with 17p 11q with 12q (interstitial) 12p with 6p and 20q (both faint) 12p with 6p and 20q (both faint) 15q with 1q and 15q (both interstitial) 17p with 17q (two interstitial sites) 17q with 1p and 6q (both faint) 20q with 6p (faint) 22q with 2q (interstitial)	GS-580-L5 GS-112-N13 GS-908-H22 GS-770-G7 GS-496-A11 GS-8-M16 GS-124-O5 GS-202-L17 GS-362-K4 GS-81-F12 GS-99-K24

<sup>a</sup>Adapted from knight, S. J. L. and Flint, J., *J. Med. Genet.* 2000, **37**, 401–409. With permission from the BMJ Publishing Group.

20. This section is not applicable if the Chromoprobe Multiprobe<sup>®</sup>-T System Kit (Cytocell Ltd., Adderbury, UK) or any other commercially available kit is being used.
21. As indirectly labeled probes keep well for approx 2 yr, it may be desirable to increase the amounts labeled and increase the labeling reaction volume accordingly.
22. The exact concentration of DNase varies between batches and the amount of DNase may vary between different DNA templates. Ideally, with each new batch, the amount of DNase needed should be optimized for each template and the optimal period of incubation noted so that the same conditions can be applied for future labelings of the template DNA.
23. The exact amount of COT-1 used should be optimized for each batch of probe made. The amount of COT-1 to be added primarily depends on the Alu content of the probe. For the telomere clones, the standard amount of COT-1 to add to labeled cosmids with insert sizes of 30–40 kb is 625 ng for every 1  $\mu$ L of probe mix. For PACs and small BACs with insert sizes of ~100 kb, 1  $\mu$ g COT-1 should be added. For larger BACs up to 3  $\mu$ g COT-1 may be required. Notable exceptions include the the chromosome 19 clones such as F20643 and 20283 which require up to 10  $\mu$ g COT-1 per 1  $\mu$ L of probe mix).
24. If a hotplate is not available, the chromosomes may be denatured in 70% formamide/0.1 mM EDTA in 2X SSC at 70°C for 5 min followed by three washes in 2X SSC at 4°C and subsequent dehydration through an ethanol series. The probe mixture is denatured separately at 95°C for 5 min prior to adding to the slide.
25. Make antibody suspensions 1–3 appropriately, according to whether single or dual detection is required. The antibody concentration is extremely important. If the concentration of the antibody is too low there may be little or no hybridization. Conversely, if it is too high there may be a very “speckled” background making it impossible to identify the cognate signals (see Fig. 2[d]). As there can be some variation between batches it is advisable to optimize the dilution of each new batch of antibody by hybridizing reliable control probes to good quality control chromosome suspensions prior to proceeding with valuable study samples.

## References

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## Fluorescence Genotyping for Screening Cryptic Telomeric Rearrangements

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### 1. Introduction

Mental retardation (MR), defined as an intelligence quotient (IQ) less than 70, represents the most frequent serious handicap in children and young adults. Moderate to severe MR (IQ<50) encompasses 1% of the population and the prevalence increases up to 2–3% if mild mental retardation (50<IQ<70) is included (1,2). Mental retardation may result from multiple causal factors: environmental factors (fetal alcohol syndrome or perinatal hypoxic-ischaemic damage for example), chromosomal factors (such as trisomy 21), or monogenic diseases (Fra-X syndrome for example). Chromosomal abnormalities, such as deletions, duplications or uniparental disomies (UPD) that result in an alteration of normal gene dosage, are a common cause of mental retardation found in 12% of a random series of mentally retarded patients (3). The responsible chromosomal segment is usually small (< 10 Mb), but encompasses multiple genes which contribute to the phenotype independently. Routine chromosome analysis allows detection of duplications and deletions in the 10 Mb range, and therefore remains above the threshold for phenotypic effect. Despite numerous attempts to increase reliability and resolution, there is still no practical way to screen the entire genome for rearrangements, regardless of size or chromosomal localization.

#### **1.1. Telomeric Rearrangements in Children with Idiopathic Mental Retardation**

The genetic cause remains unknown in as many as 40% cases of moderate to severe mental retardation, so called idiopathic MR (4,5). In recent years, submicroscopic rearrangements of chromosome ends have been documented in patients with specific mental retardation syndromes and apparently normal karyotypes. This is the case for deletions of chromosome 4p16.3, 5p15.2 or 17p13.3 (see Note 1). However, in most cases, the deletion of other telomeric regions have a less characteristic phenotype,

making it difficult to ascertain these syndromes through specific clinical phenotypes. A strategy involving the use of hypervariable DNA polymorphisms for the detection of telomeric abnormalities was then tested in a pilot study of patients with unexplained MR (6). The results suggested that 6% of idiopathic mental retardation might be explained by submicroscopic rearrangements involving telomeres.

## **1.2. Strategies for Molecular Detection of Cryptic Telomeric Rearrangements**

Several methods have therefore been developed to detect terminal chromosome aberrations. An innovative fluorescence *in situ* hybridization technique (FISH) has been set up (7). It exploits a unique set of telomere specific probes to directly assess loss or gain of telomeric sequences. A large study performed by Knight and colleagues (8) indicated that subtle telomeric abnormalities occurred in 7.4% of children with idiopathic MR, and demonstrated this technique as a powerful diagnostic tool. However, this method remains expensive and difficult to use for routine testing. In addition, this approach excludes significant regions of the genome where submicroscopic rearrangements are also liable to occur (9,10). Therefore, a whole genome screening technique has been proposed, namely Comparative Genomic Hybridization (CGH) (10–12). However, this approach is unable to detect subtle anomalies smaller than 5 Mb and requires specific microscope and computer facilities. The development of a novel efficient and reliable method to detect small telomeric rearrangements has been a major challenge for public health.

Microsatellite markers are naturally occurring DNA polymorphisms that can be used to search for irregular allele inheritance and detection of deletions, duplications, or uniparental disomies (13). A pilot study based on conventional genotyping allowed diagnosis of two deletions of telomeric regions in a series of 27 children with idiopathic MR (14). We have recently developed a novel strategy to detect telomeric abnormalities based on automated fluorescent genotyping of telomeric specific microsatellites (15). Polymerase chain reaction (PCR) products corresponding to these loci are analyzed on an automatic sequencer and allelic profiles are determined using specific software. For each marker, the genotype of the affected child is determined and compared to the parental genotypes to detect any case of non-Mendelian segregation.

To evaluate the feasibility and sensitivity of this approach, a pilot study was performed in a selected clinical population. Ninety-two mentally retarded children, belonging to 77 independent families were tested. These patients, born from unrelated parents, had a normal high resolution karyotype. They all presented with a severe idiopathic mental retardation and dysmorphic features associated with at least one of the following criteria: (1) seizures, (2) overgrowth or failure to thrive, (3) behavioral anomalies, or (4) congenital anomalies. Eleven telomeric rearrangements have been characterized: 8 deletions, 1 duplication, and 2 uniparental disomies, corresponding to a detection rate of 12%.

Our results give strong support to the view that cryptic telomeric rearrangements significantly contribute to idiopathic mental retardation (about 12% in our series) and demonstrate that fluorescent genotyping is a very sensitive method to detect such

rearrangements. Moreover, at variance with cytogenetics, which requires a high technical expertise, genotyping offers the advantage of giving quantitative and objective results that can be automated in many respects. Genotyping offers the advantage of directly identifying the parental origin of the rearrangement. Lastly, the microsatellite technique provides the unique opportunity to detect uniparental disomies, a mechanism involved in a number of human diseases.

The set of markers described here have an average heterozygosity score of 0.75. Because only few chromosome maps encompass telomeres, several loci tested (**Table 1**) are likely to lie hundreds of kilobases away from the telomere. Sequence data derived from the human genome sequencing projects will hopefully allow us to define more informative and/or telomeric microsatellite markers, so that the primary sets of markers described here will be improved. The development of new fluorophores will also allow us to reduce the number of sets. We estimated extensive genotyping of one child and his two parents to cost 80 dollars, i.e., three to four times less than estimated for the cytogenetic method. Progress in automation will likely make this approach even more affordable.

Yet, it should be borne in mind that this approach has also its limitations. First, it cannot detect balanced rearrangements when dosage is unaltered. A second limitation is that dosage differences cannot be reliably detected. Hence, this technique may overlook cases of monosomy and trisomy when the parents share the same allele or cases of small tandem duplications. Finally, this screening technique may generate false-positive results owing to the instability of microsatellites. Thus, non-Mendelian inheritance of a single microsatellite by itself does not provide evidence that a chromosomal rearrangement has occurred, and further investigations must be carried out to assess whether the chromosomal abnormality is responsible for the MR (*see Note 2*). Genotyping of additional markers, quantitative PCR and/or FISH analyses are required to support this conclusion. In addition, FISH analyses are required to distinguish a *de novo* rearrangement from an inherited chromosome imbalance and to identify the carrier(s) of the balanced anomaly in the family. This test is now proposed as a routine exam to families.

## 2. Materials

### 2.1. PCR Amplification of Telomeric Microsatellites

1. 10 ng/ $\mu$ L Sample DNA.
2. 10  $\mu$ M PCR primers (*see Table 1*): Forward primers are labeled with either 6-FAM, HEX, or NED phosphoramidites. To minimize subsequent freeze-thawing, the primers are aliquoted into working solutions (10 pmol/ $\mu$ L).
3. 10X Concentrated PCR buffer: 200 mM Tris-HCl, pH 8.4, 500 mM KCl.
4. 50 mM Magnesium chloride solution.
5. dATP, dGTP, dCTP, and dTTP mixture (2 mM each).
6. 5 U/mL Taq polymerase.
7. 96- Wells microtiter plates for PCR and aluminium heat resistant sealers.
8. Thermal cycler.

### 2.2. PCR Verification

1. Horizontal gel electrophoresis apparatus with 6  $\times$  10 cm gel formers, combs and 250V power supply.

**Table 1**  
**Microsatellite Markers Used for Telomere Genotyping<sup>a</sup>**

Telomere	D Number	Genetic distance to telomere (cM)	Fluorophore	Size range	Heterozygosity
1p	D1S243	0	FAM	142–170	0.86
«	D1S468	6.2	HEX	173–191	0.75
1q	D1S2682	0	NED	110–150	0.76
«	D1S2836	2.6	FAM	268–281	0.79
2p	D2S2268	0	HEX	205–231	0.61
«	D2S323	3.6	FAM	177–193	0.57
2q	D2S2338	0	FAM	155–173	0.55
«	D2S140	4.5	NED	151–167	0.76
3p	D3S1270	0	NED	154–196	0.75
«	D3S1307	1.4	FAM	237–251	0.80
3q	D3S3550	0	FAM	230–270	0.74
«	D3S3707	2.2	HEX	262–278	0.65
4p	D4S3038	0	NED	195–240	0.78
«	D4S2936	0	NED	170–184	0.83
4q	D4S2930	0	NED	216–236	0.80
«	D4S426	1.1	HEX	177–191	0.56
5p	D5S678	0	FAM	252–264	0.71
«	D5S392	0.6	HEX	83–117	0.87
5q	D5S2006	0	HEX	135–165	0.74
«	D5S408	1.8	FAM	247–265	0.73
6p	D6S1600	0	FAM	165–210	0.77
«	D6S344	1.4	HEX	139–159	0.72
6q	D6S281	0	HEX	125–180	0.68
«	D6S1693	10.8	FAM	85–93	0.51
7p	D7S2477	0	FAM	132–180	0.66
«	D7S2563	1.7	HEX	91–111	0.78
7q	D7S2423	0	NED	230–270	0.71
«	D7S2465	1.9	HEX	158–181	0.83
8p	D8S504	0	FAM	193–203	0.71
«	D8S264	0.7	FAM	121–145	0.83
8q	D8S1836	0	HEX	121–160	0.84
«	D8S1751	1.4	NED	149–163	0.69
9p	D9S1779	0	NED	114–154	0.63
«	D9S1858	0.1	HEX	124–144	0.58
9q	D9S1838	0	NED	155–175	0.83
«	D9S158	3.5	FAM	213–233	0.71
10p	D10S249	0	NED	106–152	0.74
«	D10S558	1.9	NED	192–212	0.80
10q	D10S1700	0	HEX	107–148	0.64
«	D10S212	1	HEX	189–201	0.70
11p	D11S1363	0	FAM	240–260	0.59
«	D11S4177	3.1	FAM	183–213	0.80

(continued)

**Table 1** (continued)

Telomere	D Number	Genetic distance to telomere (cM)	Fluorophore	Size range	Heterozygosity
11q	D11S968	3.3	HEX	131–165	0.81
«	D11S4125	3.3	FAM	231–253	0.81
12p	D12S352	0	HEX	145–170	0.72
«	D12S341	0.6	FAM	114–130	0.79
12q	D12S357	0	HEX	260–315	0.84
«	D12S1638	0	NED	120–130	0.68
13q	D13S293	0	NED	80–106	0.50
«	D13S1295	4.6	HEX	102–120	0.74
14q	D14S1007	0	HEX	110–135	0.77
«	D14S293	3.9	HEX	153–165	0.65
15q	D15S966	0	HEX	225–265	0.82
«	D15S203	0.6	FAM	116–140	0.62
16p	D16S521	0	FAM	156–172	0.71
«	D16S3024	5	HEX	208–248	0.86
16q	D16S3121	0	HEX	65–90	0.62
«	D16S3407	0	FAM	195–205	0.77
17p	D17S1866	0	HEX	144–195	0.80
«	D17S926	0.6	FAM	243–260	0.80
17q	D17S928	0	NED	76–96	0.75
«	D17S1830	11	HEX	108–122	0.79
18p	D18S59	0.1	HEX	127–182	0.81
«	D18S476	1.6	HEX	263–275	0.76
18q	D18S70	0	FAM	115–135	0.83
«	D18S1141	1.9	NED	263–293	0.77
19p	D19S886	0	NED	105–165	0.63
«	D19S883	5.5	HEX	158–170	0.72
«	D19S878	6.5	FAM	208–230	0.83
19q	D19S890	0	HEX	190–210	0.68
«	D19S218	2.5	FAM	240–256	0.60
20p	D20S864	0	FAM	215–235	0.52
«	D20S103	0	FAM	92–106	0.70
20q	D20S173	0	HEX	135–195	0.66
«	D20S171	2.1	FAM	123–149	0.73
21p	D21S1911	0.1	HEX	105–145	0.69
«	D21S1904	0	HEX	152–170	0.52
21q	D21S1897	0	FAM	178–224	0.74
«	D21S1903	0.7	FAM	243–265	0.83
22q	D22S1169	0	FAM	110–135	0.78
«	D22S1161	2.2	HEX	235–245	0.68
Xp	DXYS233	0	HEX	260–300	0.81
«	DXYS234	2	NED	234–254	0.72
Xq	DXS8087	0	FAM	270–300	0.69
«	DXS1073	1.6	HEX	217–243	0.80

<sup>a</sup>Genetic distance from centromere and heterozygosity score were obtained from the Genethon map, see <http://www.genethon.fr>.

2. Agarose.
3. 10 mg/mL ethidium bromide solution.
4. Gel loading buffer.
5. Electrophoresis buffer (1X TBE): 10.8 g Tris base, 5.5 g boric acid, and 4 mL 0.5 M EDTA, pH 8.0 in 1 L distilled water.
6. UV transilluminator.

### **2.3. Gel Analysis of Amplified Products**

1. Long Ranger™ Gel solution (FMC; 50% stock solution).
2. Urea.
3. 0.45 µm membrane.
4. Gel loading buffer.
5. *N,N,N',N'*-Tetramethylene-ethylenediamine (TEMED).
6. Ammonium persulfate: 10% aqueous solution.
7. GS-400 HD ROX.
8. ABI 377 automated sequencer equipped with GeneScan™ and Genotyper™ software.

## **3. Method**

### **3.1. PCR Amplification of Telomeric Microsatellites**

1. Thaw primers on ice, then gently mix and centrifuge the tubes.
2. For each marker, prepare a 12 reactions mix containing: 196 µL autoclaved distilled water, 32.5 µL 10X PCR buffer, 9.75 µL MgCl<sub>2</sub>, 32.5 µL of dNTP mixture, 2 µL of each primer forward and reverse, 1U of Taq polymerase. Keep on ice.
3. Add 15 µL of the PCR mix to 10 µL of DNA. Seal the plate using aluminium adhesive cover.
4. Place the 96-well microtiter plate in the thermal cycler and then incubate for 5 min at 94°C to denature the DNA prior to amplification.
5. Cycle at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s for 35 cycles, with a final elongation time of 7 min at 72°C. The samples can be stored at -20°C until use.

### **3.2. PCR Verification**

1. For gel electrophoresis, mix 3 µL of loading buffer to 5 µL of PCR product and load on a 1.5% horizontal agarose gel in 1X TBE containing 5 µg/mL ethidium bromide.
2. Run at 100V for 30 min. Use appropriate molecular weight standard.
3. Visualize the DNA in the gel by transilluminating with UV light to ensure proper amplification of the microsatellite.

### **3.3. Gel Analysis of Amplified Products**

1. Assemble glass plate and spacers in the cassette following the method described in the ABI Automated sequencer Manual.
2. Mix 3 mL Long Ranger™ Gel solution, 3 mL 10X TBE, 10.8 g Urea and deionized H<sub>2</sub>O to a final vol of 30 mL. Filter through 0.45 µm membrane.
3. Add 150 µL of 10% APS and 21 µL of TEMED and pour the gel. Allow 1 h for gel polymerization.
4. Mount the gel cassette onto the sequencing apparatus according to the manufacturer's instructions.
5. After PCR amplification pool the amplified products as indicating in **Table 2**. Pool equal amount of each product (*see Note 3*).

**Table 2**  
**Sets of PCR-Amplified Dinucleotide Repeat Microsatellite Markers**  
**Suitable for Automated Fluorescent Genotyping**

	Locus	Telomere	Labeling	Allele size range
SET 1	D1S243	1p	FAM	142-170
	D1S2682	1q	NED	110-150
	D2S2268	2p	HEX	205-231
	D4S3038	4p	NED	195-240
	D3S3550	3q	FAM	230-270
SET 2	D2S2338	2q	FAM	155-173
	D3S1270	3p	NED	154-196
	D5S678	5p	NED	252-264
	D5S2006	5q	HEX	135-165
	D6S281	6q	HEX	203-219
SET 3	D4S2930	4q	NED	216-236
	D6S1600	6p	FAM	165-210
	D7S2423	7q	HEX	230-270
	D8S1836	8q	HEX	121-160
	D9S1779	9p	NED	114-154
SET 4	D7S2477	7p	FAM	132-180
	D8S504	8p	FAM	193-203
	D9S1838	9q	NED	155-175
	D11S968	11q	HEX	131-165
	D12S357	12q	HEX	260-315
SET 5	D10S249	10p	NED	106-152
	D10S1700	10q	FAM	107-148
	D11S1363	11p	FAM	240-260
	D12S352	12p	HEX	145-170
SET 6	D14S1007	14q	HEX	110-135
	D15S966	15q	HEX	225-265
	D16S521	16p	FAM	156-172
	D16S3407	16q	FAM	195-205
SET 7	D13S293	13q	NED	80-106
	D17S1866	17p	FAM	144-195
	D17S928	17q	NED	76-96
	D18S59	18p	HEX	127-182
	D18S70	18q	FAM	115-135
SET 8	D19S886	19p	NED	105-165
	D19S890	19q	HEX	190-210
	D20S864	20p	FAM	215-235
	D21S1911	21p	HEX	105-145
	D22S1169	22q	FAM	67-85
SET 9	DXYS233	Xp	HEX	260-300
	D20S173	20q	HEX	135-195
	D21S1897	21q	FAM	178-224
	DXS8087	Xq	FAM	270-300

6. Freshly prepare a loading cocktail based on the following ratio: 0.5  $\mu$ L GS-400 HD ROX, 1  $\mu$ L Loading buffer, 5  $\mu$ L dionized formamide.
7. Mix 2  $\mu$ L of the pooled PCR products with 3  $\mu$ L of loading cocktail.
8. Denature at 90°C for 3 min; quick chill on ice.
9. Load onto gel lanes.
10. For data collection, use run module GS 36D-2400.
11. Analysis is performed using the GeneScan™ as described in the manufacturer's manual. Check manually for each lane the sizing of the ROX-labeled size standard.
12. Determine allele sizes using the Genotyper™ software.

#### 4. Notes

1. Cytogenetically invisible unbalanced translocations have been shown to be the cause of mental retardation in the  $\alpha$ -thalassemia mental retardation syndrome (ATR-16, **[16]**), Wolf-Hirschhorn syndrome (**17**), Miller-Diecker syndrome (**18**) and in cri-du-chat syndrome (**19**).
2. One problem that may arise using this method is determination of the phenotypic consequences of the telomeric rearrangements characterized. The correlation is clear when there is more than one affected offspring and when the rearrangement co-segregates with MR. In the absence of family history for MR, evidence for the biological effects of the rearrangements can be obtained either when the rearrangement includes regions previously found deleted in known MR syndromes or when the size of the deletion is large. In all other cases, the ascertainment of the pathogenicity of small rearrangements may be more problematical.
3. To achieve even peak heights across all loci, one may have to adjust pooling ratios for each pool.

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## Microarray CGH

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### 1. Introduction

Comparative genomic hybridization (CGH) to metaphase chromosome targets (1,2) has significantly contributed to our understanding of the cancer cytogenetics of more complex malignancies such as solid tumors (3,4). This molecular cytogenetics-based technique (hereafter referred to as “chromosome CGH”) is capable of defining genome-wide DNA copy number imbalances in sample cells relative to a normal reference in a single experiment. Chromosome CGH has greatly increased our understanding of tumor biology and progression since the minimal recurrent regions of chromosomal gain and loss are likely to contain novel oncogene(s) and tumor suppressor gene(s) respectively.

The unique advantage of chromosome CGH is its whole-genome screening capability which is significantly faster and less laborious than low-throughput methods for examining single-target dosage changes such as Southern analysis, PCR, and fluorescence *in situ* hybridization (FISH). Chromosome CGH is now a well-established molecular cytogenetic method, but there are two technical limitations that restrict its usefulness as a comprehensive screening tool. First, because the target DNA within the chromosome is highly condensed and supercoiled, the resolution for determining copy number changes is no less than 10 Mb for loss (1). For copy number gains, the minimal detectable size is probably no less than 2 Mb, which is a function of both amplicon size and copy number (1,5). This resolution, while capable of providing a starting point for positional cloning studies, will still encompass too many genes to precisely localize a sequence of interest. Second, the analysis of the images obtained following chromosome CGH is only partly automated and experienced cytogeneticists must identify each chromosome to determine regions of imbalances.

Recent developments in microarray methods have circumvented some of the limitations of chromosome CGH. Complementary DNA (cDNA) microarray technology, realized through advances in the Human Genome Project (HGP) as well as robotic arraying technology on glass slides, has facilitated high-throughput analysis of

differential gene expression in tumors (6–8). An emerging platform that addresses the shortcomings of chromosome CGH couples the technique to microarray expression technology, and is generally referred to as “microarray CGH”. Instead of using metaphase chromosomes, CGH is applied to arrayed short sequences of DNA bound to glass slides (herein defined as the “targets” for hybridization) and probed with genomes of interest (herein defined as the “probe”) (see **Note 1**). With sufficient representation on the microarray, this system significantly increases resolution for localizing regions of imbalance. Furthermore, just as with expression microarray screening, analysis is straightforward and automated. Two technology platforms have recently been published: (1) cDNA-based array CGH (9,10); and (2) genomic DNA-based array CGH (also referred to as “matrix CGH” and “array CGH” [11,12]). This chapter will provide an overview of the currently published methods, but readers should be aware that microarray CGH is an emerging technology and there are likely to be continual refinements to the protocols described below. For updated protocols, please visit <http://www.utoronto.ca/cancyto/>.

### 1.1. cDNA Array CGH

Microarray CGH using cDNA targets (hereafter referred to as “cDNA array CGH”) was first described by Pollack et al. (9). This platform makes use of conventional cDNA microarrays, normally employed in expression screening, for examining genomic copy number imbalances. As depicted in **Fig. 1**, this has the advantage that duplicate arrays may be used in parallel to provide a comprehensive overview of both expression and gene copy number change in a tissue (9). The increasing availability of a variety of different cDNA microarray expression formats means that modification of protocols to interrogate these cDNA targets by CGH is immediately accessible for high-throughput analysis of gene dosage changes.

#### 1.1.1. Application of cDNA Array CGH to Cancer Genomics

Pollack et al. examined breast cancer cell lines and tissues using a 3360 feature microarray by cDNA array CGH (9). With optimization, they demonstrated that the technique was capable of detecting copy number gains and single deletion losses. Analysis of the tumors and cell lines showed that not all amplified genes were overexpressed, nor were most highly overexpressed genes amplified; however, a subset of the genes, including *ERBB2*, were observed to be both amplified and overexpressed. They proposed that these genes might be important mediators of the tumor initiation and progression.

The utility of cDNA array CGH for detecting gene amplifications was recently shown by Heiskanen et al. (13). In this study, cell lines with known gene amplifications were used to establish the sensitivity limits of the technique. In contrast to the protocol used by others (9,10), genomic DNA is biotin labeled and a tyramide amplification protocol (14) is employed (13). Progressive dilution from 100 to 2% of genomic DNA from the neuroblastoma cell line NGP with normal DNA during labeling corresponded to decreasing *MYCN* signal intensity on the microarray. Amplifications of 5-fold and greater were readily detected by this method, and at 2% dilution, *MYCN* intensity was

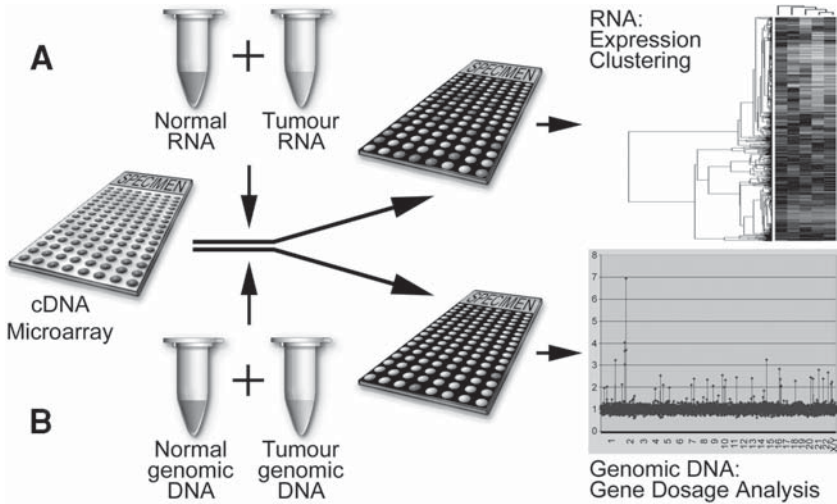


Fig. 1. Schematic depiction of the utility of cDNA microarrays in expression and CGH analyses. cDNA microarrays are screened with labeled probes derived from RNA and/or DNA of normal (Cy5) and tumor (Cy3) tissue. Analysis of the red:green signal intensity ratios indicate the level of (A) gene expression or (B) gene dosage change, respectively. Analyses may require data mining techniques for optimal interpretation of the results. (A) Two-dimensional hierarchical clustering (32,37) is applied to the results to identify patterns of gene expression and establish clinical correlates. (B) *in silico* cDNA chromosome localization and arrangement into sequential order allows the results of cDNA array CGH to be depicted as an ideogram type plot across the genome, facilitating identification of regions of gene dosage change.

observed at 2.5-fold relative to other non-amplified genes. However, the main limitation of this method is its inability to allow two-color CGH and thus necessitates the use of two microarrays (test, control) per experiment.

Recently, we have demonstrated the suitability of cDNA array CGH for gene amplification screening of patient samples (10). In this study, the *MYCN* (chromosome region 2p24) amplification status in neuroblastoma patients and cell lines was confirmed by cDNA array CGH on a high density 19,200 feature microarray. In the cell line IMR32, cDNA array CGH confirmed a recently described co-amplified oncogene, *MEIS1* (15,16). Importantly, the technique was able to distinguish three tumor genotypes in patient samples not previously described (Fig. 2). This study demonstrates not only the high-throughput advantage of examining thousands of genes by cDNA array CGH over conventional methods such as FISH and Southern analyses, but also the increase in resolution in contrast to chromosome CGH.

In another study by Pei et al. (17), the increased resolving power of cDNA array CGH for delineating amplicon boundaries was demonstrated in pediatric carcinomas. This work clearly shows the limited resolution of chromosome CGH when contrasted to cDNA array CGH. These results are depicted in Fig. 3.

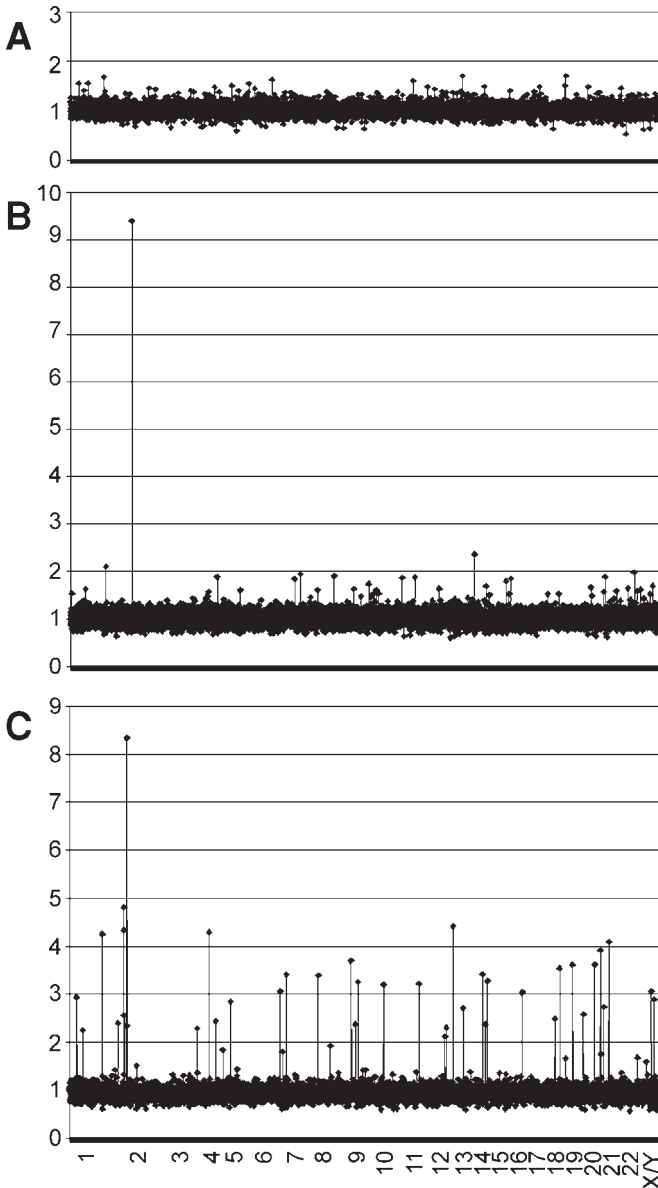


Fig. 2. Normalized cDNA array CGH of neuroblastoma patients identified three tumor genotypes: **(A)** No high copy gains or amplification of genomic DNA; **(B)** *MYCN* amplification as the sole genomic copy number imbalance; and **(C)** *MYCN* amplification with previously undetected co-amplified 2p24 genes and high copy number gains of mitochondrial DNA sequences and numerous other genes, suggesting an underlying genetic instability. This third clinical genotype was not previously described, as these regions are not resolvable by chromosome CGH (10).

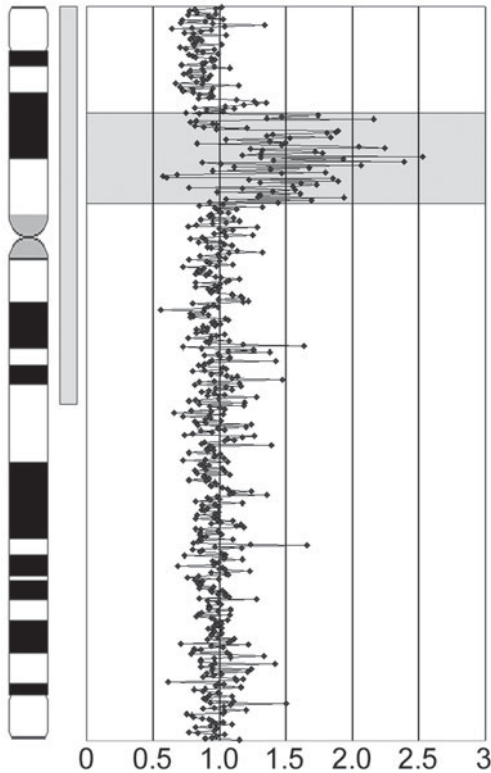


Fig. 3. High resolution detection of gene dosage changes on chromosome 17 using high density cDNA array CGH. Chromosome CGH detected high copy gain of the chromosome region 17p–17q21 (vertical gray bar) in an osteosarcoma sample. Corresponding normalized cDNA array CGH using genomic DNA from the same sample significantly resolved the boundaries of this gain to the region 17p12–17p11.2 (horizontal gray bar). Chromosome ideograms are constructed by *in silico* assignment of microarray cDNAs to chromosomes, then arranging cDNAs into sequential order along each chromosome (10).

### 1.1.2. Current Limitations of cDNA Array CGH

There are at least three limitations to current cDNA array CGH methods. Firstly, target cDNA sequences are of low complexity in content in comparison to genomic sequences, lacking intronic and other nontranscribed elements such as repetitive DNA and control sequences. Thus, many regions of the genome being interrogated will not hybridize with uniform efficiency, so that the specificity of the technique may be low or poorly reproducible. Secondly, target cDNA sequences are typically only 0.5–2 kilobases in size (9,10,13). This is many orders of magnitude smaller than the smallest chromosome, and 1–2 orders of magnitude smaller than genomic insert sequences in bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs), and cosmids. Although this may be suitable for expression mapping by microarray

where the probe is comparable in size, reduced signal sensitivity may become a concern when using labeled genomic probes. Although Pollack et al. (9) described detection of both copy number gains and losses by cDNA array CGH, it is likely that genomic DNA-based arrays are more robust for detection of single copy changes, including copy losses. Finally, the last issue with cDNA microarray technology, and therefore also with cDNA array CGH, is that currently there is a significant number of gene misannotations in the commercially available clone sets (18). This may take the form of wrongly identified sequences, incorrect chromosomal locations, or even the complete absence of human sequences in the cDNA targets (e.g., owing to clone contamination, heterologous sequences). In practical terms, this manifests as inconsistent results or findings that cannot be substantiated when other methods are applied. To eliminate this shortcoming, commercial sources of clone sets and many institutions with array fabrication capabilities are sequence confirming their clone sets. Overall, these limitations contribute to the high rate of false positive (15%), and false negative (15%) results reported for this technique (9).

## 1.2. Array CGH

The second microarray CGH platform (hereafter referred to as “array CGH”) uses genomic DNA sequences as targets on the microarray. Array CGH was first established by Solinas-Toldo et al. (11), and further refined by Pinkel et al. (12). As described in these studies, the DNA targets for the microarray can be derived from genomic clones including yeast artificial chromosome (YAC; 0.2–2 Mb in size), BAC (up to 300 kb), P1 (~70–100 kb), PAC (~130–150 kb), and cosmid (~30–45 kb), and are of several orders of magnitude smaller than chromosome targets. This decrease in target size increases the resolution of copy number imbalance detection over chromosome CGH (Fig. 4). Given the differences in the structural complexity in the target DNA with respect to chromosome CGH, modifications to the hybridization conditions are necessary (11,12). The advantage of array CGH over cDNA array CGH is that there is more uniformity in hybridization and subsequent signal fidelity because the DNA targets have a greater complexity and coverage, containing intronic and other non-transcribed genomic sequences.

### 1.2.1. Application of Array CGH to Cancer Genomics

To date, several groups have published results using array CGH (11,12,19–25). Pinkel et al. (12) detected genomic imbalances within a sub-band of chromosome 20 in breast cancer that had failed to be observed using chromosome CGH. Using array CGH, precise genomic mapping of the position of amplicon boundaries within 20q13.2 was performed (19). This allowed *CYP24* to be localized within the minimal amplified region, identifying it as a new candidate oncogene in breast cancer (19). In another study, array CGH was used to examine neurofibromatosis type 2 (NF2) patients and determined the extent and frequency of deletions around the *NF2* locus on chromosome 22q (20). This microarray was constructed from a 7 Mb tiling path of 104 BAC and PAC genomic clones around *NF2*, and included smaller cosmids for mapping copy number changes at higher resolution. Both single copy losses and homozygous deletions were detectable in the patient samples by this system (Fig. 5). Further refine-



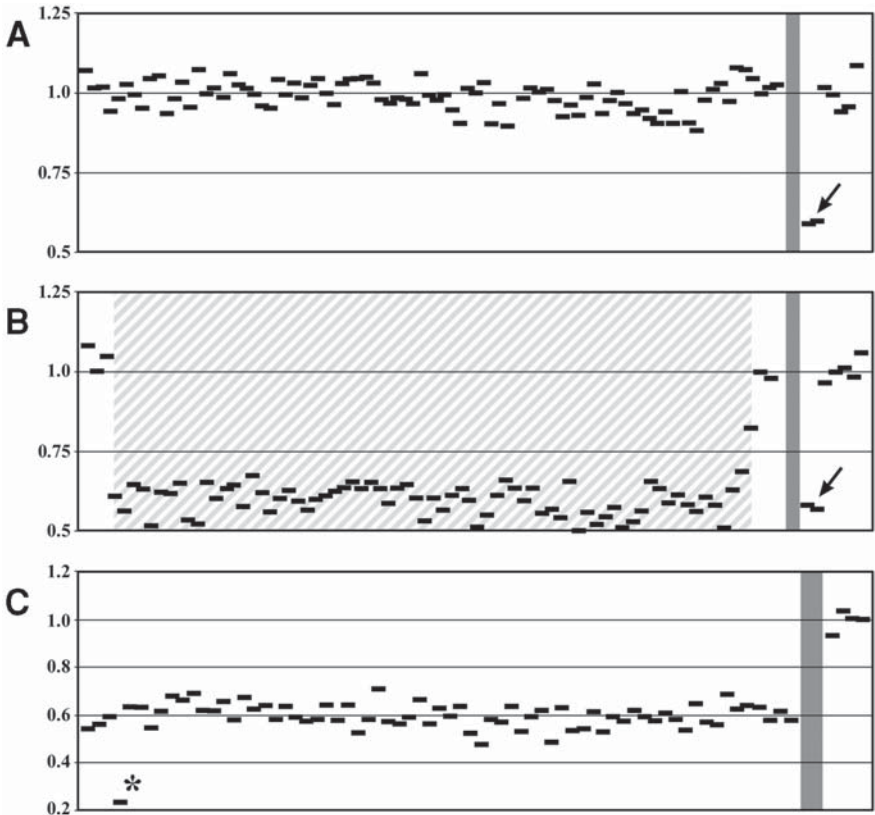


Fig. 5. Histogram showing the copy number of the genomic clones comprising a 7 Mb tiling path on chromosome 22q, represented from the centromeric (left) to the telomeric (right) direction. Each black bar represents an individual genomic clone. Chromosome X and Y control genomic clones are separated (gray bar) on the right of the histogram. (A) Array CGH comparing normal male and female DNA shows expected single copy loss of chromosome X clones (arrows). (B) Comparison of a male NF2 patient against normal female control delineates boundaries of heterozygous loss along the *NF2* locus and surrounding region (stippled region). (C) The detection of homozygous interstitial deletion (asterisk) within a region of single copy loss in a heterozygous female NF2 patient against a normal female control demonstrates the sensitivity and the resolution of array CGH. The accuracy of the technique is reflected by the deviation of the ratio from the expected values. Adapted from Bruder et al., 2001 (20) with permission.

hemispheres. Gene dosage changes in patients with Klinefelter's syndrome (karyotype: XXY) were examined with a DNA microarray constructed with cosmids covering the pseudoautosomal region of the sex chromosomes, and findings were correlated with anomalous dominance and other cognitive or behavioral phenotypes.

#### 1.2.4. Current Issues with Genomic DNA-based Array Fabrication

Although array CGH still has some limitations, most of these relate to array production and will be addressed as the technology matures. While modifications to existing array fabrication systems are possible, current production limitations are mainly associated with difficulties in automating batch preparation DNA from genomic clones. For example, published array CGH studies involve the use of laborious DNA extraction methods such as maxi prep kits (Qiagen) and phenol/chloroform extractions from genomic clones (11,12). However, commercially available batch extraction kits (e.g., R.E.A.L. System™, Qiagen) from genomic clones coupled with DOP-PCR may aid in automation (see Note 2). A second difficulty is related to the generation of adequate amounts of DNA for batch microarray production. While cDNA expression clones have universal primer sites amenable to large scale PCR synthesis of genes and expressed sequence tags for subsequent purification and arraying, the same is not true for genomic clones. In addition, the larger genomic inserts require long PCR which has more exacting amplification conditions (27), which may be confounded by the presence of repetitive DNA elements in template sequences. The third difficulty is the viscosity of large size genomic sequences in solution that may cause clogging of spotting pins of some arrayers, although new split pin designs may circumvent this problem (28). Finally, as with the cDNA clone sets, there is also the concern that a small but significant number of commercially available genomic clones are misannotated in their localization (e.g., owing to source clone plate contamination, mislabeling). Currently the solution is to FISH-confirm cytogenetic mappings of clones used for array CGH, although this is not a trivial task when dealing with tens or hundreds of genomic clones. The BAC/PAC resources (<http://www.chori.org/bacpac/>), further described in chapter 27, is an ongoing project to FISH-map all clones (25) that will largely alleviate this problem.

#### 1.2.5. Current Accessibility to Genomic DNA-based Arrays

While cDNA microarrays can be obtained both commercially and from array fabrication core facilities within research institutions, array CGH is not yet immediately accessible to most researchers. At present, scientists wanting to study a chromosomal region of interest by array CGH will require custom array production. Progress in the HGP has facilitated construction of a tiling path of genomic clones that cover chromosomal loci of interest (e.g., MapViewer resource at the National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>). While the associated costs of genomic DNA-based microarray production are not practical for individual research laboratories, it is likely that institutional core microarray facilities will be able to modify production to address this need. Conceivably, the post-HGP era will facilitate production of whole genome arrays (29), and even higher-resolution chromosome-specific and chromosome band-specific microarrays. Notably, the first high-density whole genome microarray (approx 2000 BAC clones) was recently introduced (25) and demonstrated its ability to precisely delineate genome-wide segmental aneuploidy breakpoints in tumor cells.

### 1.2.6. Commercial Sources of Genomic DNA-based Arrays

An alternative to custom arraying of genomic targets may be to obtain commercially available microarrays. One such system is produced by Vysis Corporation (<http://www.vysis.com>), called the GenoSensor System™. The AmpliOnc I™ array from Vysis contains BAC, PAC, and P1 genomic clones from 59 known oncogenes spotted in triplicate (30), and has been used by groups studying breast cancer (21) and glioblastoma multiforme (24). This microarray complements their GenoSensor™ microarray reader and analysis software package. The next generation genomic microarray from Vysis will comprise 250–300 features, including genomic clones from the AmpliOnc I™ array, subtelomeric regions of all chromosomes, major tumor suppressor genes, and major microdeletion syndrome loci (30). Recently, Spectral Genomics (<http://www.spectralgenomics.com/>) has produced a commercially available whole-genome human BAC microarray kit. This microarray is spotted in duplicate with 1003 human BAC clones, spaced at regular intervals along the genome, giving an effective resolution of 3 Mb for defining genomic aberrations. It is expected that both higher resolution (1 Mb and less) human and mouse BAC microarrays will become available for purchase in the near future.

### 1.3. Detection and Analysis

Analysis of microarray CGH involves three components, namely: (1) image acquisition; (2) quantification of fluorescence intensity; and (3) interpretation. These can be accomplished using the system developed for expression microarrays with minimal or no modification.

#### 1.3.1. Image Acquisition

Image acquisition for microarray CGH requires systematic scanning of all gridded features on the microarray. Commercially available microarray scanners are typically laser-based scanning systems that can acquire the two differential wavelengths sequentially (e.g., Packard BioScience, <http://www.packardbiochip.com>) or simultaneously (e.g., Virtek Vision Inc., <http://www.virtek.ca>; Axon Instruments Inc., <http://www.axon.com>). Alternatively, resources for the development of in-house microarray scanning systems are also available (e.g., <http://brownlab.stanford.edu/>; [31]). The technical details underlying these systems are specific to the hardware package, and are beyond the scope of this chapter.

#### 1.3.2. Fluorescence Quantification and Ratio Analysis

Software for fluorescence quantification and ratio analysis of gridded spots is usually included with the scanner hardware. Alternatively, there are less sophisticated programs publicly available (e.g., ScanAnalyze: <http://rana.stanford.edu/>; [32]). Quantified fluorescence intensities require normalization and establishment of the fluorescence ratio baseline. Often, microarray features are spotted in duplicate or triplicate for assessing result reproducibility. For array CGH, inclusion of genomic clones onto the microarray from regions that are known not to be involved in copy number change are recommended as internal controls for these purposes. In addition, parallel experiments in which differentially labeled normal genomic DNA is compared against itself can serve to establish the specificity of the system. Overall, there is an obvious need

for statistical analysis of the conformity of the results (33). Global normalization approaches such as those used in expression microarray experiments may also be used for establishing baseline thresholds (10,34).

Previous reports indicate that the relationship between the fluorescence ratio and copy number changes (1,9,11,12) deviates from linearity at low copy numbers. For this reason, it is important for users to independently establish this relationship for interpretation of CGH results and to confirm imbalances by direct FISH analysis of tissue sections.

### 1.3.3. The Role of Bioinformatics in Microarray CGH

As representation on the microarrays increases in density, data storage (35) and bioinformatics will become an important aspect of the CGH analysis. In addition, the increase in resolution will make the task of identifying consensus regions of genomic imbalance amongst samples more challenging. Overall, this will necessitate data mining techniques that can handle many data points on multiple dimensions between experiments. Moreover, for cDNA array CGH, *in silico* determination of chromosomal localizations of cDNA targets is essential for providing a comprehensive ideogram type schematic of chromosomal copy number changes (Fig. 3 [10]). As microarray CGH technology becomes more prevalent, more standardized informatics and analysis tools will appear.

## 2. Materials

### 2.1. cDNA Array CGH

#### 2.1.1. Array Preparation

1. 20X Sodium saline citrate (SSC): Dissolve 175.32 g of NaCl, 88.23 g of sodium citrate-2H<sub>2</sub>O in 1 L water, titrate to pH 7.0. Store at room temperature.
2. cDNA Microarray. Store in dessicator at room temperature.
3. Blocking solution: 3% bovine serum albumin (BSA), 4X SSC, 0.1% Tween-20. Store at -20°C.
4. Glass coverslips.

#### 2.1.2. Probe Preparation by Random Primer Labeling of Genomic DNA

1. High molecular weight genomic DNA.
2. *Eco*RI or *Dpn*II (New England Biolabs).
3. Qiaquick PCR purification kit (Qiagen).
4. BioPrime labeling kit (Gibco-BRL). Store at -20°C.
5. dNTP Mixture: 4.8 mM each of dATP, dGTP, dTTP.
6. 2.4 mM dCTP.
7. 1 mM Cy5-dCTP, Cy3-dCTP (Amersham). Store in the dark at -20°C.
8. Microcon 30 filter (Amicon).
9. Yeast tRNA (Gibco-BRL). Store at -80°C.
10. Poly(dA-dT) (Sigma). Store at -20°C.
11. Cot-1 DNA (Gibco-BRL).
12. Hybridization buffer: 3.4X SSC and 0.3% SDS. Prepare fresh per experiment.

#### 2.1.3. Probe Denaturation and Hybridization

1. Rubber cement.
2. Hybridization oven.

#### 2.1.4. Washes

1. Heated waterbath.
2. Coplin jars.
3. Slide centrifuge.

### 2.2. Array CGH

#### 2.2.1. Array Preparation

1. DNA extracted and purified from genomic clones.
2. Maxiprep DNA extraction kit (Qiagen).
3. Glass slides.
4. Glass capillary tubes or robotic arrayer.
5. Blocking solution: 10  $\mu\text{g}/\mu\text{L}$  salmon sperm DNA (Life Technologies) in 50% formamide (Gibco-BRL), 10% dextran sulphate, 2X SSC, 0.2% SDS, 0.2% Tween-20. Store at  $-20^{\circ}\text{C}$ .

#### 2.2.2. Probe Preparation by Nick-Translation of Genomic DNA

1. High molecular weight genomic DNA.
2. DNA polymerase I (Roche).
3. DNase I (Gibco-BRL).
4. 10X Cy3 dNTPs: 0.1 mg/mL BSA (Sigma), 0.1 M  $\beta$ -mercaptoethanol (Sigma), 0.5 M Tris-HCl, 50 mM  $\text{MgCl}_2$ , 0.08 mM Cy3-dCTP (Amersham), 0.2 mM dATP, 0.12 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP; dissolved in water. pH 7.4. Store in the dark at  $-20^{\circ}\text{C}$ .
5. 10X Cy5 dNTPs: 0.1 mg/mL BSA, 0.1 M  $\beta$ -mercaptoethanol, 0.5 M Tris-HCl, 50 mM  $\text{MgCl}_2$ , 0.08 mM Cy5-dCTP, 0.2 mM dATP, 0.12 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP; dissolved in water. pH 7.4. Store in the dark at  $-20^{\circ}\text{C}$ .
6. DNase I dilution buffer: 50 mM Tris-HCl, 5 mM  $\text{MgCl}_2$ , 1 mM  $\beta$ -mercaptoethanol, 100  $\mu\text{g}/\text{mL}$  BSA; dissolved in water. pH 7.4. Store at  $-20^{\circ}\text{C}$ .
7. DNA size standard ladder (e.g., *Hind*III ladder).
8. 0.3 M Ethylenediaminetetraacetic acid (EDTA) (Gibco-BRL).
9. Sephadex G50 spin column (Amersham).
10. COT-1 DNA (Gibco-BRL).
11. Hybridization buffer: 50% formamide, 10% dextran sulphate, 2X SSC, 2% SDS. Store at  $-20^{\circ}\text{C}$ .

#### 2.2.3. Probe Denaturation and Hybridization

1. Hybridization oven.

#### 2.2.4. Washes

1. Heated waterbath.
2. 0.1 M Sodium phosphate buffer.
3. NP-40 (Vysis).

### 3. Methods

#### 3.1. cDNA Array CGH

##### 3.1.1. Array Preparation

1. Block cDNA microarray under a glass coverslip for 1 h at  $37^{\circ}\text{C}$  with blocking solution prior to hybridization with denatured probe (see **Note 3**).

### 3.1.2. Random Primer Labeling of Genomic DNA

1. 2  $\mu\text{g}$  each of high molecular weight tumor and normal genomic DNA is separately digested with *DpnII* for 1–1.5 h (*see Notes 4–6*). The digestion products are purified (Qiaquick PCR kit), vacuum dried, and resuspended in 25  $\mu\text{L}$  of water.
2. Random primer labeling is performed using the Bioprime labeling kit, according to manufacturer's instructions, with modifications. Denature the DNA and 20  $\mu\text{L}$  Random primers (included in kit) at 100°C for 5 min. Immediately chill on ice, and add 2.5  $\mu\text{L}$  dNTPs, 1.25  $\mu\text{L}$  dCTP, 1  $\mu\text{L}$  Cy5/Cy3-dCTP, and 1  $\mu\text{L}$  Klenow fragment (included in kit). Incubate at 37°C for 90 min.
3. Combine Cy3- and Cy5-labeled products and load onto a microcon 30 filter. After centrifuging at 2000g for 10 min, check the sample reservoir for the presence of labeled product (purple color). Add directly to the sample reservoir 30  $\mu\text{g}$  Cot-1 DNA, 100  $\mu\text{g}$  yeast tRNA, and 20  $\mu\text{g}$  poly(dA-dT), and centrifuge for 20 min at 5000g. To recover the sample, add 15  $\mu\text{L}$  hybridization buffer, and invert microcon filter into a fresh collection tube and centrifuge for 1 min at 16,000g.

### 3.1.3. Probe Denaturation and Hybridization

1. Denature the probe at 100°C for 90 s in heated waterbath or PCR machine. Chill probe on ice, and allow probe to preanneal at 37°C for 0.5–1 h.
2. The probe is added to the microarray, covered with a glass coverslip and sealed with rubber cement. Hybridization is at 65°C for 16–20 h in a moist chamber humidified with hybridization buffer (*see Notes 3 and 7*).

### 3.1.4. Washes

1. The cDNA microarray is washed at 65°C (*see Note 7*) for 5 min in 2X SSC, 0.03% SDS, followed by successive washes in 1X SSC and 0.2X SSC at room temperature (5 min each).
2. The microarray is centrifuged at low speed (50g) for 5 min to dry.

## 3.2. Array CGH

### 3.2.1. Array Preparation

1. Genomic clones (BACs, PACs, cosmids, etc.) are grown with appropriate antibiotic and isolated using commercially available maxi kits. Typical yield is tens of micrograms of DNA. Standard protocols using phenol/chloroform may be used to further purify the DNA (*see Note 2*).
2. Size and quality of DNA is assessed by 1% agarose gel electrophoresis, and quantified with a UV-spectrophotometer.
3. This target DNA is sonicated to 1.5–15 kb fragments, precipitated, diluted to appropriate concentrations and spotted down on glass slides in a clean environment with capillary tubes at approx 200–400  $\mu\text{m}$  diameter spots (*see Note 8*).
4. Arrays are preannealed for 1 h at 37°C with 20  $\mu\text{L}$  Blocking solution under a glass coverslip in a hybridization chamber (*see Notes 3 and 9*).

### 3.2.2. Probe Preparation by Nick-Translation of Genomic DNA

1. 2  $\mu\text{g}$  each of high molecular weight tumor and normal genomic DNA (*see Note 5*) is separately labeled by nick translation. The reaction mixtures are as follows:  
A) Cy3 reaction (to total 100  $\mu\text{L}$  with water):

1. Tumor genomic DNA: 2  $\mu\text{g}$
  2. 10X Cy3 dNTPs: 10  $\mu\text{L}$
  3. DNA polymerase I: 1  $\mu\text{L}$
  4. DNase I (*see Note 10*)
- B) Cy5 reaction (to total 100  $\mu\text{L}$  with water):
1. Normal genomic DNA: 2  $\mu\text{g}$
  2. 10X Cy5 dNTPs: 10  $\mu\text{L}$
  3. DNA polymerase I: 1  $\mu\text{L}$
  4. DNase I (*see Note 10*)
2. The labeling reaction proceeds for 1.5 h at 16°C (refrigerated waterbath or PCR machine), following which the reaction mixtures are put on ice.
  3. The size of the labeled product is assessed by 1% agarose gel electrophoresis (*see Note 11*). Optimum fragment length for CGH is 500–2000 base pairs. If the size range is too large, reaction mixtures are returned to 16°C with additional DNase I and polymerase I to incubate further.
  4. Labeling reaction is stopped with addition of 0.1 vol 0.3 M EDTA.
  5. Unincorporated nucleotides are removed from the labeling mixtures using a Sephadex G50 spin column.
  6. Labeled products are mixed together, supplemented with 50  $\mu\text{g}$  Cot-1 DNA, and precipitated with 0.1 vol 3 M sodium acetate and 2 vol cold 100% ethanol. Precipitate is rinsed with 70% ethanol and air dried, then redissolved in 20  $\mu\text{L}$  hybridization buffer.

### 3.2.3. Probe Denaturation and Hybridization

1. Denature probe for 5 min at 75°C, and allow preannealing of the probe for 0.5–1 h at 37°C to ensure sufficient blocking of repetitive elements.
2. Apply the probe to the microarray after preannealing of the microarray is completed, cover with glass coverslip and seal with rubber cement. Arrays are hybridized for 24 h at 37°C in a chamber humidified with hybridization buffer (*see Note 3*).

### 3.2.4. Washes

1. Arrays are washed at 55°C in 50% formamide, 2X SSC pH 7.0 (3X, 10 min each), then in 0.1 M sodium phosphate buffer with 0.1% NP-40, pH 8, at room temperature, 5–10 min.
2. Drain excess liquid and mount slide in DAPI/Antifade under a glass coverslip.

## 4. Notes

1. Controversy exists in establishing a standard nomenclature. Although the term “probe” correctly refers to the known nucleic acid sequence tethered on the microarray while “target” is the unknown sequence in the sample (36), for the sake of conformity this chapter is following the convention used by all current microarray CGH publications.
2. Until automated and practical batch methods are developed, many groups are using maxi kits for obtaining target DNA for genomic DNA-based microarrays. This is a labor- and time-intensive process that needs repeating when the target DNA is exhausted over multiple arrayings. If purified, target DNA is available (at least several hundred nanograms template, from either maxi or mini preps), DOP-PCR (26) may be used to ensure an indefinite supply of target DNA.
3. It is very important that the microarray does not dry during any hybridization step. Ensure that the hybridization chamber remains humidified with hybridization buffer to prevent

evaporation of the probe or blocking mixture. If the microarray does dry, the results are invariably unusable.

4. The protocol herein is optimized for cDNA microarrays with approx 3500 features arrayed over an area of approx  $18 \times 16 \text{ mm}^2$  (**9,10**). The amount of DNA, as well as the final hybridization volume, must be scaled up when using higher density microarrays covering a larger spotting area (**10**).
5. As expected, the size and purity of the unlabeled genomic DNA is very important for obtaining high quality results using microarray CGH. Low quality DNA used in labeling can result in high background and low signal intensity on the microarray. The protocol stated herein is optimized for genomic DNA extracted from fresh tissues.
6. The choice of restriction enzyme for digestion is important for labeling efficiency. It has been noted that decreasing the average fragment size prior to labeling may increase labeling efficiency (**9**). This has to be balanced against excessive digestion producing fragments that are too small to be suitable for hybridization to the cDNA targets. In our hands, *EcoRI* has produced consistently satisfactory results for human genomic DNA.
7. When beginning the technique, a range of different hybridization and wash temperatures should be tested to determine the optimal sensitivity and specificity for the specific cDNA microarrays used. In our hands (**10**), we have found that hybridization at  $37^\circ\text{C}$  and wash at  $55^\circ\text{C}$  allows sufficient sensitivity for detection of high copy number gains and amplifications. We have observed that  $65^\circ\text{C}$  washes reduced signal intensity on our microarrays. Too low a wash temperature will result in nonspecific binding (too many yellow signals). We recommend that these tests be performed using differentially labeled DNAs from different samples to ensure optimization of the technique specificity.
8. To date, the protocols for array fabrication have not yet been standardized. The published works specify target DNA concentrations of 400–1000  $\mu\text{g/mL}$  hand spotted on glass slides coated with poly-L-lysine (**11**), or 2  $\mu\text{g}/\mu\text{L}$  target DNA on aminopropyltrimethoxy silane-coated slides (**12**). It is important to note that both the concentration as well as the slide preparation is likely to change as automation procedures with robotic arrayers emerge.
9. The protocol specified herein for array CGH assumes a maximum gridded feature area that can be covered with a  $22 \times 20 \text{ mm}^2$  glass coverslip. In addition, it is assumed that the target DNA are denatured during array fabrication (**12**). Otherwise, a microarray denaturation step of 2 min in 70% formamide/4X SSC (**11**) must be included prior to probe hybridization.
10. The final probe length depends on the DNase I concentration. For CGH, the suitable length for hybridization ranges from 500–2000 base pairs. Initially, stock solutions of  $1 \times 10^{-4} \text{ U}/\mu\text{L}$ , prepared fresh in DNase I dilution buffer, may be used to obtain the final concentration of  $5 \times 10^{-5} \text{ U}/\mu\text{L}$ . However, this should be adjusted as necessary to obtain optimal fragment length.
11. Approximately 0.05–0.1 vol of each labeling mixture is loaded onto the gel with DNA stain (e.g., ethidium bromide). Assessment of labeling by agarose gel is recommended as it can aid in troubleshooting array CGH results.

## Acknowledgments

The authors' are grateful to Paula Marrano, Bisera Vukovic, Monique Albert, and Pascale Macgregor for critical reading of the manuscript. This work was supported by the National Cancer Institute of Canada, with funds from the Canadian Cancer Society. PCP was supported by the AFUD and the Imclone Systems, Inc.

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## Molecular Approaches for Delineating Marker Chromosomes

Michael D. Graf and Stuart Schwartz

### 1. Introduction

A marker chromosome is defined as a structurally abnormal supernumerary chromosome that can not be identified by routine cytogenetics. Examples of marker chromosomes include rings, derivatives, dicentrics, and minute chromosomes. Current literature suggests that supernumerary marker chromosomes are found in 0.14–0.72/1000 newborns and more commonly in prenatal testing at 0.65–1.5/1000 (1). The significance of a marker chromosome depends on several factors, including inheritance, mode of ascertainment, chromosomal origin, and morphology as well as structure of the marker.

### 2. Clinical Significance of Marker Chromosomes

About 40% of markers are familial. In such cases, usually there are no phenotypic consequences for individuals who inherit the marker. There are exceptions, such as uniparental disomy (UPD), or if the parent has an unidentified, low-level, tissue specific mosaicism for the marker chromosome without phenotypic manifestations (1–4). Most of the postnatally obtained marker chromosomes are associated with a phenotypic abnormality owing to the manner of their ascertainment. Marker chromosomes ascertained prenatally are much more problematic since only a small proportion of cases will have associated abnormalities.

There are numerous published reports of individual cases or a few cases of particular marker chromosomes and associated phenotypes. However, the more influential studies are those that collect data from consecutive analyses or from a large number of individual cases involving marker chromosomes. Some of the most recent and most useful studies perform molecular cytogenetic analyses on the marker chromosomes

allowing them to be grouped by chromosomal origin, morphology, and phenotype. The first major study to offer general risk figures for marker chromosomes analyzed data from over 377,000 amniocentesis studies (5). Data from that study suggested there is a 13% chance for associated phenotypic abnormalities in prenatally ascertained, *de novo* cases of supernumerary marker chromosomes. However, this study was completed before the advent of FISH technology, limiting the ability to characterize the markers. Since then, several studies have collected cases of marker chromosomes in an attempt to make phenotype/karyotype correlations based on molecular cytogenetic analysis of the marker chromosomes.

The largest study since Warburton (5) to characterize risk for phenotypic abnormality for marker chromosomes was completed by Crolla in 1998 (6). He utilized FISH analysis and combined data from all existing reported cases of autosomal marker chromosomes (168 total cases). This study excluded sex chromosome markers as well as markers that are found more commonly, such as those derived from chromosome 15, the marker derived from chromosome 22 that is associated with Cat-eye syndrome, and isochromosomes for the short arms of chromosomes 9, 12, and 18, as these markers are clinically well delineated (*see Subheadings 4.3. and 4.4.*). In addition to providing information from markers derived from specific chromosomes, several general conclusions were made. As expected, *de novo* markers were more often associated with phenotypic abnormalities compared to markers that were inherited from phenotypically normal parents. In addition, this study reinforced the fact that the majority of marker chromosomes originate from acrocentric chromosomes. Even when markers derived from chromosome 15 were excluded, the remaining acrocentric chromosomes still accounted for the majority (60%) of the marker chromosomes in this study. In addition, it was found that markers derived from the acrocentric chromosomes (in contrast to other chromosomes) were much less likely to be associated with a phenotypic abnormality (7.1 vs 28.6%) (excluding those that cause Cat-eye syndrome, those that were derived from chromosome 15, and those determined to be isochromosome 18p).

A collaborative study is in progress that will have the largest collection of cases of marker chromosomes to date (over 500). Preliminary data from this study suggests that risks for associated phenotypic abnormalities when a *de novo* marker chromosome is detected prenatally are higher than current literature suggests (7).

Although precise phenotype/karyotype data regarding marker chromosomes is still incomplete, it is nevertheless imperative to characterize each marker chromosome to optimize the potential clinical correlations. In this chapter, we have two objectives. First, we will provide information concerning known phenotype/karyotype correlations of marker chromosomes. Secondly, we will describe approaches to efficiently characterize a marker chromosome in an attempt to make these phenotype/karyotype correlations.

### 3. Available Technology

In addition to standard FISH analysis, there are several molecular cytogenetic techniques that can efficiently characterize marker chromosomes. Some of these include Spectral Karyotyping (SKY), M-FISH, CGH, and microdissection followed by reverse

painting. M-FISH and SKY can be used to determine the chromosomal origin of a marker by using methodology that simultaneously labels each chromosome with a different color (8,9). CGH and microdissection, can also be used to delineate the origin of chromosomal material (10,11). Most of these methods are described in detail elsewhere in this volume, but are not available to every laboratory.

There are several different types of FISH probes that prove useful in delineating the structure of marker chromosomes, including centromere specific (alpha satellite) probes, whole chromosome paints, commercially-available chromosome-specific probes, and BACs (bacterial artificial chromosomes) which are probes generated from information gained from the Human Genome Project. The advent of this technology and collection of cases based on findings from this technology has led to several well known phenotypic correlations when a particular marker chromosome is ascertained.

## 4. Phenotype/Karyotype Correlations

### 4.1. *Dicentric 15*

This marker chromosome consists of two short arms and a portion of the long arms of a chromosome 15 and is often named the “inverted duplication 15” or “inv dup(15).” There are several variations of this marker that can be delineated by molecular cytogenetic techniques. If the isodicentric 15 is relatively large, it may include the critical region for Prader-Willi/Angelman syndromes, and have an associated phenotype that can consist of mental retardation, developmental delay, behavioral disturbances, seizures, hypotonia, and strabismus. This phenotype is similar if the marker is trisomic or tetrasomic for the PWS/AS critical region (12,13). If the isodicentric region is smaller and is determined not to contain the PWS/AS region, a normal phenotype is usually seen, unless uniparental disomy (UPD) occurs (14–16).

### 4.2. *Dicentric 22*

This marker chromosome consists of two short arms and a portion of the long arms of chromosome 22 and is sometimes named the “inverted duplication 22” or “inv dup(22).” When the dicentric 22 contains the critical region for Cat-eye syndrome (22q11), the individual will often have a variable phenotype that includes coloboma of the iris and/or retina, preauricular skin tags and pits, anal atresia, branchial clefts, heart defects, renal malformations, urogenital defects, mild to moderate mental retardation, and dysmorphic features (17–20).

### 4.3. *Iso 12p*

This marker chromosome is usually found in mosaic form and is associated with Pallister-Killian syndrome. It is usually found less commonly and in a lower frequency in lymphocytes as compared to amniotic fluid or skin. Most individuals with Pallister-Killian syndrome have this marker chromosome, which consists of two short arms of chromosome 12 and, therefore, have tetrasomy for the short arm of chromosome 12. The phenotype can consist of severe mental retardation, seizures, hypotonia, pigmentary abnormalities, sparse hair, and a coarse face (21,22). However, the phenotype can

be extremely variable as demonstrated by the report of a mild case in association with a relatively high percentage of cells containing the marker (23).

#### **4.4. Iso18p**

Individuals with this marker chromosome are tetrasomic for the short arm of chromosome 18 and often have a range of features including low birth weight, significant mental retardation, hypotonia, short stature, seizures, microcephaly, and characteristic facies (24,25).

#### **4.5. Sex Chromosome Markers**

Markers involving the X or Y chromosome are often found in karyotypes with 45,X or with mosaicism for 45,X or 45,Y and a normal cell line. These markers do not fit our precise definition of a marker chromosome since they are not supernumerary. However, such structurally abnormal chromosomes are still often referred to as marker chromosomes.

It should first be determined whether these marker chromosomes originate from the X or from the Y chromosome. Marker chromosomes derived from the X chromosome should be evaluated for the presence of XIST since studies have shown that the absence of XIST is often associated with mental retardation/developmental delay. However, phenotypic outcome is also dependent on the amount of genetic material the marker contains as well as if the karyotype is mosaic (26,27).

Marker chromosomes originating from the Y chromosome are often found in conjunction with 45 X mosaicism in individuals exhibiting features of Turner syndrome. It is important to determine whether the gonadoblastoma region (GBY) near the centromere of the Y chromosome is present, as literature suggests the presence of this region in phenotypic females can be a risk factor for gonadoblastoma (28,29).

### **5. Approaches to Effectively Delineate a Marker Chromosome**

#### **5.1. Inheritance**

It is imperative to first determine whether the marker is familial or inherited since the majority of inherited marker chromosomes will not have an associated abnormal phenotype.

#### **5.2. Routine Cytogenetics**

Initial G-banding will often provide several useful pieces of information in cases of marker chromosomes. G-banding can sometimes suggest the morphology or structure of the marker chromosome, which can aid in making decisions as to what additional tests should be performed. For example, if morphological satellites are seen by cytogenetics, one should focus additional testing on the acrocentric chromosomes. In addition, G-banding can often suggest a particular chromosomal origin, such as in the cases of tetrasomy 12p or isochromosome 18p. In such cases, FISH should always be used to confirm the origin of the marker chromosome. Finally, G-banding will also demonstrate mosaicism, if present. If the marker chromosome is found in low frequency, this will often make additional FISH analyses more difficult because there

may be few metaphases that contain the marker or the marker may be lost in culture over time.

### **5.3. If Cytogenetics Does Not Suggest a Chromosomal Origin**

#### **5.3.1. Existence of Centromere**

A centromeric probe (alpha satellite) should initially be used to determine if the marker chromosome contains a centromere. This will allow the proper triage to follow as approaches for delineation will differ. In addition, if the marker does not contain a centromere, this increases the likelihood that the marker contains euchromatic material, and therefore, the risks for associated phenotypic abnormalities is increased.

#### **5.3.2. Acentric or Large Marker Chromosomes**

Use SKY, M-FISH, CGH, or chromosome microdissection if possible to determine chromosomal origin in those cases which do not have a centromere or where there is euchromatic material in addition to a centromere.

#### **5.3.3. Centric or Small Marker Chromosomes**

If the marker is small, contains mostly centromeric material, or if it is present in only a small proportion of cells, the above technology may not prove useful. Technology that uses a set of probes such as the Chromoprobe Multiprobe system by Cytocell, Inc., that utilizes a set of alpha satellite probes for all the chromosomes can be used as long as there is centromeric material present (Cytocell, Inc, United Kingdom). However, this methodology is best used on peripheral blood samples and not prenatal samples or other cultured cells. If any of the above technology is not available, one can use chromosome-specific, alpha satellite DNA probes for all the chromosomes. However, one can initiate such studies using the probes from the chromosomes most often associated with marker chromosomes (e.g., 13, 14, 15, 18, 21, 22, X, Y). If the origin is still unknown, other chromosome specific probes can be used.

#### **5.3.4. Known Chromosomal Origin**

Once the chromosomal origin is known, it is often beneficial to use commercially available chromosome specific probes or BACs to further determine the significance of the marker chromosome. For example, if the marker is determined to be from chromosome 15, use probes found within the PWS/AS critical region (SNRPN, D15S11, or GABRB3). If the marker contains the PWS/AS critical region, it is more likely that there will be associated abnormalities.

## **6. Special Cases**

### **6.1. Chorionic Villus Sampling (CVS)**

Approximately 1–2% of all cytogenetic analysis from a CVS are mosaic (30). Some of these cases may involve a marker chromosome. Regardless of whether the mosaicism is determined to be confined placental mosaicism or true fetal mosaicism, additional studies are often warranted. Confined placental mosaicism has been associated with intrauterine growth retardation, and depending on chromosomes involved, the

fetus can have an abnormal phenotype if uniparental disomy occurs (31). When a CVS shows mosaicism, an amniocentesis should be recommended since it is often impossible to determine whether the abnormal cell line is fetal or placental in origin. If the subsequent amniocentesis reveals a normal karyotype or confirms the mosaic karyotype, uniparental disomy (UPD) studies should be considered if the marker originated from a chromosome known to have substantial imprinting effects: chromosomes 6, 7, 11, 14, 15 (32).

### **6.2. Amniocentesis**

Mosaicism in an amniocentesis sample is more likely to be real, meaning the fetus is mosaic. It is not recommended to repeat the amniocentesis. If a marker is involved, it is imperative to attempt to determine its chromosomal origin, since prognosis may differ. However, after the chromosomal origin of the marker is determined, UPD should be considered if the marker originated from a chromosomes known to have substantial imprinting effects: chromosomes 6, 7, 11, 14, 15 (32).

### **6.3. Mosaicism (general)**

A proportion of marker chromosomes, especially those ascertained prenatally, are found in mosaic form. When only a small proportion of cells contain the marker chromosome, it might not be possible to do further studies since the marker can be lost over time in culture. For prenatal cases, it is recommended to obtain postnatal samples (cord blood, placenta, and/or foreskin) to determine if the marker is present in other tissues. Additional tissue samples (other than peripheral blood) may offer more information for cases of marker chromosomes ascertained postnatally as well. Although the results of these analyses might not change the prognosis, it may help better delineate the significance of the marker chromosome.

### **6.4. Scarce Resources**

In some cases, one may need to perform numerous tests in an attempt to characterize a marker chromosome. Each test is often labor intensive and costly. However, any piece of additional information obtained from additional analyses can be extremely helpful to the physician and family to help determine the significance of the marker chromosome. Such information is even more helpful in prenatally ascertained cases. If a laboratory is having difficulty characterizing a marker, or if they do not have the resources to perform additional studies, it is highly encouraged that they contact another laboratory to see if they might be able help with the characterization of the marker. Even after an exhaustive search, a minority of cases involving marker chromosomes will not allow characterization of the marker. This can result from low levels of mosaicism or owing to the fact that the marker is small, or contains only centromeric material. It is encouraged that laboratories save genetic material on the proband, and possibly the parents, so further studies can be initiated if new resources or technology becomes available.

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## Interphase FISH for Prenatal Diagnosis of Common Aneuploidies

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### 1. Introduction

#### 1.2. Interphase FISH Analysis for Prenatal Diagnosis

Cytogenetic analysis is currently a standard prenatal diagnostic test. It is routinely offered to pregnant patients who have an increased risk of carrying chromosomally abnormal fetuses. The traditional “gold standard” for prenatal diagnosis of chromosome abnormalities is metaphase analysis by G-banding. The primary advantages of standard cytogenetic analysis are the ability to detect aneuploidies as well as structural chromosomal aberrations with great accuracy (>99.5%). Traditional karyotyping, however, requires isolation of metaphase chromosomes from cultured fetal cells and therefore is time consuming. Although reporting time has decreased dramatically during the last 3 decades, conventional karyotyping still requires 7–12 d of which culture time is the most time consuming (1).

In certain clinical situations, especially in prenatal diagnosis, waiting for chromosome analysis may place a significant emotional stress on the patient, and clinical burden on the referring physician (2–6). The worldwide acceptance of chromosome analysis as routine prenatal diagnostic test, and the significant improvements in karyotyping technology have only increased the demand for immediate answers, and have made clinicians and patients even more intolerant of delays in receiving results (7,8). For that reason alone, interphase FISH analysis for the most common aneuploidies was accepted with great expectations and enthusiasm by many obstetricians and cytogeneticists. They felt that rapid FISH analysis might offer an opportunity to reduce anxiety and facilitate earlier decision making (7,8).

Fluorescence *in situ* hybridization (FISH) involves hybridization of fluorescently labeled specific probes to the patient’s chromosomal DNA and signal detection using a fluorescence microscope. FISH is applicable in interphase as well as metaphase cells and therefore does not require cultured cells for diagnosis. The technique was introduced

as a potentially powerful tool in clinical cytogenetics about 15 years ago (9,10). It has been found to be highly effective for rapidly determining the number of chromosomes in interphase cells using chromosome specific probes (11–17).

Aneuploidies of only 5 chromosomes (13, 18, 21, X, and Y) account for about 65% of all chromosomal abnormalities and for 85–95% of the chromosomal aberrations causing live born birth defects (18–20). Therefore, rapid enumeration of these five chromosomes would potentially be of great value to prenatal diagnosis. In the early 90s, a rapid prenatal test to detect the most common chromosomal abnormalities in uncultured interphase fetal cells was indeed designed (21–23). Since then, the application of FISH to prenatal screening for common autosomal trisomies (13, 18, and 21) and sex chromosome anomalies is becoming increasingly common worldwide (24–33).

## **1.2. Historical Background and Recognition**

The potential ability of FISH to identify specific chromosomes in uncultured interphase nuclei was determined by early studies (9,11–17,34–36). The potential application of FISH technology for rapid routine prenatal diagnosis of the most common aneuploidies was also established in the early 1990s (21–23,37,8). However, early attempts at aneuploidy detection in uncultured amniocytes suffered from significant limitations caused by probe design, sample preparation, and assay conditions. Klinger et al. (37), and later Ward et al. (38), reported the results of the first major studies comparing aneuploidy detection by FISH with standard cytogenetic analysis. Their studies formed the basis of the clinical protocols for the application of FISH to prenatal diagnosis. However, these two and most of the following early studies had several obstacles that delayed wide acceptance of FISH as a highly reliable method for routine prenatal diagnosis. Many studies were artificially blinded at best, and did not evaluate the test in actual clinical settings. The final diagnosis in a significant number of early FISH studies was unsuccessful due to failed hybridization, or problematic results owing to insufficient number of nuclei for analysis of one or more chromosomes. Of special concern was the fact that, in many studies, FISH misdiagnosed several false negatives and positives.

These concerns on the sensitivity, specificity and predictive values of the test, especially in the lack of uniform laboratory methods and diagnostic standards, produced profound skepticism in the genetics community (39). As a result, the American College of Medical Genetics (ACMG) stated in 1993, that FISH for clinical cytogenetic studies should be considered investigational (40). The ACMG also stated that until accepted as a standard laboratory technique, FISH should be used in prenatal interphase cytogenetics only in conjunction with standard cytogenetic analysis, and that irreversible therapeutic action should not be initiated on the basis of prenatal FISH analysis alone.

The ACMG in its original policy statement (40) recognized that appropriate quality assurance and quality control for reagents, as well as techniques in the development of standardized protocols must be established for FISH analysis. The policy statement called for well designed, multicenter, prospective, clinical trials to assess the analytical performance as well as the clinical applicability of FISH analysis in prenatal diagnosis.

One major obstacle in establishing standard protocols and wide acceptance of the test were the chromosome specific probes used for interphase FISH. Authors (37,38,41–50) used different probes constructed by their own laboratories, or commercially available probes from different sources, while most cytogenetic laboratories are not qualified to synthesize DNA probes or to perform the necessary quality control studies for each probe used. Furthermore, the assay conditions should be modified for each set of probes since the quality and characteristics of the probes are the key factors for successful FISH analysis. The introduction of multicolor, commercially available, highly specific and reliable probes significantly changed the performance of the test and its wide acceptance by many clinical programs.

In 1997, the AneuVysion™ assay (Vysis, Inc., Downers Grove, IL, USA) was cleared by the American Food and Drug Administration (FDA) to enumerate chromosomes 13, 18, 21, X and Y in amniocytes. The assay kit includes two sets of multicolor probes. One set is a mixture of probes directed to centromeric alpha satellite of chromosomes 18, X, and Y, and the other is a mixture of two probes designed to recognize specific loci on chromosomes 13 and 21. Many laboratories worldwide adopted the AneuVysion™ kit after it was reviewed and cleared by the American FDA, and nearly 30,000 reported cases established its accuracy and reliability since then (51) (see **Note 1**).

In 2000, the ACMG published a new policy statement regarding clinical applications of FISH for cytogenetic analysis (52). It stated that it is clear that the high analytical sensitivity and specificity of interphase FISH testing for prenatal diagnosis or screening provides highly accurate results for those abnormalities detectable by the test. They further concluded that it is reasonable to report positive FISH results. Decision-making regarding clinical management of the fetus should be based on information from two of three of the following: positive FISH results, confirmatory chromosome analysis, or consistent clinical information (52).

Interphase FISH analysis for prenatal diagnosis is currently validated for uncultured amniocytes only. We believe, as many others, that the test is applicable to uncultured chorionic villus cells with comparable accuracy to amniocytes (7,32,43–45,53–59). Unfortunately, the reported number of CVS cases is much smaller and far from allowing reliable calculation of analytical and clinical performances.

### 1.3. Analytical Performance

The ACMG in its recently published policy statement concluded that FISH technologies provide highly analytical accurate test systems (52). The tests are standardizable and controllable. The statement clearly recognizes that most of the unresolved analytical concerns expressed 7 years ago were satisfactorily answered. Tepperberg et al. (51) have recently reviewed the literature extensively, and presented the overall analytical performance of interphase FISH testing for the prenatal diagnosis of the most common aneuploidies in their report. If all informative cases reported in the literature are counted (7,26–29,31,33,37,38,41–43,45–51,53,59–69) (47,312 specimens), the test has sensitivity of 99.39%, specificity of 99.98%, and positive and negative predictive values of 99.81 and 99.94%, respectively. If only the most recently published papers, reporting on studies with the FDA reviewed probes, are counted

(7,27–29,31,33,51,53,59,62–69) (29,039 informative cases), the performance is even better with sensitivity of 99.75%, specificity of 99.99%, and positive and negative predictive values of 99.96 and 99.97%, respectively.

#### **1.4. Clinical Performance**

Interphase FISH analysis for prenatal detection of the common aneuploidies (chromosomes 13, 18, 21, X, and Y) is very accurate and reliable test. However, those requesting the test should be fully aware of what that specific test can, and more importantly, cannot do. The test has extremely high analytical sensitivity, specificity, and predictive values. However, what is truly relevant for clinical management of the fetus is its clinical sensitivity and predictive values, which are dramatically different.

Aneuploidies of chromosomes other than 13, 18, 21, X, and Y, structural aberrations, ring or marker chromosomes and many mosaic states are theoretically undetectable by routine interphase FISH testing. The laboratory, however, is currently requested to detect all cytogenetically visible chromosomal abnormalities, where all refers to those aberrations detectable by the “gold standard” traditional karyotyping. By that clinically relevant definition, routine FISH analysis will miss about 25–30% of the detectable cytogenetic abnormalities, even with 100% accuracy of the test (8,25,70).

One factor that may influence the different clinical sensitivity of FISH versus classical cytogenetics, is the indication for chromosomal analysis (7). Since aneuploidy is the primary risk, which increases with maternal age, it is expected that FISH tests will have their greatest clinical sensitivity among the oldest individuals in the population to be screened. Based on data from several large series of prenatal diagnostic cases, the likelihood that a prenatal FISH test using probes from chromosomes 13, 18, 21, X, and Y would detect the abnormality can be determined (8,25,70). For all patients tested prenatally, approx 70% of all clinically significant cytogenetic abnormalities would be detected by the FISH test. Among patients presenting with advanced maternal age, clinical sensitivity approaches 80%, while in those cases ascertained by abnormal ultrasound or triple marker prenatal screening, a group which includes younger patients, the test sensitivity is reduced. In this context, sensitivity is defined as the detection of abnormalities associated with abnormalities in live births.

#### **1.5 Interphase FISH—Adjunct or Substitute to Karyotyping?**

A detailed economic and social analysis regarding that question is beyond the scope of this chapter. It is important however to emphasize the major clinical arguments in favor of using routine interphase FISH analysis, at present time, as an adjunct to standard cytogenetics analysis and not as its replacement. Most if not all authors currently share that view (58,70).

Interphase FISH for detection of the common aneuploidies misses about 30% of all chromosome abnormalities detectable by standard cytogenetics. It can be argued that considerable percentage of the “missed” cases would be clinically insignificant. Balanced translocations or marker chromosomes are good examples, which account for many such missed diagnoses. However, some *de novo* balanced translocations and

marker chromosomes are associated with clinically significant abnormalities, and the identification of all rearrangements is important for accurate genetic counseling and planning of future pregnancies.

A confirmatory cytogenetic result is important in “positive” FISH cases as well. The classical karyotyping will reveal the mechanism of aneuploidy (translocation vs trisomy), necessary for counseling on recurrence risk in future pregnancies.

One may also argue that cost benefit issues will direct towards abandoning the expensive cytogenetic analysis in favor of the faster, less expensive FISH technique. Many feel, however, that the cost of the “missed” cases far outweighs the savings (70).

FISH is an evolving exciting technology. Improvements and new technologies may change the balance of the equation in the near future.

## **1.6. Indications**

### **1.6.1. Low Risk Pregnancies**

In principle, all pregnant patients going through invasive prenatal testing for chromosome analysis may benefit from the rapid information provided by prenatal interphase FISH testing. However, the most common indications for such testing is advanced maternal age and increased risk of Down syndrome based on maternal serum multiple marker screening. Although the risk for a numerical chromosomal abnormality increases with advanced maternal age and abnormal biochemical screening, the total number of abnormalities detected in this population is relatively low (approx 3–4%, [7]). Other relatively low risk indications for invasive prenatal diagnosis, such as maternal request, studies for fetal infections or single gene genetic diseases, are certainly not indications for interphase FISH testing.

We usually do not offer adjunctive prenatal interphase FISH to patients with uncomplicated pregnancies in which prenatal diagnosis is performed at gestational ages of < 20 wk. On the other hand, if a patient or a her referring physician specifically request for it, or a patient expresses unusual anxiety, the option of rapid FISH testing is discussed by the genetic counselor at the time of the preprocedure counseling session.

### **1.6.2. Abnormal Ultrasound Findings.**

A fetal anomaly detected by ultrasound is the single most significant risk factor for chromosome aberrations, particularly aneuploidy (7,28,51,59,60). The incidence rate of chromosome aneuploidy detected by G-banding is ranging between 10–20% depending on the specific ultrasound finding. Ultrasound finding is also the single most significant factor for emotional distress to the patient and her family. This combination of significant anxiety and high rate of numerical chromosomal abnormalities makes pregnancies complicated by ultrasound findings, the most common indication for rapid interphase FISH analysis in many programs. In these circumstances, many will consider the rapid test, regardless of gestational age.

### **1.6.3. Late Gestational Age**

Several time sensitive prenatal situations can benefit from prenatal interphase FISH testing more than others. In these situations, waiting for results may be critical for

clinical decision making. One relatively common scenario is impending deadline for decision on pregnancy termination. Another scenario is pregnancy at 30 wk or more, complicated by ultrasound findings (28). At the very least, rapid prenatal interphase results can provide limited information that may improve the way the delivery of a fetus with multiple anomalies is managed

#### 1.6.4. Confirmation

Couples who have received abnormal results from prenatal chromosome analysis may ask if the findings could either be incorrect or have been mistaken for another patient's sample. They may insist upon a confirmatory chromosome analysis before making any decisions about terminating the pregnancy. Provided the previous abnormal chromosome result is detectable using the prenatal interphase FISH test, FISH can be extremely useful in quickly verifying results without having to wait the additional 7–14 d for chromosome analysis. It is important to initiate cultures, however, in case the FISH results do not agree with results of the earlier chromosome analysis.

In cases of mosaicism detected on cultured cells, the laboratory may request confirmatory interphase FISH testing. In such cases, the proportions of normal vs abnormal cells, or the distribution of normal/abnormal cells in fetal and extra-embryonic membranes is an important determinant of abnormality. Interphase FISH testing affords greater statistical accuracy because of larger cell samples, scorable in much shorter time, when compared with traditional cytogenetic analysis (71).

In all cases of confirmation, the standard chromosome analysis precedes the interphase FISH testing, so it is not necessary to repeat metaphase karyotyping.

#### 1.6.5. Indications for “Diagnostic” Interphase FISH Analysis

Some ultrasonographically identified fetal anomalies may be conclusive for a syndromic diagnosis. Families with known parental carrier of a relevant rearrangement can also indicate a fetus at significant risk for one of the common aneuploidies. These cases will benefit greatly from prenatal FISH studies and may be approached by a diagnostic FISH test (52).

### 1.7. Limitations and Pitfalls

#### 1.7.1. Inherent Limitations

Interphase FISH analysis provides information regarding only the specific probe loci used. The established interphase FISH test for prenatal screening of the most common aneuploidies uses probes, which are designed to hybridized to loci on chromosomes 13, 18, 21, X, and Y. The test allows counting of loci and has no power to detect rearrangements.

The test does not detect structural chromosomal aberrations, or numerical abnormalities of chromosomes other than 13,18,21,X, and Y, and therefore, it does not substitute for complete standard cytogenetic analysis. These inherent limitations should be emphasized and stated in all test reports (*see Note 2*).

#### 1.7.2. Technical Artifacts

Technical artifacts may cause misinterpretation of the results. The most common artifacts are overlapping or splitting signals. Overlapping signals are usually the result

of overlapping chromosomes. Splitting signals are either inherent to a specific probe or owing to separation of sister chromatids. Suboptimal denaturation or hybridization, and inappropriate wash conditions may cause high background or weak signals.

An effective quality control system, a training program for technicians, and assay validation by establishing a database of reportable reference ranges as described by the ACMG Standards and Guidelines for Clinical Genetics Laboratories can significantly reduce misinterpretation of results due to technical artifacts ([72], see **Notes 3** and **4**).

### 1.7.3. Delay of Results

Speed of diagnosis and rapid reporting is the most distinct advantage of prenatal interphase FISH test. Most laboratories report results within 24 h from receipt of the sample, and when indicated can complete the test in 6 h. However, delays in reporting can occur. In most cases the delay is owing to technical difficulties and the need for rehybridization of a sample. Technical problems such as insufficient number of cells for signal detection would usually delay results by 24 h at the most. Whenever results are not available in 48 h, the laboratory director should consider reporting a specimen uninformative by FISH, especially since standard chromosome analysis results should be available in 5–10 d.

### 1.7.4. Maternal Cell Contamination

Chorionic villus sampling (CVS) and amniocentesis are designed to exclusively obtain fetal cells for prenatal genetic testing. Therefore, one of the most significant challenges in interpreting results of any prenatal genetic test is maternal cell contamination.

The presence of maternal cells in a small proportion of amniotic fluids cultured for cytogenetic analysis is well documented (73), but rarely results in misdiagnosis. There is, however, dramatic difference in the frequency of detection of maternal cells between cultured and uncultured fluids (74). The overall frequency of maternal cell contamination (MCC) in cultured amniotic fluids is estimated to be 0.4% (73), while the reported detection rates of MCC in uncultured samples is 10–20% (74–76). These observations suggest that one should be extra careful when using uncultured fetal cells for direct genetic testing, such as direct DNA or rapid interphase FISH analyses.

There is a strong correlation between visible blood in the fluid or the pellet and frequency and intensity of MCC (74,75). Therefore, the best way to avoid misinterpretation of results due to MCC is to accept only clear, yellow amniotic fluid for FISH. Bloody or brownish fluid, or pellet contaminated with numerous red blood cells should not be analyzed by interphase FISH and tested by standard cytogenetics only.

It was also suggested by some authors, (77–79) that MCC can be significantly reduced by improvements in obstetrical techniques. Using smaller gauge needles, avoiding the placenta if possible, and most importantly, discarding the first 5 mL of amniotic fluid aspirated, may reduce MCC significantly. Most experienced obstetricians obtain no more than 1–3% bloody samples.

Although most MCC cases are associated with maternal blood in amniotic fluid, it has been recently reported that approx 10% of prenatal interphase FISH samples from pregnancies complicated by significant oligohydramnios have overwhelming mater-

nal cell contamination (80). The FISH results of these samples should also be interpreted with caution.

Maternal cell contamination following CVS is caused by blood cells but more frequently by decidua. To minimize the risk of MCC following CVS, an experienced technician should meticulously clean the specimen under the microscope, and only very clean pieces of chorionic villi should be chosen for FISH.

The risk of MCC is significantly reduced but not eliminated by taking the described above measures. Feldman et al. (59) reported on prospectively studied 301 specimens of high risk pregnancies. The obstetricians were very experienced prenatal geneticists who kept very strict obstetrical technique, and did not use the first 5 mL of amniotic fluid for FISH. Specimens with visible blood in the fluid or pellet were excluded. Despite all this measures, five of their 301 (1.7%) cases were suspected for MCC and needed further studies (59). Maternal cell contamination should be suspected in the following circumstances: (1) A sample with a mixture of XX, XY signals, (2) cells with unusual morphology on the slide, (3) any interphase FISH results suspicious for chromosomal mosaicism of any type.

#### 1.7.5. Other Causes of Mosaicism

Other causes of mosaic states are confined placental mosaicism, true fetal mosaicism, disappearing twin syndrome, or technical error. Pseudomosaicism is not considered since the test is performed on uncultured cells.

Detection of mosaicism by prenatal interphase FISH can be extremely difficult. We suspect mosaicism whenever 10–85% of the cells express the same signal pattern suggestive of aneuploidy. In those cases, we recommend extension of the FISH study and analyze as many as possible cells, but no less than 100. We also extend the standard cytogenetic analysis as well.

The question of reporting FISH results suspected for mosaicism vs call the test uninformative, is an open one. We believe that considering the extremely low number of cases suspected for mosaicism, and if the indication for FISH test was not time sensitive, it is wise to report inconclusive tests and wait for confirmation by standard karyotyping.

#### 1.7.6. Uninformative Tests

The incidence of uninformative cases is very low and ranges between 3–5%. The most common reasons for uninformative cases are inadequate volume of amniotic fluid or chorionic villi cells and maternal cell contamination detected by visible blood in the sample or pellet. Hybridization failure and inconclusive results are by far less frequent.

We usually request extra 10 mL of amniotic fluid for FISH, but will try to perform the test even if only 2–5 mL is available.

#### 1.7.7. False Positive and Negative Results

Tepperberg et al. (51) have recently published a multicenter study. They also extensively reviewed the literature for false results and present the overall analytical performance of the test in their report. Nine (0.019%) of 47,312 informative cases, included

in population based, published studies, enabling frequency calculation were false positives (7,26–29,31,33,37,38,41–43,45–51,53,59–69). The false interpretation of results was additional 13/21 signal in 3 cases (45–47), and additional or absent signal for one sex chromosome (X or Y) in the 6 other cases (38,43,48,51,64). Few other false positives have been reported, but these cases were published as case reports and are not included in the frequency calculations (81–87).

The risk of 0.019% false positive interphase FISH results is very small. In prenatal diagnosis, however, there is zero tolerance for false positive results that can lead to termination of an unaffected fetus. For that reason, the ACMG originally stated in 1993 that irreversible therapeutic action should not be initiated on the basis of FISH analysis alone (40). Based on the very high accuracy and reliability of the test, the ACMG changed its policy recently, and stated in 2000 that clinical decision making should be based on information from two of three of the following: positive FISH results, confirmatory chromosome analysis, or consistent clinical information (52).

The false negative rate, calculated by Tepperberg et al., is also very low (51). Twenty-three (0.049%) of the 47,312 cases reported in population studies, were false negatives. Twelve cases of trisomy-21 (38,42,46,50,66), 5 cases of trisomy-13 (38,50,51,66), 4 cases of trisomy-18 (29,38,51,66), 1 case of monosomy-X (42), and 1 false negative of undetermined nature (68) were missed by interphase FISH analysis. Few other false negatives have been reported, but these cases were published as case-reports and are not included in the frequency calculations (65,86,88,89).

False negatives are somewhat less disturbing, since negative results do not lead to irreversible termination of unaffected pregnancy. The interphase FISH test has very effective back up, as the standard chromosome analysis will reveal all false negative results in 7–10 d. False negative FISH results can be, however, of clinical importance in some cases, especially when the indication for rapid FISH analysis was a time sensitive situation, and a decision of continuing an affected pregnancy may also be irreversible one. It is also important to emphasize that the test has an inherent clinical false negative rate of 2–3%, as the test obviously misses all those abnormalities it is not designed to detect.

Tepperberg et al. suggest that the use of the FDA reviewed probes (AneuVysion™ Assay) is safer regarding the false positive and negative rates of the test, compared to other non-FDA reviewed probes. The calculated false positive rates for the FDA reviewed probes and non-FDA reviewed probes are 0.003 and 0.044%, respectively, and the calculated false negative rates are 0.024 and 0.049%, respectively. However, the studies with the FDA-reviewed Vysis probes are all recently published (7,27–29,31,33,51,53,59,62–69), while most studies with other probes were published earlier (26,37,38,41–43,45–50,60,61). Other improvements rather than better probes, such as more experienced technicians and clinicians, validated protocols, and availability of reference ranges and databases may be responsible for the better performance of the test in recent years.

The causes of most false results were extensively studied. Most misdiagnoses were attributed to probably preventable causes such as maternal contamination, and non-specific or failure of probe hybridization. However, some of them are nonpreventable, which resulted from true genetic polymorphism, or abnormal structure of chromosomes (51,64,81,5–89).

## 2. Materials

### 2.1. Laboratory Equipment

1. Fluorescence microscope and recommended filters (*see Note 5*).
2. Phase contrast light microscope
3. Refrigerator and freezer (1–4 and –20°C).
4. Centrifuge.
5. Microcentrifuge.
6. Magnetic stirrer.
7. Slide warmer (45–50°C).
8. Waterbaths (37°C and  $73 \pm 1^\circ\text{C}$ ).
9. Air incubator (37°C).
10. Humidified hybridization chamber.
11. PH meter with glass electrode.
12. Vortex mixer.
13. Coplin jars (9) [Suggested type: Wheaton Product. No. 900620, vertical stair].
14. Thermometers (2) (*see Note 6*).
15. Timers.
16. Forceps.
17. Diamond tipped scribe.
18. Test tube racks.
19. Pipetors (1–20  $\mu\text{L}$ ).
20. Sterile tips.
21. Microscope glass slides.
22. 22  $\times$  22 mm Glass coverslips.
23. Disposable syringe (5 mL).

### 2.2. Reagents

#### 2.2.1. Reagents Provided with the AneuVysion™ Kit (*see Note 7*)

1. LSI®-13/21 DNA probe mix: a mixture of SpectrumGreen™ and SpectrumOrange™ directly labeled fluorescent DNA probes (*see Note 8*) that contain unique DNA sequences corresponding to the 13q14 region, and the D21S259, D21S341 and D21S342 loci located in the 21q22.13 to 21q22.2 region on chromosomes 13 and 21, respectively. The DNA-probes are pre-denatured and pre-mixed (10 ng/ $\mu\text{L}$ ) with blocking DNA and hybridization buffer [dextran sulfate, formamide (*see Note 9*), SS].
2. CEP®-18/X/Y DNA probe mix: a mixture of SpectrumAqua™, SpectrumGreen™, and SpectrumOrange™ directly labeled fluorescent DNA probes (*see Note 8*) specific for the alpha satellite DNA sequences at the D18Z1, DXZ1 and DYZ3 regions of chromosomes 18, X, and Y, respectively. The DNA probes are pre-denatured and premixed (15 ng/ $\mu\text{L}$ ) with blocking DNA and hybridization buffer [dextran sulfate, formamide (*see Note 9*), SSC].
3. DAPI II counterstain: 600  $\mu\text{L}$ /vial of 4,6-diamidino-2-phenylindole in phenylenediamine dihydrochloride, glycerol, and buffer (*see Note 10*).
4. NP-40: 4 mL of nonionic detergent.
5. 20X Saline-sodium citrate (SSC): 66 g of sodium chloride and sodium citrate mixture.

#### 2.2.2. Reagents Not Provided with the AneuVysion™ Kit

1. Water-purified (distilled, deionized or Milli-Q), store at room temperature.
2. Ethanol (100%), store at room temperature.

3. HCl (12 *N*).
4. NaOH (1 *N*).
5. Formamide, ultrapure grade. Store at 4°C for up to 1 mo from delivery.
6. Methanol, reagent grade, anhydrous.
7. Glacial acetic acid, reagent grade.
8. Sodium citrate.
9. KCl.
10. Trypsin/EDTA: 0.05% trypsin, 0.53 mM EDTA·4Na in Hanks' balanced salt solution without CaCl<sub>2</sub>, MgCl<sub>2</sub>·6H<sub>2</sub>O and MgSO<sub>4</sub>·7H<sub>2</sub>O.
11. Rubber cement.

### 2.2.3. Preparation of Working Solutions

1. Hypotonic solution 1 (0.8% sodium citrate): Dissolve 0.8 g of sodium citrate in 100 mL of purified water. Mix well and store at room temperature. Expires after 1 mo.
2. Hypotonic solution 2 (0.56% KCl): Dissolve 0.56 g of KCl in 100 mL of purified water. Mix well and store at room temperature. Expires after 1 mo.
3. Cell dissociation solution (60% acetic-acid): Mix thoroughly 1.5 mL glacial acetic-acid and 1 mL of purified water. Prepare fresh for each chorionic villi cell preparation.
4. Fixative (3:1 Methanol:acetic-acid): Mix 3 parts methanol and 1 part acetic acid. Prepare fresh fixative for each batch of slides. Keep the fixative capped in a glass bottle to minimize hydration.
5. 20X SSC (250 mL, pH 5.3): Mix thoroughly 66 g of the 20X SSC mixture, supplied with the AneuVysion™ kit, in 200 mL purified water. Adjust pH with a pH-meter to 5.3 at room temperature using HCl (12 *N*). Add purified water to bring final volume of 250 mL. Store up to 6 mo at room temperature.
6. 2X SSC (250 mL, pH 7.0 ± 0.2): Mix thoroughly 25 mL of 20X SSC (pH 5.3) with 200 mL of purified water. Adjust pH with a pH-meter to 7.0 ± 0.2 at room temperature using NaOH (1 *N*). Add purified water to bring final volume of 250 mL. Store up to 6 mo at room temperature.
7. Denaturing solution [70% formamide in 2X SSC (70 mL, pH 7.0–8.0)]: Add 49 mL formamide, 7 mL 20X SSC (pH 5.3), and 14 mL purified water to a Coplin jar and mix well. Verify that the pH is between 7.0–8.0 at room temperature using pH meter. Use each batch of denaturant for 7 d only, then discard. Store at 4°C between uses. Verify pH prior to each use.
8. Ethanol wash solutions (70, 85, and 100%): Prepare v/v dilutions of 70 and 85% ethanol using 100% ethanol and purified water. Store stock solutions for 6 mo at room temperature in tightly capped containers. Dilutions may be used for 1 wk unless evaporation occurs or the solution becomes diluted due to excessive use.
9. 0.3% NP-40 in 0.4X SSC wash solution (1000 mL, pH 7.0–7.5): Mix thoroughly 20 mL 20X SSC (pH 5.3) and 950 mL purified water. Add 3 mL of NP-40. Adjust pH to 7.0–7.5 with NaOH (1 *N*). Adjust volume to 1 L with purified water. Store stock solution in a covered container at room temperature for up to 6 mo. Discard solution that was used in the assay at the end of each day.
10. 0.1% NP-40 in 2X SSC wash solution (1000 mL, pH 7.0–7.5): Mix thoroughly 100 mL 20X SSC (pH 5.3), and 850 mL purified water. Add 1 mL of NP-40. Adjust pH to 7.0–7.5 with NaOH (1 *N*). Adjust volume to 1 L with purified water. Store unused solution in a covered container at room temperature for up to 6 mo. Discard solution that was used in the assay at the end of each day.

### 3. Methods

#### 3.1. Preparation of Uncultured Fetal Cells for FISH

##### 3.1.1. Preparation of Uncultured Amniocytes

1. Thaw 4 mL of trypsin-EDTA.
2. Centrifuge 3–5 mL of clear amniotic fluid at 400 g for 10 min (*see Note 11*).
3. Remove the supernatant.
4. Resuspend the pellet in 4 mL of trypsin-EDTA.
5. Vortex the suspension gently.
6. Incubate at 37°C for 20 min.
7. Centrifuge at 400g for 10 min.
8. Remove the supernatant
9. Resuspend the pellet by slowly adding prewarmed (37°C) hypotonic solution (0.8% sodium citrate or 0.56% KCl) up to a final volume of 10 mL.
10. Incubate at 37°C for 20 min.
11. Centrifuge at 400g for 10 min.
12. Remove the supernatant
13. Resuspend the pellet by adding 2 mL of fixative (3:1 mixture of methanol and glacial acetic acid) and gentle mixing.
14. Keep the suspension in refrigerator (2–8°C) for at least 1 h or until ready to prepare slides.
15. Adjust volume of cell suspension according to pellet size: Centrifuge at 400g for 10 min, remove the supernatant and resuspend the pellet in 0.8–2.0 mL of fixative (3:1 mixture of methanol and glacial acetic acid).
16. Mark 2 hybridization areas on the backside of 1 or 2 glass slides with a diamond tipped scribe (*see Note 12*).
17. Drop about 20  $\mu$ L of cell suspension on each of 2 separate hybridization areas (*see Notes 13 and 14*).
18. Continue directly to denaturation and hybridization (*see Note 15*) or keep the slides in a covered slide box at –20°C until ready to run the assay (*see Note 16*).

##### 3.1.2. Preparation of Uncultured Chorionic Villous Cells

1. Clean the chorionic villi thoroughly under the microscope (*see Note 17*).
2. Transfer 1–2 pieces of cleaned villi to a 35-mm Petri dish for FISH.
3. Pipet off the medium.
4. Add 2 mL of fixative (3:1 mixture of methanol and glacial acetic acid) drop by drop, on the villi.
5. Incubate at RT for 10 min.
6. Replace the fixative by 2 mL of fresh solution.
7. Incubate at RT for 10 min.
8. Remove the fixative.
9. Air dry the villi.
10. Add 0.5–0.8 mL of dissociation solution (60% acetic acid).
11. Incubate at RT for 5 min. You can facilitate dissociation of cells by gentle mixing using thin glass pipet. Slides can be prepared starting 2 min after the dissociation solution was added.
12. Mark 2 hybridization areas on the backside of 1 or 2 glass slides with diamond tipped scribe (*see Note 12*).

13. Drop about 20  $\mu\text{L}$  of cell suspension on each of 2 separate hybridization areas (*see* **Notes 13 and 14**).
14. Continue directly to denaturation and hybridization (*see* **Note 15**) or keep the slides in a covered slide box at  $-20^{\circ}\text{C}$  until ready to run the assay (*see* **Note 16**).

### **3.2. FISH Assay Procedure**

#### **3.2.1. General Preparations (see Note 18)**

1. Prewarm the humidified hybridization chamber by placing it in a  $37^{\circ}\text{C}$  incubator.
2. Prewarm one waterbath to  $37^{\circ}\text{C}$ .
3. Prewarm another waterbath to  $73 \pm 1^{\circ}\text{C}$ .
4. Prewarm the slide warmer to  $45\text{--}50^{\circ}\text{C}$ .

#### **3.2.2. Pretreatment of Slides (see Note 19)**

5. Add 40 mL of 2 X SSC to a Coplin jar. Prewarm the jar in a  $37^{\circ}\text{C}$  waterbath. Place the slides in the Coplin jar for 30 min at  $37^{\circ}\text{C}$ .
6. Remove the slides from the Coplin jar and dry them.
7. Prepare 3 Coplin jars with 70, 85, and 100% ethanol at room temperature.
8. Immerse the slides in the 70% ethanol jar for 2 min.
9. Remove the slides from the 70% ethanol jar and immediately immerse them in the 85% ethanol jar for 2 min.
10. Remove the slides from the 85% ethanol jar and immediately immerse them in the 100% ethanol jar for 2 min.
11. Remove the slides and air dry them.

#### **3.2.3. Denaturation of Sample DNA (see Note 20)**

1. Verify that the pH of the denaturing solution is 7.0–8.0.
2. Add denaturing solution to a Coplin jar and place it in a  $73 \pm 1^{\circ}\text{C}$  waterbath for at least 45 min prior to denaturation of slides. Verify the solution temperature inside the Coplin jar immediately before use (*see* **Note 21**).
3. Immerse the prepared slide(s) (no more than 3) in the denaturing solution at  $73 \pm 1^{\circ}\text{C}$  for 5 min. Do not agitate the jar.
4. Prepare 3 Coplin jars with 70, 85, and 100% ethanol wash solution at room temperature.
5. Remove the slides, using forceps, from the denaturing solution and immediately place them into the 70% ethanol. Agitate the slide to remove the formamide. Allow the slides to stand in the ethanol wash for 2 min.
6. Remove the slides from the 70% ethanol jar and immediately immerse them in the 85% ethanol jar for 2 min.
7. Repeat **step 6** with 100% ethanol.
8. Drain the excess ethanol from the slide by touching the bottom edge of the slide to a blotter and wiping the underside of the slide with a laboratory wipe.
9. Place the slides on a  $45\text{--}50^{\circ}\text{C}$  slide warmer no more than 2 min before you are ready to apply the probe solution (*see* **Note 22**).

#### **3.2.4. Probe Mixture Preparation**

1. Allow probes to thaw gradually to room temperature.
2. Vortex the probe tubes.
3. Spin the tubes briefly (3–5 s) in a microcentrifuge.

### 3.2.5. Hybridization

1. Apply 10  $\mu\text{L}$  of CEP-18/X/Y probe mix to one hybridization area. Immediately place a coverslip (22  $\times$  22 mm) over the probe solution. Allow the solution to spread evenly under the coverslip and avoid air bubbles.
2. Apply 10  $\mu\text{L}$  of LSI-13/21 probe mix to the other hybridization area. Place a coverslip over the probe solution as described in **step 1**.
3. Seal the coverslips with diluted rubber cement as follows: Draw the rubber cement into a 5 mL syringe. Eject a small amount of cement overlapping the coverslip and the slide, thereby forming a seal around the coverslip.
4. Place the slide(s) in the pre-warmed 37°C hybridization chamber and cover the chamber with a tight lid and incubate at 37°C for 6–24 h.

### 3.2.6. Post-Hybridization Washes (see **Note 20**)

1. Add 0.3% NP-40/0.4X SSC in a Coplin jar.
2. Prewarm the 0.3% NP-40/0.4X SSC solution by placing the Coplin jar in a 73  $\pm$  1°C waterbath for at least 30 min.
3. Add 0.1% NP-40/2X SSC to a second Coplin jar and place at room temperature.
4. Remove the slides from the hybridization chamber.
5. Remove the rubber cement seal and coverslips from one slide.
6. Immediately place the slide in the Coplin jar containing 0.3% NP-40/0.4X SSC at 73  $\pm$  1°C.
7. Agitate the slide for approx 3 s.
8. Repeat **steps 5–7** with the next slide.
9. Repeat **steps 5–7** with the third slide. Do not wash more than 3 slides in one batch.
10. Incubate for 2 min. Begin timing the 2 min incubation period when the last slide has been placed in the jar.
11. Remove each slide from the jar and immediately place it in the jar of 0.1% NP-40/2X SSC at room temperature for 5–60 s.
12. Allow the slides to air dry in the dark (closed drawer is sufficient).
13. Apply 10  $\mu\text{L}$  of DAPI-II counterstain to each target area of the slide and cover with a glass coverslip.
14. Store the slides in the dark until ready for signal detection (see **Note 23**).

## 3.3. Signal Detection

### 3.3.1. Assessing Slide Adequacy

1. Use  $\times 10$ ,  $\times 25$ , and  $\times 40$  objectives to evaluate the general appearance of the slide. Switch filters to evaluate the different signals according to the color detected (aqua, orange or green).
2. The background should be dark and almost free of fluorescence particles or haziness.
3. Scan the slide with the  $\times 25$  objective and select the areas most suitable for signal detection. The cells at the selected zone should be sparsely distributed. Several cells can be scanned within a viewing field, while only few cells are overlapping. Avoid areas with dense distribution of cells, many cells overlapping, or clumps of cells.

### 3.3.2. Signal Enumeration

1. Choose one filter, and begin analysis in the upper left quadrant of the selected area, using a  $\times 40$  objective. Scan from left to right, while skipping areas with a high cell density.
2. Count the number of signals of one specific probe within the nuclear boundary of each interphase cell. Probe signals should be bright, distinct and easily detectable. Signals are

bright compact or stringy diffuse oval shapes. Signals are sometimes split into two close round bright shapes. These split signals should be counted as one signal.

3. Continue the scanning until 50 nuclei are enumerated and analyzed for the specific target.
4. Repeat **steps 1–3** for each of the 5 probes. Use filters and scan the relevant hybridized areas accordingly.
5. Record the results on special record file.
6. Take sample pictures (computerized or slides) for each probe.
7. Report the results on specifically designed report page. Enumeration of 50 interphase nuclei per target are reported as the number and percentage of nuclei with 1, 2, 3, 4, and >4 signals for LSI-13, LSI-21, and CEP-18. The sex chromosomes complement should be reported as the number and percentage of nuclei with X, Y, XX, XY, XXY, XYY, XXX, and others.

### 3.4. Interpretation of Results

1. A target with less than 50 evaluated nuclei should be either supplemented with an additional slide, or considered uninformative.
2. Diagnose euploidy if at least 85% of the cells are euploids.
3. Diagnose aneuploidy if at least 85% of the cells have the same aneuploid signals.
4. If more than 10% of the cells have the same aneuploid signals, the test should be extended and 100–200 cells should be analyzed. The final diagnosis by FISH is determined by the signals detected in the majority of cells. Mosaicism is suspected in these cases, and the standard cytogenetic analysis should be extended as well.

## 4. Notes

1. Our protocols were established based mainly on the published experience of others, combined with our own extensive 10-yr experience. The presented denaturation, hybridization, and washing protocols are based on the commercially available AneuVysion™ assay kit, and generally follow the protocols recommended by Vysis.
2. Rapid FISH analysis on uncultured fetal cells for prenatal diagnosis of the most common aneuploidies is very useful tool if used in the appropriate clinical settings. Clinicians however, should be educated and patients should be carefully counseled about the advantages and limitations of routine prenatal cytogenetic analysis by FISH. It should be emphasized that the test does not detect structural chromosomal aberrations, mosaicism, or numerical abnormalities of chromosomes other than 13, 18, 21, X, and Y. These limitations should be also detailed in the final report of FISH results.
3. The assay requires good laboratory practice and experience, and has its mandatory learning curve. Prior to adoption of the technique as a clinical tool, it is advisable to run the assay in the cytogenetic laboratory as a pilot study for at least 50–100 cases. During that study the FISH results are compared to the standard cytogenetic analysis for internal control and learning purposes only, and are not reported to clinicians or patients.
4. ProbeChek™ positive and negative control slides are included in the AneuVysion™ kit. Normal male and mosaic amniocytes serve as negative and positive controls, respectively. The control slides can be used for quality control and in training technologists to interpret FISH on interphase cells.
5. A presumed failure of reagents in an *in situ* assay may actually indicate that a malfunctioning or sub-optimal fluorescence microscope or incorrect filter set is being used to view a successful hybridization assay. Routine microscope cleaning and periodic “check-ups” by technical representative are good practice. The excitation light source should be

properly aligned. Its life time should be recorded and kept in the range of about 200 h. Objectives should be used with or without immersion oil according to the magnification. The immersion oil used should be one formulated for low auto fluorescence and specifically designed for use in fluorescence microscopy. It is advised to use the multi-bandpass and single bandpass fluorescence microscope filter sets optimized for use with the AneuVysion™ kit. Filters are available from Vysis for most microscope models. CEP-18/X/Y and LSI-13/21, aqua, green and orange labeled chromosomes can be viewed with single bandpass Aqua, Green and Orange, or with triple bandpass DAPI/Green/Orange filter sets. The SpectrumAqua signals are aqua-blue, the SpectrumOrange signals are pinkish orange, and the SpectrumGreen signals are greenish-yellow. All other DNA will be blue with the DAPI stain.

6. The use of calibrated thermometers is strongly recommended for measuring temperatures of solutions, waterbaths, and incubators as these temperatures are critical for optimum product performance.
7. Store an unopened AneuVysion™ kit as a unit at  $-20^{\circ}\text{C}$  protected from light and humidity. The different components of an opened kit should be stored as follows: The DNA probe mixtures, DAPI-II counterstain, and the control slides at  $-20^{\circ}\text{C}$  protected from light and humidity; The 20X SSC salts and NP-40 detergent may be stored at room temperature. Expiration dates for each of the unopened kit components are indicated on the individual component labels. These storage conditions apply to both opened and unopened components.
8. Fluorophores are readily bleached by exposure to light. To limit their degradation, handle all solutions containing fluorophores in reduced light. This includes all steps involved in handling the hybridized slides. Carry out all steps, which do not require light for manipulation (incubation periods, washes, etc.) in subdued lighting to avoid direct light projecting onto the fluorophore.
9. Formamide is a known teratogen. Formamide is used for preparation of the denaturing solution and also contained in the DNA probe mixtures. Avoid contact with skin and mucous membranes.
10. The DAPI-II Counterstain contains DAPI (4,6-diamidino-2-phenylindole) and free base 1,4 phenylenediamine. DAPI is a possible mutagen based on positive genotoxic effects. Avoid inhalation, ingestion, or contact with skin. 1,4 phenylenediamine is a known dermal sensitizer and a possible respiratory sensitizer. Avoid inhalation, ingestion, or contact with skin.
11. Accept only clear, yellow amniotic fluid for FISH. Bloody or brownish fluid, or pellet contaminated with numerous red blood cells may interfere with the results and lead to false interpretation.
12. The slides should be clean and cold. Keep the slides in refrigerator and wash them with cold fixative immediately before dropping cells. Not properly cleaned slides may cause high background and hamper reading.
13. Technicians in most cytogenetic laboratories are very experienced with dropping cells on slides. However, dropping slides for interphase FISH analysis is an important step for good quality test. Sparsely distributed cells limit the ability of the examiner to choose the best cells for counting signals. Hybridization areas crowded with too many cells lead to substantial difficulties in relating signals to cells. Properly dropped slides will ensure easy and straightforward reading. Adjusting the volume of cell suspension according to pellet size before dropping the slides, and getting experience by “try and error”, is the best way to ensure good quality slides.

14. Since the AneuVysion™ kit is made of two sets of probes (LSI-13/21 and CEP-18/X/Y), the minimal requirements for each case are two separate hybridization areas, one for each set. It is not too technically demanding to prepare slides with 2 hybridization areas each, however one may find designing one area per slide much simpler. It is good practice to prepare at least one backup slide for each tested slide. The backup slides should be stored without hybridization at  $-20^{\circ}\text{C}$ , in a covered slide box.
15. Slide quality is one of the most important factors affecting the degree of hybridization. It is advised to examine the prepared slides under phase contrast light microscope prior to denaturation and hybridization. Evaluate the density of cells on the slide and the presence or absence of cytoplasm around the interphase nuclei.
16. Long term storage of specimens can be done at a few stages of preparation and processing. Fixed amniocytes or chorionic villi cells can be stored before dropping on slides at  $-20^{\circ}\text{C}$  in fixative. Prepared slides can also be stored before hybridization in a covered slide box. The box should be sealed in a plastic bag and stored at  $-20^{\circ}\text{C}$ .
17. Choose only very clean pieces of chorionic villi. A specimen contaminated with maternal blood or decidua may interfere with the results and lead to false interpretation. The appearance of decidua is usually white balls of tissue with no budding or blood vessels. They can have a spongy or “mushy” appearance. Good villi have white branches with buds and have blood vessels coursing through at least some branches.
18. HYBrite™ denaturation/hybridization system is a keypad-operated instrument manufactured by Vysis. It performs denaturation and hybridization for FISH assays on up to 12 slides simultaneously. The instrument eliminates the need for denaturation reagents, waterbath and incubator for FISH assays, and saves about 45 min of hands-on time required for the test. HYBrite™ is user friendly and safer for the technician. However, we feel that the bench procedure is more accurate and gives better results in tough cases.
19. Pretreatment of slides is optional. One may use pretreatment only for amniotic fluids of relatively late gestational age (>18 weeks). We use the method described above for all slides of all specimens prepared for interphase FISH analysis.
20. Do not denature or wash more than 3 slides at one time per Coplin jar.
21. Stringency of temperature and buffer concentration is important for denaturation. The temperature of the denaturation solution must be  $73 \pm 1^{\circ}\text{C}$ , and should be verified immediately before each use. Verify that the pH of the denaturation solution is between 7.0–8.0 before each use, as well.
22. Timing of the last ethanol wash, removing the slide to the slide-warmer, and applying the probe mix is very important for successful hybridization. If the timing of the hybridization is such that the slide is ready more than 2 min before the probe is ready, the slide should remain in the jar of 100% ethanol.
23. Hybridized slides can be stored with coverslips at  $-20^{\circ}\text{C}$ , in the dark. Under these conditions, the slides can be stored for up to 12 mo without significant loss in fluorescence signal intensity.

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## Prenatal Diagnosis of Chromosomal Abnormalities Using Maternal Blood

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### 1. Introduction

Fetal chromosomal aneuploidies can be detected by fluorescent *in situ* hybridization (FISH) analysis of intact fetal cells circulating in maternal blood. Given the low number of circulating fetal cells (1 in  $10^4$  to  $10^6$  maternal cells) at any given time during pregnancy and given that no fetal-specific markers can unambiguously distinguish maternal from fetal cells, success in fetal cell analysis relies on hybridization and detection efficiency of probes in cells recovered following some formal enrichment. Consequently, many different protocols have been developed using one or more techniques to enrich and improve purity of fetal cells for subsequent FISH. FISH analysis is complicated by enrichment not being 100 percent efficient, yielding heterogeneous mixtures of cell types that require different FISH conditions for optimal signal detection. In addition, certain methods used during enrichment, namely fixation and storage of cells, can adversely affect the quality of cells recovered for FISH analysis. The focus of this chapter will be to highlight variables that impact FISH analysis of fetal cells recovered from maternal blood. Detailed description of the methods developed at Baylor College of Medicine is provided. Recovery of fetal cells for DNA analysis using PCR techniques will not be reviewed; however, several recent reviews are recommended (1–3).

#### 1.1. Application of FISH on Fetal Cells in Maternal Blood

In the United States, offering prenatal cytogenetic diagnosis is considered “standard of care” of pregnant women 35 yr or older at the time of delivery. Fetal cells are thus obtained invasively using techniques such as chorionic villus sampling or amniocentesis. The threshold for these procedures has long been 35 yr of age, a largely arbitrary figure taking into account the relative risk of the most common autosomal aneuploidy (trisomy 21) and the likelihood of a miscarriage secondary to the procedure.

From: *Methods in Molecular Biology, Vol. 204: Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

Despite the accuracy and safety of chorionic villus sampling and amniocentesis, there has been relatively little impact on the incidence of trisomy 21 because 80% of newborn infants with Down syndrome are born to women below the age of 35 (4,5). Moreover, only about half of U.S. gravidas over age 35 undergo an invasive prenatal diagnostic procedure. For this reason, women under age 35 yr must be offered maternal serum analyte screening, which may or may not lead to invasive procedure. This noninvasive method detects about 65% in the overall general population, but fewer in younger women (6–8). Application of FISH to detect fetal chromosomal abnormalities (mainly aneuploidy) using maternal blood offers an alternative noninvasive means of testing all pregnancies with the potential for a lower false-positive detection than the 5% accepted for maternal serum screening (9–12). Unlike other screening methods, fetal cells from maternal blood can also provide direct evaluation of fetal cells from an ongoing pregnancy, rendering genetic counseling less ambiguous for most couples.

### 1.1.1. Aneuploidy Detection

Diagnosis of fetal aneuploidy, primarily trisomy 13, 18, and 21, has been accomplished by FISH (13–17). Early studies employed sequential or multi-color FISH, enabling detection of up to three chromosomes simultaneously (X, Y, and usually either 21 or 18). However, effective prenatal testing in the general population will require concurrent assessment of all the common aneuploidies: which involve chromosomes X, Y, 13, 18, and 21 (18). Thus, we have developed a five-color FISH protocol that enables simultaneous analysis of all five-chromosomes following a single hybridization reaction (19). Based on analysis of more than 300 maternal specimens, our FISH strategy yields >90% hybridization efficiency of all five probes with a very low false-positive detection rate of XY (4%) and autosomal aneuploid (<1.5% for each) cells (11,20). Among cases in which the fetus was subsequently confirmed to be trisomy 13, 18, or 21, we diagnosed 33% (1 in 3 cases), 55% (5 of 9 cases), and 37% (6 of 16 cases), respectively; the absolute number of aneuploid cells ranged from 1–2 (most cases) to 32. Our low false-positive rate largely reflects the use of direct labeled probes and the relatively stringent conditions employed during enrichment. Current clinical trials are in progress to further assess the feasibility and reliability of this attractive approach.

### 1.1.2. Mosaicism

Low grade fetal cell mosaicism has been observed of fetal cells with different chromosomal constitutions in a given specimen that are unlikely to be maternal. In a case we reported, XY [2] and XXY [7] cells were detected among 2000 flow sorted XX-maternal cells (21). Although cytogenetic analysis of cultured chorionic villus cells indicated a normal XY-male karyotype, more extensive FISH analysis of 250 cultured cells demonstrated low grade mosaicism of XXY cells (4 cells) on one coverslip, but not on a second. Therefore, detection of abnormal fetal cells in some maternal cases may reflect biological mechanisms involved in selecting against abnormal cell lines, presumably the phenomenon observed with confined placental mosaicism (22).

### 1.1.3. Balanced Translocation

FISH has also been used to exclude fetal aneuploidy in a case in which the mother has a balanced translocation (46,XX,t(1;6)(p31;q14) (23). Using FACS to select for gamma-globin or zeta-globin positive cells, recovered nuclei were analyzed for gender and number of chromosomes 1 and 6. Based on the finding of only two signals for each X, 1 and 6, fetal aneuploidy was excluded but not confirmed until birth. Analysis of fetal cells in maternal blood by FISH may serve as an alternative means of prenatal diagnosis in couples who have a history of multiple miscarriages and decline prenatal testing.

## 1.2. Gestational Time

In nested PCR studies, fetal Y-specific DNA is consistently detected by a six week gestation (4 weeks embryogenesis) (24). This observation may or may not indicate presence of intact fetal cells, in light of evidence reported by Lo and colleagues that cell free fetal DNA is present in maternal blood (25). However, PCR detection of Y-specific sequences is also consistently observed at 10–15 wk gestation (26,27). By this stage of gestation, intact cells are present and detectable by FISH (28,29). A variety of different enrichment methods have been used to recover and confirm intact fetal cells by FISH analysis.

## 1.3. Fetal Cell Types

Several different fetal cell types exist in maternal blood: trophoblasts, granulocytes, lymphocytes, and erythroblasts (30–36). Trophoblasts may be multinucleated or anucleated, which would obviously interfere with aneuploidy detection by interphase FISH (37). A problem with lymphocytes is that cells could persist from prior pregnancies and lead to diagnostic error (38,39). This especially becomes relevant for women with prior history of chromosomally abnormal spontaneous abortions. Fetal nucleated red blood cells (nRBCs or erythroblasts) are thus considered the most attractive candidate cell type, given that these cells have unique morphologic features, comprise about 10% of red cells in the 8-wk fetus, and are rare in peripheral adult blood. Use of erythroid specific markers can enhance the detection of nRBCs (40). No fetal specific markers exist, as evident by PCR studies showing that only as many as 50% of nucleated RBCs recovered from maternal blood are fetal in origin (41). Additionally, fetal nucleated RBCs are unlikely to persist from a prior pregnancy, since these cells lose their nucleus after 5–7 d. Therefore, we and many other investigators have focused on developing methods to recover fetal erythroblasts for FISH testing.

## 1.4. Frequency of Fetal Cells

### 1.4.1. During Pregnancy

The ratio of fetal to maternal nucleated cells has been reported to be  $1:4.75 \times 10^6$  to  $1.6 \times 10^7$  based on cell staining (42). Using FISH on whole blood smears, frequencies of fetal cells (all types) in maternal blood in the first, second and third trimesters were 0.27, 3.52, and  $8.56 \times 10^6$ , respectively (27). Similar estimates were made by Bianchi et al. (43) who used quantitative PCR to estimate that 1–2 fetal cells are present per cc of maternal blood. These authors further demonstrated that a significantly greater num-

ber of fetal cells were detected when the fetus was aneuploid, as we have observed as well (44). Recovering increased numbers of fetal cells in pre-eclampsia and pregnancies in which fetal growth is severely restricted has been observed (45–47). Nonetheless, the relatively few fetal cells mean that some form of enrichment is necessary to increase the purity of fetal cells, thus reducing the number of adventitious maternal cells that complicate fetal FISH analysis. Purity need not reach 100% for accurate and practical diagnosis.

#### *1.4.2. Persistence in Non-Pregnant Women*

Several studies have reported persistence of lymphoid fetal cells in women postpartum and up to 27 yr after pregnancy and may play a role in some autoimmune diseases (39,48–50). Although fetal cells may persist from prior pregnancies, with appropriate techniques, this potential source of error can be minimized. In particular, we have used the CD71 transferrin receptor or gamma-globin of fetal hemoglobin to select for fetal cells by FACS in 33 maternal cases confirmed to have a female fetus at the time of testing and have had prior male pregnancies (14 mo–19 yr) (51). Using FISH to detect X and Y, we found no male cells in 32 of 33 cases. In one case, five XY-cells were scored. These results suggest that fetal NRBCs from prior pregnancies are rare and unlikely to be recovered in most future pregnancies using the selection criteria outlined.

#### *1.4.3. Sampling Prior to Invasive Procedure*

The actual number of fetal cells circulating in maternal blood may plausibly be increased following an invasive procedure e.g., chorionic villus sampling (52). The rationale is that invasive procedures result in transfer of blood between the fetus and mother. Therefore, in evaluating fetal cell detection frequencies, maternal blood must be drawn prior to the procedure or at least two weeks after.

### **1.5. Strategies Involved in Enrichment of Fetal Cells that Influence FISH**

The low frequency of fetal cells dictates that enrichment is often necessary for FISH analysis. For most strategies, enrichment is accomplished sequentially. There are many steps at which cells are subject to conditions that affect subsequent FISH.

#### *1.5.1. Initial Separation*

A preliminary separation like Ficol-Paque or Percoll-gradient density centrifugation is typically employed to remove mature RBCs and granulocytes, leaving peripheral mononuclear cells. Different gradient densities are likely to influence the type, number and quality of cells recovered (53–55). Indeed, we have observed that increasing Ficol density (from 1.070 to 1.090 to 1.119 g/mL) typically results in a higher proportion of target cells but with increased clumping.

#### *1.5.2. Enrichment*

Further enrichment is usually achieved using magnetic activated cell sorting (MACS) (56,57), ferrofluid suspension with magnet (58), charge flow separation (59) or fluorescence-activated cell sorting (FACS) (60–63). The general principle is to

employ negative and/or positive selection criteria to remove unwanted maternal cells and retrieval of desired fetal cells using monoclonal antibodies (mAb) that recognize cell surface antigens and/or intracellular proteins. Although all of the methods listed above have been used successfully to isolate fetal cells, none result in 100% enrichment.

### 1.5.3. Antibody Selection

Our group and many other investigators have selected the transferrin receptor, CD71, and/or the gamma globin protein chain of HbF for positive selection of candidate cells (63–66). Many other antibodies have been suggested, but few if any have proved consistently suitable for enrichment and subsequent FISH (67–70). Initially, we had salutary results with GPA, but later we reported the clumping effects of several different batches of antibodies against this commonly used erythroid marker, glycophorin A (62,71) (see Note 4).

### 1.5.4. Fixation and Storage of Cells Prior to Sorting

Depending on the strategy and methods used, cells can unavoidably be subjected to many washing steps, harsh fixations, centrifugations, and storage conditions that can negatively affect quality for subsequent FISH. Prior to sorting by FACS, antibody-fluorescence is preserved by fixing cells in paraformaldehyde and storing at 4°C for batch processing. If the concentration of paraformaldehyde exceeds 3%, Ab-staining and nuclei are well preserved; however, recovered sorted cells will likely be impermeable to FISH probes. Lower concentrations (1–2%) of paraformaldehyde make nuclei more permeable; however, storage time at 4°C is limited for cell preservation given the relatively mild fixation (72). These observations reflect the dilemma of cytogenetists in balancing fixation, centrifugation and storage of cells prior to slide preparation. Our group has concluded that 3% paraformaldehyde fixation for 1 h is optimal for sorting and FISH (72). We further concluded that FISH quality deteriorates when cells are stored for more than 10 d prior to sorting. Other enrichment methods, such as MACS, appear not to require so stringent labeling and fixation conditions for selection of cells. However, under these more mild conditions, the purity of the cell population will be adversely affected such that many more maternal cells will be recovered and processed for FISH.

### 1.5.5. Unique Features of Enriched Cells Affecting FISH

FISH is a sensitive technique that enables detection and analysis of rare cells, potentially allowing direct independent analysis of every fetal cell present. As stated, enrichment is unlikely to reach 100%, and a contaminating admixture of maternal mature red cells, lymphocytes, and granulocytes is inevitably present. Automated slide scanning has been proposed for localization of the few nucleated RBCs, but no apparatus is available commercially (73–75). Until a fetal specific marker is found, analyzing all or most of nuclei after enrichment is thus necessary. Developing a FISH method specific for enriched cells is complicated, however, by the majority of cells recovered not being of the target cell type. Moreover, cytogeneticists know that different cells types often require different FISH conditions for optimal and consistent probe hybridization and signal quality. Unlike multicolor FISH analysis of uncultured amniotic fluid cells, in which cells are more homogenous, FISH analysis using a combination of locus-

specific and repeat-sequence probes will be compromised when multiple cell types are present. In performing five-color FISH to detect X, Y, 13, 18, and 21 on FACS enriched cells, we observed decreased hybridization efficiency of the locus-specific probes (75–85%) as compared to the alpha satellite repeat probes (>95%, [19]). Although reduced hybridization efficiency theoretically might be influenced by probe (signal) size, we did not observe this among the control cells scored. Given that different cell types cannot be avoided after enrichment, it is important to use FISH methods that permit analysis of all cells, despite differences in signal quality (intensity and size). This will allow interpretation of each cell.

### **1.6. Strategies to Distinguish Fetal from Maternal Cells**

Accurate genetic diagnosis of rare fetal cells that exist in maternal blood depends on the ability of the observer to identify fetal cells in a background of maternal cells. Attempting to further reduce the number of nuclei to be analyzed by FISH, immunohistochemical staining techniques have been described for distinguishing fetal erythroblasts from maternal erythroblasts (76–83). Zheng et al. (76) described simultaneous immunohistochemical staining using a mouse anti-fetal hemoglobin antibody and FISH using a chromosome Y-probe. However, a fraction of maternal cells isolated also stained positive for HbF. Given that as many as 50% of circulating nucleated RBCs are fetal in origin and desirable for FISH (41), automated methods to scan and locate candidate cells based on morphology and staining for subsequent FISH would be attractive (75). Unfortunately, detection of the globin chains and/or morphology requires staining solutions that often contain preservatives capable of interfering with FISH.

## **2. Materials**

### **2.1. Flow Sorting**

#### **2.1.1. General Reagents**

1. IsolympH ficoll gradient (density 1.077) (Gallard Schlesinger, Carle Place, NY, cat. no. T1000).
2. 10X Phosphate buffered saline (PBS) solution: 137 mM NaCl, 27 mM KCl, 43 mM  $\text{KH}_2\text{PO}_4$ , 14 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  dissolved in deionized water, sterilize through 0.22  $\mu\text{m}$  filter, and store at room temperature for up to 1 mo.
3. 1X PBS solution: Dilute 10X PBS in deionized water, sterilize through 0.22  $\mu\text{m}$  filter, and store at 4°C. Solution must be warmed to room temperature prior to use.
4. PBS-EDTA (PBSE): Dissolve 0.0372 g of EDTA (reagent grade) in 100 mL of 1X PBS, sterilize through 0.22  $\mu\text{m}$  filter, and store at 4°C. Solution must be warmed to room temperature prior to use.
5. Trypan Blue (0.4%) solution: Dissolve 0.4 g of Trypan Blue (Sigma, St. Louis, MO, cat. no. T0776) in 100 mL of deionized water, sterilize through 0.22  $\mu\text{m}$  filter, and store at room temperature.

#### **2.1.2. Depletion of CD3<sup>+</sup> and CD13<sup>+</sup> Maternal Cells**

1. CD3 and CD13 antibodies both labeled with biotin (Biosdesign International, Kennebunk, ME, cat. no. P01106B and P01150B, respectively).
2. Quantum/Streptavidin magnetic particles (Clemente Associates, Madison, CT, cat. no. SAB250). Store at 4°C.

3. Rare earth magnet (Clemente Associates, Madison, CT).
4. PBSE/2% FBS solution: Add 10 mL of heat inactivated FBS to 490 mL of PBSE, sterilize through 0.22  $\mu\text{m}$  filter, and store at 4°C for up to 1 mo. Solution must be warmed to room temperature prior to use.
5. PBSE/5% FBS solution: Add 25 mL of heat inactivated fetal bovine serum (FBS) to 475 mL of PBSE, sterilize through 0.22  $\mu\text{m}$  filter, and store at 4°C for up to 1 mo. Solution must be warmed to room temperature prior to use.
6. Prepare Quantum magnetic particles by calculating the particle amount needed for each sample, a final concentration of 5  $\mu\text{g}$  of particles for  $10^6$  cells is needed. Add the desired amount of particles (should be in the range of 300–400  $\mu\text{L}$ ) to a 15 mL polypropylene conical tube. Dilute 1:100 with PBSE/5% FBS and mix well. Separate particles with magnet and carefully transfer particles using a pipet to a new 15-mL tube. Discard supernatant.

### 2.1.3. Cell Surface Staining for CD71 and CD45

1. Fluorescein isothiocyanate (FITC)-labeled CD71 mAb (Becton Dickinson, San Jose, CA, cat. no. 347513); phycoerythrin (PE)-labeled CD45 mAb (KC56-RD1) (Coulter Corp., Hialeah, FL, cat. no. 6603839) dilute to 0.1 mg/mL using PBSE/2% FBS and store at 4°C for up to 1 mo.
2. Paraformaldehyde (4%) solution: Add 4.0 g of paraformaldehyde (reagent grade) to 90 mL deionized water. To dissolve, adjust to pH 10.0. Once in solution, lower to pH 7.0. Add 10 mL of 10X PBS (for a final concentration of 4.0 g in 100 mL of 1X PBS), sterilize through 0.22  $\mu\text{m}$  filter and store at 4°C.
3. Prepare 3% paraformaldehyde by diluting 4% paraformaldehyde 3:1 with 1X PBS and store at 4°C.

### 2.1.4. Flow Cytometry for Isolation of CD71<sup>+</sup> and CD45<sup>-</sup> Cells

1. EPICS Model 753 (Coulter Corp.) - equipped with the Cicero High-Speed Sort System (Cytomation, Inc., Ft. Collins, CO).
2. An argon ion laser (Coherent, Inc., Palo Alto, CA).
3. 3X Quartz SortSense flow cell tip with 100  $\mu\text{m}$  orifice diameter and Profile pickup lens (Coulter Corp.).
4. Optical filters include a 457–502-nm longpass laser blocking filter, a 550-nm longpass dichroic filter to transmit the FITC emission to a 525-nm bandpass and bend the PE emission to a 575-nm bandpass filter.
5. Isoton II sheath solution (Coulter Corp.).
6. Autoclave sterilized microcentrifuge tubes and clean silylated (positive charge) glass slides.

## 2.2. FISH

### 2.2.1 Cellular Fixation, DNA Dehydration and Denaturation

1. Carnoy's fixative: Standard (v/v) 3 parts methanol : 1 part acetic acid (100 mL) prepared fresh (within 30 min of use).
2. Ethanol solutions (70, 90, and 100%): Using 100% absolute ethanol, prepare 70 and 90% ethanol (v/v) with deionized water. Prepare two bottles of each (500 mL). Store one at room temperature and the other at -20°C.
3. 20X SSC solution: Premeasured powder (Oncor, Gaithersburg, MD, cat. no. S4073) dissolved in 1 L of deionized water.
4. 70% formamide/2X SSC denaturation solution: Prepare fresh prior to use in a 50 mL glass Coplin jar with lid by combining 28 mL formamide [Fisher Scientific (ultrapure

molecular biology grade) (Pittsburg, PA, cat. no. F84-1), 4 mL 20X SSC and 8 mL deionized water. To prevent cracking of glass Coplin jar, gradually prewarm this solution in a 43°C waterbath then transfer to a 70°C waterbath.

### 2.2.2 Five-Color FISH

1. Purchase direct fluorophore labeled chromosome specific probes. In comparing several manufacturer FISH probe products, we have found the specificity and quality of direct-labeled probes (Vysis, Inc.; Downers Grove, IL) to be best for our application. These probes are of suitable size, which generate bright fluorescence signals that are visually distinct, enabling simultaneous analysis of chromosomes X, Y, 13, 18, and 21. Three alpha-satellite centromere-specific (CEP) and two locus-specific (LSI) probes are combined: CEP X SpectrumGreen™, CEP X SpectrumOrange™, CEP Y SpectrumAqua™, CEP 18 SpectrumAqua™, CEP 18 SpectrumOrange™, LSI 13 SpectrumGreen™, and LSI 21 SpectrumOrange™ (Vysis, Inc.). Probes must be protected from light and stored frozen at -20°C. Aliquoting is not necessary.
2. In optimizing our five-color FISH strategy, chromosomes X, Y, 13, 18, and 21 are efficiently detected as yellow, blue, green, pink, and red/orange fluorescent colors, respectively. Therefore, prior to preparing the five probe cocktail, combinatorial mixing of the CEP X and CEP 18 probes is necessary for generating two of the five fluorescent colors, yellow and pink, respectively. This is done by premixing equal amounts (premix only 5 µL of each probe at a time) of CEP X SpectrumGreen™ and CEP X SpectrumOrange™ to create a yellow fluorescence signal. Similarly, premix equal amounts of CEP 18 SpectrumAqua™ and CEP 18 SpectrumOrange™ to create a pink fluorescence signal. Store premixed probes at -20°C.
3. For a hybridization area of 22 × 22 mm per slide, prepare a 10 µL five probe cocktail in a sterile microcentrifuge tube by combining 0.5 µL of each LSI 13 and 21 probes, 0.3 µL of each CEP X (premixed), CEP 18 (premixed) and CEP Y probes, 1.0 µL deionized water, and 7.1 µL LSI™ probe buffer (provided with purchase of LSI probes).
4. Prior to performing FISH, prepare a 37°C humidity chamber by placing an empty plastic pipet tip rack in the center of a small plastic container with a tight fitting lid. Fill approx 10% of volume with water and store in 37°C incubator.
5. 2X SSC solution: Dilute 20X SSC in deionized water, adjust to pH 7.0. Store at room temperature for up to 6 mo.
6. 1X PBD solution: Dilute 10X PBD™ (Oncor, Gaithersburg, MD, cat. no. S1370-7) with deionized water. Store at 4°C for up to 6 mo.
7. DAPI (4',6-Diamidino-2-phenylindole) II counterstain in antifade solution (125 ng DAPI/mL) (Vysis, Inc., Downers Grove, IL, cat. no. 32-804831).
8. Slide boxes for storage of processed slides at 4°C.

### 2.2.3. Microscope Analysis

1. Axiophot or Axioskop fluorescent microscope equipped with camera and fluorescence objectives of ×40 and ×100 magnification (Carl Zeiss, Inc., Thornwood, NY).
2. Appropriate DAPI single bandpass filter [Omega Optical, Brattleboro, VT) and Aqua/Green/Orange triple bandpass filter set (Vysis Inc., Downers Grove, IL).
3. High pressure light source, 100 W Hg lamp (Carl Zeiss, Inc., Thornwood, NY).

## 3. Methods

### 3.1. Blood Separation

1. Collect approx 30 mL of venous blood in sodium heparin vacutainers (*see Note 2*).

2. Transfer 15 mL of blood to a 50-mL polypropylene conical tube and add 15 mL of PBSE (final ratio is 1:1 blood:PBSE). A 30 mL blood sample is divided into two tubes.
3. Underlay each mixture of blood:PBSE with 10–15 mL Isolymp using a sterile syringe and Pasteur pipet. Tightly cap the tubes and centrifuge for 25 min at 1600-rpm (200g) with the brake off.
4. Remove tubes carefully. Using a vacuum and Pasteur pipet, carefully remove the top layer (plasma and platelets) and discard. Leave behind approx 1 cm of this layer.
5. With a 10-mL pipet, combine the mononuclear cell layer from the two tubes in a 50-mL polypropylene tube. Centrifuge for 10 min at 1600-rpm (200g) with the brake on.
6. Pour off the supernatant and resuspend the pellet in 50 mL of PBSE. Centrifuge for 5 min at 1200-rpm (100g). Pour off the supernatant and resuspend pellet in PBSE in a volume equal to the initial blood vol (30 mL) (*see Note 3*). Vortex for an even suspension of cells.
7. Transfer 50  $\mu$ L of each cell suspension to a clean 5-mL polypropylene conical tube and add 50  $\mu$ L of 0.2% Trypan blue. Place cells on hemacytometer for cell count. Resuspend cells at a concentration of  $20 \times 10^6$  cells/mL in PBSE/2% FBS.

### 3.2. Quantum Magnetic Particle Depletion

1. Stain cells by mixing 1  $\mu$ g of each CD3-biotin and CD13-biotin with  $10^6$  cells. Incubate on ice for 20 min.
2. Wash cell suspension twice with PBSE. Resuspend the cells in a 15 mL polypropylene tube at  $10 \times 10^6$  cells/mL in PBSE/5% FBS.
3. Add appropriate amount of washed avidin magnetic particles (amount calculated in **Subheading 2.1.2.**). Incubate on ice for 15–20 min, swirling at 5 min intervals to ensure uniform suspension of particles and cells.
4. Place tube against magnet for 5 min. Carefully remove unbound cells with an automatic pipetter and dispense into a clean 15-mL tube. Repeat the magnetic separation with this second tube to ensure the removal of all magnetic particles. Dispense unbound cells into a third clean 15-mL tube.
5. Centrifuge the unbound cells and resuspend in 10–20 mL of PBSE for a postdepletion hemacytometer cell count as described in **Subheading 3.1., step 7**. Resuspend cells at a concentration of  $20 \times 10^6$  cells/mL in PBSE/2% FBS.

### 3.3. Cell Surface Staining

1. Add 1  $\mu$ g of CD71-FITC (half the amount suggested by manufacturer) and 10  $\mu$ L of diluted CD45-PE (*see Subheading 2.1.3.*) per  $1 \times 10^6$  cells. Incubate on ice for 20 min.
2. Wash cells with PBSE/2% FBS and resuspend in 3% paraformaldehyde at a concentration of  $10 \times 10^6$  cells/mL. Incubate on ice for 60 min.
3. Wash cells twice with PBSE/2% FBS and resuspend in PBSE/2% FBS at a concentration of  $10 \times 10^6$  cells/mL.
4. Store tubes at 4°C, keep dark until ready for sort.

### 3.4. Flow Sorting

1. Tune argon laser to 488-nm and operate at 50 mW.
2. Color compensation is 25–30% of FITC signal from the PE signal, 5% of PE signal from the FITC, and is monitored by using samples stained with the appropriate single- and dual- color fluorochromes.
3. Crystal drive frequency is increased to 30 kHz to create more and smaller droplets. Sheath pressure is at 12 psi.
4. Sorting is performed by using two-droplet deflection with the anticoincidence engaged.

Samples are suspended in a volume ranging from 0.2–2.0 mL of PBSE/2% FBS to create a flow rate of 1000–6000 cells/s, depending on the cell concentration after separation and depletion.

5. Forward-angle light scatter is collected as a linear signal, and all fluorescence emissions are collected on a four-decade logarithmic scale. A light scatter gate is set to eliminate debris and aggregates. Target cells are CD45<sup>-</sup>,CD71<sup>+</sup>.

### 3.5. Cell Collection

1. A silylated slide is set in the path of the sort stream for collecting the cells of interest. Slides are allowed to air dry completely. Dry slides are then incubated in fresh Carnoy's fixative twice for 15 min each to remove flow sorting Isoton II solution and to adhere cells to slide. Dry slides are stored at –20°C until FISH analysis. In place of a slide, a sterile microcentrifuge tube can be used for collecting cells in suspension. In this case, sorted cells are washed once in PBSE/2% FBS (62) to remove residual saline, then resuspended in 20–50 µL supernatant and stored at 4°C until FISH (*see Note 4*).

### 3.6. Five-Color FISH

1. Incubate slides in 2X SSC at room temperature for 5 min.
2. Dehydrate cellular DNA by incubating slides serially in 70, 90, and 100% ethanol, 2 min each, and air dry completely.
3. Denature cellular DNA by incubating slide in 70% formamide/2X SSC for 20 min. This denaturation time is based on 3% paraformaldehyde fixation. If less than 3% fixative was used, denaturation time can be lowered by 5–10 min. Also note, if more than one slide is to be denatured at a time, the temperature of the 70% formamide/2X SSC solution will decrease by 1°C for each additional slide. Therefore, increase the waterbath temperature accordingly.
4. During the 20 min denaturation time, begin preparing the five-probe cocktail and store on ice protected from light.
5. Immediately transfer slide to ice-cold (–20°C) 70% ethanol for 2 min followed by 2 min incubation in ice cold (–20°C) 90 and 100% ethanol. Air-dry slide completely.
6. Denature five-probe cocktail by incubating in a 70°C waterbath for 5 min.
7. Apply probe cocktail immediately to target area on slide, cover with a 22 × 22-mm glass coverslip and seal with rubber cement.
8. Place slide in 37°C humidity chamber and allow for overnight (16 h) hybridization.
9. Next morning, prewarm approx 40 mL 2X SSC in a glass Coplin jar with lid to 70°C in a waterbath.
10. Processing only one slide at a time, remove coverslip and rubber cement. Place slide in 2X SSC at 70°C for only 10 s.
11. Immediately transfer slide to 1X PBD for 1 min.
12. Apply 18 µL DAPI II counterstain to a 24 × 50-mm glass coverslip. Drain excess PBD from slide using a paper towel then place slide on top of coverslip/DAPI. Gently press out any large air bubbles. Protect from excess exposure to room light by placing slides in a slide box.
13. Allow DAPI to soak into cells for 10 min prior to viewing under fluorescence microscope. Once analysis is complete, slides are stored at 4°C in a slide box.

### 3.7. Microscope Analysis

1. Each chromosome is identified by a probe-specific color and each cell is analyzed for the presence of 0, 1, 2, 3 or more signals per probe. Although we have arbitrarily chosen to

score 3000 nuclei per hybridization reaction, on average only 1500 nuclei are available after flow sorting for FISH analysis (*see Note 5*).

2. Fetal male cells are identified by the presence of one signal each for the X (yellow signal) and the Y (blue signal) chromosomes (*see Note 6*).
3. Detection of fetal trisomy 13, 18, and 21 relies on scoring three distinct signals for the autosome involved. Consequently, close attention must be given to distinguish three signals from split-domain signals. Because chromosome condensation is variable in certain stages of interphase and that FISH probes are typically composed of several DNA fragments, non-fluorescent gaps will appear within a probe signal on either one or both homologs. Therefore, signals are considered to represent split-domains' when (1) the size and intensity of each of the two signals is less than that of the signal for the other homologue, and (2) the distance between the two signals is less than the diameter of either of the two signals (*see Note 6*).

### 3.8. FISH on MACS Cells

There are some alterations in the FISH protocol when analysis is to be performed on MACS enriched cells from maternal blood (*see Note 7*).

## 4. Notes

1. Antibodies detected by fluorescence can affect FISH analysis when using multiple probes for aneuploidy detection. Using FITC-labeled antibody to stain for an abundant intracellular protein (i.e., gamma globin) will result in nuclei that are fluorescent all over. Thus, DNA probes labeled with fluorophores that emit in the green spectrum will be difficult to detect, limiting the number of chromosome specific probes available for FISH. Therefore, it is important to know prior to FISH those fluorophores used to label cells for FACS enrichment.
2. Temperature control of blood specimens prior to enrichment is important in maintaining suitable sample quality for subsequent separation enrichment and FISH. For overnight storage, specimens should be kept at room temperature. Extreme hot or cold temperature conditions will result in cell clumping or cell lysis, making enrichment inefficient. For shipping blood specimens, ice packs may be necessary during the summer months of the year.
3. Independent of type and density of gradient used to separate cells, it is important that the recovered cell layer be thoroughly washed to avoid cytoplasmic background and clumping of nuclei that would interfere with FISH analysis.
4. Specious particles in cytoplasm may exist, making nuclei appear to be engulfed in a haze or cloud. Probe hybridization is nearly zero. As in conventional cytogenetics, washes are inadequate and cell drying is not performed under controlled temperature and humidity conditions. To avoid this problem, cells sorted directly onto silylated slides must be allowed to dry slowly (30–60 min) on a 37°C warm plate followed by Carnoy's fixation. Following Carnoy's fixation, similar precautions need to be taken. Cytoplasmic particulate background can be especially problematic when humidity is low and rooms heated (i.e., winter). To overcome this, slides are allowed to dry slowly after Carnoy's fixation either flat or upright while placed within a small box to maintain the humidity level. Alternatively, cells collected (by FACS or MACS) in a plastic tube can be washed prior to placement on slides. To avoid cell loss, cells should not be cytocentrifuged or suspended in greater than 100–200  $\mu\text{L}$  of volume for spreading onto slides.

5. Enrichment probably increases the relative frequency of fetal cells only 100 fold; thus, perhaps 1 fetal cell per  $10^3$  maternal cells exists rather than the 1 per  $10^5$  prior to enrichment. Depending on the separation and enrichment method used, the number of total cells recovered and the number of cells scanned will vary. Our group scores up to 3000 nuclei per specimen. If we fail to detect a single fetal cell after scoring 3000 nuclei, we find it unlikely that scoring additional cells will detect fetal cells.
6. Each observer should use a score sheet to record the interpretation of each cell at the time of analysis. As in clinical cytogenetics, cells are analyzed in a systematic manner from left/right and down. An experienced observer can analyze 3000 cells on one slide in 2–3 h when three probes are used or 3–5 h when five probes are used. In conventional prenatal cytogenetics, the diagnosis of fetal aneuploidy is dependent on detection in multiple colonies, but in fetal cell analysis, the detection of only one trisomic cell among enriched cells is considered sufficient as shown by correlation with subsequent fetal karyotype. Of course, this is possible only when hybridization efficiency is optimal and signals can be detected among all or most cells.
7. FISH on MACS cells is relatively easier to perform given that fixation using paraformaldehyde is either absent or done using low 1–2% concentration. After MACS, recovered nuclei should be evaluated under phase contrast. If nuclei are gray and flat, then denaturation time should be reduced considerably to 1–3 min using an 80°C oven (not formamide). If paraformaldehyde fixation is used, then denaturation time may need to be increased. If FISH signals are weak or absent, pretreatment using proteinase K or pepsin may be required.

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## Preimplantation FISH Diagnosis of Aneuploidies

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### 1. Introduction

Preimplantation genetic diagnosis (PGD) has been introduced as a principally new approach to the prevention of genetic disorders, avoiding prenatal diagnosis and potential termination of pregnancy (1,2). It is based on the genetic testing of oocytes or embryos, with the purpose of establishing an unaffected pregnancy, rather than performing fetal diagnosis after at risk patients become pregnant, facing a risk for pregnancy termination if the fetus is affected. Although PGD was originally introduced for pre-existing genetic disorders (3,4), its application appears to be of special relevance for sporadic conditions, such as chromosomal abnormalities, which contribute significantly to pregnancy loss and infertility.

PGD has presently been performed in more than 3000 clinical cycles worldwide, resulting in 700 clinical pregnancies and births of approximately 600 unaffected children (5). At least three quarters of these clinical cycles were done for the age related aneuploidies by the method of fluorescent *in situ* hybridization (FISH) analysis, described in detail previously in an Atlas of Preimplantation Genetic Diagnosis (6), and briefly summarized in the present chapter.

#### 1.1. Approaches to Preimplantation Genetic Diagnosis

PGD is currently performed by two major approaches, which involves the testing of either blastomeres biopsied from the cleaving embryos, or polar bodies removed from matured and fertilized oocytes. Blastomere biopsy can be done as early as the 6–8-cell stage and allows testing for both maternally and paternally derived genetic abnormalities (3,6). First and second polar body (PB1 and PB2) removal, on the other hand, allows testing exclusively for maternally derived abnormalities (4,6). Each of the two approaches has advantages and disadvantages, and are applied depending on the clinical circumstances. For example, despite a reduction in embryo cell number, which may have an influence on the embryo viability, blastomere biopsy is the method of choice for paternally derived dominant conditions and translocations, as well as for

gender determination and HLA typing. On the other hand, removal of PB1 and PB2, naturally extruded from oocytes in the process of maturation and fertilization, respectively, should not have any effect on the embryo viability, however provides no information on gender and paternal mutations. PB1 and PB2 genetic analysis is of great value for testing of the autosomal recessive conditions and the maternally derived dominant mutations and translocations. It is also a method of choice for specific diagnosis of X-linked diseases, avoiding the discard of 50% male fetuses currently seen in blastomere based gender determination. Finally, because over 90% of chromosomal errors originate from maternal meiosis, the PB approach may be of special value for PGD of aneuploidies. This approach is also of importance due to a relatively high rate of mosaicism at the cleavage stage, which is recognized as the major limitation of the blastomere based PGD for chromosomal disorders (*see below*).

## **1.2. Preimplantation FISH Diagnosis**

PGD for the age related aneuploidies is currently done by FISH analysis, using commercially available chromosome specific probes (Vysis, Downers Groves, IL). It was first applied in 1991 for gender determination using DNA probes specific either for the X or Y chromosome (7). Since testing for only one of the sex chromosomes could lead to misdiagnosis of gender due to a possible failure of hybridization, a dual FISH was introduced, involving the simultaneous detection of X and Y, each in different color (8). Further, the dual FISH analysis was combined with a ploidy assessment to improve the accuracy, by adding a centromeric probe specific for chromosome-18 (9,10). Testing was then extended to up to five autosomes, including chromosomes 13, 16, 21, and 22 (11,12), although it is currently possible to analyze up to a dozen chromosomes, using additional rounds of rehybridization (12,13).

The overall experience of preimplantation FISH analysis currently involves more than 2000 clinical cycles, resulting in an improved pregnancy rate in poor prognosis IVF patients (5). Approximately half of these cycles were performed by FISH analysis of blastomeres and half by FISH analysis of PB1 and PB2, resulting in hundreds of unaffected pregnancies and healthy children born at the present time. The follow up confirmation studies of the preselected abnormal embryos, and the babies born following the procedure, demonstrated an acceptable accuracy of the FISH analysis, which is described below. Examples of normal and abnormal FISH patterns in PB1, PB2 and blastomeres are presented in **Fig. 1**.

### **1.2.1. FISH Analysis in Blastomeres**

The reliability of the FISH technique for aneuploidy detection in blastomeres has been extensively studied (14–17). By comparing the FISH results in the cleaving embryos to morphological abnormalities and maternal age, it was established that the observed chromosomal abnormalities were not related to the limitations of the FISH technique, but were due to the embryo variables (14,15). However, a high rate of mosaicism was observed at the cleavage stage (15–17), which was particularly high in slow embryos exhibiting an arrested development. An overall 12% mosaicism rate was shown to be a common feature in cleaving embryos (18), which represents a major limitation of the FISH analysis of aneuploidies performed at this stage. This may affect

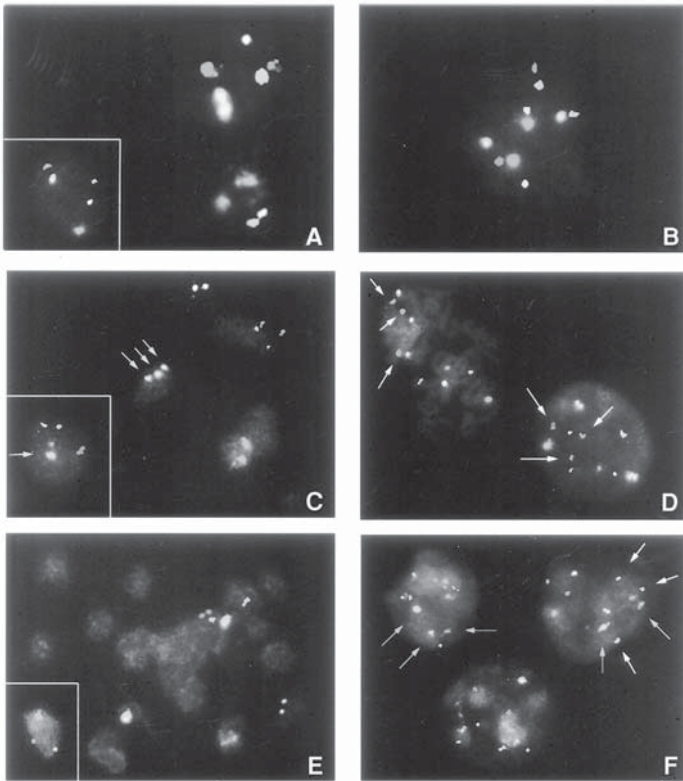


Fig. 1. Normal pattern of FISH signals and abnormalities detected by five-color probe in the first and second polar bodies and blastomeres. **(A)** Normal paired signals for chromosomes 13, 16, 18, 21, and 22 in the first polar body (a total of ten signals), and one signal for the same chromosomes in the second polar body (a total of 5 signals; on the bottom left corner). **(B)** Normal blastomere nucleus, two signals for each pair of chromosomes studied (a total of ten signals). **(C)** Extra signal for chromosome 18 in the first polar body (shown by three arrows) and a normal pattern of signals for other chromosomes in the first and all five chromosomes in the second polar body (one signal for chromosome 18 in the second polar body is shown by one arrow). The extra signal for chromosome 18 in the first polar body indicates a lack of one of the chromosome 18 in the corresponding oocyte, which will lead to monosomy 18 in the resulting embryo, following the extrusion of this one chromatid to the second polar body. **(D)** Three signals for chromosome 21 in blastomere nucleus and metaphase chromosomes (shown by three arrows) indicating trisomy 21 in the embryo. All other chromosomes probed showed a normal signal pattern (a total of 11 signals instead of 10). **(E)** Missing signals for chromosomes 13 and 18 in the second polar body (a total of three signals instead of the expected 5; bottom left corner). **(F)** Double trisomy 13 and 18 in the resulting embryo, originating from the oocyte shown in (E). The embryo is mosaic for trisomy 18, evident by the presence of three signals for chromosome 18 in the cell on the left (three arrows, showing only chromosome 18 in this nucleus) and five signals for this chromosome 13 and 18 in the cell on the right (five arrows showing only signals for chromosomes 13 and 18), instead of the expected six signals for the chromosomes 13 and 18 in this double trisomy embryo.

a diagnostic accuracy only partially, because the abnormal cell detection from a mosaic embryo will not lead to transfer. An average clinically relevant proportion of mosaicism has been calculated to be in the range 5% (19). Mosaicism at the cleavage stage is shown in **Fig. 1F**. It has recently been shown that mosaicism will also present diagnostic problems at the blastocyst stage (20) despite the initial prediction that the abnormal cells are deviated mainly to trophectoderm.

### 1.2.2. FISH Analysis of the First Polar Body

PB1 is the by product of the first meiotic division, extruded during maturation of oocyte. The first attempt to use FISH analysis for testing PB1 was undertaken in 1994 (21–23). In this work, 130 unfertilized M-II oocytes were tested simultaneously with their PB1 using X-chromosome and chromosome 18 specific probes. It was demonstrated that PB1 FISH data allows an exact prediction of the chromosome set in the corresponding oocytes (21,23). Each chromosome in PB1 was represented by double dots (signals), corresponding to two chromatids in each univalent (**Fig. 1A**). The data suggested that the number of signals (chromatids) in PB1 reliably predicts the corresponding number of signals (chromatids) in the MII oocytes, thereby providing an excellent tool for the genetic pre-selection of oocytes. It was also of interest, that in addition to a normal distribution of signals in PB1 and the corresponding MII oocytes, meiotic errors were also detected, confirming the accuracy of PB1 diagnosis for predicting the genotype of the corresponding oocyte. For example, in one PB1, four signals for chromosome 18 were detected, perfectly in accordance with the lack of the chromosome 18 signals in the corresponding MII oocyte (chromosome 18 nondisjunction). This suggested that the chromosomal complements of the oocyte could be inferred from the testing of PB1, which can be removed following its extrusion from the mature oocyte with no potential influence on the embryo viability. Another interesting phenomenon was the observation of chromatid mal-segregation as a possible cause of chromosomal aneuploidy in the resulting mature oocytes (*see* an example of extrachromatid signal in **Fig. 1C**). In four oocytes, instead of the expected two signals, three were found in the MII oocytes, which perfectly corresponded to a single signal in the corresponding PB1. Similar results were reported by another group, confirming diagnostic significance of the PB1 FISH analysis for predicting the genotype of the preimplantation embryo (24).

### 1.2.3. FISH Analysis of the Second Polar Body

PB2 is the by product of the second meiotic division, extruded following fertilization of oocyte. The need for FISH analysis of PB2 for PGD of aneuploidies was first proposed in 1995, when it was demonstrated that PB1 testing alone does not allow predicting the resulting genotype of the oocyte (22). It was shown that in contrast to the paired dots in PB1, each chromosome in PB2 was represented by a single dot (*see* left upper panel in **Fig. 1A**), so the lack of or addition of a signal for a particular chromosome provided evidence of a second meiotic division error. Although only 19 of 55 oocytes in this first study were tested by both PB1 and PB2, evidence for a possible error in both PB1 and PB2 was presented. This data suggested that some

oocytes selected as normal, based on the PB1 FISH analysis, could still appear to be abnormal following non-disjunction in the second meiotic division (**Fig. 1E**). Therefore, FISH analysis for both PB1 and PB2 has become the basic requirement for PGD of aneuploidies, which allows detection of errors in both the first and second meiotic divisions. Currently, more than 5000 oocytes have been analyzed by FISH analysis, showing the accuracy and reliability of PB1 and PB2 testing for predicting the karyotype of the embryo, resulting from the corresponding oocyte. The data demonstrated 50% aneuploidy rate in oocytes from IVF patients of advanced maternal age (25–27), resulting from the errors in both the first and second meiotic divisions, in contrast to the previously believed concept that aneuploidies mainly originate from meiosis I (28).

## 2. Materials

### 2.1. Preparation of PB1 and PB2 and Blastomeres, Following their Removal from Oocytes and Embryos

#### 2.1.1. Preparation of PB1 and PB2

1. Microscope slides (25 × 75 × 1 mm) (Fisher Healthcare, Houston, Texas).
2. Culture flask (50 mL) (Fisher Healthcare, Houston, Texas).
3. 5 3/4" Pasteur pipets.
4. Mouthpiece with tubing to hold micropipette (Sigma, St. Louis, MO, Cat. no. A-5177).
5. Carbide marker for engraving glass (Fisher Healthcare, Cat. no. 13-378).
6. Lead pencil and permanent marker.
7. Rubber bulb for blow drying.
8. Glacial acetic acid \*.
9. Methanol \*.
10. HPLC grade H<sub>2</sub>O (Mallinckrodt Baker Inc., Paris, Kentucky).
11. Stereomicroscope.
12. Inverted microscope with phase contrast optics, ×10 and ×20 objectives (Olympus CK 40, Olympus America Inc., Melville, NY).
13. Microtorch (Blazer Corporation, New York, NY).
14. Micropipets (1–5 µL) (Drummond Scientific, Broomall, Penn.).
15. Hanging drop slide (engrave a circle in the bottom center using a carbide marker) Fisher Healthcare, Cat. no. 12-560.

#### 2.1.2. Preparation of Blastomeres

The items 1–13 under **Subheading 2.1.1.** and the following additional materials are needed:

1. Micropipets (25–50 µL) (Drummond Scientific).
2. 35 × 10 mm Petri dishes (Falcon) (Fisher Healthcare, Cat. no. 08-757-100A).
3. Bovine serum albumin (BSA) (Sigma, Cat. no. A-3311).
4. Sodium citrate.
5. Hypotonic solution containing 6 mg/mL of BSA and 1% sodium citrate is dissolved in HPLC H<sub>2</sub>O, filtered through a 0.2 µm filter and stored at 5°C.

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\*Store previously opened bottles at 5°C to reduce water absorption from the atmosphere.

## 2.2. Pretreatment

1. Graduated cylinders and serological pipets.
2. Coplin jars (8).
3. Pepsin (Vysis, Downers Grove, IL, Cat. no. 30-801265).
4. Hydrochloric acid (2*N*).
5. Magnesium chloride.
6. Phosphate buffered saline (Irvine Scientific, Santa Ana, CA).
7. Neutral buffered formalin (10%) (w/v 4% Formaldehyde) (VWR, Chicago, IL Cat. no. 3239).
8. 20X SSC Solution: Dissolving 175.3 g of sodium chloride and 88.2 g sodium citrate in 1 L of HPLC H<sub>2</sub>O and adjusting pH to 5.3. pH adjustments are made using a HCl (1 *N*) solution. Filtered through a 0.45 μm pore filtration unit, and stored at room temperature for up to 6 mo.
9. 2X SSC Solution: Adding 100 mL of 20X SSC to 900 mL of HPLC grade water and pH to 7.0–7.5; filtered and stored at room temperature for up to 6 mo, with working solution to be changed weekly.
10. 1% Formaldehyde: Combining 30 mL of buffered neutral formalin (10%), 90 mL of 1X PBS and 0.54 g MgCl. This 1% formaldehyde solution can be stored at 5°C and the working solution changed weekly.
11. 0.5 mg/mL Pepsin solution: Combining 25 mg of pepsin per 50 mL of 0.01 *N* HCl. Fresh pepsin solution is prepared every 1–2 wk and maintained at 37°C.
12. Ethanol series: (70, 85, 100%): Diluting ethyl alcohol with HPLC grade water.
13. Methanol.

## 2.3. Application of Probes, Denaturation and Hybridization

1. Slide warmer (capable of consistent temperature, i.e., 68–73°C).
2. Air incubator (set at 37°C).
3. Microcentrifuge.
4. Vortex.
5. Calibrated thermometers (for slide warmer, incubator and waterbath).
6. Micropipetors (2–20 μL, 20–200 μL) and appropriate sterile tips.
7. Glass dishes with removable tray (Fisher Healthcare, Cat. no. 08-812).
8. Coverslips (22 × 30 mm) (Corning) (Fisher Healthcare, Cat. no. 12-531A).
9. Microcentrifuge tubes.
10. Forceps (fine tip).
11. Parafilm (Fisher Healthcare, Cat. no. 13-374-10).
12. Directly labeled probes commonly used for PGD of aneuploidies Vysis: Chromosome 13 Locus Specific Identifier (LSI) # 32-192018 Spectrum green; Chromosome 18 Centromeric Enumeration Probe (CEP) Cat. no. 32-131018 Spectrum aqua; Chromosome 21 Locus Specific Identifier (LSI) Cat. no. 32-190002 Spectrum orange; X & Y Centromeric Enumeration Probe Cat. no. 32-131051 Green X, Orange Y; MultiVysion PGT Chromosomes X, Y, 13, 18, and 21 Cat. no. 32-131080; MultiVysion PB Panel, Chromosomes 13, 16, 18, 21, and 22 Cat. no. 32-131085.

## 2.4. Washing and Counterstain Application

1. Sodium chloride.
2. Sodium citrate.
3. HPLC grade H<sub>2</sub>O (*see Subheading 2.1.1*).
4. NP-40 (Vysis, Cat. no. 32-804818).

5. Sodium hydroxide solution (1 *N*).
6. Hydrochloric acid (1 *N*).
7. Water bath (capable of maintaining temperatures up to 100°C).
8. pH meter and standards.
9. Vectashield antifade mounting medium (Vector Labs, Cat. no. H-1000).
10. DAPI counterstain (Sigma, Cat. no. D1388).
11. 0.4X SSC/0.3% NP-40: combine 20 mL 20X SSC, pH 5.3, 977 mL HPLC grade water and 3 mL NP-40. Mix thoroughly until NP-40 is dissolved. Adjust pH to 7.0–7.5 with NaOH. Add water to bring the final vol to 1 L, and filter through a 0.2 µm pore filtration unit. Store unused solution at room temperature for up to 6 mo.
12. 2X SSC/0.1% NP-40: 100 mL 20X SSC, pH 5.3, 899 mL HPLC grade water and 1 mL of NP-40. Mix thoroughly and measure pH at room temperature. Adjust pH to 7.0–7.5 with NaOH. Add water to bring the final vol to 1 L, and filter through a 0.2 µm pore filtration unit. Store unused solution at room temperature for up to 6 mo. Discard used solutions at the end of each day.
13. 0.7X SSC/0.3% NP-40: 35 mL 20X SSC, pH 5.3, 962 mL HPLC grade water and 3 mL NP-40. Mix thoroughly until NP-40 is dissolved. Adjust pH to 7.0–7.5 with NaOH. Add water to bring the final vol to 1 L.
14. DAPI stock solution: Prepare a stock solution of DAPI counterstain (1 mg/mL) in HPLC H<sub>2</sub>O.
15. DAPI working solution: The working solution (0.5 µg/mL) is prepared by adding 50 µL of DAPI stock solution to 100 mL of HPLC H<sub>2</sub>O. Store at –20°C.
16. DAPI /antifade: DAPI working solution is combined with Vectashield antifade mounting medium, 1: 1, and stored at –20°C until needed.

## 2.5. Signal Evaluation

1. Microscope (Microphot FXA - Nikon) with epifluorescence attachment.
2. Single bandpass filters (Chroma Tech.) for all fluorophores: DAPI (blue), FITC (green), TRITC (red), AQUA (aqua), F15 (gold), and F6 (blue).
3. Dual - bandpass FITC/TRITC (red and green), Aqua /Blue and triple-bandpass DAPI/FITC/TRITC (red, green, and blue) filters are also recommended.
4. Photographic equipment: 35-mm camera loaded with P400 Kodak Ektachrome film or a CCD camera, filter wheel and computer with imaging software (Quips Imaging Workstation, Applied Imaging, Santa Clara, CA).
5. Immersion oil (fluorescence) (Cargille Laboratories, Ins, Cedar Grove, NJ, Cat. no. 16212).

## 3. Methods

### 3.1. Preparation of PB1 and PB2 and Blastomeres for FISH Analysis, Following Their Removal from Oocytes and Embryos

#### 3.1.1. Preparation of PB1 and PB2

1. Prepare fresh fixative (methanol and glacial acetic acid, 3:1) in a 50 mL culture flask and store in the freezer until needed.
2. Pull micropipettes over the flame of the microtorch to obtain an attenuated tip with an inner diameter of approximately 50 µm. A larger diameter may lead to a potential PB loss.
3. Clean again, already precleaned glass slides using fixative to remove any grease or dirt, which may be on the slide. This is achieved by dropping a few drops of fixative and wiping with a lint free tissue.

4. Fill the hanging drop slide with HPLC H<sub>2</sub>O using a pasteur pipet.
5. After oocytes/zygotes have been manipulated and PB removal has been accomplished, the PB(s) can be found in the micromanipulation dish in a 20  $\mu$ L drop of culture medium under oil (6) using an inverted microscope with phase contrast optics ( $\times 10$  and  $\times 20$  objectives).
6. Once located, using the micropipet, a small amount of water is aspirated to insure proper control of fluid in and out of the pipet.
7. After aspiration of a small amount of water, the PB is aspirated into the pipet and brought to the hanging drop slide containing water. The PB is released from the pipet by blowing gently until it is visualized in the center circle of the hanging drop slide. Do not allow the polar body to settle and stick to the glass surface of the hanging drop slide.
8. Once rinsed in the water, the PB is aspirated again into the pipet. Any oil surrounding the pipet usually is removed by going through the water. The PB is transferred to a clean slide and a drop of water containing the PB is released from the pipet by gently blowing.
9. After partial evaporation of the water, the PB will swell and begin to flatten out onto the slide. Prior to complete drying of the water, a drop of fixative is placed on top of the PB from the same micropipet to obtain spreading of the chromatin.
10. Prior to complete drying, another drop of fixative is placed on top of the polar body and repeated if necessary until the cytoplasm dissolves. Careful attention is paid to observe this process and to thoroughly remove all cytoplasm (see **Notes 1** and **5**).
11. The location of the chromatin is defined by encircling using the carbide marker, taking care to lightly engrave the glass to avoid interference from glass fragments. A second heavier circle is engraved on the bottom of the slide to easily locate the smaller circle and thus the chromatin at the time of analysis.
12. The slide is marked with the patient's name and the corresponding oocyte number, along with pertinent information such as the number of pieces of chromatin.
13. Additional drops of fixative are added to the slide and blown dry using a rubber bulb.
14. Slides can be stored at room temperature overnight before hybridization or if hybridized immediately, a pretreatment method is recommended. Results can be obtained well within a reasonable time frame for embryo transfer.
15. Using a permanent marker, the area in which the PB chromatin is enclosed is marked from the back of the slide. This determines the area in which the probe is to be placed.

### 3.1.2. Preparation of Blastomeres

1. Prepare fresh fixative (methanol and glacial acetic acid, 3:1) in a 50 mL culture flask and store in the freezer until needed.
2. Engrave a circle in the bottom of a 35  $\times$  10 mm Petri dish and add 3 mL of hypotonic solution. The circle allows easy location of blastomeres.
3. Using a microtorch, 25–50  $\mu$ L micropipets are pulled to obtain an attenuated tip approximately 70  $\mu$ m in diameter.
4. Aspirate a small amount of hypotonic solution into the pipet.
5. The blastomere contained in a microdrop of culture medium is located using a stereomicroscope. The size of the pipet opening is checked prior to picking up the blastomere to insure it is larger than the blastomere. Gently aspirate the blastomere into the pipet and transfer it to the Petri dish containing hypotonic solution.
6. After 3–5 min, the blastomere is aspirated into the pipet and transferred to a microscope slide with a small amount of hypotonic solution.
7. Under the control of the inverted microscope with phase contrast optics, the blastomere is constantly observed until drying is almost complete. Just prior to crystallization of the

hypotonic solution, the fixative is aspirated into the pipet and dropped onto the blastomere. While constantly observing the cell, just before drying occurs, fixative is dropped again. This is done several times until the cytoplasm has dissolved with only the nucleus remaining. Complete removal of the cytoplasm is essential to hybridization (*see Note 1*).

- Using the carbide marker, the nucleus is encircled for easy location after hybridization.
- After recording pertinent information on the frosted area of the slide, several drops of fixative are added to the slide and blown dry using a rubber bulb.
- The slides are pretreated. Just prior to probe application, the exact location of the nucleus is indicated on the bottom of the slide using a permanent marker. This defines the area in which to apply the probe.

### 3.2. Pretreatment

- Incubate slides 10 min in 2X SSC, pH 7.0–7.5, at 37°C.
- Fix slides for 5 min in 1% formaldehyde at ambient temperature.
- Wash slides 5 min in 1X PBS, pH 7.0–7.5, at ambient temperature.
- Incubate slides 5 min in 0.5 mg/mL pepsin in 0.01 N HCl at 37°C.
- Wash 5 min in 1X PBS at ambient temperature.
- Drain slides and wipe the back of the slides to remove excess PBS.
- Immerse slides sequentially in 70, 85, and 100% ethanol at ambient temperature for one minute, air dry and proceed with hybridization.
- For the purpose of rehybridization, prior to pretreatment, coverslips are removed from the slides and the slides are placed in methanol for 5 min to insure fixation of the chromatin to the slide.

### 3.3. Probe Application, Hybridization and Wash

#### 3.3.1. Probe Mixtures

Probe mixtures are prepared according to the manufacturer specifications.

- Prepare a 3 color probe mixture by first centrifuging, then vortexing each of the probes of interest and 1 vial of LSI hybridization buffer for 10 s. For 10  $\mu$ L of working probe, i.e., chromosomes 13, 18 and 21, add 1  $\mu$ L of each probe to 7  $\mu$ L of LSI hybridization buffer in a small microcentrifuge tube. Vortex and centrifuge to mix.
- For a single chromosome probe mixture, add 1  $\mu$ L of probe and 2  $\mu$ L of HPLC H<sub>2</sub>O to 7  $\mu$ L of hybridization buffer. For an X & Y CEP probe mixture, vortex and centrifuge the probe and 1 vial of CEP hybridization buffer. For 10  $\mu$ L of working probe, add 1  $\mu$ L of X and Y probe to 7  $\mu$ L of CEP hybridization buffer and then add 2  $\mu$ L of HPLC H<sub>2</sub>O. Vortex and centrifuge for 10 s.
- MultiVysion 5 color probe mixtures are ready to use. Centrifuge and vortex prior to probe application. All working probe mixtures can be stored at –20°C for several months according to the manufacturers expiration date.

#### 3.3.2. Hybridization Procedure

- Prior to probe application, 22  $\times$  30 mm coverslips are cut into 8  $\times$  8 mm squares using a carbide marker and stored in a glass Petri with lid.
- Parafilm is cut into approximately 22  $\times$  22 mm squares and stored in a Petri dish.
- A humidification chamber for hybridization is prepared by placing a paper towel moistened with sterile filtered water into a glass (staining) dish and the slide rack is placed inside and covered. The humidification chamber is warmed in an air incubator at 37°C prior to the application of the working probe.

4. The working probe is removed from the freezer, centrifuged for 10 s, and mixed by vortexing.
5. Using a micropipetor, 2  $\mu$ L of working probe is applied to the marked area in which the chromatin is located. Immediately after application of the probe, an 8  $\times$  8 mm coverslip is placed on top using forceps. Avoid air bubbles, which interfere with hybridization. If any air bubbles should appear, depress the coverslip lightly using forceps.
6. Coverslips can be sealed using rubber cement or parafilm. When applying parafilm, insure adhesion to the slide by pressing down around the coverslip (*see Note 2*).
7. The slides are placed on the slide warmer at 69°C for 8 min for simultaneous denaturation of both probe and specimen nucleic acids. Afterward, they are removed from the slide warmer and quickly transferred to the humidification chamber, which is placed in a 37°C air incubator for hybridization to occur (*see Notes 3 and 4*).

### 3.3.3. Washing Procedures

The following two wash protocols save time, do not require formamide and are routinely used in our laboratory.

#### 3.3.3.1. RAPID WASH I

This wash is used for 1–3 color probe mixtures and MultiVysion PGT—Chromosomes 13, 18, 21, X, Y

1. Fill a Coplin jar with 0.4X SSC/0.3% NP-40, pH 7.4, and place in a 73°C waterbath. Using a calibrated thermometer, check the temperature of the solution inside the jar before adding slides for the wash procedure. The solution temperature should be 73  $\pm$  1°C.
2. Fill a second Coplin jar with 2X SSC/0.1% NP-40, pH 7.4, and place at room temperature.
3. Place slide(s) in the 0.4X SSC/0.3% NP-40 immediately after removing the coverslip. When all the slides are in the jar (maximum of 4) incubate for 5 min.
4. After 5 min, remove the slide(s) from the wash solution and place in the Coplin jar, containing 2X SSC/0.1% NP-40 at room temperature. Incubate for 1 min.
5. Remove the slide(s) from the wash solution, dip slides in a Coplin containing HPLC grade water and place vertically to drain on a paper towel in a dark area (such as a drawer).

#### 3.3.3.2. RAPID WASH II

This wash is used for MultiVysion PB panel probe—Chromosomes 13, 16, 18, 21, and 22.

1. Fill a Coplin jar with 0.7X SSC/0.3% NP-40, pH 7.4, and place in a 73°C waterbath. Using a calibrated thermometer, check the temperature of the solution inside the jar before adding slides for the wash procedure. The solution temperature should be 73  $\pm$  1°C.
2. Fill a second Coplin jar with 2X SSC/0.1% NP-40, pH 7.4, and place at room temperature.
3. Place slide(s) in the 0.7X SSC/0.3% NP-40 immediately after removing the coverslip. When all the slides are in the jar (maximum of 4) incubate for 7 min.
4. After 7 min remove the slide(s) from the wash solution and place in the Coplin jar, containing 2X SSC/0.1% NP-40 at room temperature. Incubate for 1 min.
5. Remove the slide(s) from the wash solution, dip slides in a Coplin containing HPLC grade water and place vertically to drain on a paper towel in a dark area (such as a drawer).

### 3.3.4. DAPI Counterstain

1. Apply approx 10  $\mu$ L to each slide and covered with a 22  $\times$  30 mm coverslip.
2. Slides are blotted using a paper towel or paper blotter to evenly spread and remove excess medium from the slide. (For hybridizations using MultiVysion 5-color probe mixtures, Vectashield antifade mounting medium is applied without DAPI).

### 3.4. Signal Evaluation

1. Signals are determined under  $\times 600$  magnification using fluorescence immersion oil. Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions containing fluorophores in reduced light. This includes all steps involved in handling the hybridized slide. Carry out all steps, which do not require light for manipulation (incubation periods, washes, etc.) in the dark (see **Note 2**).
2. Fluorescence microscopy is accomplished using a 100 W mercury lamp. Although the manufacturer recommends no more than 200 h of use, hybridization signals begin to appear dim after 175 h, creating potential for misdiagnosis. Replacement bulbs must be on hand.
3. For the MultiVysion PGT probe, chromosome X is seen in blue, chromosome Y is seen in gold, chromosome 13 is seen in red, chromosome 18 is seen in aqua and chromosome 21 is seen in green (see **Notes 7 and 8**).
4. For the MultiVysion PB Panel probe: chromosome 13 is seen in red, chromosome 16 is seen in aqua, chromosome 18 is seen in violet blue, chromosome 21 is seen in green and chromosome 22 is seen in gold (**Fig. 1**) (see **Notes 7 and 8**).
5. Visualization of signals is performed using the appropriate single band pass filters for the probe fluorophores (red, green, blue, gold, aqua and DAPI). Dual or triple band pass filters are useful in distinguishing signals from nonspecific fluorescence or bleed through usually seen with centromeric enumeration probes, which hybridize to alphoid repeat sequences resulting in large bright signals (see **Note 7**).
6. When determining the number of signals present, consider the size and intensity of each signal especially when in close proximity to one another. Chromosomes 13, 21, and 22 are identified using locus specific identifier DNA probes that contain specific sequences homologous to the chromosomes of interest as well as unlabeled blocking DNA to suppress common sequences. These result in smaller round signals when compared to signals from centromeric enumeration probes which hybridize to a greater number of alpha repeat sequences identifying chromosomes 16, 18, X, and Y (see **Notes 6 and 7**).
7. Signals for different chromosomes which are within close proximity of one another to the point of overlapping, are distinguished from nonspecific hybridization by close examination using both single and dual bandpass filters. Upon imaging, the overlapping portion of the signals may appear yellow or even white because of the fluorophore combination (see **Notes 7 and 8**). Use of an imaging system is highly recommended when using probe mixtures containing greater than 3 colors.
8. PB1 contains two chromatids for each chromosome, so the signals for each chromosome are paired, at least 1 domain apart and of equal size (see **Fig. 1A, C, E**). However, if chromatids are in close proximity of one another, the signals may appear as one fluorescent strip (see **Fig. 1E** for chromosome 16 signals in PB1), representative of two chromatids (see **Notes 6 and 9**).
9. PB2 contains single chromatids for each chromosome, therefore, resulting in one fluorescent signal for each chromosome tested (see **Fig. 1A, C, E**).

10. Two, single dot signals for each chromosome studied are normally observed in each blastomere nucleus (*see Fig. 1B, D, F*). However, signals can appear as double dots depending upon the stage of the cell cycle, degree of chromatin condensation and overspreading. After replication, a signal may appear as paired dots < 1 domain apart or as a strip consisting of two interconnected dots. These signals are counted as one signal according to standard criteria (*see Notes 7 and 8*).
11. If signals are of equal size and intensity and are 2 domains apart they are counted as two separate signals. However, proximity of two separate signals may be even closer than two domains in highly condensed nuclei and must be considered in order to avoid a misdiagnosis. Size and intensity of the signals of the individual nuclei as well as additional nuclei on the same slide must be taken into consideration when distinguishing nonspecific fluorescence from actual signals. Hybridization spots of lower intensity, smaller in size or with a flat, nonfluorescing appearance are not counted (*see Notes 6 and 7*).

#### 4. Notes

1. Prior to performing FISH, it is imperative that chromatin of blastomeres and PB1 and PB2 is completely isolated, free of cytoplasm, and thoroughly affixed to the microscope slide. Therefore, prior to applying fluorescent probes, slides with fixed PB or blastomeres are pretreated to maintain chromatin morphology and remove any residual cytoplasm. Residual cytoplasm poses the greatest interference to the formation of new specific hybrids during the reannealing of nucleic acids and appears as nonspecific fluorescence, making diagnosis difficult. This is an important step to overcome. Inadequate isolation of the chromatin and presence of cellular debris may result in nonspecific fluorescence after hybridization, rendering interpretation of signals difficult or even impossible.
2. Standardization and quality control monitoring of all steps involved in the FISH process, including fixation, is crucial to signal quality and interpretation. A lymphocyte control slide must be run with every assay to insure specificity.
3. Hybridization can vary from 2 h (CEP and LSI probes) to 16 h (whole chromosome paints and subtelomeric probes) depending upon the probe mixture used. Hybridization with MultiVysion probes, in which DAPI counterstain is not used, should not exceed 3 h. This is due to the strong background intensity of the chromatin that may occur with aqua labeled placental DNA present in the probe mixture. The recommended hybridization time for MultiVysion probes is 2 h for blastomeres and should not exceed 3 h for polar bodies.
4. The Hybrite (Vysis) can be used instead of a slide warmer and incubator oven for the simultaneous denaturing and hybridization of nucleic acids (maximum of 12 slides). A program is edited for a melting temperature of 69°C for 8 min followed by hybridization at 37°C for 3–16 h. A few milliliters of water are placed in each trough along the slide warmer for humidity, especially important for overnight hybridizations. Slides are placed on the Hybrite slide warmer after the probe is applied and covered with a coverslip and parafilm. The cover is closed and the program is started. Slides are left undisturbed until the hybridization period is over. A slide thermometer should be used to insure proper function, i.e., accurate melting temperature, prior to initiating a program.
5. Inadequate PB1 fixation in which residual cytoplasm is present may result in hybridization failure due to the inability of probe nucleic acids to access nucleic acids in the fixed specimen. If new hybrids should form, signals may appear faint and /or pulverized with multiple dots. This is often seen with excessively degenerate 1<sup>st</sup> polar bodies. Inadequate fixation of the chromatin along with cellular debris may result in nonspecific fluorescence after hybridization rendering interpretation of signals difficult or even impossible.

6. Diffuse or multiple dot signals (pulverization) may be seen from time to time involving one probe or all probes, regardless of procedure standardization, making interpretation complex. This is usually a result of chromatin degradation. The quality of both PB1 and PB2 signals must be taken into consideration, particularly when diffuse or split signals are present.
7. Nonspecific hybridization will fluoresce on two or more single band pass filters at the same, exact location and usually appears dull. Occasionally, bleed through of extremely bright signals can be seen with filters not intended for the specific fluorophore. They are seen as dull, flat fluorescing areas distinguishable from specific signals. This is often seen with probes that target alpha satellite repeat sequences, which generally result in diffuse bright signals.
8. Intense nonspecific hybridization is also ruled out by examination with the appropriate single and dual bandpass filters. Blastomere nuclei without hybridization signals, which are free of cytoplasm and located on the same slide with other nuclei in which signals are visible, are considered abnormal. Under these conditions, the absence of signals cannot be attributed to hybridization failure. This is not uncommon, especially if variation in size between nuclei is evident or if nuclei are fragmented.
9. In evaluating fluorescent signals in PB1, removed following fertilization assessment, e.g., 24 h after retrieval, signals representing each chromatid may be located further apart as separation of the chromatids occurs with aging. These pre-divided signals are considered normal, making signal number even easier to ascertain (*see* the chromosome 22 gold signals in PB1 in **Figure 1A**, and also the chromosome 18 signals in PB1 in **Fig. 1E**).
10. A high accuracy and reliability of preimplantation FISH diagnosis for aneuploidies, using blastomeres and PB1 and PB2 were demonstrated by confirmation studies of the whole embryos which were predicted abnormal and by the follow-up cytogenetic testing of outcomes of more than 500 clinical pregnancies yielded from approx 2000 PGD cycles (5).

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## Microdeletion Syndromes

### *Characteristics and Diagnosis*

**Stuart Schwartz and Michael D. Graf**

#### **1. Introduction**

Schmickel first defined the Contiguous Gene syndromes in 1986. These are syndromes that involve the deletion of a contiguous stretch of DNA, including multiple genes, on a chromosome. They are also referred to as microdeletion syndromes or segmental aneusomy. These syndromes are clinically recognized with distinct physical, behavioral, and mental characteristics and often involve some individual features that can be inherited in a Mendelian fashion (i.e., lissencephaly, heart defects, etc.). No other type of cytogenetic abnormality has benefited more from the introduction of fluorescence *in situ* hybridization (FISH) than these syndromes. While some of the abnormalities can be detected cytogenetically, FISH has added dramatically to the detection of these disorders and should be used whenever possible to confirm the deletions (**Table 1**).

#### **2. Common Microdeletion Syndromes**

##### **2.1. Smith-Magenis Syndrome**

Smith-Magenis syndrome (SMS), with an estimated incidence of 1 in 25,000 births, is caused by a deletion at 17p11.2. This deletion causes a consistent pattern of physical features, developmental delay, and behavior problems. The physical features include a distinctive face including brachycephaly, prominent forehead, synophrys, epicanthal folds, broad nasal bridge, ear anomalies, and prognathism. In addition, there is often brachydactyly, peripheral neuropathy, a deep, hoarse voice, and decreased sensitivity to pain. Individuals with SMS have varying degrees of mental retardation (MR) and there is often speech delay. Perhaps the most recognizable features of SMS are the sleep disturbances (difficulty falling and staying asleep) and behavioral problems. Commonly observed behavior problems include head-banging, wrist-biting, auto-amplexation (self-hugging), onychotillomania (pulling out of one's fingernails or toenails), and polyembolokoilomania (inserting objects into body orifices) (**1**).

From: *Methods in Molecular Biology, Vol. 204: Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

**Table 1**  
**Microdeletion Syndromes**

Syndrome	Phenotypic features	Cytogenetic location	FISH probes <sup>a</sup>
Prader-Willi Syndrome	Hypotonia, Hyperphagia, Obesity, Short Stature, Small Hands and Feet, Hypopigmentation, Mental Retardation	15q11-q13	Commercial (SNRPN)
Angelman Syndrome	Hypotonia, Microcephaly, Ataxic Gait, Inappropriate Laughter, Seizures, Hypopigmentation, Mental Retardation	15q11-q13	Commercial (D15S10)
Miller-Dieker Syndrome	Type I Lissencephaly, Dysmorphic Facies	17p13.3	Commercial (D17S379)
Velo-Cardio Facial Syndrome (Del-22q)	Abnormal Facies, Cleft Palate, Thymic Hypoplasia, Hypocalcemia, Heart Defect (Conotruncal Defect)	22q11	Commercial (TUPLE1)
Langer-Giedion Syndrome	Trichorhinophalangeal Syndrome (Sparse Hair, Bulbous nose, Cone-shaped Phalangeal epiphyses), Multiple Exostoses, Mental Retardation	8q24.1	Investigator
AWTA (WAGR)	Aniridia, Wilms Tumor, Genitourinary Dysplasia, Mental Retardation	11p13	Investigator
Smith-Magenis Syndrome	Dysmorphic Facial Features, Behavioral Abnormalities, Self Destructive Behavior, Peripheral Neuropathy, Mental Retardation	17p11.2 (FLII, TOP3, SHMT1)	Commercial

Williams Syndrome	Dysmorphic Facial Features, Infantile Hypercalcemia, Congenital Heart Disease, Gregarious Personality, Premature Aging of the Skin, Mental Retardation	7q11.23	Commercial (ELN)
Rubinstein-Taybi Syndrome <sup>b</sup>	Dysmorphic Facial Features, Broad Thumbs and First Toes, Mental Retardation	16p13.3	Investigator
Alpha-Thalassemia and Mental Retardation (ATR-16)	Dysmorphic Facial Features, Alpha-Thalassemia, Mental Retardation	16p13.3	Investigator
Alagille Syndrome <sup>b</sup>	Dysmorphic Facial Features, Chronic Cholestasis, Vertebral Arch Defects, Pulmonic Stenosis	20p11.23-p12.2	Investigator
Greig-Cephalopoly-syndactyly Syndrome <sup>b</sup>	Craniosynostosis, Polysyndactyly, Mental Retardation	7p13	Investigator
Albrights Hereditary Osteodystrophy-Like Syndrome	Short stocky build, Abnormal Facies, Brachymetaphalangism, Seizures, Developmental Delay	2q37	Investigator
1p36 Deletion Syndrome	Hypotonia, Developmental Delay, Growth Abnormalities, Craniofacial Dysmorphism, Minor Cardiac Malformations	1p36	Investigator
Xp22.3 Deletion	X-Linked Ichthyosis, Mental Retardation, Epilepsy, Ocular Albinism, Kallman Syndrome	Xp22.3	Investigator
Xp21 Deletion	Muscular Dystrophy, Glycerol Kinase Deficiency, Congenital Adrenal Hypoplasia, Mental Retardation	Xp21	Investigator
Saethre—Chotzen Syndrome <sup>b</sup>	Saethre-Chotzen Syndrome, Learning Difficulties	7p21.1	Investigator

(continued)

**Table 1 (continued)**

Syndrome	Phenotypic features	Cytogenetic location	FISH probes <sup>a</sup>
Y Chromosome Deletion	Unilateral Cryptorchidism, Idiopathic Infertility	Yq13	Commercial (PCR Primers)
van der Woude Syndrome	Cleft Lip With or Without Cleft Palate; Bilateral Lip Pits; Hypodontia	1q32–1q41	Investigator
Diamond-Blackfan Anemia	Red Blood Cell Hypoplasia, Macrocephalia, Hypotonia and Psychomotor Retardation	19q13.2	Investigator
NF1 Microdeletion Syndrome <sup>b</sup>	Neurofibromatosis, Early Age Onset of Cutaneous Neurofibromas, Facial Dysmorphism, Learning Disabilities, Mental Retardation	17q11.2	Investigator
Distal 22q Microdeletion	Hypotonia, Severe Language Delay, Mild Facial Dysmorphism	22q13–22qter	Commercial (D22S39, ARSA)
17p11.2 Duplication	Charcot-Marie-Tooth (CMT) Disease, Developmental Delay	17p11.2	Commercial (FLII, TOP 3, SHMT1)
Cat-Eye Syndrome	Coloboma, Choanal Atresia, Learning Disabilities, Mental Retardation	22q11.2	Investigator
Dicentric (15)	Seizures, Autism, Mental Retardation, Mild Facial Dysmorphism	15q11–15q13	Commercial (SNRPN)

<sup>a</sup>FISH probes are available either from Commercial Companies or through Investigators/Published Articles.

<sup>b</sup>The occurrences of these syndromes are primarily owing to mutations, not chromosomal deletions.

The critical region of SMS is estimated to contain about 5 Mb of DNA that usually allows cytogenetic detection of the deletion. However, some patients may have smaller deletions and FISH is often necessary to confirm small cytogenetic deletions.

## **2.2. Miller-Dieker Syndrome**

Miller-Dieker syndrome (MDS), a rare contiguous gene deletion, is caused by a deletion at 17p13.3. The phenotype of MDS consists of type I lissencephaly, mental retardation and characteristic facies including, prominent forehead, bitemporal hollowing, microcephaly, short nose with upturned nares, protuberant upper lip, thin vermilion border, and small jaw (2).

A few cases of MDS are autosomal recessive, but most have a deletion at 17p13.3. Studies have delineated a gene responsible for the lissencephaly (LS1) seen in this syndrome, while deletions of other putative genes are suspected to cause the dysmorphic features in the syndrome. This deletion can occur by a number of mechanisms including recombination of a parental balanced translocation or inversion of chromosome 17, a ring chromosome 17, or by a *de novo* deletion. Approximately 50% of these deletions can be detected by routine cytogenetics, but FISH with an MDS probe localized to 17p13.3 can detect or confirm whether the critical region for MDS is deleted. Additionally, some cases of isolated lissencephaly can be detected with this probe.

## **2.3. Prader-Willi Syndrome and Angelman Syndrome**

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) each have incidences of about 1 in 10,000–15,000 births and are caused by alterations at the region of 15q11–q13. The majority of cases (70%) of both PWS and AS are caused by a deletion of that region. The other causes not discussed in detail here are uniparental disomy (UPD), imprinting mutations, and, in the case of AS, gene mutations. PWS and AS exhibit genetic imprinting, meaning that the phenotype is determined by parental origin of the abnormal chromosome 15. If the deletion is on the chromosome 15 inherited from the mother, AS will be the result, whereas a paternally inherited deletion will cause PWS.

The clinical phenotypes of these two conditions are quite distinct. PWS consists of severe hypotonia in infancy, developmental delay, hypogonadism, short stature, and mental retardation. Beginning in early childhood, severe hyperphagia (over eating) leading to obesity may be one of the most distinctive behaviors, although there can also be temper tantrums, stubbornness, and obsessive-compulsive behaviors. There is also a typical facial appearance, especially in childhood, consisting of almond-shaped eyes, narrow bifrontal diameter, narrow nasal bridge, and a down-turned mouth with a thin upper lip. The characteristic features of AS include milder hypotonia, early seizures, developmental delay, delayed or absent speech, ataxia, and severe mental retardation. Typical behaviors include periods of inappropriate laughter, hand-flapping, tongue-thrusting, sleep abnormalities, hyperactivity, and aggressiveness. Common physical features include a large, wide mouth, widely spaced teeth, a prominent pointy chin, scoliosis, contractures, and hypopigmentation (3).

High-resolution chromosome analysis will often detect PWS/AS deletions, but FISH with a *SNRPN* probe is often needed to confirm cytogenetic findings or to detect

submicroscopic deletions. However, FISH can not determine the parental origin of the chromosomes so further molecular analysis (methylation analysis or uniparental disomy studies) is required to determine whether the deletion will lead to PWS or AS.

#### **2.4. Velocardiofacial Syndrome and DiGeorge Syndrome**

Velocardiofacial syndrome (VCF) and DiGeorge syndrome are both caused by a deletion at 22q11. They are syndromes with overlapping clinical features and recent literature has suggested using the general nomenclature of 22q11 Deletion syndrome when describing the condition. This may be the most common microdeletion syndrome, with an estimated incidence of 1 in 4000 births. Clinical features of the 22q11 Deletion syndrome are extremely variable, but can include hypernasal speech, learning disabilities, hypocalcemic seizures, and hypoplastic thymus leading to T-cell abnormalities. The typical physical features of the 22q11 Deletion include long, tapered fingers, cleft palate, prominent nose with squared nasal root, and microcephaly. In the more serious cases (DiGeorge syndrome), there are abnormalities in the third and fourth brachial arches leading to both thymic hypoplasia and parathyroid hypoplasia. Congenital heart disease is commonly associated with this deletion (85% of patients), most often a conotruncal defect. Therefore, it is suggested that if certain structural defects are found prenatally, FISH for the 22q deletion is warranted.

Only about 20–30% of the 22q11 deletions are detected by routine cytogenetics. It is necessary to utilize FISH to document a deletion. Most commonly a commercially available TUPLE1 probe is used to detect the deletion (4).

#### **2.5. Williams Syndrome**

Williams syndrome is caused by a deletion of the q11.23 region of chromosome 7, which includes the elastin gene. Williams syndrome is a developmental disorder involving both the central nervous system and vascular connective tissue. Its physical features often include prominent lips, epicanthal folds, blue eyes with a stellate pattern of the iris, anteverted nares, long philtrum, renal and dentition abnormalities, and premature aging of the skin. There is commonly a heart defect, most often supravalvular aortic stenosis (5–8). Infants with Williams syndrome are often colicky and have hypercalcemia. Children with this syndrome have gregarious personalities as they are often outgoing, loquacious, and very friendly. Persons with Williams syndrome are usually moderately mentally retarded and can have some behavior problems.

This disorder is not detectable by routine or high-resolution chromosome analysis. FISH using a DNA probe of the elastin gene will detect the deletion.

### **3. Less Common Microdeletion Syndromes**

#### **3.1. Langer-Giedion Syndrome**

Langer-Giedion syndrome (tricho-rhino-phalangeal syndrome type II–TRPS II) is a contiguous gene syndrome involving the deletion of at least two genes (TRPS I and EXT I) that are responsible for autosomal dominant disorders (TRPS I and multiple exostoses). The Langer-Giedion syndrome includes phenotypic features seen in both of these autosomal dominant syndromes. The phenotypic findings consists of developmental delay/mental retardation, sparse hair, bulbous nose, cone shaped phalangeal

epiphyses (all seen in TRPS I), and multiple cartilaginous exostoses. Mapping studies have suggested that the developmental delay/mental retardation is caused by genes outside of the TRPS I gene. Thus, the microdeletion of the TRPS I, EXTI, and MR genes, localized to 8q24.1, is responsible for the resultant phenotype (9).

In many cases, this deletion can be detected by routine or high-resolution analysis. While no commercially available probes are available, cosmid, phage, PAC, and YAC probes are available for deletion detection by FISH, as well as microsatellite markers within the deleted region (10).

### **3.2. Rubinstein-Taybi Syndrome**

Rubinstein-Taybi syndrome (RTS) is an autosomal dominant disorder, which has been localized to 16p13.3. In some cases this disorder has been due to a deletion of the RTS gene in this region. Rubinstein-Taybi syndrome consists of a number of phenotypic features including growth and mental retardation, broad thumb and halluces, short stature, and a typical facies (beaked nose, prominent columella, hypoplastic maxilla, and down-slanted palpebral fissures). Molecular analysis has demonstrated that this syndrome is due to disruption of the CREB binding protein (CBP), due to point mutation or chromosomal rearrangement (including deletions, translocations and inversions). The CBP gene codes for a large nuclear protein that is involved in transcriptional regulation, chromatin remodeling, and the integration of several different transduction pathways (11–13).

This deletion is usually not detectable by either routine or high-resolution chromosomal analysis. Initial studies utilizing the cosmid RT1, which includes the CREB binding protein, had suggested that up to 25% of RTS patients had a chromosomal syndrome; however more recent studies have suggested that a lower figure (8–12%) is more likely. A work by Petrij et al. has indicated a group of 5 cosmid probes (rather than just RT1) should be used for complete FISH analysis (12).

### **3.3. Aniridia Wilms Tumor Association**

Aniridia Wilms Tumor Association (AWTA), also referred to as the Wilms tumor, Aniridia, Genitourinary dysplasia and Retardation syndrome (WAGR) is due to a deletion in the short arm of chromosome 11 (11p13). Children with this deletion present with aniridia (absence of iris) and mental retardation. Some of the individuals with this deletion and an XY karyotype will have sex reversal and be phenotypic females. A portion of the individuals with deletions will also have a Wilms tumor, a kidney tumor that is the most common childhood tumor. Due to the common association of the various features, any child with a deletion, without a Wilms tumor at ascertainment, must be monitored for this cancer. Genes for aniridia (AN2), Wilms tumor (WT1) and sex reversal have all been identified in this region (14).

This deletion can usually be detected with routine or high-resolution cytogenetic analysis. A series of cosmid probes are available that can be used for FISH confirmation of deletions in this region (15).

### **3.4. Alpha-Thalassemia and Mental Retardation Syndrome (ATR-16)**

Alpha-Thalassemia and Mental Retardation syndrome (ATR-16) is a rare disorder that is owing to the deletion in 16p13.3 in the telomeric region of the short arm. The

loss of the two alpha-hemoglobin genes in this region results in the individuals being a carrier for alpha-thalassemia, while loss of other contiguous genes is responsible for the minor dysmorphic features and mental retardation (16). If the other parent is a carrier, they could have alpha thalassemia or Hemoglobin H. A recent study by Pfeifer et al. has indicated that the *SOX8* gene, which codes for a 446 amino acid protein expressed strongly in the brain, is deleted in an ATR-16 patient and is a good candidate gene contributing for the mental retardation in these patients (17).

This syndrome is rarely initially detected by high-resolution chromosome analysis. Rather, it is usually ascertained by phenotypic findings and confirmed by FISH or molecular analysis.

### 3.5. Alagille Syndrome

Alagille syndrome is a genetic disorder localized to 20p11.23–20p12.2. Mild dysmorphic facial features, chronic cholestasis (associated with intrahepatic bile duct paucity), cardiovascular anomalies, ocular anomalies, and minor skeletal malformations, characterize this condition. The Jagged 1 (*JAG1*) gene has been identified as the gene responsible for Alagille syndrome. This gene encodes a ligand in the Notch signaling pathway and is believed to be involved in cell fate determination. Approximately 70% of the identified cases of Alagille syndrome are thought to be owing to a *JAG 1* mutation, which includes protein truncation, splicing, and missense mutations. Whole gene deletions can also cause Alagille syndrome. However, these are infrequent, as they account for only approx 6% of the cases (18,19). Approximately 95% of the cases of Alagille syndrome have a cardiac defect. Most involve right-sided defects, which can range from mild peripheral pulmonic stenosis to the severe forms of Tetralogy of Fallot. Recent studies have shown that individuals with isolated heart defects (e.g. Tetralogy of Fallot) can have mutations in the *JAG1* gene (20).

The finding of a deletion associated with Alagille syndrome is an infrequent event, but most can be detected by routine cytogenetics or high-resolution chromosomal analysis. A contig consisting of YACs and BACs is available for FISH analysis.

### 3.6. Greig-Cephalopolysyndactyly Syndrome

Greig-Cephalopolysyndactyly syndrome is an autosomal dominant disorder, which has in some cases been associated with a deletion in 7p13. This syndrome has been characterized by craniosynostosis and polysyndactyly. It has been shown to be owing to point mutations and translocations involving the zinc finger gene *GLI3*. Further analysis has revealed that while mutations of *GLI3* results in Greig-Cephalopolysyndactyly syndrome, mutations in this gene may lead to Pallister-Hall syndrome or post-axial polydactyly type A. Patients with Greig-Cephalopolysyndactyly resulting from a deletion of the *GLI3* gene and contiguous region demonstrate significant developmental retardation and mental retardation, in addition to the phenotypic findings (polysyndactyly, bifid thumb, peculiar skull shape, hip dislocation, and advanced bone age) (21,22).

Most of the deletions that have been detected in association with this contiguous gene have been detected by utilizing standard cytogenetics. For more detailed analysis, both investigator developed FISH probes and molecular markers are available.

### **3.7. Albright Hereditary Osteodystrophy-Like Phenotype**

Albright hereditary osteodystrophy-like phenotype is a reported syndrome in patients with a subtle deletion in the telomeric region of the long arm of chromosome 2 (2q37–2qter). The features seen in this syndrome includes a short stocky build, abnormal facies (including a round face, sparse hair, deep set eyes, bulbous nose, thin vermillion border), brachymetaphalangism, seizures, and developmental delay. Behavior findings include periods of hyperkinesia and aggressive behavior. Although a number of these patients have been reported, no candidate gene region has been implicated to date (23,24).

A number of patients have been ascertained and identified using both routine cytogenetic analysis and high-resolution studies. Several YACs and BACs are available for a more detailed molecular analysis of this region.

### **3.8. 1p36.3 Deletion Syndrome**

A microdeletion syndrome involving a deletion of the 1p36.3 had been reported with increasing frequency over the past several years and appears to have increasing importance in genetics. Phenotypic features seen in these patients include: hypotonia, developmental delay and mental retardation, growth abnormalities (growth retardation, microcephaly, obesity), craniofacial dysmorphism (large anterior fontanelle, prominent forehead, deep set eyes, flat nasal bridge, midfacial hypoplasia, ear asymmetry, pointed chin, orofacial clefting), and minor cardiac malformations (25). A neurodevelopmental profile has been suggested for this syndrome in which severe learning disability, motor delay with hypotonia, markedly delayed visual maturation, and postural asymmetry together with epilepsy is seen (26). In some instances, patients with this deletion have been ascertained because of a suspicion of Prader-Willi syndrome, while many have been referred because of the hypotonia. Shapira et al. have studied a group of 14 patients, using both FISH and DNA polymorphisms and have found that there is no uniform region of deletion and the cases contain a spectrum of different deletion sizes (27).

Many of these patients have been detected by routine and high-resolution cytogenetics because of the distinctive double dark band seen in 1p36. Also, as the phenotype is so broad, it is difficult to specifically look for a deletion by FISH in a specific subgroup of patients. However, several patients have been reported in which 1p36.3 deletions have been detected using a subtelomeric probe kit. Several groups have utilized a repetitive probe (1DZ2) in 1p36.3 to confirm deletions. As indicated above, this region has been studied with a series of BACs, which are available for FISH analysis (27).

### **3.9. Xp22.3 Deletion**

The Xp22.3 syndrome is a classical contiguous gene deletion syndrome that is seen in males. It involves the deletion of several genes in the pseudoautosomal region of the X chromosome, each resulting in a specific phenotype. This syndrome involves the deletion of genes resulting in X-linked ichthyosis, mental retardation, epilepsy, ocular albinism, and Kallman syndrome. In some cases this will also involve short stature

owing to chondrodysplasia punctata. The patients may be ascertained because of any of the above phenotypes and subsequently determined to have a deletion covering several genes (28,29).

This deletion is usually detectable by standard cytogenetics analysis as it can encompass several megabases of DNA. However, some smaller deletions can exist, which can be detected by FISH using commercial probes for X-linked ichthyosis (STS) or Kallman syndrome (KAL) (28,29).

### **3.10. Xp21 Deletion**

Similar to the above-mentioned Xp22.3, the contiguous gene deletion involving Xp21 is also seen in males. This contiguous gene deletion syndrome also involves the deletion of specific genes, in this case in Xp21, each again resulting a specific phenotype. Most of these patients are males ascertained due to the presence of Duchenne muscular dystrophy. However, they have additional phenotypic findings that may include: Aland Island eye disease, adrenal hypoplasia, glycerol kinase deficiency, retinitis pigmentosa, and ornithine transcarbamylase deficiency. The exact phenotype again depends on the precise breakpoint and which genes are deleted (30,31).

While some of these patients have been detected cytogenetically, many of the deletions are submicroscopic and must be delineated using FISH probes and microsatellite markers localized to this region.

### **3.11. Saethre-Chotzen Syndrome**

Saethre-Chotzen syndrome is an autosomal dominant craniosynostosis disorder that is relatively common. This syndrome is characterized by brachydactyly, soft tissue syndactyly and mildly dysmorphic facial features (ptosis, facial asymmetry, and prominent ear crura). Molecular analysis has revealed that a mutation in the coding region of the *TWIST* gene (which encodes a basic helix-loop-helix transcription factor) accounts for a number of these cases. Approximately 80% of the cases of this syndrome result from an abnormality in the *TWIST* gene. Gripp et al. have suggested that up to 37% of the cases might be due to large deletions (32). Cases, which involve a deletion of more than just the *TWIST* gene, also have developmental delay. This gene has been localized to 7p21.1 (32).

This is not a syndrome that can usually be detected with routine or high-resolution cytogenetic analysis, with the exception of translocations. However several probes for FISH are available to determine whether a deletion is present and the extent of that deletion. It is important to differentiate whether the underlying cause is a mutation or deletion, due to the association of developmental delay with deletions.

### **3.12. Van der Woude Syndrome**

Van der Woude syndrome is a highly penetrant autosomal dominant form of syndromic cleft lip and palate. It is estimated that it accounts for approx 2% of all cleft lip and palate cases. Phenotypic features associated with this syndrome include: cleft lip with or without cleft palate, bilateral lip pits, and hypodontia. Patients with this syndrome have normal intelligence, however, those patients with a deletion have developmental delay. The gene for this syndrome has not been identified, but has been

localized to a 1.6 cM region in the 1q31–1q41 region. This is also the region that has been implicated in the deletion cases (34,35).

There have only been a few cases with this syndrome identified as having a deletion. Those which have a deletion, have been detected by routine chromosome analysis. A 4.4 Mb YAC contig through the critical region has been constructed, with both BACs and PACs available for a more thorough analysis (35).

### **3.13. Diamond-Blackfan Anemia**

Diamond-Blackfan anemia is a rare constitutional blood disorder in which there is pure red-cell aplasia in the neonatal period or in infancy. Both its etiology and pathogenesis are unknown. Linkage analysis has localized this disorder to a 4.1 cM region at 19q13.2. Analysis of over 50 individuals with this syndrome has revealed that in some cases, a small microdeletion is associated with this anemia. These patients, in addition to the red blood cell hypoplasia, had other phenotypic findings including macrocephaly, hypotonia, and psychomotor retardation. These additional findings in microdeletion patients suggest that this is a contiguous gene deletion. Analysis of the microdeletion patients has reduced the critical region for this syndrome to about 1 Mb (36,37).

The initially detected deletion patients identified with this syndrome were ascertained using standard or high resolution chromosomal analysis. Subsequent to this, a number of investigators have utilized FISH to better characterize the deletions (37).

### **3.14. Neurofibromatosis**

Neurofibromatosis is a well characterized autosomal disorder in which patients have café-au-lait spots and neurofibromas. It is a syndrome that has considerable variable expressivity. Molecular analysis has shown a variety of mutations associated with the syndrome. However, more recent studies have indicated that approx 5–10% of these patients have a deletion of 17q11.2, which includes the NF1 gene rather than a mutation. In addition to the typical findings seen in neurofibromatosis, the microdeletion patients have other findings such as earlier onset of cutaneous neurofibromas, facial dysmorphism, and learning disabilities/mental retardation. The addition of these findings to neurofibromatosis classifies the deletion as a contiguous gene syndrome. Similar to that seen in other microdeletion syndromes (Prader-Willi syndrome, Angelman syndrome, Velo-cardio-facial syndrome and Williams syndrome), specific repetitive/paralogous segments flank the deletion and are responsible for unequal meiotic crossingover leading to the consistent microdeletion (38,39).

These deletions are not readily detectable by cytogenetic analysis alone. A variety of FISH probes, including a detailed YAC map, are available for a more detailed analysis (39).

### **3.15. Distal 22q Deletion**

The utilization of subtelomeric probes will yield the detection of microdeletions not previously seen. One such terminal deletion that has been characterized by a number of groups is of the subtelomeric region of the long arm of chromosome 22. These patients present with hypotonia, severe language delay, and mild facial dysmorphism. Precise phenotypic characterization of these deletions have been difficult as many of these

result from reciprocal translocations and contain not only a deletion, but in many cases a duplication (40,41).

These patients are essentially only ascertained using FISH with a subtelomeric 22q probe (D22S39; ARSA). Several of these cases have been ascertained serendipitously using the VCF probe, and although that probe is present, the D22S39 probe that is used as a control probe is deleted, confirming this distal deletion.

### 3.16. Y Chromosome Deletions

One recent microdeletion that has been identified involves a deletion in the long arm of the Y chromosome (Yq13). This is not a contiguous gene deletion, but rather a microdeletion involving DNA important in male fertility. Thus, these men will normally present with only infertility. Deletions have been detected in three different regions of the long arm of the Y chromosome; the AZFa, AZFb, or AZFc regions. While there are many varied reported frequencies for this deletion, it is estimated that 2–4% of patients with severely impaired spermatogenesis will have a microdeletion in this region. A smaller frequency of males with less severe defects will also have a deletion in this region (42,43).

This microdeletion can not be detected by cytogenetic or FISH analysis. These deletions will only be detected using PCR analysis of markers within the three regions (42,43).

## 4. Microduplication Syndromes

Theoretically for many interstitial microdeletions, a reciprocal microduplication syndrome should exist. This does not seem to be true for the vast majority of these cases, however. They may occur, but the clinical phenotype may be too mild to lead to cytogenetic analysis or it is possible that they are cytogenetically too subtle to permit a diagnosis. In this review, three well documented microduplication syndromes (two of which involve supernumerary chromosomes) will be presented. All three of these syndromes are well established and delineated in the literature.

### 4.1. 17p11.2 Duplications

The 17p11.2 duplication is thought to be the homologous reciprocal recombination of the Smith-Magenis deletion, with a *de novo* duplication of the region deleted in Smith-Magenis. The clinical phenotype seen in this syndrome is considerably milder than that associated with the deletion of the same region. These patients can have Charcot-Marie-Tooth disease type 1A, along with developmental delay (44,45).

These patients are difficult to diagnose with either standard or high resolution cytogenetics. They can, however, be easily identified using FISH. The same commercial probes used to identify the Smith-Magenis deletion (FLII, TOP3, SHMT1) can also be used to determine the presence of a duplication. In addition, detailed genomic maps of this region have been constructed, indicating the availability of other FISH probes or microsatellite markers.

### 4.2. Cat-eye Syndrome

Cat-eye syndrome (CES) is a disorder with a variable phenotype of multiple anomalies. Some of these findings include: downward slanting palpebral fissures, micro-

ptthalmia, congenital heart defect, urinary tract anomalies, and skeletal anomalies. All of these features may be seen simultaneously in only 1/3 of the cases, however both anal atresia and ocular colobomas must be present to make a clinical diagnosis. Mild to moderate mental retardation is seen in about 1/3 of the cases. Cytogenetically, this syndrome is characterized by the presence of a supernumerary bi-satellited dicentric chromosome derived from chromosome 22 (46,47).

The supernumerary marker chromosome is easily identified by routine cytogenetics. Nevertheless, not all dicentric chromosomes derived from chromosome 22 will be associated with the Cat-eye syndrome phenotype. The critical region for this syndrome, localized to 22q11.2, must be present. This area has been heavily studied and a number of probes are available to confirm this region's presence. It should be noted that the probes for VCF (i.e., TUPLE1) are distal to this region and are not appropriate to use to delineate the CES syndrome (47).

### 4.3. Dicentric (15) Syndrome

The dicentric chromosome 15, better known as an inv dup(15), is also a supernumerary dicentric chromosome. This is the most common structurally abnormal supernumerary chromosome seen cytogenetically. It has a variable phenotype and usually only mild facial dysmorphism is seen. Most often this syndrome is associated with seizures, autism, and mental retardation. Similar to the above mentioned syndrome (CES), this chromosome is bisatellited and dicentric and can involve breaks in at least four different areas in 15q. The abnormal phenotype is believed to only be associated with those cases in which the Prader-Willi/Angelman syndrome (PWS/AS) critical region in 15q is present. In those cases where the breakpoint is proximal to this critical region, the individual will have a normal phenotype (except for where uniparental disomy occurs) (48,49).

Again, this marker is routinely ascertained with standard cytogenetic analysis. However, no phenotype will be present with the absence of the PWS/AS critical region. The presence of this region cannot be done by cytogenetic analysis, but must be examined with FISH. Utilization of a probe used to diagnoses the deletion in Prader-Willi syndrome (i.e., SNRPN) must be used. Both a number of commercial and noncommercial probes are available for these studies (49).

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## Molecular Cytogenetics in Reproductive Pathology

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### 1. Introduction

Conventional cytogenetic analysis has contributed for the last 35 yr to establishing the etiology of spontaneous abortions (SAs), representing an important diagnostic aid for reproductive pathologists, geneticists, and physicians. There is however a problem in obtaining a cytogenetic result from 10–30% of abortion specimens owing to culture failure. In addition, growth of maternally-derived cells in culture can result in a false negative result. Nowadays, new molecular cytogenetic tools are available to get cytogenetic information in almost all cases of SA submitted to the Cytogenetics laboratory.

Pregnancy loss is common in human reproduction, with over 50% of all conceptions and 15–20% of recognized pregnancies resulting in failure. The majority of the losses occurs early in gestation. Cytogenetic abnormalities represent the major etiology for reproductive failure, responsible for >70% of early losses (before 6 wk of gestation), around 50% of losses between 8–15 wk of gestation, and 5–10% of stillbirths (1). Nearly all these chromosomal defects happen *de novo*, as a result of either a meiotic or a postzygotic division error or due to an abnormal fertilization. The meiotic errors can occur in both maternal and paternal gametes, but are more frequently maternal in origin.

One of the most common causes of pregnancy loss is chromosomal trisomy. It may be present in all cells of the developing embryo, or, in some cases, one of the three chromosome copies may be lost by anaphase lagging or non-disjunction (2) or chromosome demolition (3) at an early postzygotic cell division. This event, called “trisomy rescue”, leads to chromosomal mosaicism, the coexistence of two cell lines in the conceptus, one disomic and the other trisomic. The trisomic cell line can be present in both the embryo and the placenta. This situation represents generalized mosaicism and can be detected in about 10% of spontaneous abortions when specifically looked for (4). Viable pregnancies with generalized mosaicism can be diagnosed by amniotic fluid analysis, but a subset may remain cryptic (5,6). If the trisomic cell line is limited

to the placenta, this situation is called confined placental mosaicism (CPM) (7). At the time of chorionic villus sampling at 10–12 wk of gestation, about 1–2% of pregnancies present such a situation (8–11). Three types of CPM are defined, based on cell lineage involved: Type I: the trisomic cell line is confined to the trophoblast; Type II: the trisomic cell line is confined to the chorionic villus mesenchyme; Type III: the trisomic cell line is detected in both cell lineages.

*In utero* fetal growth retardation and a poor perinatal outcome are commonly associated with CPM (12,13). Furthermore, when CPM is detected, the chromosomally normal fetus has a risk of one third of having uniparental disomy (the same parental origin for a given chromosome pair) for the chromosomal pair that is trisomic in the placenta. Uniparental disomy involving specific chromosomes (i.e., 7, 14, and 15) leads to an abnormal fetal phenotype (14,15).

### **1.1. Spontaneous Abortion**

Abortion is defined as the premature expulsion or removal of the conceptus from the uterus before it is viable. A gestational age of 20 wk of gestation or a fetal weight of 500 g are currently considered to be the lower limits of fetal viability.

The main cause of SAs is chromosomal abnormality. Trisomies are found in 26% of pregnancy losses, triploidy and tetraploidy in 10% and sex chromosome monosomy in 9% (1). About 10% of the cases show mosaicism for aneuploidy (4). Less frequently, an unbalanced structural rearrangement is found (2%), or double trisomy (0.6–1.9%) (16–18). Therefore, over 50% of all SAs are chromosomally abnormal.

Monosomy X represents the most common single chromosomal anomaly found in cytogenetically abnormal SAs. The most common trisomy is trisomy 16, which occurs in a third of the trisomic conceptuses. The frequency of other trisomies is summarized in **Table 1**.

Histological and morphological examination is not helpful in distinguishing between chromosomally normal and abnormal abortion specimens (19). For that reason, cytogenetic testing is essential in order to establish the etiology of SA and provide genetic and reproductive counselling.

### **1.2. Stillbirth**

Stillbirth is defined as a fetus dying intrapartum or antepartum after 20 wk of gestation or having a weight over 500 g. This event occurs in about 0.4–4% of total births (20), with developing countries having the highest rates. Stillbirths in North America represents about 1% of births (21). Incidence of chromosomal aberrations in stillbirth is about 4% in non-macerated fetuses (1,22), and more than twice (9%) in macerated stillborns (22,23).

### **1.3. Limitations of Conventional Cytogenetic Testing**

Conventional cytogenetic studies of products of conception rely on obtaining viable tissues, establishing primary cultures, and harvesting metaphase chromosomes for analysis. Although the contribution of conventional cytogenetic testing to reproductive pathology has been immense, there are several limitations:

**Table 1**  
**Trisomy Frequency in First Trimester Trisomic Spontaneous Abortions, Arranged from Ref. (1)**

Trisomy	(%)
16	31.4
22	11.2
21	10
15	7.1
18	4.6
13	4.6
2	4.6
14	4
7	3.8
8	3.3
4	3.3
9	3
20	2.5
10	2
3	1.3
6	1.3
12	0.8
11	0.4
5	0.4
17	0.4

1. The major problem of cytogenetic analysis of tissues from products of conception is obtaining dividing cells. Cytogenetic testing on tissues from SA has a high rate of tissue culture failure (10–40%). Placenta has a very poor growth rate after 34 wk of gestation. Tissues of macerated stillbirth also fail to grow in more than half of the cases.
2. The selective growth of maternal decidua may lead to the cytogenetic diagnosis of a normal female karyotype not representative of the true fetal chromosome complement.
3. Cultural artifacts may occur in long-term cultures of chorionic stroma.

To avoid these problems, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH), two recently developed molecular cytogenetic techniques, utilize uncultured cells and tissues to overcome the limitations of cell culture. They provide an alternative approach for cytogenetic analysis of products of conception and stillbirths.

## 2. Molecular Cytogenetic Techniques

### 2.1. Fluorescence In Situ Hybridization

Fluorescence *in situ* hybridization (FISH) allows the detection of the presence (or absence) of a DNA sequence of interest directly on metaphases or interphasic nuclei. A probe (a DNA sequence complementary to the region of interest), labeled directly or

indirectly with a fluorochrome, is hybridized with the clinical material after denaturation of both the probe's and the specimen's DNAs. The fluorescent signal can then be visualized by a fluorescence microscope. This technique is now widely used in clinical cytogenetic laboratories.

### 2.1.1. Application

A chromosome defect is usually strongly suspected when the pathological examination of a late abortion reveals a fetus with focal anomalies. Confirmation of a suspected chromosomal abnormality can be done by FISH. For example, the presence of a posterior cervical cystic hygroma is an indicator of a cytogenetic abnormality in 85% of cases, mostly monosomy X, but also trisomy 21 and 18 (24). Therefore, for this fetal defect, the use of probes for chromosomes 13, 18, 21, X and Y can provide definitive diagnosis.

When a specific fetal defect is not present, the range of possible chromosome anomalies is wider. Although a complete chromosome scan is theoretically possible, the use of fluorescent probes for chromosomes 13, 15, 16, 18, 21, 22, X and Y allows the detection of the most common autosomal trisomies, as well as sex aneuploidies, triploidies and tetraploidies. This approach can potentially establish an etiology for more than 80% of chromosomally abnormal SAs (see **Table 1**). Weremowicz et al. (25) showed that it is a rapid and cost effective approach, avoiding the need to continue culture for 42% of the samples. In another study (26), FISH probes for chromosome 13, 15, 16, 18, 21, X and Y were selected according to ultrasound findings or autopsy results and applied to specimens that failed to grow in culture. This approach led to a result in 28 out of 30 cases, with an aneuploidy detected in 46% of the cases. The 2 cases with FISH failure represented severely macerated fetuses.

In the case of pathological examination of stillbirth, FISH performed with probes for chromosomes 13, 18, 21, X, Y can reveal all types of aneuploidy usually compatible with late gestation (1). Such probes are now commercially available as kits of aneuploidy detection (Cytocell®, Adderbury, UK, Vysis®, Downers Grove, IL).

### 2.1.2. Limitations

Valuable information is provided on the copy number of specific chromosomes corresponding to the probe used in the FISH assay. Most of the time, enumeration for a chromosome is done with a centromeric probe, as this kind of probe provides large signals that are easy to count. Unfortunately, it does not provide any information on the structure of the chromosome analyzed. When three signals are detected, the sample is assumed to be trisomic, whereas it also could be triploid or contain an unbalanced structural rearrangement. The simultaneous use of two or more probes allows one to distinguish between aneuploidy and polyploidy, which are far more common than unbalanced structural abnormalities. In addition, although several probes with different fluorescent dyes can be used in one or two experiments, FISH does not provide a screen for the entire genome, and only the most common trisomies can be tested for.

The presence of chromosomal mosaicism with an abnormal cell line present in less than 10% of the cells cannot be established with confidence. To increase their efficiency in detecting mosaicism, cytogenetic laboratories providing clinical FISH analy-

ses need to establish cut-off values for the detection of a mosaic aneuploid cell line (27). The cut-off value varies according to the tissue, to the probe and potentially to the observer.

## **2.2. Comparative Genomic Hybridization**

The technique of CGH allows the entire genome to be screened for extra or missing chromosome material in a single hybridization (28). It involves the simultaneous hybridization of test and reference DNA, each labeled with a different fluorochrome, to normal target metaphase chromosomes. The fluorescence intensity ratio of the test and reference DNA is quantitated and analyzed by using a digital image analysis system. It reveals gains and losses of the test DNA relative to the reference DNA.

### **2.2.1. Application**

Recently, Daniely et al. (1998) used CGH to study 38 products of conceptions of undetermined etiology from couples experiencing recurrent spontaneous abortions (29). Eighteen cases were analyzed both cytogenetically and by CGH. Consistent results were found in 15 cases, while 2 partial unbalances were revealed by CGH only and one case of mosaicism for trisomy 16 was detected by routine cytogenetic analysis but missed by CGH. Twenty samples were analyzed by CGH only, with abnormal results in 12 cases. Ploidy level was not tested in this study (29). Lomax et al. (2000) (30) analyzed 301 spontaneous abortions from couples with RSA or advanced maternal age by both traditional cytogenetic analysis and CGH coupled with flow cytometry. Forty-eight of their cases failed to grow in culture, but 96% of these were successfully analyzed by CGH. Two-third of these specimens were cytogenetically abnormal. Out of 253 samples successfully analyzed by both methods, correlation of results was found in 235. The discrepant results were due to maternal contamination and tetraploidy in 14 cases. Hypertriploidy was identified cytogenetically in 3 cases, whereas CGH showed only aneuploidy. One case showed a gain for chromosome 18, whereas cytogenetics showed a tetraploid karyotype with an unbalanced rearrangement of chromosome 1. This case was interpreted as resulting from sample mislabeling. CGH has been also shown as a valuable technique for cytogenetic analysis of macerated stillbirth (31). Sixteen fetuses with a known karyotype, including 5 macerated fetuses, were successfully analyzed by CGH. Ten stillborn fetuses were also analyzed after tissue culture failure for conventional cytogenetics. Nine fetuses had normal results. The authors' did not provide any explanation for an abnormal CGH pattern with gains of both chromosome 17 telomeric regions, which were not confirmed by FISH with the chromosome 17 long arm telomeric probe. Several probes spanning a region may be needed in some cases to confirm an abnormal CGH result.

### **2.2.2. Limitations**

Comparative genomic hybridization detects chromosomal unbalance but does not allow the diagnosis of a change in the ploidy level. This is of more concern in cases of early spontaneous abortions, for which polyploidies represent 20% of the cytogenetically abnormal cases. However, it is much rarer in stillbirth. Therefore, another test should be added for the detection of polyploidy, whenever CGH shows balanced result.

Several options are possible: a) Flow cytometry, which has been used extensively for the quantitative determination of cellular DNA content of molar pregnancies. It is easy to perform, rapid, accurate and inexpensive (32). It has recently been successfully used in SA analysis (30,31). b) FISH analysis with probes for at least 2 different chromosomes. c) Microsatellites analysis, which also provides further insight into the parental origin of the extra haploid set of chromosomes. The limitation of this process is that the ploidy level cannot be established in cases with combined aneuploidy and polyploidy (e.g., 70,XXY,+2). As polyploidy is not associated with an increased recurrence risk, the genetic counselling based on the report of the aneuploidy only will not be affected. CGH allows the detection of unbalanced rearrangements over 10 Mb and has the same resolution as conventional cytogenetic analysis of cultured tissues from spontaneous abortions (300–350 bands). Although small rearrangements may be missed by CGH, it gives a more precise extent of the genomic imbalance. However, the exact nature of the rearrangement requires conventional cytogenetic analysis. Mosaicism may be missed by CGH testing when the unbalanced cell line is present in <30–40% of the cells (29,33,34). The level of detected mosaicism is likely to depend on the experience of the examiner. Balanced structural rearrangements cannot be detected by CGH. CGH analysis is suboptimal for telomeric regions because of low fluorescence intensity and for centromeric and heterochromatic repeat regions because they are blocked by the unlabeled cot-1 DNA needed to perform this technique (33).

### 3. Collection of Tissues for Molecular Cytogenetic Techniques

Tissue for molecular cytogenetic studies may be obtained from fresh, frozen, formalin-fixed and paraffin-embedded samples. The typical size of the sample is 0.5 cm<sup>3</sup>. Preparations for FISH studies usually consist of making touch preparations on glass slides using fresh or frozen cellular organs such as thymus, lung, kidney, and chorionic villi. If fixed or paraffin-embedded tissues are to be used, a special procedure allowing paraffin removal and rehydration of the tissue should be used prior to making touch preparations (35). DNA extraction for comparative genomic hybridization analysis can be easily done using the same tissues as listed above. Special preparation procedure is again required for fixed and paraffin-embedded tissues (36). For studies of first trimester spontaneous abortion, the pathologist usually submits chorion or chorionic villi. These should be carefully cleaned to avoid maternal contamination. Only when mosaicism is studied, all three cell lineages present in the conceptus (trophoblast, extra-embryonic mesenchyme, and embryonic/fetal tissues) need to be collected. Routinely, chorionic villi are the tissue of choice for both FISH and CGH as they are cellular and, even in a small sample, contain sufficient amounts of DNA.

There is a great temptation to cytogenetically examine only fetal tissue in second trimester spontaneous abortion, or in stillbirth. However, the most common cause of intrauterine death in later stages of pregnancy is an abnormal function of the placenta. Therefore, placental examination should be an integral part of cytogenetic testing of late pregnancy loss. This is essential when the intrauterine death is unexplained and the fetus is retained for a long time after intrauterine death. Samples of both fetal and placental tissue should be submitted for DNA extraction and CGH analysis.

Mosaicism studies are most likely indicated in case of late *in utero* death, severe intrauterine growth retardation and confirmation of a prenatally diagnosed mosaicism. Cytogeneticists require close collaboration with clinicians and pathologists to get as many tissues as possible. Henderson et al. (37) have shown that when confined placental mosaicism is present, the trisomy level may vary between placental sites. Therefore, to confirm the presence of CPM, the pathologist should submit villi from several different placental sites. Detection of a CPM should prompt fetal uniparental disomy studies on the specific chromosome, which is trisomic in the placenta, particularly if uniparental disomy for this chromosome has phenotypic consequences (38).

#### 4. Summary

This chapter presents the summary of two molecular cytogenetic techniques—FISH and CGH—with their applications and limitations in the studies of pregnancy loss. These molecular techniques clearly represent a significant advantage over the traditional cytogenetic technique and likely will become the predominant cytogenetic techniques in reproductive cytogenetics of the future.

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## Interphase FISH Studies of Chronic Myeloid Leukemia

Gordon W. Dewald

### 1. Introduction

#### 1.1. Chapter Goals

This chapter deals with the application of fluorescence *in situ* hybridization (FISH) to study interphase nuclei from patients with chronic myeloid leukemia (CML). In our experience, FISH for *BCR/ABL* fusion detects all forms of the Philadelphia (Ph) chromosome (1,2). FISH is a valuable adjunct to conventional cytogenetic studies and can be applied to the same preparations. Because FISH is useful for quantifying proliferating neoplastic cells in metaphase and non-proliferating cells in interphase, it is particularly useful in assessing response to therapy using either peripheral blood or bone marrow (2,3). This chapter discusses ways to gain experience and validate all aspects of FISH-based testing with *BCR* and *ABL* probes. In addition, methods for assuring quality are discussed for FISH-based testing with the *BCR* and *ABL* probes used in routine clinical practice.

#### 1.2. Definitions

To best appreciate this chapter, the reader should be familiar with the following terminology:

- Abnormal reference range is expressed as the range in percentage of cells with an expected abnormal pattern among patients with CML.
- Atypical pattern refers to unusual signal patterns produced by FISH in cells of some patients with a t(9;22)(q34;q11.2).
- Analytical sensitivity of the FISH test is defined as the percentage of scorable interphase nuclei or metaphase cells with the expected signal pattern for normal or abnormal with *BCR/ABL* fusion.
- Normal cutoff refers to the maximum percentage of scorable cells with false-positive signals for *BCR/ABL* fusion.
- Signal pattern designation: The Ventana *BCR* probe has a red signal (R) and the *ABL* probe has a green signal (G). The Vysis™ *BCR* probe has a green signal (G) and the *ABL*

probe has a red signal (R). The background chromatin is blue with both of these products. A *BCR/ABL* fusion (F) is observed as touching red and green signals or as a yellow signal. The probe letter is used to designate signal patterns, for example, 1R1G2F indicates one red, one green and two fusion signals.

- Typical pattern refers to the most common signal pattern produced by FISH in cells with a  $t(9;22)(q34;q11.2)$ .

### 1.3. Building a New FISH Test

Building a new clinical test involving FISH with chromosome specific probes requires systematic validation of the method and the probes, practice to assure proficiency, experience with many cases to assure consistent and accurate interpretation, and careful documentation of all work to meet the expectations of accrediting agencies (4). To date, no interphase FISH test for CML has been approved by the United States Food and Drug Administration. Under these circumstances, the final report for any patient should indicate “this test was developed and its performance characteristics were determined by your Laboratory Name, and has not been approved by the United States Food and Drug Administration”. Some recommended procedures to validate interphase FISH studies in CML are summarized in the note section of this chapter.

Standards for validating and applying FISH tests in clinical practice are still in the formative stages. Some recommendations for performing FISH testing have been suggested by the American College of Medical Genetics (5). In addition, certain accrediting agencies such as the College of American Pathologists (6), and New York State Department of Health (7) have defined certain quality assurance criteria which they expect laboratories to use in clinical practice. Although probe manufacturers do extensive testing and quality control on their products, it is important to be aware that reliable validation, analysis, and interpretation of FISH results rests with the laboratory that performs the assay.

Several different FISH strategies and commercial products are available for interphase studies in CML. It is important to consider the pros and cons of these various methods when choosing a FISH technique for clinical practice. Although each of these methods are summarized in this chapter, only D-FISH will be described in detail since it provides the best analytical sensitivity.

### 1.4. The Philadelphia Chromosome

A proliferation of cells with a  $t(9;22)(q34;q11.2)$  occurs in the bone marrow and blood of more than 90% of patients with CML (8). This translocation is also observed in 3% of children and 20% of adults with acute lymphoblastic leukemia, and 1% of patients with *de novo* acute myeloid leukemia with immature granulocytes (9–12). The abnormal chromosome 22 associated with  $t(9;22)(q34;q11.2)$  is known as the Ph-chromosome (13).

In our experience and that of others, 5% of the remaining patients with CML have a Ph-chromosome that is derived from complex variants of  $t(9;22)(q34;q11.2)$  (14–16). Complex variants involve three or more chromosomes, but they include break and fusion points at 9q34 and 22q11.2 (15,17). The remaining 5% of patients with CML have normal chromosomes by conventional cytogenetic studies, but are abnormal by

molecular techniques (18). These patients have a masked Ph-chromosome resulting from a submicroscopic insertion involving chromosomes 9 and 22. Therefore, all patients with CML have a  $t(9;22)(q34;q11.2)$  or a variant of this anomaly and "Ph-negative CML" is a nonentity (19).

The  $t(9;22)(q34;q11.2)$  results from a break in the Abelson oncogene (*ABL*) at 9q34 and the breakpoint cluster region (*BCR*) at 22q11.2, and produces a DNA fusion of part of the *ABL* and *BCR* genes on the Ph-chromosome (20). In most patients with CML, the  $t(9;22)(q34;q11.2)$  involves a breakpoint in a 5.8-kb region of the *BCR* gene known as the major *BCR* (21,22). In acute lymphoblastic leukemia, approx 50% of patients with a  $t(9;22)(q34;q11.2)$  have a breakpoint in the major *BCR*; the remaining patients have a breakpoint in the minor *BCR* (23). In a few patients with CML, the Ph-chromosome involves a breakpoint that is outside either the major or minor *BCR* (24,25). The breakpoints on chromosome 9 associated with  $t(9;22)(q34;q11.2)$  vary among patients with CML, but usually occur within a 200-kb region on the centromeric side of the tyrosine kinase domain within the *ABL* locus (26).

### 1.5. *BCR/ABL Fusion and the Ph-Chromosome*

With conventional cytogenetic methods, the observation of a Ph-chromosome may be evidence of CML, but it is not direct evidence of *BCR* and *ABL* fusion. An abnormal *BCR* or *BCR/ABL* fusion can be demonstrated by Southern blot analysis, polymerase chain reaction (PCR), and western blot (27–29). Because these molecular procedures use isolated DNA, mRNA, or protein, they do not demonstrate *BCR/ABL* fusion in individual cells. Several methods are now available to detect *BCR/ABL* fusion using FISH with DNA probes for *ABL* and *BCR* which have been labeled with differently colored fluorophores (1,2,30–32). These FISH methods permit visualization of *BCR/ABL* fusion in individual interphase and metaphase cells (1,2,33,34).

### 1.6. *Ph-Chromosome and Treatment of CML*

Modern treatments for CML have produced several important new challenges for those working in laboratory genetics. First, genetic tests must detect all forms of the Ph-chromosome to accurately diagnose CML as well as Ph-positive acute lymphoblastic leukemia and Ph-positive acute myeloid leukemia. Second, genetic tests must accurately quantify the abnormal cells before and after treatment to help physicians assess the effectiveness of their treatment of the disease. Third, genetic tests must detect and quantify very low levels of abnormal cells in order to address important questions of cure or to predict relapse early.

At the time of this writing, the only known curative therapy in CML is HLA-compatible allogeneic bone marrow transplantation (35). Unfortunately, many patients with CML are not candidates for bone marrow transplantation because of advanced age or lack of a suitable donor. For such patients, treatment options include interferon- $\alpha$ , hydroxyurea, and busulfan. Recent reports suggest that interferon- $\alpha$  and other therapies may prolong duration of the chronic phase and improve survival (36–40). Exciting new treatments involving *BCR/ABL* tyrosine kinase inhibitors such as STI571 appear to hold great promise for the treatment of patients with CML (41–43). These methods are currently in clinical trials and may change the algorithm for treating patients with

CML. Nevertheless, the detection and quantification of *BCR/ABL* fusion continues to be an important monitor of therapy for CML.

### 1.7. Methods of FISH Using DNA Probes for *BCR* and *ABL*

Some investigators have created their own “home brew” probes to detect fusion of *BCR/ABL*. However, commercial probes are preferred by most laboratories because using them avoids the rigorous quality control processes needed to develop and validate “home brews”. Therefore, this chapter will focus on commercial DNA probes for *BCR* and *ABL* as these are the products widely used in clinical practice today.

The first commercial FISH strategies to detect *BCR/ABL* fusion were reported in the early 1990s (1). Since then, several other commercial FISH strategies have become available to detect fusion of *BCR* and *ABL* loci (2,32,44). As a consequence, various laboratories employ different strategies today. Because the performance characteristics of the various strategies differ, the application of FISH technology can appear to produce inconsistent results. To appreciate the results of FISH studies from any laboratory, it is important to know the strategy used and to be familiar with its strengths and weaknesses.

The initial commercial FISH strategy for *BCR/ABL* fusion produced a single *BCR/ABL* fusion signal on the Ph-chromosome (1). This method is referred to as single fusion FISH or S-FISH (Fig. 1 and Fig. 2A). A second strategy produced two *BCR/ABL* fusion signals, one on the Ph-chromosome and one on the abnormal chromosome 9 (2). This strategy is referred to as double fusion FISH or D-FISH (Fig. 1 and Fig. 2B). A third strategy produced a *BCR/ABL* fusion signal on the Ph-chromosome and an extra signal on the abnormal chromosome 9 (45). This strategy is referred to as extra signal FISH or ES-FISH (Fig. 1 and Fig. 2C).

The normal cutoff of the different FISH strategies for detecting *BCR/ABL* fusion varies significantly. In the analysis of 500 nuclei, D-FISH detects neoplastic cells at levels that exceed 1%, ES-FISH at levels over 3% and S-FISH at levels greater than 10% (46). The most widely used strategies in clinical practice today are S-FISH and D-FISH. Compared with other strategies, D-FISH has the best sensitivity in detection of disease, the best precision in estimation of tumor burden and detection of atypical forms of *BCR* and *ABL* signal patterns that may have special prognostic importance.

#### 1.7.1. S-FISH

S-FISH uses *ABL* and *BCR* probes derived from DNA sequences that flank the *BCR/ABL* fusion site on the Ph-chromosome. One of the original commercial probe mixtures utilized an *ABL* probe with two overlapping biotin-labeled cosmids hybridizing to 9q34, telomeric to the 200-kb breakpoint region of *ABL* between exons Ib and II (1). The mixture also included three overlapping digoxigenin-labeled cosmids hybridizing to the major *BCR* at 22q11.2. This probe contained part of the 5.8-kb major *BCR* and adjacent regions extending to the centromeric side of the major and minor *BCR*.

Specimens were processed with fluorescein-conjugated avidin and rhodamine-conjugated anti-digoxigenin to visualize the *ABL* and *BCR* signals in different colors.

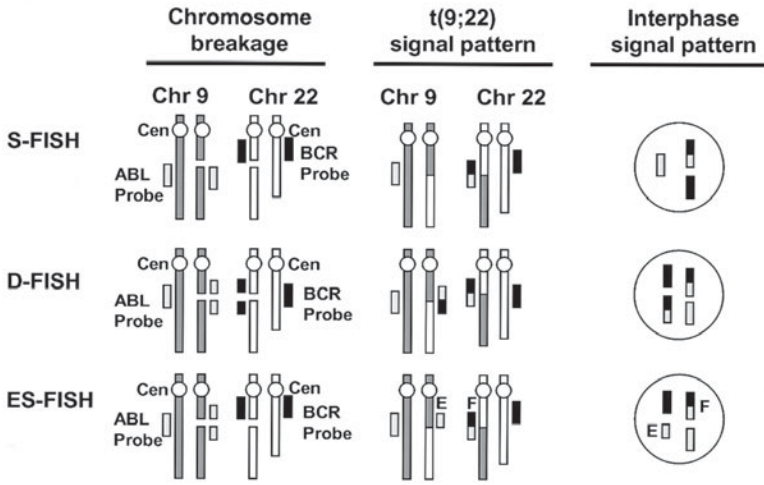


Fig. 1. Illustration of three common commercial strategies that use FISH to detect *BCR/ABL* fusion. S-FISH uses *ABL* probes that hybridize to the telomeric side of the breakpoint on chromosome 9 and *BCR* probes that hybridize to the centromeric side of the breakpoint on chromosome 22. A reciprocal 9;22 translocation produces a *BCR/ABL* fusion signal on the abnormal chromosome 22. In interphase nuclei, one red, one green, and one yellow signal is observed (**Fig. 2A**). D-FISH uses *BCR* and *ABL* probes that hybridize to regions that span the breakpoints on chromosomes 9 and 22. A reciprocal 9;22 translocation produces adjacent *BCR* and *ABL* signals on both the abnormal chromosome 9 and 22. In interphase nuclei 1R1G2F signals are observed (**Fig. 2B**). ES-FISH uses *ABL* probes that hybridize to a region that spans the breakpoint on chromosome 9, and *BCR* probes that hybridize to the centromeric side of the *BCR* on chromosome 22. A reciprocal 9;22 translocation produces a *BCR/ABL* fusion signal on the abnormal chromosome 22, and a small *ABL* signal on the abnormal chromosome 9. In interphase nuclei two red, one green, and one yellow signal is observed (**Fig. 2C**).

The chromosomes were counter-stained with 4'-6'-diamidino-2-phenylindole dihydrochloride (DAPI). The cells were viewed through a fluorescence microscope with either a dual-pass filter for fluorescein isothiocyanate (FITC) and TexasRed, or a triple-pass filter for DAPI, FITC, and TexasRed (Chromotek, Battleboro, VT). With this method, the *ABL* probe had a green signal, the major *BCR* probe had a red signal, and the background chromatin was blue. Any translocation involving the fusion of *ABL* and major *BCR* regions resulted in two close red and green signals or a single fused red/green signal which usually appeared yellow (**Fig. 2A**).

This method detected all forms of the Ph-chromosome, but randomly overlapping signals were frequent, and the normal cutoff to detect neoplastic nuclei with *BCR/ABL* fusion was 10% (**1**). Many investigators found the fluorescence of the *BCR* and *ABL* signals with this method small and dim. These problems were associated primarily with using indirect labeling techniques and small probes. These *BCR* and *ABL* probes are no longer available commercially.

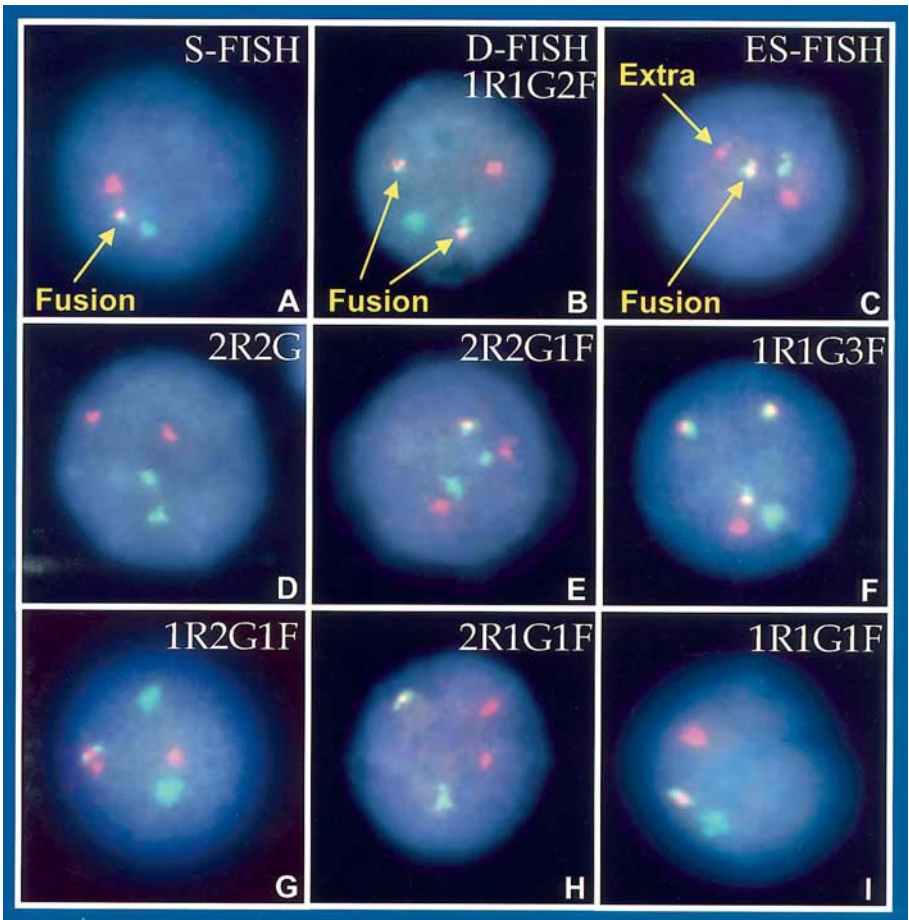


Fig. 2. Three commercial FISH strategies can detect fusion of *BCR* and *ABL* in neoplastic nuclei of patients with CML. (A) In nuclei with a  $t(9;22)(q34;q11.2)$ , S-FISH produces one red, one green, and 1 yellow fusion signal. (B) D-FISH produces 1R1G2F signals. (C) ES-FISH produces one red, one green, one small red, and one yellow fusion signal. Representative Vysis™ D-FISH patterns for *BCR* and *ABL* in interphase nuclei from different patients with CML. (D) Normal nuclei have 2R2G signals. (E) Nuclei with a complex Ph-chromosome have 2R2G1F signals. (F) Nuclei with a  $t(9;22)(q34;q11.2)$  and an additional Ph-chromosome have 1R1G3F signals. (G–I) Representative nuclei with atypical D-FISH patterns produced by cytogenetic mechanisms described in Fig. 3.

Subsequently another commercial company produced a product to detect large and bright *BCR/ABL* fusion signals with an S-FISH method (44). These probes are available today and widely used to study specimens from patients with CML. The method utilizes a mixture of *BCR* probes, directly labeled with SpectrumGreen™ fluorophore,

and *ABL* probes, directly labeled with SpectrumOrange™ fluorophore. The hybridization site of the *ABL* probes cover about 200 kb extending from exons 4 and 5 of the *ABL* gene toward the telomere of chromosome 9. The hybridization site of the *BCR* probes cover about 300 kb from exons 13 and 14 of the *BCR* gene (major *BCR* exons 2 and 3) toward the centromere on chromosome 22, crossing well beyond the minor *BCR* region.

Cells can be viewed with a fluorescence microscope equipped with a triple-pass filter. With this method, the *ABL* signal appears orange, the *BCR* signal green, and the background chromatin blue. Any translocation involving the fusion of *ABL* and *BCR* regions results in adjacent or fused orange and green signals that sometimes appear yellow.

Because the *BCR* and *ABL* signals are large, the incidence of randomly overlapping signals is great. Consequently, the normal cutoff of this method for detecting neoplastic cells with *BCR/ABL* fusion is 12.5% (44).

With S-FISH it is important to use strict scoring criteria to study interphase nuclei (1). The most consistent results are obtained when investigators score only interphase nuclei in which it is possible to account for both copies of the *ABL* and the major *BCR* probes. Thus, most qualifying interphase nuclei either have two red and two green signals (normal nuclei) or one red, one green, and one fusion signal (nuclei with *BCR/ABL* fusion). When *BCR* and *ABL* signals are overlapped or immediately adjacent to one another, they are scored as a fusion signal. Most investigators also score interphase nuclei with one red, one green, and two fusion signals because this observation is consistent with an extra Ph-chromosome.

### 1.7.2. D-FISH

The original D-FISH strategy using commercial *BCR* and *ABL* probes is widely used in clinical practice today (2). The method uses differently colored, direct-labeled probes for *BCR* and *ABL*. The *ABL* probe set includes several DNA sequences that hybridize to 9q34 and span the 200-kb breakpoint region of *ABL*. The *BCR* probe set includes several DNA sequences that hybridize to 22q11.2 and span the common breakpoints in both the major and minor *BCR*.

Nuclei can be viewed with a fluorescence microscope equipped with a dual-pass filter for FITC and TexasRed, or a triple-pass filter for DAPI, FITC, and TexasRed. The *BCR* probe has a red signal, the *ABL* probe a green signal and the background chromatin is blue. Based on extensive studies, criteria have been developed for D-FISH that limit the scoring process to cells that have either normal or abnormal signal patterns (2). Normal nuclei have 2R2G signals (Fig. 2B). Abnormal nuclei with one Ph-chromosome either have 1R1G2F or 2R2G1F signals (Fig. 2D–F). Nuclei with two copies of the Ph-chromosome have 1R1G3F or 2R2G2F signals (Fig. 2F).

Some investigators suggest that the scoring process is complex for D-FISH. However, with experience this method is highly sensitive, consistent and can be mastered by most laboratories (47). The normal cutoff of the D-FISH test for detecting neoplastic nuclei with a t(9;22)(q34;q11.2) or a complex t(9;22) is 1% when 500 nuclei are examined, and 0.079% when 6000 nuclei are studied (2).

An important observation from early studies of patients with a  $t(9;22)(q34;q11.2)$  indicated that approx 20% of patients with a  $t(9;22)(q34;q11.2)$  had atypical D-FISH patterns (48). It is important to use different normal cutoff criteria for detection of neoplastic disease when studying such patients with D-FISH. Some investigators suggest that patients with atypical D-FISH patterns may have a different prognosis than patients with typical D-FISH patterns when treated with standard therapies for CML (49).

Until recently, a commercial test using the D-FISH strategy with *BCR* and *ABL* probes was available from only one company (Ventana Medical Systems, Tucson, AZ). However, a similar test with *BCR* and *ABL* probes has become available from another company (Vysis Inc., Downers Grove, IL). The Vysis™ *ABL* probe spans a genomic target of approx 650 kb extending from an area centromeric of the argininosuccinate synthetase gene (*ASS*) to well telomeric of the last *ABL* exon. The *BCR* probe target spans a genomic distance of approx 1.5 Mb. The *BCR* probe begins within the variable segments of the immunoglobulin lambda light chain locus, extends along chromosome 22 through the *BCR* gene, and ends at a point approx 900 kb telomeric of *BCR*. Both the *ABL* and *BCR* probes span their respective breakpoints associated with  $t(9;22)(q34;q11.2)$ .

We compared the Vysis™ and Ventana D-FISH probes and found that they had the same normal cutoff and abnormal reference range. These probes produced similar percentages of abnormal nuclei and the same signal patterns in patients with CML. This comparison was based on bone marrow and blood specimens from 10 normal individuals and 13 patients with various forms of the Ph-chromosome. This series included three patients who had responded to treatment and ten patients at diagnosis of CML.

### 1.7.3. ES-FISH

In our experience the normal cutoff for ES-FISH is 3%. This method detects most, but not all, forms of atypical signal patterns associated with the *BCR* and *ABL* hybridization sites (46,48). ES-FISH is not widely used in clinical practice today.

One "home brew" ES-FISH method uses three differently colored DNA probes for *BCR*, *ABL*, and a probe for the *ASS* gene which is located on the centromeric side of *ABL* on chromosome 9 (32). The method uses SpectrumGreen™-labeled probes extending from *BCR* exons 13 and 14 (b2 and b3) for approx 300 kb in the 5' direction and a SpectrumOrange™-labeled probe extending for approx 200 kb in the 3' direction from exons 4 and 5 on *ABL*. These probes are supplemented by another probe such as the *ASS* gene, which is labeled with a fluor of a color other than SpectrumGreen™ or SpectrumOrange™, for example aqua. The *ASS* probe hybridizes to 9q34 on the centromeric side of the *ABL* breakpoint and remains on the abnormal chromosome 9 derived from the  $t(9;22)(q34;q11.2)$ . A  $t(9;22)(q34;q11.2)$  rearranges the various probe hybridization sites so that in metaphases the observer sees a yellow fusion signal on the Ph-chromosome, and a small aqua signal on the abnormal chromosome 9. In interphase nuclei, one red, one green, one aqua, and one yellow signal is observed.

One commercial ES-FISH method uses two differently colored probes, one probe spans the *ABL* breakpoint and the other probe hybridizes to the centromeric side of the

*BCR* (**Fig. 1, [50]**). The t(9;22)(q34;q11.2) rearranges the hybridization sites so that the observer sees a yellow fusion signal on the Ph-chromosome, and a small red signal on the abnormal chromosome 9. In interphase, two red, one green, and one yellow signal is observed (**Fig. 2C**).

## 2. Materials

1. Coverslips, circle 12-mm (Fisher Scientific, Pittsburgh, PA).
2. DAPI1 Counterstain (Vysis, Inc., Downers Grove, IL): Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
3. DAPI1 Working solution: Mix 1 part DAPI1 with 9 parts Vectashield. This is a 10% solution. Use within 6 mo.
4. Ethanol (70%): Add 300 mL of distilled water to 700 mL of 100% ethanol. Mix well and store at room temperature. Use within 1 yr.
5. Ethanol (85%): Add 150 mL of distilled water to 850 mL of 100% ethanol. Mix well and store at room temperature. Use within 1 yr.
6. Ethanol (100%): Pure grain alcohol (ethanol). Store a 1000 mL aliquot at room temperature. Use within 1 yr.
7. 70% Formamide solution: Mix in a ratio of 7 parts formamide, 2 parts distilled water, and 1 part 20X SSC solution. Adjust pH to 6.3. Store at  $4^{\circ}\text{C}$ . Use within 3 mo.
8. Formamide ( $\text{CH}_3\text{NO}$ ) (US Biochem, Cleveland, OH): 500 g lots. Store at  $4^{\circ}\text{C}$ . Use within 1 yr.
9. Fixative (2.5:1): 25 mL methanol and 10 mL Glacial acetic acid. Prepare fresh each time.
10. Glacial acetic acid: Reagent grade, meets ACS specifications. 2.5 L lots. Use within 1 yr.
11. LSI<sup>®</sup> *BCR/ABL* dual color dual fusion probe (Vysis, Inc., Downers Grove, IL.): Direct labeled probes that span *BCR* (green) at 22q11.2 and *ABL* (red) at 9q34. Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
12. LSI<sup>®</sup> *BCR/ABL* probe working solution: Make up probe solution in a ratio as follows: 8 parts LSI/wcp<sup>®</sup> hybridization buffer: 1 part distilled water, 1 part LSI<sup>®</sup> *BCR/ABL* dual color dual fusion probe. Store at  $-20^{\circ}\text{C}$ .
13. LSI/wcp<sup>®</sup> hybridization buffer (Vysis, Inc., Downers Grove, IL.): 150  $\mu\text{L}$  lots. Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
14. Methanol: methanol anhydrous (absolute) acetone free. Analytical reagent. 4 L lots. Use within 2 yr.
15. Nonidet P40 (NP40) Vysis, Inc., (Downers Grove, IL.): 1 mL lots. Store at  $-20^{\circ}\text{C}$ . See product label for expiration date.
16. 0.4X SSC Solution: Add 20 mL of 20X SSC solution (pH 6.3) to 980 mL distilled water and mix thoroughly. Adjust pH to 7.0. Store at room temperature. Use within 6 mo.
17. 2X SSC Solution, pH 7.0: 17.53 g sodium chloride, 8.82 g sodium citrate, and bring to 1 L with distilled water or 100 mL 20X SSC solution, pH 6.3, with 900 mL distilled water, adjust pH to 7.0. Store at room temperature. Use within 6 mo.
18. 2X SSC/0.1% NP40: Mix 100 mL 20X SSC, pH 5.3, with 850 mL distilled water. Add 1 mL NP0 and adjust pH to 7.0. Bring final vol to 1 L. Store at room temperature. Use within 6 mo.
19. 20X SSC Solution, pH 6.3: 175.3 g sodium chloride, 88.2 g sodium citrate, bring to 900 mL with distilled water; or add 132 g of 20X SSC powder (Vysis, Inc., Downers Grove, IL) to final vol of 500 mL distilled water. Adjust pH to 6.3 with concentrated HCl. Store at room temperature. Use within 6 mo.

20. 20X SSC (powder) (Vysis, Inc., Downers Grove, IL.): 500 g lots. Store at room temperature. See product label for expiration date.
21. Vectashield (Vector Laboratories, Inc. Burlingame, CA.): Store at 4°C. See product label for expiration date.

### 3. Methods

Purpose: The method described here uses a Vysis™ D-FISH *BCR/ABL* probe strategy with probes that span the common breakpoints associated with *BCR* and *ABL* for patients with a t(9;22)(q34;q11.2).

#### 3.1. Specimens

1. The best results are obtained with bone marrow and peripheral blood that have been cultured and harvested according to the standard cytogenetic procedure for hematological malignancies.
2. It is important to use fresh slide preparations for FISH studies. Cells fixed in methanol and glacial acetic acid can be stored at -70°C until FISH studies are needed.
3. Blood and bone marrow smears can be processed with this method.
4. A Ph-positive control slide preparation containing both normal and abnormal cells should be run with each batch of patient samples.

#### 3.2. Slide Preparation

1. Use a phase contrast microscope to scan the microscope slide to locate a suitable hybridization area that contains an adequate number of interphase cells. The cells should have good morphology and overlap of cells should be minimal. If the slide is unsatisfactory, it is best to prepare another slide rather than continue with the procedure.
2. Incubate freshly prepared slide for 1 h in a 65°C drying oven or 10 min in a 90°C drying oven. Avoid prolonged baking of the slide at high temperatures (60–90°C).
3. Place the slide in a Coplin jar of 2X SSC solution at 37°C for 1 h.
4. Dehydrate the slide preparation for 1 min each in a series of Coplin jars with 70, 85, and 100% ethanol at room temperature. Jet air dry slide.

#### 3.3. Denaturation and Hybridization

Select either the Coplin jar method at in **Subheading 3.3.1.** or the HYBrite™ method in **Subheading 3.3.2.**

##### 3.3.1. Coplin Jar Method

1. Denature the slide preparation in a Coplin jar with 70% formamide solution at 74°C for 1 min for metaphase studies or 2 min for interphase studies.
2. Dehydrate the slide preparation for 1 min each in a series of Coplin jars with 70, 85, and 100% ethanol at room temperature. Jet air dry slide.
3. Pipet 3 µL of LSI® *BCR/ABL* probe working solution into a 0.65 mL microcentrifuge tube. Centrifuge for 10 s.
4. Denature the probe mix for 5 min by floating the centrifuge tube in a 74°C waterbath.
5. Remove the centrifuge tube from the waterbath and immediately apply the probe mix to the slide preparation and place the slide on a 45°C slide warmer.
6. Apply a round 12-mm coverslip on the hybridization area. Seal the coverslip with rubber cement. Incubate the slide preparation in a humidified chamber in a 37°C oven for 8–20 h.
7. Go to **Subheading 3.2.6.** to proceed with the post-hybridization wash.

### 3.3.2. HYBrite™ (Vysis, Inc., Downers Grove, IL) Codenaturing Method

1. Pipet 3  $\mu\text{L}$  of LSI® *BCR/ABL* probe working solution directly on the slide preparation. Apply a round 12-mm coverslip on the hybridization area. Seal the coverslip with rubber cement.
2. Flood HYBrite™ canals with water. Be careful to avoid overfilling the channels as this can compromise the DNA denaturation process.
3. Set HYBrite™ settings as follows: melt temperature, 80°C; melt time, 5 min; hybridization temperature, 37°C; and hybridization time, 20 h.
4. Place slides in the HYBrite™ and allow the machine to perform the hybridization process.
5. Remove slides from the HYBrite™ and go to **Subheading 3.4.** to proceed with the post-hybridization wash.

### 3.4. Post-Hybridization Wash

1. Remove the rubber cement. Carefully remove the coverslip and discard.
2. Place the slide in a Coplin jar with 0.4X SSC solution at 74°C for 2 min.
3. Remove the slide and rinse it in a Coplin jar with 2X SSC/0.1% NP40 solution at room temperature for 1 min.
4. Remove the slide from the Coplin jar and touch the edge of the slide to a paper towel to remove the excess 2X SSC/0.1% NP40.
5. Apply 10  $\mu\text{L}$  of DAPII working solution to the hybridization area.
6. Place a coverslip over the area. Gently apply pressure to the coverslip to remove air bubbles. The slide is now ready for analysis by microscopy.

### 3.5. Microscopy

1. Use a high quality fluorescence microscope equipped with a 100 W mercury lamp.
2. Use a dual-pass FITC and TexasRed set to simultaneously view signals. Use a single-pass FITC and TexasRed sets to view individual fluorors.
3. Use a computer based imaging system with appropriate software to capture images of cells for documentation.

### 3.6. Scoring Criteria

1. The Vysis™ *BCR* probe has a green signal (G), the *ABL* probe has a red signal (R) and the background chromatin is blue. A *BCR/ABL* fusion is observed as touching red and green signals or as a yellow signal. Hereafter the *BCR/ABL* fusion signal is referred to as F in this chapter.
2. Normal interphase or metaphase cells have 2R2G signals and no F signal (**Fig. 2**).
3. Abnormal nuclei with one Ph-chromosome either have 1R1G2F or 2R2G1F. Nuclei with two Ph-chromosomes have 1R1G3F or 2R2G2F (*see Notes 4.1 and Fig. 2*).
4. Ph-positive metaphase or interphase cells with atypical abnormal D-FISH patterns will have five or less signals and at least one fusion signal. Atypical signal patterns include 2R1G1F, 1R2G1F and 1R1G1F (*see Notes 4.4 and Fig. 2*).
5. If there is any doubt as to whether or not a cell should be scored, or as to whether or not a fusion signal is present, do not score the cell.

### 3.7. Analysis of Slide Preparations

1. Score 100 qualifying nuclei for a positive control before analyzing patient samples to be sure the method worked.
2. If possible, analyze ten or more Ph-positive metaphases prior to treatment in order to establish the signal pattern of the Ph-chromosome. This information is important to know

whether typical scoring criteria or atypical scoring criteria should be used to analyze interphase nuclei (see **Notes 4.1** and **4.4**).

3. Capture at least two representative metaphases to document these studies.
4. Two observers should independently score 250 consecutive qualifying interphase cells from two or more separate areas of the hybridization site.
5. If the results of the two observers differ by more than 5%, a third observer should score 250 consecutive interphase cells, or a second hybridization should be considered.
6. If the results of the two observers are acceptable, the results may be averaged together to calculate the percentage of cells with *BCR/ABL* fusion in the specimen.
7. If the patient has a Ph-chromosome associated with a typical signal pattern and the results are within normal limits (<1% abnormal nuclei), then each observer should analyze 2750 more nuclei for a total of 6000 nuclei to look for minimal residual disease (see **Notes 4.2** and **4.6**).
8. Capture an image of at least one area of the slide to document several nuclei that represent the final result.

### 3.8. Interpretation of Results

1. Experienced observers should rarely, if ever, observe nuclei with false-positive signal patterns in normal specimens (see **Notes 4.7–4.15**). For these observers, the normal cutoff for analysis of 500 nuclei is less than 1%, and for 6000 nuclei is less than 0.079% (see **Notes 4.1–4.4**).
2. Experienced observers should detect more than 90% abnormal interphase cells in most patients with untreated CML (see **Notes 4.1**).
3. The frequency of false-positive nuclei is different for patients with atypical signal patterns. The normal cutoff for patients with 1R2G1F is 1.2%, 2R1G1F is 1.8%, and 1R1G1F is 23.0% (see **Notes 4.4**).

### 3.9. Quality Control (see **Notes 4.16**)

1. Any new reagents should be tested on a control specimen before use in clinical practice.
2. A Ph-positive control slide should be run with each batch of patient specimens.
3. Each specimen should be analyzed independently by two scorers, and their results should be within 5% of one another.
4. A chart summarizing the results of each scorer for the control specimen should be maintained and routinely evaluated for trends that might suggest technical problems.
5. All test failures should be investigated and documented to establish the source of the problem.
6. A monthly result report is produced to assess test performance.
7. For more information on quality assurance (see **Notes 4.7–4.16**).

## 4. Notes

### 4.1. Signal Patterns Vary According to Type of Ph-Chromosome

In our experience, the best testing method to detect all the various forms of the Ph-chromosome uses the D-FISH strategy (2). The typical D-FISH pattern in nuclei with t(9;22)(q34;q11.2) is 1R1G2F (**Fig. 2B**). This observation corresponds with two fusion signals, one on chromosome 22 and one on chromosome 9 and occurs in approx 80% of patients with a t(9;22)(q34;q11.2). When more than one Ph-chromosome is present, D-FISH will produce a *BCR/ABL* fusion signal for each copy of the Ph-chromosome.

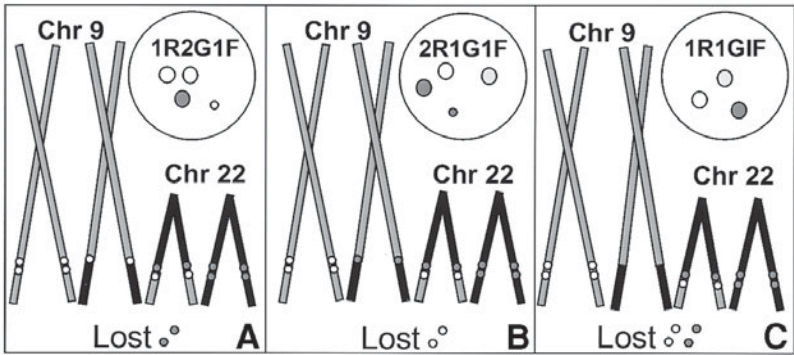


Fig. 3. Representative interphase nuclei showing the origin of three atypical D-FISH patterns in cells with a  $t(9;22)(q34;q11.2)$ . (A) Nuclei that have one red, two green, and one fusion signal, as in Fig. 2G, have loss of the *BCR* hybridization site that is normally translocated to the abnormal chromosome 9. (B) Nuclei that have two red, one green, and one yellow fusion signal, as in Fig. 2H, have loss of the *ABL* hybridization site that normally remains on the abnormal chromosome 9. (C) Nuclei that have one red, one green, and one fusion signal as in Fig. 2I have loss of the *ABL* and *BCR* hybridization sites that are normally observed on the abnormal chromosome 9.

Patients with complex Ph-chromosomes have different signal patterns by D-FISH. For example, consider a patient with a  $t(5;9;22)(q31;q34;q11.2)$ . In metaphases a *BCR/ABL* fusion signal would be observed on the Ph-chromosome, a small *ABL* signal on the abnormal chromosome 9, and a small *BCR* signal on the abnormal chromosome 5. In interphase the predominant signal pattern would be 2R2G1F (Fig. 2E). The *ABL/BCR* fusion that is normally observed on the abnormal chromosome 9 with D-FISH does not occur in complex translocations.

Unusual *BCR/ABL* signals are also observed in patients with masked Ph-chromosomes. FISH studies show that masked Ph-chromosomes originate from small insertions involving the *BCR* and *ABL* loci. These insertions are not visible by conventional cytogenetic studies, but are readily detectable by FISH (2). In our experience, approx 50% of patients with masked Ph-chromosomes have a *BCR/ABL* fusion signal on the abnormal chromosome 22. The remaining 50% have *BCR/ABL* fusion on the abnormal chromosome 9. In interphase nuclei, the predominant D-FISH patterns of masked Ph-chromosomes are 1R2G1F or 2R1G1F. It is important to use a FISH technique to monitor response to therapy for patients with a masked Ph-chromosome because conventional cytogenetic methods cannot detect this abnormality.

Three kinds of atypical D-FISH patterns occur in approximately 20% of patients with a  $t(9;22)(q34;q11.2)$  (48). In these patients, there is loss of a portion of *BCR* or *ABL* or portions of both of these hybridization sites normally associated with the break and fusion point on the abnormal chromosome 9 (Fig. 2G-I and Fig. 3) (see Notes 4.4).

Laboratory personnel need to be aware of these variant signal patterns as they will need to adjust scoring criteria and will need to utilize different normal values. It is useful to examine a few metaphases at diagnosis to establish the exact *BCR/ABL* signal

pattern for each patient. In studies after therapy, it is important for the technologist to be aware of the signal pattern for each patient so that an appropriate study can be made.

The literature is mixed with regard to the clinical significance of patients with CML who have variant Ph-chromosomes compared to those with typical Ph-chromosomes (15,49,51). Several clinical trials are underway to test new forms of therapy for CML. This will be an important opportunity to establish the clinical significance of patients with variant translocations and to determine if new therapies are equally effective for all patients with different variant Ph-chromosomes.

#### **4.2. FISH to Monitor Therapy in CML**

Conventional cytogenetic studies of bone marrow are widely used in clinical practice to monitor the effectiveness of various forms of treatment for patients with CML, especially for interferon and STI571 therapy (36,39,52–54). Considerable evidence demonstrates a strong correlation between prognosis and changes in percentage of Ph-positive metaphases after interferon therapy. The best outcome for survival and prolonged chronic phase seems to be enjoyed by patients with CML in whom the percentage of Ph-positive metaphases is reduced to < 33% of all metaphases (36–38,40,55).

In clinical practice, physicians usually collect bone marrow aspirates from patients with CML on interferon therapy at 3–6 mo intervals to obtain cytogenetic data. For technical reasons, such as packed bone marrow or hypoplasia, it is not always possible to obtain suitable bone marrow specimens for chromosome studies. Although peripheral blood is easier to collect from patients, the number of mitotic cells in blood after treatment is usually inadequate to accurately quantify the proportion of neoplastic cells by conventional cytogenetic studies.

The analysis of interphase nuclei from blood and bone marrow with FISH is also useful to study patients with CML before and after treatment to assess the effectiveness of therapy (2,3,50,56,57). At diagnosis the percentage of neoplastic cells is usually high, whereas after effective therapy the percentage of neoplastic cells progressively decreases (Fig. 4). The best FISH tests to assess response to therapy are those methods that have a high analytical sensitivity, such as D-FISH and ES-FISH (3,50). With such tests, the percentage of Ph-positive nuclei in bone marrow prior to treatment is usually 85–99%. When treatment is successful, the post-treatment percentage of neoplastic nuclei can be accurately tracked down to 1% by D-FISH and to 3% by ES-FISH.

A few studies have investigated the utility of FISH to study blood to monitor response to therapy in CML (3,50,58). One such study involved 37 paired sets of bone marrow and blood specimens collected within 24–96 h of each other, from 10 patients before and during treatment of CML with interferon (3). The investigators learned that analysis of 500 nuclei with D-FISH from bone marrow and peripheral blood could detect less than 1% neoplastic cells and produced results far more sensitive than quantitative cytogenetics. Thus, the use of D-FISH to score 500 interphase nuclei from either bone marrow or blood can substitute for quantitative cytogenetics for purposes of monitoring response to therapy for CML.

The percentages of neoplastic nuclei detected by D-FISH in blood and bone marrow prior to treatment differs in approx 70% of patients (Fig. 5; [3]). Although it is

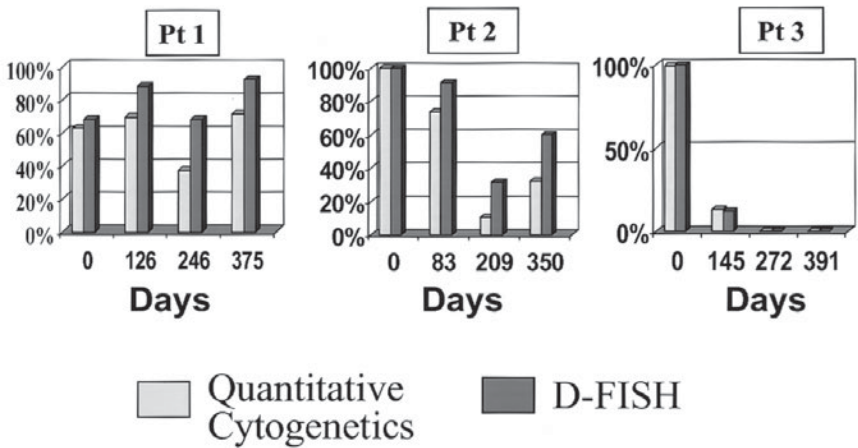


Fig. 4. D-FISH and conventional cytogenetic studies are useful to monitor patients with CML who undergo treatment with interferon. The results of these two methods may differ in their estimates of the percentage of Ph-positive cells, but changes in response to therapy over time correlate closely. The results of D-FISH and cytogenetics for patient 1 are consistent with a non-responder. Patient 2 initially responds to therapy but then relapses. Patient 3 is a complete responder and achieves a cytogenetic remission with no Ph-positive metaphases and normal D-FISH results.

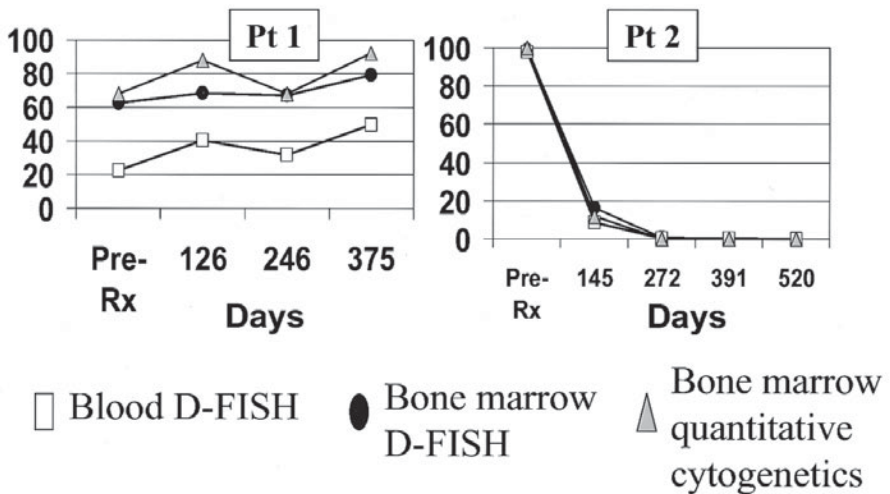


Fig. 5. D-FISH can be used to study peripheral blood to monitor response to interferon therapy in CML. In some patients, such as patient 1, the percentage of neoplastic cells in bone marrow is higher than blood. In other patients, such as patient 2, the tumor burden is similar in blood and bone marrow. Over time, changes in percentage of neoplastic cells are reflected in a similar fashion in both bone marrow and blood.

possible to use a simple linear regression model to predict the changes in percentages of Ph-positive nuclei in bone marrow using data collected from D-FISH studies of peripheral blood, the accuracy of such predictions is not great. Nevertheless, similar changes occur in the percentage of neoplastic nuclei in blood and bone marrow over the course of therapy, and correspond relatively closely to the results of quantitative cytogenetic studies of cells from bone marrow. This indicates that D-FISH is useful to test blood from patients with CML to monitor therapy.

Based on analysis of 6,000 nuclei with D-FISH, very low levels of abnormal cells can be identified in both bone marrow and blood from patients in complete cytogenetic remission (3). Consequently, analysis of interphase nuclei from blood with D-FISH can substitute for quantitative cytogenetic studies on bone marrow, even when the patient is in complete cytogenetic remission. Thus, it may not be necessary to collect bone marrow samples frequently to monitor therapy in patients with CML.

One important new challenge for genetic testing is becoming apparent as more patients with CML are treated with STI571. Some of these patients become “drug resistant” owing to amplification of the *BCR/ABL* fusion gene. This outcome can be detected in individual neoplastic cells with FISH by the observation of clusters or multiple *BCR/ABL* fusion signals. Organized studies of series of patients with STI571 drug resistant for *BCR/ABL* fusion need to be performed to understand how to accurately score amplification signals for *BCR/ABL* fusion and to learn the clinical relevancy of amplification of *BCR/ABL* fusion.

### **4.3. Metaphase vs Interphase FISH Studies**

Most physicians working with CML patients compare the percentage of Ph-positive metaphases before and after therapy to assess the effectiveness of that therapy. Different classification schemes for response to therapy based on percentage of Ph-positive metaphases have been proposed (36,39,40,52,53). One of the more popular schemes is used by investigators from M. D. Anderson Cancer Center and classifies response to therapy as complete, partial, minor, or absent (40). These categories are based on observing a Ph-chromosome in 0% of metaphases, 1–34% of metaphases, 35–95% of metaphases, and >95% of metaphases, respectively.

A common misconception is that patients with untreated CML always have 100% Ph-positive metaphases before treatment and that the percentage of metaphases observed after treatment translates directly into the change in percentage of Ph-positive metaphases after therapy. However, only 90% of patients with CML actually have a Ph-chromosome in each metaphase before treatment by conventional cytogenetic studies (14). The remaining 10% of patients with CML have masked Ph chromosomes or mosaicism, i.e., a mixture of normal and Ph-positive metaphases. To adjust for mosaicism when using quantitative cytogenetics and D-FISH, it may be useful to standardize the percentage of neoplastic cells after therapy to the percentage of neoplastic cells before treatment. One approach is to divide the percentage of neoplastic cells before therapy into the percentage of neoplastic cells after therapy and then multiply by 100.

A second misconception is that the percentage of interphase nuclei with *BCR/ABL* fusion, as determined by FISH, can be applied to the same “response-to-therapy”

scheme applied to results of conventional cytogenetic studies. Unlike conventional cytogenetic studies, the results of D-FISH investigations indicate that all patients with CML have both normal and neoplastic cells in their bone marrows even before treatment. This observation is owing to the ability of highly sensitive FISH methods to detect small percentages of normal cells in patients with CML before treatment.

Moreover, studies with FISH of interphase nuclei evaluate non-proliferating cells. By comparison, standard cytogenetics tests evaluate proliferating cells. Cytogenetic studies usually involve up to 25 metaphases and, therefore, would be significantly affected by sampling error. For example, if 25 metaphases are examined, a sampling error of only one Ph-positive metaphase results in 4% error in the estimate of tumor burden. By comparison, FISH studies commonly analyze 500 interphase nuclei. Thus, a sampling error of one nucleus with *BCR/ABL* fusion results in an error of only 0.20% in the estimation of tumor burden.

Because of these and other differences between metaphase and interphase analyses, it is not surprising that the percentage of metaphases with a Ph-chromosome is not the same as the percentage of interphase nuclei with *BCR/ABL* fusion. Nevertheless, several investigations have shown that for patients whose percentage of Ph-positive metaphases is reduced because of therapy, a corresponding reduction in percentage of nuclei with *BCR/ABL* fusion also occurs (3,50). Thus, the results of FISH can be used to assess response to therapy, but the percentage of Ph-positive metaphases may not be the same as the percentage of nonproliferating cells with *BCR/ABL* fusion.

The CML National Study Group recently compared the percentage of metaphases with a Ph-chromosome with the percentage of interphase nuclei that had *BCR/ABL* fusion in a series of patients with CML (59). This work involved 65 patients from a randomized, double blind clinical trial of interferon- $\alpha$  versus interferon- $\alpha$  plus cytosine arabinoside. Progress of the patients was assessed by computing the change in percent of Ph-positive metaphases as measured by conventional cytogenetic studies and nuclei with *BCR/ABL* fusion as measured by D-FISH. The patient's "best response" was defined for each series of specimens from any patient as the smallest percentage of neoplastic cells after therapy relative to the baseline study. These investigators also classified each patient's best response at any given time according to the percentage decrease in Ph-positive metaphases or interphase nuclei using the M. D. Anderson cytogenetic classification scheme for response to therapy.

The Spearman correlations for the best percent change for neoplastic cells were moderately strong between chromosome studies and D-FISH on bone marrow or peripheral blood. Best-percent-change refers to the lowest percentage of Ph-positive cells that each patient achieved over 12 months of their initial treatment for CML. When best-percent-change was classified into the M. D. Anderson response categories there was a trend toward disagreement between chromosome studies of bone marrow and D-FISH studies on either the same bone marrow or blood. The results of chromosome studies on bone marrow tended to "downgrade" the best-response category. This is not an unexpected outcome given that standard cytogenetics establishes the percentage of dividing neoplastic cells and D-FISH establishes the percentage of nonproliferating neoplastic interphase nuclei.

Since neoplastic cells in CML divide more frequently than normal cells, the percentage of Ph-positive metaphases is often higher than the percentage of interphases with *BCR/ABL* fusion. Thus, care should be exercised for interpreting data when different methods are employed. No one has produced a response-to-therapy scheme for CML based on the results of interphase FISH studies. Most patients that achieve a complete cytogenetic remission usually have less than 1% abnormal nuclei when studied by D-FISH (3).

Although FISH studies can detect very low levels of abnormal nuclei, these methods are at their best when assessing responses to therapy involving more than 1% of neoplastic nuclei. This author is not aware of any direct comparisons of FISH studies and molecular genetic studies such as RT-PCR to assess CML. Therefore, the comparative effectiveness of these methods to predict relapse when very low levels of abnormal nuclei are involved, such as after bone marrow transplantation, are not known.

#### **4.4. Atypical D-FISH Patterns in CML**

D-FISH detects two *BCR/ABL* fusion signals in cells with a  $t(9;22)(q34;q11.2)$  from patients with CML. A recent D-FISH study involving 147 patients with CML demonstrated considerable macro genetic variation of the *BCR* and *ABL* hybridization loci (Fig. 2G–I and Fig. 3) (48). Among these patients, typical D-FISH patterns were observed in 81%, but the remaining 19% of patients had any one of three different atypical patterns. Each of the atypical patterns was consistent with loss of the 3' portion of *BCR* that is usually translocated to chromosome 9, loss of the 5' segment of *ABL* that usually remains on the translocated chromosome 9, or loss of both the 3' translocation *BCR* and 5' segment of *ABL* that usually remains on the translocated chromosome 9.

These atypical patterns were associated with all forms of Ph-chromosomes including  $t(9;22)(q34;q11.2)$ , complex translocations, and masked translocations. The normal cut-off for 500 interphase nuclei for patients with typical patterns is <1%. In contrast, the normal cut-off for patients with 1R2G1F and 2R1G1F is less than 1.8% and for patients with 1R1G1F is <23%. Thus, special scoring criteria are needed to detect and quantify nuclei with atypical patterns using D-FISH or ES-FISH (48). The proportion of patients that responded to therapy with interferon- $\alpha$ -2b and cytosine arabinoside for 36 patients with typical D-FISH pattern was similar to seven patients with atypical patterns (48).

Sinclair et al. (49) recently studied survival among 11 patients with CML who had large deletions of DNA adjacent to the break and fusion points on the abnormal chromosome 9 in patients with  $t(9;22)(q34;q11.2)$  or variants of this translocation. They found that the deletions spanned up to several megabases and had variable molecular breakpoints. They reported that these deletions were detected at diagnosis and were present in all Ph-positive metaphases. This suggested that these deletions most likely originate early in disease, possibly even at the same time the Ph-chromosome was formed. Kaplan-Meier analysis showed a median survival of 36 mo in patients with deletions. By comparison, patients without a detectable deletion showed a median

survival of >90 mo. The difference in survival was statistically significant and multivariate analysis demonstrated that the prognostic importance of the molecular deletion was independent of age, sex, percentage of peripheral blood blasts, and platelet count. These investigators suggest that a  $t(9;22)(q34;q11.2)$  may not only produce a dominantly acting oncogene by fusion, but also may result in the loss of one or more genes that influence progression of the disease. If this observation holds true after analysis of a much larger series of patients with CML, then D-FISH will be particularly useful in clinical practice because it detects atypical signal patterns (48,50).

#### **4.5. Cytogenetics and FISH for Accelerated Phase and Blast Crisis of CML**

It is possible to use various FISH techniques to study chromosome evolution associated with the onset of the accelerated phase or blast crisis in CML. However, given the success of new treatments for CML, little effort has been expended to develop and test such FISH techniques as they have little practical utility in clinical practice. Nevertheless, it is important to be aware that abnormalities other than Ph-chromosomes do occur in CML. Some of these abnormalities, such as extra Ph-chromosomes, may affect the results of testing for *BCR/ABL* fusion. Other chromosome abnormalities would not be detected by FISH studies for *BCR/ABL* fusion, but would require other FISH strategies that are beyond the scope of this chapter. However, physicians could order these tests on a patient-by-patient basis to maximize their utility for specific clinical situations.

Approximately 71% of patients with CML in blast crisis have at least one of the following chromosome anomalies: +8,  $i(17q)$ , +19 or an extra Ph-chromosome (60). Approximately 15% of patients with CML in blast crisis have  $-7$ ,  $-17$ , +17, +21,  $-Y$  or  $t(3;21)(q26;q22)$ . The study of chromosome evolution has been helpful for predicting the type of impending blast crisis (61,62). Chromosome anomalies such as  $del(7)(q22)$  and  $-7$  are often associated with lymphoid blast crisis. Other anomalies such as  $i(17q)$  and +8 generally suggest myeloid blast crisis.

Chromosome evolution may explain why response to therapy and median duration of survival has been inversely proportionate to the extent of aneuploidy. Moreover, chromosome evolution may be a cytogenetic mechanism that interferes with attempts to cure disease using specific genetic therapies such as STI571. It is not expected that chromosome abnormalities other than those producing a simple *BCR/ABL* fusion would respond to *BCR/ABL* tyrosine kinase inhibitors.

Certain chromosome anomalies manifest an altered genetic expression, even in the presence of a Ph-chromosome (63). For example, the observation of a Ph-chromosome with  $inv(3)(q21q26)$  is usually associated with CML in megakaryoblastic transformation. A Ph-chromosome with  $inv(16)(p13q22)$  is associated with CML in blast crisis with abnormal eosinophils. A Ph-chromosome with a  $del(13)(q12q14)$  is associated with CML in blast crisis with myelofibrosis. A Ph-chromosome with  $t(15;17)(q22;q12)$  is associated with CML in blast crisis with phenotype similar to acute promyelocytic leukemia. Thus, the study of chromosome evolution in patients with CML can help explain unusual cellular morphology in certain patients.

#### **4.6. Genetic Testing Strategies for CML**

Cytogenetic, molecular genetic, and FISH are all useful techniques to study specimens from patients with CML. Each of these methods has strengths and weaknesses (64). However, no single testing method fulfills all the needs of clinicians who treat patients with CML. It is important to develop combinations of testing strategies that are accurate and cost effective for any given clinical situation. Moreover, tests change over time as methodologies improve and as new clinical challenges arise. The review process to develop better genetic testing methods should be an ongoing activity.

Several alternative genetic testing strategies for CML could be suggested, but the best combinations of methods would differ among various institutions depending upon the technologies available locally. Nevertheless, the following testing strategy for CML would work for many institutions. At diagnosis, a quantitative cytogenetic analysis of 25 or more metaphases from bone marrow can be done. This test helps identify the kind of Ph-chromosome and, in the event the patient has a disease other than CML, may reveal chromosome abnormalities that would help make the correct diagnosis. In specimens showing apparently normal chromosomes, but showing cellular morphology consistent with CML, FISH studies could detect a masked Ph-chromosome.

If the patient has CML, it is important to perform FISH studies to establish a pretreatment base line for the percentage of interphase nuclei with *BCR/ABL* fusion. It is useful to perform FISH studies on bone marrow and blood to permit appropriate comparisons with post-treatment specimens. Pretreatment specimens provide a good opportunity to examine a few Ph-positive metaphases to establish the type of signal pattern and thus to assure appropriate scoring criteria for subsequent specimens. For patients on therapy, D-FISH can be performed on peripheral blood at periodic intervals to assess the effectiveness of therapy. Consequently, bone marrow may not need to be collected to monitor therapy as frequently as in current practice. If the results fall below 1% based on analysis of 500 nuclei, it is useful to study up to 6000 nuclei to increase the sensitivity of the test.

Studies involving direct comparisons of FISH and RT-PCR to detect very low levels of residual disease in the same specimens and to correlate the results with survival and prediction of relapse have not been performed. Some publications suggest that RT-PCR may be more sensitive for detecting residual disease in a patient when it involves low levels of neoplastic cells (65–67). Perhaps answers to questions such as absolute cure may be best addressed using molecular genetic procedures, but this is not known.

#### **4.7. Multi-Institutional Experience with FISH Studies for *BCR/ABL* Fusion**

Recently, 28 laboratories in the United States worked together to investigate D-FISH to detect *BCR/ABL* fusion in bone marrow cells (47). Although these laboratories tested D-FISH, many of their experiences would also apply to S-FISH and ES-FISH.

In a three-part study, D-FISH probes were used to study *BCR/ABL* fusion in interphase nuclei from bone marrow (47). Initially, each laboratory familiarized itself with the procedure by applying D-FISH to known normal and abnormal specimens. Then,

collectively, the laboratories studied 20 normal and 20 abnormal specimens in a blinded fashion and measured the time it took to do the studies. Finally, each laboratory and two experts from one of the participating institutions studied six serial dilutions with 0–98% abnormal nuclei.

Using the reported normal cutoff of 1% abnormal nuclei in 500 nuclei analyzed by D-FISH, participants reported no false-negative cases and 15 false-positive cases (3). Each false-positive case involved low levels of abnormal nuclei ranging from 1.0–6.6%. These errors were attributed to inconsistent application of the scoring criteria and lack of experience scoring specimens with low levels of abnormal nuclei. Results provided by participants for serial dilutions approximated the expected percentages of abnormal nuclei, but those from the experts exhibited greater precision.

This multi-institutional study demonstrated the need for pilot studies and the utility of carefully designed clinical evaluations using blinded techniques to establish normal values and abnormal reference ranges. This investigation also demonstrated the importance of gaining experience with FISH before performing tests in clinical practice. It is clear from these results that precise quantitative evaluations of specimens require more skill and experience to score nuclei objectively than do qualitative evaluations.

The results of this multi-institutional study indicate that D-FISH is a powerful new method for detecting low levels of nuclei with *BCR/ABL* fusion. For those who have not used D-FISH, considerable practice with unknown coded specimens before using the method in clinical practice is a necessity. Moreover, observations of interphase nuclei alone make it difficult to be certain about specific hybridization sites of probes used with FISH; for example, simple, complex, and masked Ph-chromosomes can produce some of the same patterns as associated with classical Ph-chromosomes. To be certain about the exact interpretation of signal patterns observed in interphase nuclei, it is necessary to examine a few metaphases initially for each case. Studies before treatment should be performed to establish the D-FISH signal pattern for any patient. Nevertheless, it is useful in the learning process for studies with FISH in CML to begin by using the scoring criteria offered by the manufacturer and to analyze at least 500 nuclei.

For any bone marrow or blood sample that has an atypical signal pattern cannot be analyzed using the standard scoring criteria. An atypical pattern should be suspected when >20% of nuclei demonstrate a recognized atypical signal pattern. Atypical patterns must be confirmed by analysis of a few Ph-positive metaphases to be sure of the chromosomal basis for the signal pattern.

The results of this multi-institutional study indicate that normal cutoffs for FISH must be established locally in each laboratory rather than by convention, because scoring fluorescent signals is not as straightforward as counting chromosomes. Consequently, each laboratory must establish a normal and an abnormal reference range for each FISH-based test before reporting any clinical results. When such reference ranges are in place, each clinical specimen can be classified as normal or abnormal.

#### **4.8. Estimate of Effort to Perform FISH Studies for CML**

Twenty-eight laboratories worked together and measured the effort associated with D-FISH by timing the work required to mix four solutions, to hybridize the nuclei on

prepared slides, and to score 500 nuclei (47). Although the time varied significantly among laboratories, the average of time for all participants was 128 min per specimen for D-FISH. In our experience, this compares favorably with an average of 236 min for a standard cytogenetic study of 25 metaphases for a bone marrow. Thus, D-FISH significantly reduces the time invested in monitoring therapy in patients with CML.

Some of the variation in effort among laboratories may have been due to inexperience with timing their work and to timing some steps of short duration. However, the most probable explanation for most of the variation in time is differing efficiencies. Performing workload studies is useful to help establish the cost of labor associated with FISH and to estimate the personnel needed for these studies.

#### **4.9. Validation of BCR and ABL Probes with FISH**

Using D-FISH, metaphases can be more accurately analyzed than interphase nuclei because the morphology of individual chromosomes can be used to help assess *BCR* and *ABL* hybridization sites (1,2). Moreover, metaphases lie flat and individual chromosomes are well spread, thereby reducing many of the problems associated with scoring interphase nuclei such as overlapping signals (68). However, scoring interphase nuclei has advantages over scoring metaphases, for example, interphase nuclei are far more numerous than metaphases and do not require culturing cells. Nevertheless, it is important for any laboratory that performs FISH with *BCR* and *ABL* probes to use personnel that are skilled at scoring both metaphase and interphase cells.

The results of FISH are objective and can be evaluated statistically if data is collected in a consistent fashion and cells are scored consecutively in an unbiased manner. It is important to achieve these skills and develop confidence with data collection prior to performing FISH studies with probes for *BCR* and *ABL* in clinical practice (4). To this end, it is necessary for each laboratory to validate the FISH procedure and gain experience with known and unknown specimens. It is not good laboratory practice to simply read the literature concerning FISH studies in CML, purchase commercial probes for *BCR* and *ABL*, and then apply the test in clinical practice without first validating the procedure locally.

Laboratories can validate the application of a FISH test with *BCR* and *ABL* probes in different ways. This chapter will suggest one approach to validate and gain experience with FISH using DNA probes for *BCR* and *ABL*. With some modifications, this approach may be useful for validating many other FISH-based tests as well. The proposed method involves the following series of steps: familiarization with FISH, learning to score cells consistently, determining the analytical sensitivity for metaphase and interphase cells, performing pilot studies, and then doing clinical evaluations.

#### **4.10. Familiarization with FISH**

Laboratory personnel should read the literature on FISH studies in CML to appreciate the applications of the technology. A commercial testing system should be chosen which best addresses the clinical needs of the laboratory. A testing system using the D-FISH strategy is currently preferable and is available from at least two commercial vendors. Laboratory personnel should use one or two known normal and abnormal specimens to obtain initial experience with the testing system. During this process of

familiarization, laboratory personnel should develop impressions of size, brightness and integrity of signals in cells from various stages of the cell cycle. Laboratory personnel should assess their equipment for applying the testing system and for visualizing the results to look for potential interfering factors.

#### **4.11. Learning to Score Signals Consistently**

Two experiments can be performed to gain initial experience with variations in signal patterns among interphase nuclei. It is advisable that two or more independent scorers perform the same experiments because comparison of their results will permit preliminary impressions of potential inter-technologist differences in perceiving color patterns, interpreting signal integrity (e.g., split signals and fusion signals), and in other unexpected outcomes of the test.

In the first experiment, each technologist should record all signal patterns observed in 100 consecutive interphase nuclei from a normal individual and from a patient known to have CML. The results will be important to appreciate different FISH signal patterns that may be encountered with the test.

In the second experiment, laboratory personnel can begin to learn how to apply consistent scoring criteria and to collect data to document this accomplishment. The three most common sources of scoring problems result from overlapping signals, separation of signals, and occasional lack of probe hybridization. Laboratory personnel should review carefully the scoring criteria suggested by the commercial supplier. Then, they should use the proposed scoring criteria to analyze 100 consecutive interphase nuclei from a normal individual and a patient known to have CML. It is then useful to calculate the percentage of nuclei that meet the scoring criteria and the percentage of nuclei with other signal patterns.

#### **4.12. Analytical Sensitivity for Cells in Metaphase**

Once laboratory personnel are familiar with the FISH test for *BCR/ABL* fusion, the performance characteristics can be determined for both *BCR* and *ABL* probes by analysis of metaphase cells. Analytical sensitivity for metaphases is the percentage of *BCR* and *ABL* signals that hybridize to the correct loci. This experiment can be done on proliferating cells by scoring 100 metaphases from phytohemagglutinin-stimulated cultures of peripheral blood. Each technologist should record the number of signals observed in each metaphase and identify the locus to the extent possible by standard cytogenetic methods. This can be accomplished by using chromosome morphology, the reverse DAPI banding pattern produced by some imaging systems, or sequential banding techniques.

#### **4.13. Analytical Sensitivity for Interphase Nuclei**

A separate experiment should be performed to determine the performance characteristics of the FISH test for interphase nuclei. For CML, these studies should be performed on blood and bone marrow separately, as these will be the tissues that will be examined in clinical practice. In the case of interphase nuclei, analytical sensitivity is defined as the percentage of scorable nuclei with the expected signal patterns. For example, if 100 interphase nuclei are analyzed from a normal individual and each

nucleus has two *BCR* and two *ABL* signals as expected, then the analytical sensitivity is 100%. On the other hand, if 75 of 100 interphase nuclei have any number of signals other than two *BCR* and two *ABL* signals, then the analytical sensitivity would be 75%. It is important that a very high percentage of nuclei meet the scoring criteria; for example, it is best if the interphase analytical sensitivity is approx 90%.

Analytical sensitivity should be calculated from an equal number of interphase nuclei from each of five different known normal or abnormal individuals. Each specimen should be set-up, processed, and scored by two or more technologists on different days in order to assess inter-run variations. The analysis of a total of 500 interphase nuclei is usually adequate; for example, 100 interphases from each of five individuals with known FISH signal patterns. Analytical sensitivity is lower for interphase nuclei than for metaphase cells because interphase nuclei are smaller and have greater three-dimensional depth than metaphase spreads. This results in more opportunity for the overlap of signals and for interference with probe and genomic DNA hybridization in interphase nuclei than in metaphases (68).

#### **4.14. Pilot Study**

Once these initial experiments are complete, the laboratory personnel should perform a pilot study to gain further experience with the FISH test using *BCR* and *ABL* probes, and to gain confidence that they are performing the test correctly. This can be done by two or more independent scorers who perform a blinded pilot study involving five normal and five abnormal specimens. In the scoring process, each technologist should record the signal pattern in 100 consecutive interphase nuclei which met the scoring criteria. In clinical practice, the number of cells analyzed may differ depending upon the desired statistical power of sensitivity expected for the test. In our experience, the sensitivity of FISH methods such as D-FISH can best be achieved in a cost-effective manner by analyzing 500 nuclei that meet the scoring criteria.

Upon completion of the pilot study, it is useful to calculate the percentage of interphase nuclei that meet the scoring criteria for each patient. It is also useful to calculate the percentage of cells that do not meet the scoring criteria. Subjectively assess whether the results for any patient appear different from the expected analytical sensitivity and other previous experiments.

It is very useful to assess the inter-observer variation in both the initial and future validation experiments. This can be done objectively for each specimen by subtracting the percentage of cells with each signal pattern between any two observers. This figure is sometimes called the inter-observer delta (47). If the delta is >5% for any given specimen, it is reasonable to suspect that the scorers applied different scoring criteria. It is important to determine the source of variation between the two scorers. This can be done when scorers examine a series of nuclei together and discuss the source of scoring discrepancies "nucleus-by-nucleus".

#### **4.15. Clinical Evaluation Study**

It is useful to perform a clinical evaluation study before using the *BCR/ABL* FISH test in clinical practice. The clinical evaluation should attempt to test all the param-

eters of the new test that might be anticipated in clinical practice. Normal values need to be established as well as an abnormal reference range. Because these figures vary among laboratories depending upon experience and proficiency, it is important that they be determined for each laboratory independently.

Before beginning the clinical evaluation study, the investigator should have completed efforts to standardize the pre-analytical, analytical and post-analytical factors including collection, transportation and preparation of specimens. In addition, the scoring criteria, analytical sensitivity, number of cells needed for a clinically pertinent power of testing sensitivity, and interpretation of results should be established. Furthermore, all safety issues should have been identified and addressed.

The clinical evaluation study could be done in various ways and may involve any number of specimens, but from a statistical point of view, the more specimens the better. A reasonable study could be based on 20 or more normal specimens and up to 20 specimens from patients known to have CML. It is important to code the specimens so that they can be studied blindly. Two technologists should score each specimen independently, making sure to record the signal patterns of scorable cells and the number of cells that do not meet the scoring criteria.

When the clinical evaluation is complete, calculate the normal cutoff using data from the normal specimens. The normal values can be calculated in several different ways. It is generally unacceptable to use the mean plus or minus two or three standard deviations, even though this is common in the literature. This statistical approach is unacceptable because it assumes the data fit a bell-shaped curve. For *BCR/ABL* fusion studies this is not the case for either patients who have 100% normal cells or patients with untreated CML who have >90% neoplastic cells. Although some statistical methods to calculate the normal cutoff are available (1,69), the following approach is relatively simple and should suffice for studies of CML using FISH.

The number of cells that should be analyzed to achieve an expected power of testing sensitivity depends on the degree of confidence required, on the level of mosaicism to be excluded, and on the analytical sensitivity of the probe (70). The closer the analytical sensitivity of any probe approaches 100%, the more a relatively few anomalous signals in any number of observations might indicate the presence of a second cell line. The scoring process represents only 52% of the total effort to perform FISH studies (47). In our experience, and that of others, it is not difficult to score 500 nuclei, record the data, and capture two or three representative images in less than 20 min. Thus, little is gained by scoring fewer cells. Moreover, the high sensitivity of the newer FISH tests becomes particularly effective when 500 or more nuclei are examined. In one study, the analysis of 500 interphase nuclei was shown to approximate the sensitivity to detect both normal and abnormal cells by conventional cytogenetic studies on 25 metaphases (3).

A good way to achieve an analytical sensitivity of FISH for *BCR/ABL* fusion to approach 100% for interphase nuclei is to score only those cells that meet the scoring criteria for normal or neoplastic signal patterns. The key to the success of this approach is defining strict scoring criteria and then consistently following them. Strict scoring criteria have been published for S-FISH and D-FISH (1,2).

One method to establish the normal cutoff and the abnormal reference range is to study a series of normal and abnormal specimens in a random and blinded fashion. This series should include 20 or more samples that are pre-designated for use in the calculation of the normal cutoff. The number of false-positive nuclei should be determined for each of the predesignated normal specimens. This analysis predicts the maximum number of abnormal appearing nuclei that was encountered in any patient. The normal cut-off can then be established by using this number to calculate the upper bound of a one-sided 95% confidence interval analysis using the binomial distribution. **Table 1** shows a series of cutoffs calculated using the binomial distribution formula for 200- or 500-nuclei samples showing from 0–15 nuclei with false-positive signal patterns. The abnormal reference range (percentage of cells with an abnormal pattern) can be calculated using data from specimens from untreated patients with CML.

As part of the clinical evaluation study, it is useful to determine the proportion of cells that do not meet the scoring criteria in a series of specimens. This figure can be calculated using the same statistical method as for the normal values and used in future studies as a quality control parameter. Whenever the proportion of unscorable cells exceeds the expected range, the test may have experienced unexpected technical problems.

The clinical sensitivity and clinical specificity can be calculated from the results of the clinical evaluation study. The clinical sensitivity refers to the percentage of correctly identified true positive specimens. The clinical specificity refers to the percentage of correctly identified true negative specimens. If the sample size is large enough, it may be useful to calculate the predictive value of the test. This is the percentage of true positive and true negative specimens that were correctly identified by the test.

When significant procedural changes are contemplated, such as a change of probes or concentration of probes, it is important to perform equivalency tests to revalidate test performance. These studies should establish that the procedure change is equivalent or better than the old procedure. Depending upon the nature of the change in procedure, it may be necessary to revalidate. For less significant changes, for example, changes in lot numbers of the same probe, a series of side-by-side analyses and evaluation of standard controls may suffice.

#### **4.16. Quality Control Studies with FISH**

The purpose of using controls in FISH studies is to assure that the procedure is working appropriately, to verify that the scoring criteria are applied consistently, to monitor technologists' competency, and to help interpret performance values. FISH studies with *BCR* and *ABL* use differently colored probes. The scoring criteria require analyzing only those cells that have an expected signal pattern. In this case, the scoring criteria should permit immediate detection of system or technical failure. For example, the inability to see both *BCR* and *ABL* signals should be an indication that the procedure is not working.

It is useful to run a positive control that contains a known percentage of normal and Ph-positive nuclei. For quantitative FISH studies, the mixture of cells from positive and negative controls is particularly valuable because it represents a challenge that is similar to the test in clinical practice, i.e., quantifying normal and abnormal cells.

**Table 1**  
**Normal Cut-Off Using One-Sided 95% Confidence Interval for the Observed**  
**Number of False-Positive Cells out of 200 or 500 Cells Scored Based**  
**on the Binomial Distribution**

Cells scored	False-positive cells	Percent false-positive cells	Normal cutoff (%)
200	0	0.0	1.5
200	1	0.5	2.5
200	2	1.0	3.0
200	3	1.5	4.0
200	4	2.0	4.5
200	5	2.5	5.0
200	6	3.0	6.0
200	7	3.5	6.5
200	8	4.0	7.0
200	9	4.5	7.5
200	10	5.0	8.5
200	11	5.5	9.5
200	12	6.0	10.0
200	13	6.5	10.5
200	14	7.0	11.5
200	15	7.5	12.0
500	0	0.0	0.6
500	1	0.2	1.0
500	2	0.4	1.2
500	3	0.6	1.6
500	4	0.8	1.8
500	5	1.0	2.0
500	6	1.2	2.4
500	7	1.4	2.6
500	8	1.6	2.8
500	9	1.8	3.2
500	10	2.0	3.4
500	11	2.2	3.6
500	12	2.4	3.8
500	13	2.6	4.2
500	14	2.8	4.4
500	15	3.0	4.6

Control specimens should be run with each specimen or batch of specimens. It is possible to make control specimens by using leftover cells obtained from patients with CML that were studied in clinical practice. Patients undergoing leukopheresis can also be a good source of control cells for CML.

The performance of the test, reagents and technologists can be monitored by graphing data from the studies of the standard control specimens on a test-by-test basis over

time. This method is also useful to monitor the performance of new lots and to obtain performance verification of probes. Moreover, graphing data from standard controls is useful to evaluate the equivalency of methodological changes such as switching from an indirect-labeled probe to a direct-labeled probe. The graph should include a mean and standard deviation based on the results from the pilot study and clinical evaluation study. It is important to update the statistics whenever there are significant changes in the procedures, including new technologists.

Any of the following new results of the standard control can indicate testing problems: (1) the run is more than three standard deviations from the mean, (2) two consecutive values are more than two standard deviations from the mean, (3) four consecutive values are more than one standard deviation from the mean, or (4) ten consecutive values fall on one side of the mean (71). Any of these situations can indicate technical or personnel problems with the test and require an investigation to identify the source of the variation and for which appropriate corrective action should be taken. Minor corrective actions may include re-testing the sample or using an additional scorer. Major corrective actions may require changes in procedure, using new reagents or new controls, reassessment of the basic statistics of the control results, remedial training of personnel, or even complete revalidation of the test.

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## Chromogenic *In Situ* Hybridization and FISH in Pathology

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### 1. Introduction

Fluorescence *in situ* hybridization (FISH) technologies enable rapid detection of chromosome aberrations in all manner of tissues, including both fresh and archival specimens. These technologies have gained broad acceptance in the clinical cytogenetic and research communities. However, these same methods are used less frequently by noncytogenetic diagnostic pathology services. This is, in part, because FISH imaging equipment is not universally available to the diagnosticians (surgical pathologists) responsible for histological diagnoses. Therefore, it is gratifying that various improvements in ISH probes and detection protocols, particularly over the past five years, have enabled routine evaluation of enzymatic ISH by light microscopy.

Virtually all malignant human tumors contain clonal chromosomal aberrations that are responsible, in part, for tumorigenic progression from nonneoplastic progenitor cell to cancer. These aberrations often affect genes involved in cellular proliferation, apoptosis, and invasiveness, and, in so doing, account for many clinicopathological properties that are hallmarks of the cancer diagnosis. Although conventional histological examination is the mainstay of surgical pathology, there are an increasing number of situations where cytogenetic markers provide critical diagnostic and prognostic information. In many cases, the cytogenetic aberrations are best interpreted in the context of histology. One well-known example is that of *ERBB2* (*HER2/NEU*) gene amplification in breast carcinoma, wherein it is important to evaluate amplification specifically in the invasive components of the tumor (1). Therefore, there is need for detection strategies which enable interpretation of molecular cytogenetic assays in histological preparations. Ideally, such assays would be read on an ordinary light microscope, rather than requiring a fluorescence system (2–4). In that way, molecular cytogenetic assays could be adopted more widely by the surgical pathology community, very much akin to immunohistochemistry, and could function as “special stains” in relationship to the routine histological evaluation of a given case.

From: *Methods in Molecular Biology*, Vol. 204: *Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

The notion of interpreting DNA-based tests in conjunction with the standard histological appraisal is not new. However, there are very few pathology/histology laboratories which perform chromogenic *in situ* hybridization (CISH) for brightfield detection, and there are even fewer laboratories which use such methods for dual-color detection of chromosomal translocations, amplifications, or deletions. The methods described herein enable reproducible FISH or CISH evaluation of various chromosomal aberrations in a wide range of pathology specimens, including paraffin sections, frozen materials, and cytopathology preparations. Importantly, these methods provide a mechanism whereby molecular cytogenetic evaluations can be routinely integrated in a standard histopathology or cytopathology work-up.

Most histopathology assays are performed in interphase cells rather than in cytogenetic metaphase preparations. Therefore, unique sequence and other locus-specific probes, e.g., alpha satellite probes, are generally more relevant as histopathology/cytopathology adjuncts than are metaphase-oriented molecular cytogenetic approaches such as comparative genomic hybridization and chromosomal painting. Notably, locus-specific ISH is equally applicable to cytogenetic or archival preparations. Almost any source of intact nuclei can be used, including: (1) histological sections; (2) touch preparations; (3) cytological preparations; (4) bone marrow smears; (5) nuclei disaggregated from thick, e.g., 25–50 micron, paraffin sections; (6) nuclei disaggregated from frozen specimens; and (7) cytogenetic preparations. As discussed above, a key advantage in using histological sections is that the tumor cells are studied in their native architecture.

### **1.1. Pros and Cons of FISH vs CISH**

FISH is more widely used for molecular cytogenetic analyses, particularly in cytogenetic laboratories, than is CISH. However, both methods have their distinct advantages and disadvantages. One substantial advantage of the FISH approach is that probe detection can be accomplished by direct detection. By contrast, chromogenic detection methods are indirect by definition, and CISH generally requires at least two additional steps beyond probe hybridization, prior to nuclear counterstaining. For example, a probe can be labeled with FITC or rhodamine, and thereby detected directly using the FISH method. The same probe might be labeled with biotin and then detected by sequential incubations with streptavidin-HRP and DAB, using the CISH method. Another advantage of FISH is that penetration of probes and detection reagents, to the target chromosomal regions, is accomplished more readily than with the larger detection proteins, e.g., alkaline phosphatase, customarily used in CISH detection. CISH, on the other hand, has several advantages that are of particular relevance in paraffin section applications. For example, paraffin section histological detail is generally better appreciated with brightfield, rather than fluorescence, counterstaining and viewing. This is in part because cellular and extracellular proteins can contribute to a dull, generalized, autofluorescence, which often obscures FISH signals in paraffin sections (see **Note 1**). Another factor is that large regions of the tissue section can be scanned rapidly after CISH counterstaining with conventional stains such as hematoxylin. Morphological detail is readily apparent using low-power objectives ( $\times 10$  or  $\times 20$ ), and

the CISH probe signals themselves can be apparent even at such low magnification. Fluorescence probe signals and counterstains on the other hand, are generally only appreciated at substantially higher magnification. A further advantage of CISH is that the probe signals are not subject to rapid fading and the slides can therefore be archived.

## **1.2. FISH/CISH in Histological Paraffin Sections**

One advantage in performing FISH or CISH in paraffin sections is that paraffin-embedded materials are readily available for virtually all pathology specimens. In addition, fixation and embedding methods can be standardized, leading to a very consistent platform for subsequent FISH or CISH detection. A unique advantage is that subtle differences in cellular morphology are often appreciated best when the cells are situated in their native architecture. It can be more difficult to identify different cell types once the individual nuclei have been extracted from paraffin sections. A disadvantage of histological paraffin sections (generally four–six micron) is that this thickness is less than the diameter of virtually all mammalian nuclei. Therefore, the nuclei are invariably incomplete, and evaluation of chromosomal deletions can be challenging. On the other hand, evaluation of gene amplifications or translocations is relatively straightforward (**Fig. 1**). Thicker sections can be used, in order to accomplish better representation of intact nuclei. However, overlapping nuclei are then more of a problem.

## **1.3 Alternate Pathology Applications: FISH/CISH in Disaggregated Nuclei, Touch Preparations, and Cytological Preparations**

The constraint of nuclear slicing, always an issue in thin paraffin sections, can be circumvented by any of several alternate approaches. The methods for these alternate approaches are beyond the scope of this chapter, but they are described in the literature and are summarized below (**5,6**). In general, all of these approaches enable evaluation of FISH or CISH probes in intact cells. However, they provide less information about cell type and tissue architecture compared to the paraffin section methods described herein.

One approach involves disaggregating intact nuclei from thick (generally 25–50 micron) paraffin sections (**7**). This approach is particularly applicable when cells do not need to be evaluated in the context of their native architecture. However, in our experience, this method is less forgiving technically, compared to hybridization in thin paraffin sections. The enzymatic treatment required for release of nuclei can sometimes damage the nuclei, and this is compounded during subsequent slide denaturation steps. Therefore, a subset of specimens will evidence poor nuclear morphology and will have suboptimal FISH or CISH signals. Intact nuclei can also be disaggregated from fresh or frozen tissue specimens, and in general such applications are more fool-proof than disaggregation from paraffin (**6**).

FISH or CISH can also be performed after pressing either fresh or thawed (frozen) tissue against a glass slide (**6**). These touch imprints require enzymatic digestion before probe hybridization. Otherwise, the labeled probes would not readily access their target

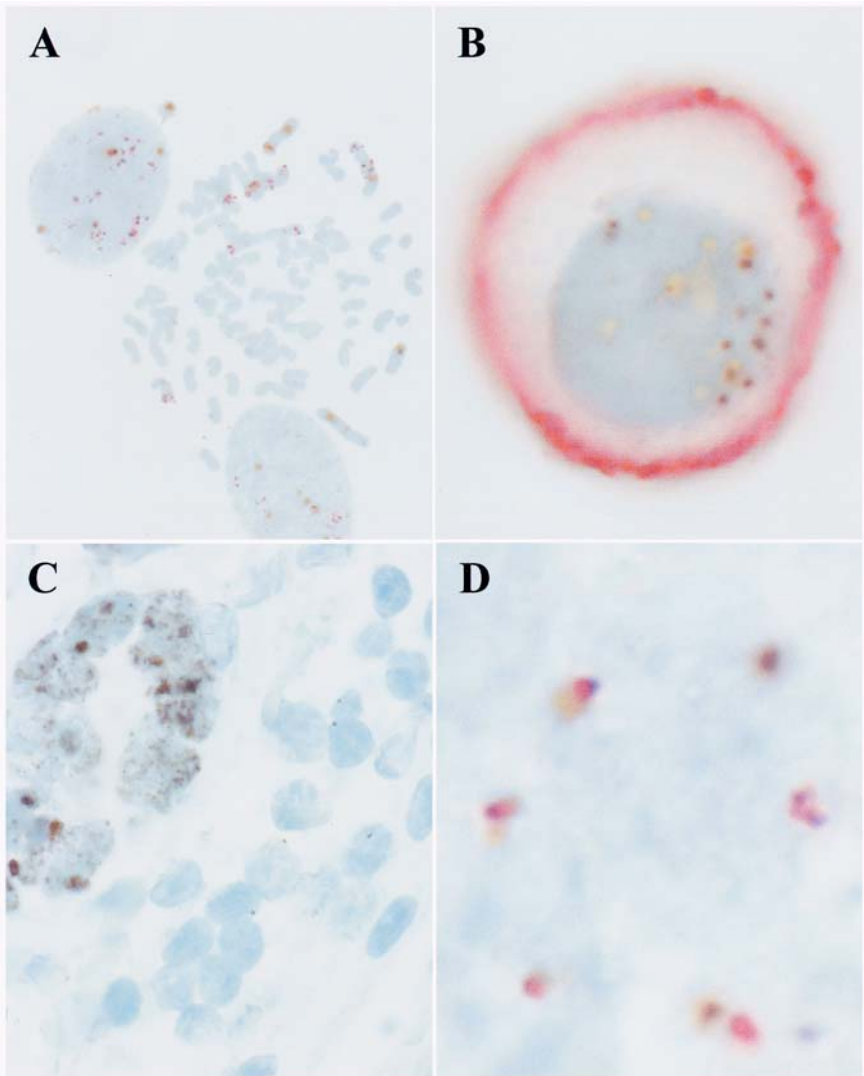


Fig. 1. (A) Dual color CISH evaluation of chromosome 17 centromere (DAB) and *ERBB2* (*HER2/NEU*) locus (Fast-Red) in breast cancer cell line, showing chromosome 17 aneusomy and high level *ERBB2* amplification. (B) Combined immunohistochemical and CISH evaluation of *ERBB2* (*HER2/NEU*) protein (Fast-Red) and genomic locus (DAB) in cell block of invasive breast cancer. Strong membrane-associated *ERBB2* expression results from high-level genomic amplification. (C) High level *MYCN* (*N-myc*) genomic amplification in paraffin section of neuroblastoma. Heterogeneity of *MYCN* amplification is apparent, with neoplastic cells in the right hand part of the field being nonamplified. (D) Dual-color FISH in Burkitt lymphoma paraffin section, demonstrating *MYC* gene region rearrangement resulting from translocation of chromosome band 8q24. Closely-associated red-brown signal pairs represent the nonrearranged chromosome 8, whereas isolated red or brown signals (at top right) represent the translocation chromosomes.

chromosomal domains through the intact cell membranes and cytoplasm. An advantage of this approach is that nuclei are intact, and therefore evaluation of chromosomal deletions is straightforward. Disadvantages are that nuclear size and shape are altered by the requisite enzymatic treatment, and it can be difficult therefore to distinguish morphologically between different cell populations, e.g., neoplastic versus nonneoplastic cells.

Cytological preparations have unique advantages for FISH or CISH purposes. Like touch preparations, they provide intact cells. However, "cytological" methods of preparation, including certain proprietary technologies such as the ThinPrep (Cytoc Corporation), are often highly standardized and very conducive to FISH/CISH applications (6).

## 2. Materials

### 2.1. Slide Treatment Before Hybridization

1. Four-micron thick paraffin sections, mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA).
2. 60°C oven.
3. Microwave oven, Model JE1390GA (General Electric Co., Louisville, NY).
4. Slide Moat, Model 240000 (Boekel Scientific, Feasterville, PA).
5. Glass Coplin jars.
6. Tris-EDTA solution: 0.1 M Tris-HCl, 40 mM EDTA, pH 7.0. Dissolve 24.2 g Tris-base and 29.22 g EDTA in 1800 mL dH<sub>2</sub>O, adjust pH with 10 N NaOH to pH 7, and QS to 2 L.
7. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub> and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in 900 mL of dH<sub>2</sub>O QS to 1 L.
8. Digest-All 3 (Zymed Laboratories, South San Francisco, CA).
9. 10% buffered formalin phosphate (Fisher Scientific Co., Fair Lawn, NJ).
10. Xylene.
11. 100, 85, and 70% ethanol.

### 2.2. Denaturation and Hybridization

1. Thermocycler with slide adapter, e.g., PTC-200/225 (MJ Research Inc., South San Francisco, CA).
2. 37°C humidified oven.
3. Plastic slide chamber.
4. Cover glass.
5. Rubber cement.
6. Biotin and/or Digoxigenin labeled DNA probes, e.g., *Her2*, *C-Myc*, *N-Myc*, (Zymed Laboratories).

### 2.3. Post-Hybridization Wash

1. 70–80°C waterbath.
2. Glass Coplin jars.
3. 20X SSC: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL H<sub>2</sub>O, QS to 1 L, and adjust pH to 7.0.
4. 2X SSC: Mix 50 mL 20X SSC with 450 mL H<sub>2</sub>O.
5. 0.5X SSC: Add 10 mL 2X SSC to 30 mL H<sub>2</sub>O.
6. PBS/T: Add 250 µL Tween-20 to 500 mL of PBS.

## 2.4. Detection

### 2.4.1. FISH

1. CAS-block (Zymed Laboratories).
2. Antibody dilution: Dissolve 10 g of lyophilized normal goat serum in 100 mL of CAS-block.
3. Streptavidin, Alexa-594 conjugate (Molecular Probes, Eugene, OR). Dilute at 1:500 in Antibody diluent.
4. Anti-digoxigenin-fluorescein, Fab-fragment (Roche Diagnostics Co., Indianapolis, IN). Dilute at 1:500 in Antibody diluent.
5. PBS/T.
6. DAPI mounting medium (Vector Laboratories, Burlingame, CA).

### 2.4.2. CISH

1. CAS-block (Zymed Laboratories).
2. Antibody dilution: Dissolve 10 g of lyophilized normal goat serum in 100 mL of CAS-block.
3. HRP-streptavidin (Zymed Laboratories). Prediluted, ready to use.
4. Anti-digoxigenin-AP, Fab-fragment (Roche Diagnostics Co., Indianapolis, IN). Dilute at 1:500 in Antibody diluent.
5. Anti-digoxigenin-fluorescein, Fab-fragment (Roche Diagnostics Co.). Dilute at 1:500 in Antibody diluent.
6. Alkaline phosphatase anti-fluorescein (Vector Laboratories). Dilute at 1:500 in Antibody diluent.
7. DAB Reagent Set (Zymed Laboratories). Prepared fresh according manufacturer's protocol.
8. Fast Red Substrate Pack (Zymed laboratories). Prepared fresh according manufacturer's protocol.
9. Ultracleaning filter units (0.45  $\mu\text{m}$ ) (Fisher Scientific).
10. PBS/T.
11. Gill's hematoxylin (Vector Laboratories).
12. Glycerogel (DAKO Co., Carpinteria, CA).

## 3. Methods

### 3.1. Slide Treatment Before Hybridization

1. Bake slides at 60°C overnight in an oven.
2. Deparaffinize the slides in 3 changes of xylene, 15 min each.
3. Dehydrate the slides in 100% ethanol, 3 $\times$ , 2 min each, and air dry.
4. Place Tris-EDTA solution in the microwave oven in a plastic Coplin jar or other microwave proof plastic slide container, then heat to 199°F using the temperature probe.
5. Place the slides in the Tris-EDTA solution and continue the microwaving at 199°F for 15 min.
6. Remove the slides and place them in the PBS.
7. Blot excess PBS from the slides and then place slides in the humidified chamber of the Slide Moat (or other humidified incubator) at 37°C.
8. Add 100–500  $\mu\text{L}$  Digest-All 3 to the sections, and let digestion proceed for 1–30 min dependent on the tissue type and fixation (*see Note 1*).
9. Stop the digestion by placing the slides in PBS.
10. Fix the slides in 10% buffered formalin for 1 min, then rinse off the formalin by placing slides briefly in 40 mL PBS in a Coplin jar.
11. Dehydrate the slides through 70, 85, and 100% ethanol, 2 min each, at room temperature. Air dry.

### 3.2. Denaturation and Hybridization

1. Add sufficient probe to the section, e.g., 10  $\mu\text{L}$  for an area corresponding to a  $22 \times 22$  mm cover slip.
2. Place coverslip over the section and seal edges with rubber cement.
3. Place the slides on the slide holder in a thermocycler and denature the slides for 3 min at  $94^\circ\text{C}$ .
4. Transfer the slides to a plastic slide chamber and place in a humidified incubator at  $37^\circ\text{C}$  overnight.

### 3.3. Post-Hybridization Wash

1. Set the waterbath temperature according to the number of slides per Coplin jar,  $73^\circ\text{C}$  for 1 slide,  $74^\circ\text{C}$  for 2 slides,  $75^\circ\text{C}$  for 3 slides, and  $76^\circ\text{C}$  for 4 slides.
2. Place glass Coplin jars containing 40 mL of 0.5X SSC in the waterbath and allow the Coplin jar SSC solution to equilibrate to the waterbath temperature. Coplin jars, and contents, should be no cooler than room temperature when placed in the waterbath. Otherwise, the jar may crack. Confirm final temperature using a thermometer.
3. Remove rubber cement and cover slips from the slides.
4. Wash the slides in the 0.5X SSC for 5 min.
5. Remove the slides and transfer to 40 mL PBS/T in a Coplin jar, at room temperature.

### 3.4. Detection

#### 3.4.1. FISH

1. Remove excess PBS/T from the slides and place in a humidified plastic slide chamber.
2. Apply 100–500  $\mu\text{L}$  CAS-block to the section and incubate for 10 min at room temperature.
3. Tap off CAS-block, then apply 100 to 500  $\mu\text{L}$  of antibody diluent containing the fluor-conjugated detection reagents, e.g., 1:500 dilutions of streptavidin-Alexa 594 and antidigoxigenin-fluorescein. Incubate for 30 min at room temperature.
4. Wash the slides  $3\times$  in 40 mL PBS/T, 2 min each wash, at room temperature.
5. Counterstain slides with DAPI mounting medium.

#### 3.4.2. CISH

1. Remove excess PBS/T from the slides and place them in a humidified plastic slide chamber.
2. Add CAS-block onto the section and incubate for 10 min at room temperature.
3. Tap off CAS-block, then add HRP-streptavidin and incubate for 30 min at room temperature.
4. Wash the slides  $3\times$  in 40 mL PBS/T, 2 min each wash, at room temperature.
5. Prepare the DAB substrate according to the manufacturer's instructions, remove the excess PBS/T from the slides and incubate the slide with DAB for 15 min.
6. Wash the slides  $3\times$  in 40 mL PBS/T, 2 min each, at room temperature.
7. Remove excess PBS/T from the slides, add antidigoxigenin-fluorescein (*see Note 2*) and incubate for 30 min at room temperature.
8. Wash the slides  $3\times$  in 40 mL PBS/T, 2 min each, at room temperature.
9. Remove excess PBS/T from the slides, add alkaline phosphatase anti-fluorescein and incubate for 30 min at room temperature.
10. Wash the slides  $3\times$  in 40 mL PBS/T, 2 min each, at room temperature.
11. Prepare the Fast Red substrate according to the manufacturer's instructions, then pass it through a  $0.45 \mu\text{m}$  filter.
12. Drain/tap excess PBS/T from the slides, then add Fast Red substrate to the section. Incubate for 30 min total with 2 changes of Fast Red substrate at 10 min intervals. This is

accomplished by tapping the Fast Red substrate from the slides every 10 min and replenishing with additional filtered substrate.

13. Wash the slides in 40 mL dH<sub>2</sub>O at room temperature.
14. Counterstain the slides with hematoxylin, being careful not to overstain (*see Note 3*). Gill's formula (without organic solvent—*see Note 4*) should be used in conjunction with Fast-Red.
15. Wash the slides in running tap water for several minutes. Do not use ammonia citrate to intensify the blue of the hematoxylin.
16. Heat the Glycergel at 50–75°C in an oven or waterbath and add a drop to the section (without use of organic solvent) then cover it with cover slip. Remove excess glycergel by gently pressing down on the cover slip, heat the slide on a hot plate (37–45°C) if the glycergel has solidified.

#### 4. Notes

1. Residual extracellular and cellular proteins can give rise to dull red-to-orange autofluorescence, which can obscure the probe FISH signals, particularly when the actual probe signals are of low intensity. Autofluorescence can often be minimized by increasing the enzymatic digestion time for the paraffin section. Inadequate tissue section digestion will also tend to impede probe access to the target chromosomal domains (resulting in weak “true” signals) and can increase nonspecific binding of the probe to nonchromosomal cellular components. Therefore, digestion time should generally be increased if cell morphology is excellent (based on the DAPI counterstain), and if the cells have scattered small nonspecific signals. A properly hybridized paraffin section should have well-preserved cell morphology (although not necessarily perfect), readily apparent probe signals, and should be devoid of nonspecific signals.
2. The CISH detection protocol here stipulates amplification of the digoxigenin-labeled probe by sequential incubations with fluorescein anti-digoxigenin and alkaline phosphatase antifluorescein. However, digoxigenin-labeled probes can also be detected without the amplification step, in which case the fluorescein antidigoxigenin is replaced by alkaline phosphatase anti-digoxigenin.
3. The different colors in dual-color CISH (brown from DAB and red from Fast-Red, in this protocol) are less distinct from each other than is the case for fluorescein vs rhodamine in dual-color FISH. It can be particularly difficult to distinguish DAB from Fast-Red when the probe signals are very small. However, the color differences can often be appreciated by focusing up and down through the section. At certain focal points, the Fast-Red will be more or less refractile and the distinct red color will be apparent. It is also important that the hematoxylin counterstain be kept to the minimum needed to define the nuclear contours and tissue architecture. Therefore, the hematoxylin should be only a light blue. Otherwise, the hematoxylin will mask the DAB vs Fast-Red color differences.
4. Organic solvents cannot be used in mounting the cover slips after staining with Fast-Red. FISH and CISH slides are ordinarily kept at 4°C, although we have found that CISH slides can be kept at room temperature for approx several weeks. FISH signals fade partially after several months in storage, whereas CISH slides are stable for approx 2 yr.
5. The dual-color CISH protocol can be modified slightly for combined CISH and immunohistochemical staining (**Fig. 1**). In this case, one chromosomal target and one protein are detected by single color CISH and immunohistochemistry, respectively.

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## FISH Detection of *HER2* Amplification in Breast Cancer

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### 1. Introduction

Gene amplification is frequently detected in human tumor cells and is thought to make an important contribution to tumorigenesis (1,2). Systematic scanning of the whole genome of tumor cells using comparative genomic hybridization has revealed that gene copy number changes occur concurrently in many areas of the genome in solid tumors (3). Detailed analysis of altered regions of DNA has revealed complex DNA rearrangements often involving multiple genes and spanning several megabases in solid tumors. Overlaid on gene rearrangements are frequent changes in chromosome ploidy (4–6). The analysis of amplified regions of DNA can lead to the identification of novel genes that contribute to tumorigenesis, but is complicated by the co-amplification of neighboring genes in these large, complex rearrangements. Many tumors show such a high degree of general DNA and chromosome rearrangement that some researchers argue the critical event in tumorigenesis is genomic instability (7–10) with gene amplification being a consequence.

#### 1.1. Amplification of the *HER2* Gene in Breast Cancer

The *HER2* gene was first identified in the DNA of chemically induced rat neuroblastomas (12), where it was designated “*neu*”. The human equivalent of “*neu*” was independently cloned from a cDNA library and called “*HER 2*” (13) for its homology to the epidermal growth factor receptor. The gene was also cloned from genomic DNA and referred to as “*c-erbB2*” (14,15) for its relation to a viral gene responsible for avian erythroblastosis. Preclinical data has shown that *HER2* gene amplification directly contributes to tumor cell growth through the function of its encoded protein called p185 or erbB2. The erbB2 protein is related to the epidermal growth factor receptor, sharing the common structure of a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane domain, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain, reviewed in (16). ErbB2 participates in a network of signaling when homodimerized or dimerized with other members of

the erbB protein family (17). Activated erbB2 enhances signaling through the MAPK pathways, which transduce growth factor signals to mediate mesenchymal–epithelial interactions (18–21). ErbB2 signaling has been shown to affect morphogenic differentiation programs in murine breast epithelial cells (22,23) and to induce cell migration and invasion of extracellular matrix in vitro (24).

*HER2* gene amplification results in overexpression of the protein p185 and occurs in about one third of breast cancers (25–28). There remain unresolved issues with respect to the technical validation of methods of assessing *HER2* alterations (11). With respect to clinical validation, an association between *HER2* amplification and an adverse prognosis in lymph node positive (LNP) disease has been consistently demonstrated since this association was first reported by Slamon et al. (25–30). The data evaluating the prognostic significance of *HER2* overexpression in lymph node negative (LNN) disease, particularly as assessed by immunohistochemistry, is controversial; some studies show a positive correlation with poor clinical outcome (31–40) whereas others show no association (41–51). For a review of immunohistochemical studies, see ref. (52). However, studies evaluating *HER2* gene amplification in lymph node negative (LNN) disease have consistently shown an association between *HER2* gene amplification and adverse prognosis. In a study by Press et al. (53), *HER2* gene amplification was assessed by fluorescence *in situ* hybridization (FISH) in a series of 240 untreated LNN breast cancer patients. In these patients, the relative risks of early recurrence, any recurrence, and disease-related death were statistically significantly associated with *HER2* amplification by FISH. A further study assessing *HER2* gene amplification using a quantitative polymerase chain reaction assay in a prospective series of 580 LNN patients demonstrated a statistically significant ( $p = 0.002$ ) increased risk of disease recurrence in patients whose tumors showed approximately a twofold amplification of the *HER2* gene (54).

## 1.2. *HER2* Overexpression and Breast Cancer Treatment

*HER2* overexpression has been demonstrated to have utility as a predictive factor in breast cancer. A predictive factor provides information on tumor response to a particular drug or therapy. *HER2* alterations provide predictive information both in the adjuvant and metastatic setting.

Several reports from randomized controlled trials suggest that patients with *HER2* positive tumors show a better response to anthracycline-based chemotherapy than patients with *HER2* negative tumors. These studies have primarily assessed *HER2* protein overexpression (55–57). *HER2* gene amplification was assessed by FISH using the Vysis PathVysion *HER2* DNA probe kit in a retrospective analysis of 524 tumor samples from patients who were enrolled in the CALGB 8869 clinical trial and and Vysis PathVysion PMA, 1998 (58). FISH analysis of the study specimens showed that there was a significant dose–response effect with anthracycline chemotherapy in patients with *HER2* gene amplification, but not in patients lacking *HER2* gene amplification. These results supported the United States Food and Drug Administration approval of the Vysis PathVysion *HER2* DNA probe kit for use with

stage II, node-positive breast cancer patients. Examples of unambiguous *HER2* gene amplification detected by FISH are shown in **Fig. 1A, B**.

There is controversial evidence as to whether the *HER2* status of tumors can predict patient response to hormonal therapy. Bianco et al. reported the results from 433 women with Stage I, II, or III (T3a) breast cancer enrolled in the Group of the University of Naples (GUN) trial (59). At a median followup of 14 yr, tamoxifen was effective in improving both disease-free survival (DFS) ( $p < 0.00001$ ) and overall survival (OS) ( $p = 0.03$ ). When the *HER2*-tamoxifen interaction was examined, it was found that tamoxifen was associated with improved DFS and OS only in *HER2* negative patients, while showing a paradoxical detrimental effect in *HER2* positive patients (59). A followup analysis in 2000 confirmed these findings (60). In contrast, other studies have not demonstrated such an association between *HER2* positivity and a decreased response to endocrine therapy (61–63).

Herceptin® (trastuzumab, Genentech, San Francisco, CA) is a modified monoclonal antibody to the *HER2* protein that is believed to induce growth arrest of tumor cells by interfering with signaling initiated by *HER2* protein on the cell surface and by triggering antibody-dependent cell-mediated cytotoxicity. In vitro studies have demonstrated that anti-*HER2* antibodies dramatically reduce the growth of tumor cells bearing high levels of *HER2* protein (64,65). Phase I and II clinical trials have demonstrated the efficacy and safety of Herceptin® when used alone or in combination with cisplatin (66,67). The pivotal phase III clinical trial (68) showed that the addition of Herceptin® to chemotherapy in patients with *HER2* overexpressing tumors was associated with a longer time to disease progression (median 7.4 vs 4.6 mo,  $p < 0.001$ ) and a lower death rate at one year (22 vs 33%,  $p = 0.008$ ) when used as a first line therapy in the metastatic setting. In this trial, the *HER2* status of patients' tumors was evaluated with an immunohistochemical assay referred to as the Clinical Trial Assay (CTA). Tumors were scored as 0, 1+, 2+ or 3+ and only patients with either 2+ or 3+ staining were considered eligible for the study (approx 1/3 patients). Results indicated that the beneficial effects of treatment were confined to the group of patients whose tumors demonstrated the strongest (3+) *HER2* immunostaining (69,70). The United States Food and Drug Administration (FDA) approved the use of the Dako HercepTest™ for the immunohistochemical detection of *HER2* overexpression in tumors of patients who are being considered for Herceptin® therapy in 1998. This test has not been directly studied in the setting of a clinical trial; however, it has been compared with the CTA in over 500 breast tumor samples obtained from the National Cancer Institute Breast Cancer Tissue Bank (69). Results indicate that, though there was a 79% concordance between the HercepTest™ and the CTA, 42% of tumors scored as 2+ by the HercepTest™ were negative (0 or 1+) by the CTA and therefore would not have met the entry criteria for inclusion in the randomized trial discussed above (70). Since its FDA approval the HercepTest™ has been widely used clinically. However, several studies have reported a high false positivity with this immunoassay (71–79). Clearly there is a need to determine which method of *HER2* status assessment best defines response in patients treated with Herceptin®.

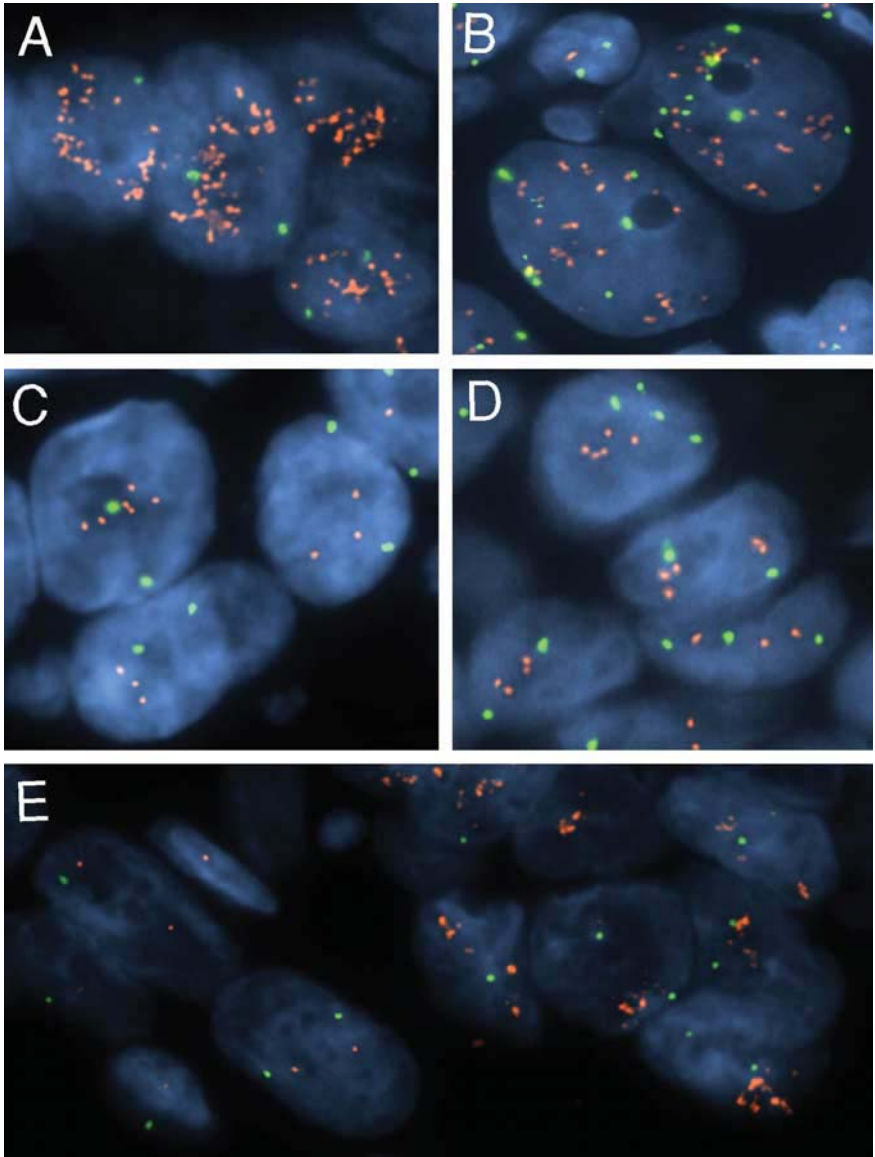


Fig. 1. HER2 FISH in breast tumor interphase nuclei. The probe for HER2 is labeled red and probe for chromosome 17 centromere is labeled green. (Path Vysion HER-2DNA Probe kit, Vysis, Downers Grove, IL). Images captured using CytoVision software from Applied Imaging, Santa Clara, CA. (A) HER2 is highly amplified and chromosome 17 is present in the normal two copies. (B) HER2 is highly amplified and chromosome 17 is present in multiple copies. (C) HER2 is present in more than two copies and chromosome 17 is present in the normal two copies. (D) Cells with  $\geq 5$  copies of HER2 per cell and polysomy of chromosome 17. (E) Intratumor heterogeneity where there is HER2 amplification in cells of the DCIS (at right), but no amplification in invasive tumor cells (at left).

### 1.3. FISH Analysis of HER2 Amplification

Because immunohistochemical (IHC) results for *HER2* status have been so variable (52), there is great interest in assessing DNA copy number using FISH as a clinical diagnostic test. However, since IHC is considerably less expensive and less labor intensive to use, it remains the test most frequently employed to assess *HER2* status in the diagnostic setting (80). Many labs propose using IHC as a screening tool with FISH as a confirmatory test for cases giving indeterminate IHC results. Reports comparing IHC results with FISH have found a high concordance in cases scored as highly positive with IHC or negative with IHC, but intermediate results (typically 2+ scores with HercepTest™) show little correlation to gene amplification (71–74,78). It has been suggested that some discordant results between IHC- and DNA-based assays can be explained by overexpression from a single copy of the *HER2* gene. A recent report (79) analyzing levels of *HER2* mRNA expression in discordant cases, however, has refuted this argument. A dilemma remains for cases with indeterminate IHC results where FISH analysis shows inconclusive results. These samples may have cells with polyploidy of chromosome 17 or borderline amplification levels of *HER2* in the context of chromosome 17 diploidy (Fig. 1C, D).

*HER2* gene copy number determined using FISH analysis correlates well with results obtained by Southern blot and dot-blot analysis (28,53), quantitative PCR (81,82) and comparative genomic hybridization (CGH) results (81,83–87). Concordance is improved when cells are microdissected to give a population enriched for tumor cells (88). FISH analysis allows assessment of gene amplification status on a cell by cell basis, so that intratumoral heterogeneity can be readily assessed, whereas Southern blotting, quantitative PCR, and CGH analysis detect gene alterations only when the majority of cells show the same change. Since FISH analysis can be performed in small populations or individual cells, it is ideal for the analysis of breast tumor core biopsy material.

Biopsy material and material from the excision of solid tumors is typically fixed in formalin, embedded in paraffin, and subsequently sectioned and mounted on microscope slides for histological examination. Formalin-fixed, paraffin-embedded tissue samples can be stored for decades (see Note 1). FISH can be readily performed on sections from this archived material owing to the stability of DNA. Preservation of the tissue architecture is a major advantage of this procedure. This allows assessment of the amplification status in the invasive tumor, rather than in ductal carcinoma *in situ* or in adjacent premalignant lesions as shown in Fig. 1E. Analysis of *HER2* amplification by FISH can be reliably performed on fine needle aspirates of breast tumors (89–92), as well as on touch preparations of fresh tissue (93). FISH analysis of disaggregated nuclei or cytological smears may prove more amenable to automated image analysis than FISH performed in tissue sections.

## 2. Materials

### 2.1. Laboratory Equipment

1. Series of three 200-mL baths of xylene (or Hemo-D) and three 200-mL baths of absolute ethanol set up in a fume hood (for removing paraffin from tissue sections) and appropriate slide carrying racks.

2. Slide dryer or hotplate set at 60°C.
3. Two waterbaths: one set at 81°C and one set at 37°C.
4. A set of 12 plastic Coplin jars.
5. Sets of micropipetor and tips for measuring 2–10  $\mu\text{L}$  and for measuring 200–1000  $\mu\text{L}$ .
6. Electronic timer for seconds.
7. Forceps.
8. Organosilane coated microscopy slides (Fisher Superfrost Plus).
9. 22  $\times$  22-mm square microscope slide coverslips.
10. Elmer's rubber cement.
11. Clear, colorless nail polish.
12. Standard lab equipment used to make solutions: thermometer, balance, pH meter, magnetic stirrer and stir bars, 1–2 L beakers, series of graduated cylinders, clean, and sealable 500-mL bottles for storage of solutions.
13. Pyrex dish or plastic container with a snap-on lid and containing a thermometer to act as a humidified chamber.
14. A reliable incubator set at 37°C.

## 2.2. Solutions, Reagents

1. 0.2 *M* HCl solution.
2. 1 *M* NaSCN solution.
3. 2X SSC stored as stock of 20X SSC (3 *M* sodium chloride, 0.3 *M* trisodium citrate, pH 7.2).
4. 2X SSC with 0.3% NP-40 detergent.
5. Deionized distilled water.
6. Pepsin (Sigma P6887, porcine pepsin) used at 72,000 units of activity per 50 mL of 0.2 *M* HCl solution).
7. Neutral buffered formalin.
8. Ultrapure formamide: 50 mL prepared as 70% formamide and 30% 2X SSC.
9. High grade absolute ethanol: 50 mL each prepared as 70%, 85%, and absolute alcohol.
10. Probe: Vysis PathVysion *HER2* DNA Probe Kit (30–161060).
11. 4',6-Diaminidino-2-phenylindole (DAPI II with antifade) counterstain (Vysis, 30-804840).

## 2.3. Microscopy

1. Microscope capable of brightfield and fluorescence microscopy (illuminated with epifluorescence from a 100 W mercury lamp).
2.  $\times 5$  objective,  $\times 40$  objective,  $\times 100$  oil immersion objective.
3. Dichroic filters (Chroma, Germany).
  1. Orange: peak excitation 559  $\pm$  38 nm, peak emission 588  $\pm$  48 nm.
  2. Green: peak excitation 509  $\pm$  31 nm, peak emission 538  $\pm$  44 nm.
  3. Blue: peak excitation 367  $\pm$  61 nm, peak emission 452  $\pm$  92 nm.
4. Appropriate immersion oil for fluorescence microscopy.

## 3. Methods

### 3.1. Cut and Mount Tissue Sections

1. Cut four micron thick sections from a formalin-fixed, paraffin-embedded tissue sample block and float in a 37°C waterbath.
2. Mount on positively charged microscopy slides.
3. Cut a corresponding tissue section adjacent in series and stain with hematoxylin and eosin (H&E) (see **Note 2**).

### 3.2. Remove Paraffin and Permeabilize Tissue Section

1. Identify appropriate areas of tumor cells on H&E slide (*see Note 3*).
2. Evaluate the slide for tissue composition, quality of fixation (*see Note 4*).
3. Bake slides in a convection oven or in a slide dryer at 60°C for 2 h (*see Note 5*).
4. Load slides into rack and remove paraffin from the tissue section by immersing the slides in a series of three xylene baths for 5 min each and then through three changes of absolute ethanol (*see Note 6*).
5. Dry slides in slide dryer or on a hotplate for 3 min at 45°C.
6. Immerse slides in 50 mL of 0.2N HCl in a Coplin jar at room temperature for 20 min.
7. Wash slides by immersing them in 50 mL of distilled water in a Coplin jar for 1 min then through two washes of 3 min each of 2X SSC.
8. Immerse slides in 1 M NaSCN at 81°C for 30 min (*see Note 7*).
9. Wash slides in distilled water for 1 min and through two changes of 2X SSC for 3 min each (*see Note 8*).
10. Immerse slides in the pepsin solution at 37°C for between 3–10 min (*see Note 4* regarding appropriate time).
11. Immerse the slides in 50 mL of fresh distilled water for 1 min and wash through two changes of 50 mL of 2X SSC.
12. Dry slides at 45°C for 3 min.
13. Secondary fixation: Immerse slides in 10% neutral buffered formalin for 10 min at room temperature (*see Note 9*).
14. Wash slides for 3 min through two changes of 2X SSC.
15. Dry at 45°C for 3 min (*see Note 10*).
16. Denature DNA of cells in the tissue section by immersing the slides in 70% formamide/30% 2X SSC solution at 81°C in the waterbath for 5 min. Immediately immerse slides into successive baths of 70, 85%, and absolute ethanol for 1 min each (*see Note 11*).
17. Allow slides to dry in 45°C heater for no more than 2 min.

### 3.3. Hybridization

1. Dim the lights in the room.
2. Using the micropipetor draw 10  $\mu$ L of probe solution in a line across a coverslip (*see Note 12*).
3. Cover the tissue sample with the coverslip and seal the edges of the coverslip temporarily with a line of rubber cement.
4. Place the slide in a closed, humidified (but not dripping) chamber at 37°C for 18 h (overnight).

### 3.4. Post-Hybridization Wash

1. Set a 71°C waterbath to heat 50 mL of 2X SSC containing 0.3% nonidet (NP-40) detergent in a Coplin jar.
2. Dim the lights.
3. Take out the slides from the incubator, peel off the rubber cement with forceps, and soak the slides for several min in a Coplin jar filled with 2X SSC/0.3% NP at room temperature (*see Note 13*).
4. Place the slides (no more than four at a time) in the heated (72°C) Coplin jar of 2X SSC/0.3% NP for 2 min.
5. Remove the slides from the heated wash, dip quickly into a Coplin jar of room temperature 2X SSC, allow to air-dry.
6. Place 10  $\mu$ L of DAPI on a coverslip, touch the slide to it, and invert (*see Note 14*).

7. Seal the edges of the coverslip with nail polish.
8. Store slides in a closed slide folder at  $-20^{\circ}\text{C}$ .
9. Allow to warm to room temperature and wipe off condensation before viewing at the microscope.

### 3.5. Analysis

1. Using brightfield microscopy and a low power objective ( $\times 10$  or  $\times 4$ ), scan the H&E microscopy slide and identify the areas of tumor cells that you wish to evaluate (*see Note 15*).
2. Count 60 tumor cells from several separated fields of view (*see Note 16*).
3. Record in a chart the red signals and green signal per cell for 60 tumor cells (*see Notes 17–20*).

## 4. Notes

1. We have had successful FISH preparations from blocks 20 yr old.
2. Tissue sections should be cut at a microtome by a skilled operator who consistently orients the sections in a uniform manner on the slides.
3. This should be done in consultation with a pathologist. The tissue sample should be checked to ascertain that it contains appropriate invasive tumor cells. Only invasive breast tumor cells should be scored. Invasive tumor cells must be distinguished from tumor cells of ductal carcinoma *in situ*, as well as lymphocytes, histiocytes, stromal fibroblasts, or adipocytes which typically appear on the same section. If your count includes proportions of cells with 2/2 counts and amplified cells, double check that you have not included normal tissue cells (especially infiltrating lymphocytes) in with the tumor cell count.

The tissue section should be scanned on low power and suitable areas of tumor cells noted along with landmarks that will be useful subsequently to orient the FISH slide. Landmarks include blood vessels, areas of adipose tissue, areas of normal ducts, and areas of DCIS. Intratumoral heterogeneity should be noted for special attention when the FISH slide is analyzed. Note any irregularities in fixation or tissue distortion (particularly if the section was originally a frozen section), which can cause confusion in interpretation. We find it very useful to circle the tumor area on the coverslip of the H&E slide with a Sharpie™ marker for future reference.

4. The optimal length of protease treatment time will depend on the age of the block, the tissue composition, and quality of tissue fixation and needs to be determined *before* undertaking the FISH preparation procedures. Protease treatment should be decreased for core biopsies and any sections that contain few tumor cells or have large areas of necrosis. These samples need to be handled particularly carefully to avoid over-digestion.
5. Meanwhile, pour 50 mL of 1 M NaSCN into a Coplin jar and set in the waterbath. Adjust the waterbath so that the temperature of the solution reaches  $81^{\circ}\text{C}$ .
6. This should be done in a fume hood. These baths may be used for many slides over several months and renewed when they appear very dirty.
7. Meanwhile pour 50 mL of 0.2 N HCl in a Coplin jar and set in a second waterbath to heat solution to  $37^{\circ}\text{C}$ , thaw a frozen aliquot of pepsin, mix well into 0.2 N HCl. Make up and set aside: 35 mL of formamide mixed with 15 mL of 2X SSC, and 50 mL each of 70%, 85%, and absolute ethanol in separate Coplin jars.
8. Upon removing slides, turn waterbath temperature down and heat Coplin jar of 70% formamide, 30% 2X SSC until  $71^{\circ}\text{C}$  in the solution.
9. Slides from blocks that are old ( $>2-3$  yr since surgery) show brighter signals if the secondary formalin fixation is omitted.
10. Take out probe to thaw. Ensure that the H&E slide is at hand for reference.

11. **Steps 16 and 17** are omitted if a HyBrite chamber (Vysis 30-144010) is used.
12. Lay the coverslip on a clean brown paper towel on the bench top (for easier visibility). Hold the slide above the coverslip and with the target area of the tissue section facing the probe, lower the slide gently to touch the probe liquid. In one quick motion, pick up and invert the slide with the coverslip clinging to it. The probe should spread out quickly and evenly without air pockets across the slide under the coverslip. Mark a very small line on the slide with a permanent-marking pen at the top and bottom corners of the coverslipped area.
13. The coverslips should float off or will need to be pried off the slides very gently with forceps. Be careful not to scrape the tissue section, which is very vulnerable to damage at this point.
14. For very small areas of tumor cells we use 5  $\mu$ L of probe and coverslip with an 18-mm coverslip, VWR Scientific, catalog number 48380046.
15. Locate any landmarks that are useful and switch over to the  $\times 25$  objective. Still using the H&E slide, turn on the fluorescence microscope and examine the area of interest noting where the landmarks are and how they appear under fluorescent light. With the previous views in mind, look at your FISH preparation slide under  $\times 25$  and try to locate the identical areas of cells. Identify areas of tumor cells and features of the surrounding tissue architecture. Switch to the  $\times 100$  objective and oil immersion and examine the field of view. The tumor cells should be free of connective tissue, but still closely associated in clusters as they appear in the H&E preparation.
16. Using a triple filter (orange/green/DAPI blue) you should be able to see complete blue staining of the nuclei with the *HER2* probe appearing as sharp orange spots and the CEP 17 probe as green spots. Confirm that hybridization has occurred correctly by analyzing some of the stromal cells or adjacent lymphocyte nuclei for 2/2 counts. Look for tumor cells that are not overlapping and where signals are bright and discrete. Look for cells where there are two green signals and count the corresponding red signals.
17. Overdigestion with protease results in ragged looking clumps or isolated tumor cells with no surrounding tissue and can be remedied by repeating the preparation with less time in protease. Poor fixation produces “hollow” cells with black spaces showing through the DAPI staining although the tissue structure appears intact. Likely this cannot be improved by repeating the preparation and will require that you scan the slide very thoroughly in search of the best preserved areas for usable results or perform FISH on another block. Rate the technical quality of the slide preparation and use this to evaluate whether there is true monosomy or polysomy in your sample or whether the preparation should be repeated.
18. Very weak signals can be seen by viewing the slide with single band pass filters one at a time, i.e., view with the triple filter, keeping in view a particular nucleus in question, switch to the orange filter and then switch to green to confirm signals that were faint with the triple filter.
19. If there is suggested heterogeneity in the H&E preparation or IHC stained slide, sample 20 cells from each of the areas in question. We and others (Ridofi et al.) have documented samples where there is discordance between DCIS and invasive tumor components (**Fig. 1E**).
20. We suggest you take advantage of the possibility with the Vysis probe kit to collect information about the number of green and red signals per cell. By keeping a record of individual cell counts you can evaluate the degree of heterogeneity and distribution of counts in your sample. This information is lost when all numbers are reported as averages. These considerations become very important when assessing borderline or low amplification cases.

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## Comparative Genomic Hybridization in Cancer Investigations

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### 1. Introduction

Comparative genomic hybridization (CGH) provides genome-scale overviews of chromosomal copy number changes in tumors (1). Unlike conventional cytogenetic analysis, it needs no cell culturing, making it applicable to practically any kind of clinical specimen from which DNA can be obtained, including archival paraffin embedded material (2). It maps the origins of amplified and deleted DNA sequences on normal chromosomes, thereby highlighting locations of important genes. However, this technique cannot detect subchromosomal changes, owing to its limited resolution, nor structural chromosomal changes such as translocations or inversions. By its nature, CGH is especially suitable for screening tumors in various stages of development, such as premalignant lesions, invasive carcinomas and metastases, pointing out the location of possible oncogenes or tumor suppressor genes that may play a role in the early onset of malignancy, tumor progression, or the process of metastasis. In addition, CGH can be used to compare different histological components within one tumor, enabling in a better understanding of the relation between phenotype and genotype, or to compare derivative cell lines with the original cell line.

The principle of the technique is shown in **Fig. 1**. Labeled tumor DNA competes with differentially labeled normal DNA for hybridizing to normal human metaphase chromosomes. Using fluorescence microscopy and digital image processing, the ratio of the two is measured along the chromosomal axes. Digital image processing includes the following steps: (a) background subtraction, (b) segmentation of chromosomes and removal of nonchromosome objects, (c) normalization of the FITC/TRITC ratio for the whole metaphase, (d) interactive karyotyping, (e) scaling of chromosomes to a standard length. Deviations from the normal ratio of 1.0 at certain chromosome regions represent amplification or deletion of genetic material in the tumor, and may sometimes already be seen in a green and red overlay image of the hybridized metaphase

From: *Methods in Molecular Biology, Vol. 204: Molecular Cytogenetics: Protocols and Applications*  
Edited by: Y. S. Fan © Humana Press Inc., Totowa, NJ

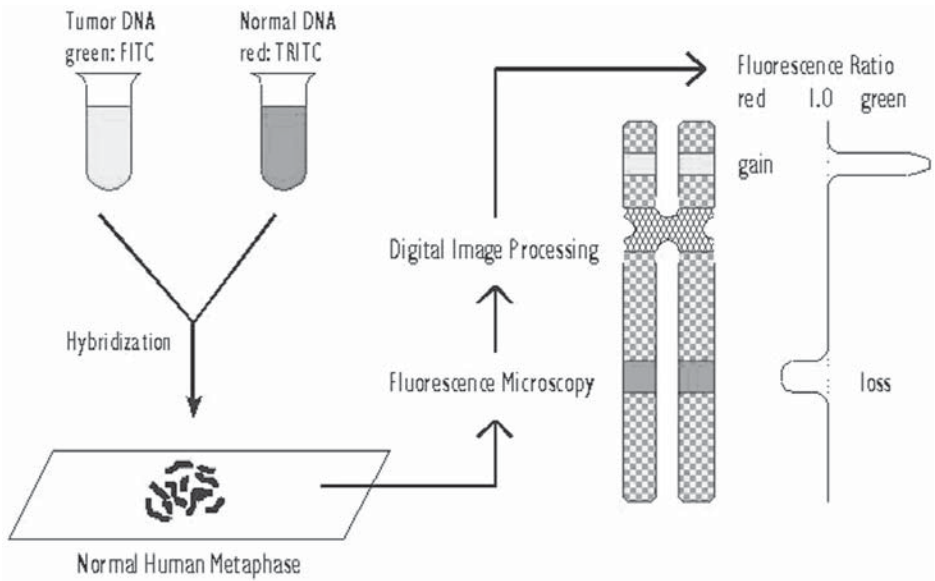


Fig. 1. Schematic overview of the CGH technique. Tumor and reference DNA are labeled with a green and red fluorochrome, respectively, and hybridized to normal metaphase spreads. Images of the fluorescent signals are captured and the green-to-red signal ratios are digitally quantified for each chromosomal locus along the chromosomal axis. (Reprinted with permission from Human Pathology (2))

(Fig. 2A). However, digital image processing is necessary for adequate evaluation. The final result is a so-called relative copy number karyotype (see Fig. 2B) that shows an overview of chromosomal copy number changes in the tumor. The sensitivity of CGH depends on the purity of the tumor sample (see Note 1); admixture with normal cells will reduce the sensitivity of CGH and its inherent resolution. For deletions the limit of detection is 10 Mb, which is about the size of an average chromosome band, but smaller amplifications (down to 250 kb) may be detected when the number of copies is high (3) (see Fig. 3). The following steps are required to perform CGH: Normal metaphase preparation, DNA labeling, hybridization and washings, fluorescence microscopy, and capturing and analyzing images with dedicated computer software, including karyotyping. Applications of CGH in cancer research include screening of tumors for genetic aberrations (4–16), searching for genes involved in the carcinogenesis of particular subsets of cancers (17), analyzing tumors in experimental models in order to get more insight in tumor progression (18), diagnostic classification, (17) and prognosis assessment (19). Besides these oncological applications, CGH analysis has also been used to study chromosomal aberrations in fetal and neonatal genomes (20–22). A recent development is microarray CGH, which will increase the possibilities to use CGH-like techniques in routine diagnostic procedures (see Note 2).

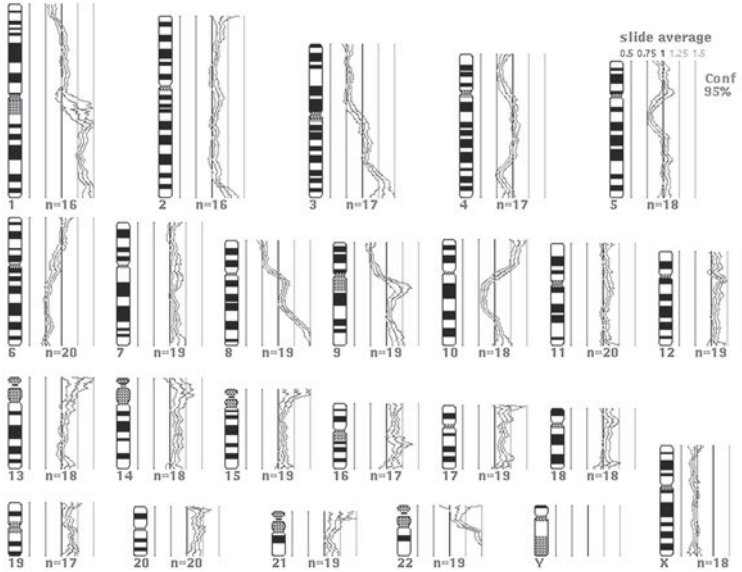


Fig. 2. Relative copy number karyotype showing the quantitative analysis of a tumor. The mean green-to-red fluorescence ratios of the chromosomes of multiple metaphase spreads are plotted in a graph corresponding to the chromosome ideograms, together with the 95% confidence interval: gains at 1p, 1q, 2q, 3q, 7q, 8q, 9q, 10p, 14q, 15q, 16p/q, 17p/q, 19q, 20q, 21q, and 22q; losses at 1p, 3p, 4p, 4q, 5q, 6p, 8p, 9p, 10q, and Xp/q.

## 2. Materials

1. Ham F10 culture medium (Gibco-BRL, Life Technologies, Paisley, UK).
2. Fetal calf serum (Gibco-BRL, Life Technologies).
3. L-glutamine (Gibco-BRL, Life Technologies).
4. Penicillin/streptomycin (Gibco-BRL, Life Technologies).
5. Phytohemagglutinin (Gibco-BRL, Life Technologies).
6. Colchicine (Gibco-BRL, Life Technologies).
7. KCl (0.075 M).
8. Methanol/acetic acid 3:1.
9. DNA polymerase I/DNase I (Gibco-BRL, Life Technologies).
10. DNase I (Gibco-BRL, Life Technologies).
11. dNTP reaction mixture: 0.2 mM dATP, dCTP, dGTP (Roche Diagnostics Nederland BV, Almere, The Netherlands); 500 mM Tris-HCl, pH 7.8; 50 mM MgCl<sub>2</sub>; 100 μM dithiotreitol; 100 μg/mL bovine serum albumin (BSA).
12. dTTP: 0.2 mM (Roche Diagnostics).
13. Biotin-16-dUTP (1 nmol/μL) (Roche Diagnostics).
14. Digoxigenin-11-dUTP (1 nmol/μL) (Roche Diagnostics).
15. Human COT-1 DNA (Roche Diagnostics).
16. Ethanol absolute.

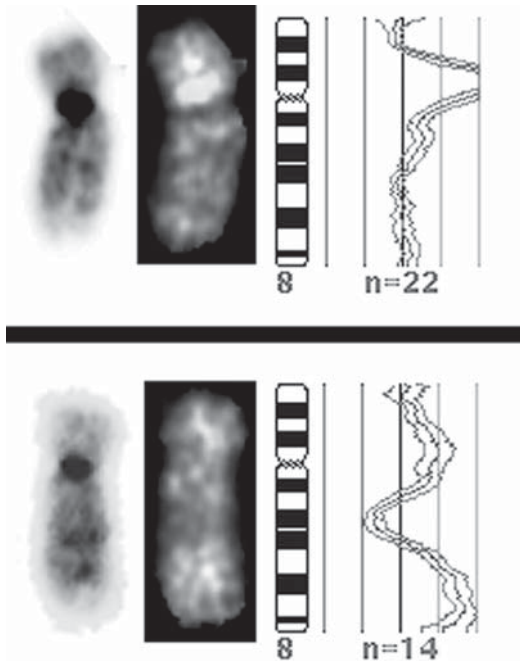


Fig. 3. An example of a small high-level amplification in chromosome band 8p11 (top); An example of a small deletion (app. 10 Mb) in chromosome band 8q21 (bottom).

17. Ethanol 99.8%.
18. 3 M Sodium acetate tri-hydrate.
19. Hybridization mixture: 50% deionized formamide (v/v), 10% dextran sulfate, 2X SSC, pH 7.0.
20. 20X SSC: 0.3 M sodium citrate, 3 M sodium chloride, pH 7.0.
21. 10X TN: 1 M Tris-HCl, 1.5M NaCl, pH 7.5.
22. TNT: 50 mL 10X TN, 450 mL H<sub>2</sub>O, 1.25 mL 20% Tween-20.
23. TNB: 0.5% (w/v) blocking reagent (Roche Diagnostics) in 1 X TN.
24. Avidin-FITC 12.5 µg/mL (Sigma-Aldrich, Poole, Dorset, UK).
25. Sheep-anti-digoxigenin-TRITC 4.0 µg/mL (Roche Diagnostics).
26. Antifade solution: Vectashield (Vector, Peterborough, Cambridgeshire, UK).
27. DAPI: 350 ng/mL 4',6-di-amino-2-phenylindole in antifade solution.

### 3. Methods

#### 3.1 Metaphase Preparation

1. Incubate 1 mL of heparinized blood with 9-mL Ham F10 culture medium containing 10% FCS, 1% L-glutamine, 1% penicilin and streptomycin, 1.5% phytohemagglutinin at 37°C in an atmosphere of 5% CO<sub>2</sub> for 72 h.
2. Arrest cells in mitosis by 0.1 µg/mL colchicine for 30 min.
3. Spin down cells at 150g for 10 min. Discard the supernatant.

4. Resuspend pellet in 10 mL hypotonic KCl and incubate 20 min at room temperature.
5. Spin down cells at 150g for 10 min. Discard the supernatant.
6. Fix cells in 10 mL of 3:1 methanol/acetic acid by carefully adding small volumes under continuous mixing.
7. Spin down cells at 150g for 10 min. Discard supernatant. Repeat **step 6**.
8. Spin down cells at 150g for 10 min. Discard supernatant.
9. Resuspend cells in approx 1 mL of 3:1 methanol/acetic acid.
10. Mount one or two drops of the cell suspension by Pasteur pipet onto ethanol-cleaned slides.
11. Post-fix the slide immediately by some drops of 3/1 methanol/acetic acid.
12. Check the quality of the metaphases by phase-contrast microscope (*see Note 3*).
13. Slides are air dried overnight at room temperature and can be stored in dry conditions at  $-20^{\circ}\text{C}$ .

### 3.2. DNA Labeling

1. Combine 1  $\mu\text{g}$  DNA, 3  $\mu\text{L}$  dNTP, 0.5  $\mu\text{L}$  dTTP, 1  $\mu\text{L}$  digoxigenin- or biotin-conjugated dUTP, 3  $\mu\text{L}$  DNA polymerase I/DNAse I, 0–1  $\mu\text{L}$  diluted DNAse I (adjust this concentration to obtain the optimal fragment lengths). Add  $\text{ddH}_2\text{O}$  to a vol of 30  $\mu\text{L}$ .
2. Incubate 1.5–2 h at  $15^{\circ}\text{C}$ .
3. Inactivate enzymes at  $70^{\circ}\text{C}$  for 15 min.
4. Visualize 5  $\mu\text{L}$  labeled DNA by gel electrophoresis through an ethidium bromide stained 1% (w/v) agarose gel.
5. Inspect the DNA fragment lengths with an ultraviolet transilluminator; the optimum smear is between 500 and 2000 kb in length (*see Note 4*).

### 3.3. Hybridization and Washing

1. Mix 10  $\mu\text{L}$  labeled (usually with biotin) tumor with 10  $\mu\text{L}$  labeled (usually with DIG) normal DNA and 40  $\mu\text{g}$  unlabeled Cot-1 DNA.
2. Ethanol precipitate the sample by adding 0.1 vol of 3 M sodium acetate and 2 vol of ethanol and centrifuging at 12,000g for 30 min.
3. Decant the supernatant and air dry the pellet.
4. Dissolve the pellet in 6  $\mu\text{L}$  hybridization mixture.
5. Denature a metaphase chromosome slide at  $72^{\circ}\text{C}$  in jar containing 70% (v/v) formamide and 2X SSC in a waterbath for 6 min (*see Note 5*).
6. Dehydrate the slide in an ethanol series (70, 96, and 100%).
7. Denature the DNA probe mixture at  $80^{\circ}\text{C}$  for 10 min.
8. Mount the probe mixture immediately on the metaphase slide.
9. Cover with a coverslip (18  $\times$  18 mm) and seal with rubber cement.
10. Hybridize for 2–3 d in a humid incubator at  $40^{\circ}\text{C}$ .
11. Remove the coverslip carefully.
12. Wash for 5 min in 2X SSC at room temperature.
13. Wash 3 times for 5 min in 0.1X SSC at  $45^{\circ}\text{C}$ .
14. Wash for 5 min in TNT at room temperature.
15. Pre-incubate for 10 min in 100  $\mu\text{L}$  of TNB under a cover slip (24  $\times$  50 mm).
16. Incubate for 60 min in 100  $\mu\text{L}$  of TNB with avidine-FITC (1/200) and sheep-anti-DIG-TRITC (1/50) under a cover slip (24  $\times$  50 mm) in humid chamber at  $40^{\circ}\text{C}$  (from now on keep the slide in the dark).
17. Wash 3 $\times$  for 5 min in TNT at room temperature.
18. Wash for 5 min in 2X SSC at room temperature.

19. Dehydrate the slide in an ethanol series (70, 96, and 100%).
20. Mount 25  $\mu$ L of antifade containing DAPI, cover with a cover slip (24 by 50 mm), and seal with rubber cement.

### 3.4. Fluorescence Microscopy and Image Analysis

A fluorescence microscope equipped with three single band pass filters is suitable for CGH. DAPI (blue) is used for chromosome identification, FITC (green) for the hybridized tumor DNA, and TRITC (red) for the hybridized normal DNA detection.

1. Screen the slide for well-spread metaphases with a homogeneous green and red fluorescent signal and a low background (*see Note 6*). Capture three images (DAPI, FITC, TRITC) of each metaphase spread.
2. The averaged ratios of approx 8–12 metaphases are plotted along the corresponding chromosomes in a so-called relative copy number karyotype (**Fig. 2**). The significance of deviations from the 1.0 ratio can be evaluated with the help of the 95% confidence interval (CI), which can be plotted along with the averaged ratios (*see Note 7*).

## 4. Notes

1. When using tissue sections to isolate DNA from tumor cells, the admixture of normal cells (stroma or infiltrating lymphocytes) may present a problem. When a sample contains more than 25% normal cells microdissection could become necessary, depending on the ploidy of the tumor (**23**). This can be done manually or by advanced laser microdissection equipment (**24,25**). However, the latter yields only limited number of cells (hence DNA), necessitating universal DNA amplification techniques (**26,27**). These techniques are time consuming, expensive, and the user must perform good control experiments to ensure the reliability of CGH results. Another approach could be cell sorting (e.g., antibodies attached to magnetic beads or flow cytometric sorting), which may enable selection (and extraction) of tumor cells, or elimination of inflammatory cells from a tissue sample.
2. New developments: A new technique currently being developed in different laboratories is the microarray technique. Using metaphase chromosomes for hybridization limits the detection of events involving small regions (less than 10–20 Mb) of the genome. The array technique allows detecting DNA copy number changes (genomic DNA) in more detail, as well as analyzing gene expression using cDNA (complementary DNA). Most publications, so far, are on cDNA arrays (**31–33**). Recently, Pinkel et al. and Albertson et al. published articles on genomic DNA microarrays, describing a method to measure copy number fluorescence ratios on a set of clones that are located on chromosome 20. The technique provides a high resolution for measurement of gains and losses of DNA sequences in genomes of mammalian complexity (**34,35**). Microarray CGH will allow analysis of genetic aberrations in cancer with a high resolution, and refining the allocation of critical genes. Moreover, in clinical applications it could enable the identification of the status of certain disease-related genes. A detailed discussion can be found elsewhere (**36,37**).
3. High-quality metaphase preparations for CGH should preferentially contain an abundance of metaphases, have little residual cytoplasm (too much cytoplasm causes background and may prevent optimal denaturation), and minimal overlapping of the chromosomes. In addition, the chromosomes should have adequate length (400–550 bands) and not contain separated chromatids. Finally, for good banding strength, chromosomes should appear

dark, not shiny, when looking through a phase contrast microscope (28). It is important to test several batches of metaphase slides from different donors when setting up CGH, because their behavior in hybridization can be very different. Alternatively, fully prepared metaphase slides are commercially available. However, these slides also still need to be tested before use, and the quality is not necessarily better than that of in-house-produced slides (prepared as described above).

4. When biotinylated and DIG-conjugated deoxynucleotides (dUTPs) are incorporated into the DNA (indirect labeling), a detection step with fluorochrome-conjugated antibodies (avidin-FITC and sheep-anti-DIG-TRITC, respectively) is required after hybridization. Directly fluorochrome-conjugated deoxynucleotides render a smoother but weaker hybridization signal along the chromosomes. It is important that the labeled DNA fragments of both tumor and reference DNA are in the same range of lengths and are within the limits of 500–2000 bp.
5. The time of denaturation is a variable that should be adjusted to each new batch of metaphase preparations. When the metaphase slides are denatured too long, the fluorescent signal probably will be strong, but the DAPI banding will be very bad, making karyotyping impossible. Conversely, when the slides are not denatured sufficiently, there will be a nice, easy-to-recognize banding DAPI pattern, but the signal will be too low and granular. The art of CGH is to find a balance between these two scenarios.
6. The selection of good-quality metaphase spreads for digital image processing is crucial in the CGH. The fluorescent signal should be strong and homogeneous over the whole metaphase spread. The user should avoid metaphase spreads with many overlapping chromosomes or metaphase spreads with very small or very large chromosomes. The background should be low. Local high backgrounds are probably caused by residual cytoplasm. An even field illumination by the microscope is essential. Uneven illumination can cause gross artifacts. Furthermore, good-quality metaphases in CGH show dark centromeric regions as a sign of good blocking by Cot-1 DNA. Centromeres contain repetitive DNA sequences that are highly variable in length between individuals (and thus between tumor and reference DNA) and can therefore interfere with the CGH analysis. These repetitive sequences also occur to a lesser but nonetheless significant extent throughout the whole genome. Suboptimal blocking by Cot-1 DNA leads to reduced sensitivity.
7. There are two ways to interpret the relative copy number karyotypes. Some researchers use fixed ratio limits, for example 0.85/1.15 or 0.75/1.25, depending on the quality of the hybridization (29). Others prefer to use the 95% CI, which takes into account the quality of the signal. According to the latter definition, deviations from normal are interpreted as loss or gain when the average ratio, together with the 95% CI, is clearly below or above ratio 1.0. In addition, the green-to-red fluorescent ratios at 1p32-pter, 16p, 19p/q, and 22q may occasionally be unreliable, leading to false positive interpretation (30). In general, these chromosomal regions are excluded from the CGH profile interpretation if it concerns low-level gains or losses.

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## Simultaneous Fluorescence Immunophenotyping and FISH on Tumor Cells

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### 1. Introduction

Chromosome aberrations are regularly detected in most hematologic neoplasms and in various solid tumors and are often associated with distinct morphologic and immunophenotypic features of certain clinico-pathologic entities of tumors (1–4). Detection of these chromosome abnormalities provides the basis for a detailed diagnosis and for the genetic classification of tumors. Especially in leukemias and lymphomas, many primary chromosome aberrations are associated with the clinical course of the disease. Moreover, during tumor progression, additional chromosome aberrations occur. Therefore, cytogenetic findings are very helpful for predicting the prognosis and for choosing risk-adapted treatment strategies (5,6).

Cytogenetic analysis by karyotyping remains the standard for the detection of numerical and structural chromosome aberrations, such as trisomies, monosomies, translocations, deletions, or inversions. This technique gives, at one glance, an overview of all microscopically visible chromosome aberrations. The molecular cytogenetic technique of fluorescent *in situ* hybridization (FISH) has been shown to be a very powerful tool for the detection of certain chromosome aberrations (7,8). By use of chromosome- or single-locus-specific DNA probes, FISH may detect these chromosome aberrations not only in metaphase, but also in interphase cells. Immunophenotyping is a routine technique in any pathology laboratory in support of a differential diagnosis. Leukemia and lymphoma cells express a defined pattern of antigens that allows one to identify the cellular origin of the tumor cells and trace them back to defined stages of the normal differentiation process.

#### 1.1. Application of Simultaneous Fluorescence Immunophenotyping and FISH

Simultaneous fluorescence immunophenotyping and FISH provides the advantages of both techniques and allows a simultaneous or sequential analysis of the phenotype

and genotype of tumor cells at the single-cell level (9,10). By use of cell lineage and differentiation-associated monoclonal antibodies for immunophenotyping as well as DNA probes specific for known chromosome aberrations of a tumor for FISH, this combined technique makes it possible to compare and correlate particular genetic aberrations directly with the differentiation capacity of tumor cells. Thus, the cell lineage and differentiation stage of tumor cells can be defined (11,12). This approach also allows one to correlate the gene copy number and its protein expression level in the same cells (13). The power and the applicability of this technique in clinical studies and basic research have been extensively demonstrated in many studies on leukemias and lymphomas (9,14).

### 1.1.1. Defining Lineage Involvement of Tumor Cells

Cytogenetic analysis, Southern blot, or PCR techniques are often used for detecting chromosome aberrations or gene rearrangements. However, with these methods, it is not possible to assign genetic changes to certain cell populations, since they either destruct the cell morphology or damage the cell surface antigens during the procedures. Simultaneous immunophenotyping and FISH allows one to define the immunophenotype of tumor cells with certain chromosome or gene aberrations. Several studies with this or a similar approach have revealed that in myeloproliferative diseases (MPDs) and myelodysplastic syndromes (MDSs), tumor cells may involve the lymphoid as well as all myeloid lineages (15–18). In most acute myeloid leukemias (AMLs) and acute lymphoid leukemias (ALLs), the chromosome abnormalities are restricted to the leukemic blasts (19–21). Using CD56 as a specific immunophenotypic marker for natural killer cells and 6q deletion or trisomy 7 as genetic markers, we performed simultaneous immunophenotyping and FISH on three patients with natural killer cell lymphomas and leukemias. In all three patients, we were able to demonstrate that these chromosome aberrations were restricted to the CD56-positive natural killer cell population, whereas CD3-positive T lymphocytes were not involved (Fig. 1A). Thus, natural killer cell lymphoma and leukemia is derived from mature natural killer cells, but not from T lymphocytes (22).

### 1.1.2. Determining the Differentiation Stage of Tumor Cells

It is believed that any tumor results from accumulated genetic changes and chromosome aberrations. One critical problem is defining which stage of cell differentiation that these genetic changes occur. Simultaneous immunophenotyping and FISH could help to determine the differentiation stage of tumor cells in many leukemias and lymphomas. By use of this combination or modified techniques, MPD, MDS, and AML in some patients were shown to be stem cell diseases with commitment to all myeloid lineages and, in some patients, to the lymphoid lineage (15,16,19,23). In CML, tumor cells are derived from a stem cell with a capacity to differentiate into all myeloid and lymphoid lineages (24–26). The *MLL* gene on 11q23 is frequently involved in different chromosome translocations in *de novo* and therapy-related leukemia. These translocations result in chimeric genes that play an important role in leukemogenesis (1). In three patients with therapy-related leukemia, we performed simultaneous immunophenotyping with CD34 as a stem cell marker and a FISH study with a YAC

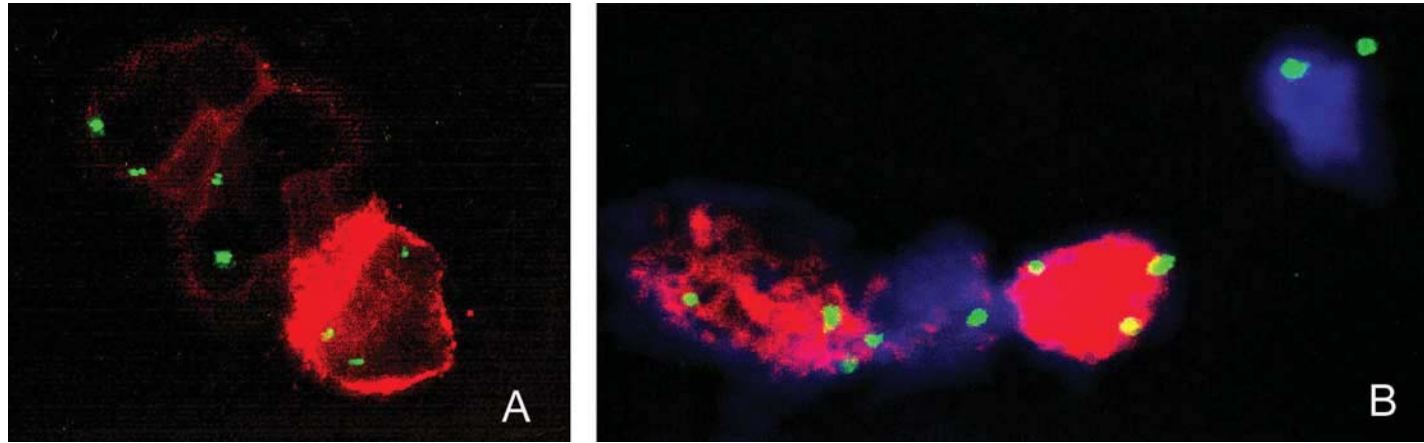


Fig. 1. Simultaneous fluorescence immunophenotyping and FISH on a natural killer cell leukemia. (A) Immunophenotyping with monoclonal mouse anti-human CD56 was performed to identify natural killer cells. Mouse anti-human CD56 was visualized with Cy3 (red). A biotinylated D7Z1 DNA probe specific for the centromere of chromosome 7 was detected with FITC (green) in FISH. The CD56-positive natural killer cell shows three hybridization signals for D7Z1, indicating trisomy 7, whereas the adjacent CD56-negative cells contain two regular hybridization signals for D7Z1. Thus, the tumor clone in the natural killer cell leukemia and lymphoma is restricted to CD56-positive natural killer cell population. (B) Monoclonal mouse anti-human Ki-67 antibody was visualized with Cy3 (red) in immunophenotyping study. A biotinylated D7Z1 probe specific for the centromere of chromosome 7 was detected with FITC (green)-conjugated avidin in the FISH study. The cells were counterstained with DAPI (blue). Two cells show three hybridization signals for D7Z1, indicating trisomy 7. These tumor cells also display a very strong fluorescence staining (red) with mouse anti-human Ki-67, indicating a high proliferation level. The adjacent cells contain two regular hybridization signals of D7Z1 and show a very weak or negative fluorescence staining with mouse anti-human Ki-67 antibody. Thus, in natural killer cell leukemia and lymphoma, chromosome aberrations are restricted to the CD56-positive tumor cell population and tumor cells have a much higher proliferation level than normal cells.

DNA probe containing the *MLL* gene. We were able to confirm that chromosome translocations involving *MLL* occurred in CD34-positive hematopoietic cells. Thus, therapy-related leukemia is derived from hematopoietic progenitor cells or even from stem cells (12).

### 1.1.3. Increasing the Detection Sensitivity of FISH

Some tumors consist of a mixture of tumor and normal bystander cells. In this case, it may be difficult to reach the threshold for the detection of chromosome aberrations by FISH (7,8). Given that the tumor cells contain an antigen that is expressed only in a minority of normal cells, simultaneous immunophenotyping and FISH can overcome these difficulties. Because the tumor cell population can be recognized by the application of defined monoclonal antibodies, only cells with positive staining will be evaluated for certain chromosome aberrations by FISH. Thus, the evaluation will be restricted to this cell population, and many normal cells around or mixed with the tumor cells will be excluded from the analysis. Therefore, the sensitivity of detection of chromosome aberrations by FISH will be greatly improved. An example was demonstrated in our previous study on 30 patients with Hodgkin's disease (27). It is known that the typical CD30-positive HRS cells in Hodgkin's disease usually comprise only ~1–2% of the total cells. When FISH only is used, this percentage is just at or even beyond the cut-off limit for the detection of defined chromosome aberrations. Using simultaneous immunophenotyping and FISH, we were able to find that only CD30-positive HRS cells contained numerical chromosome aberrations in all 30 patients with Hodgkin's disease, whereas CD30-negative lymph node cells regularly had normal numbers of hybridization signals. This provided evidence that only CD30-positive HRS cells belong to tumor clones (27). In addition, this technique may be useful in evaluating patient response to therapy by detecting minimal residual tumor cells, given that the tumor cells have an immunophenotype different from that of the normal cells.

### 1.1.4. Correlating the Gene Copy Number and its Protein Expression Level

Simultaneous immunophenotyping and FISH enable us to compare the gene copy number with its protein expression level directly at the single-cell level. We applied this technique to six breast carcinoma cell lines and compared the copy number of the estrogen receptor (*ESR*) gene and the estrogen receptor (ER) expression pattern at the single cell level (13). We found that the tumor cells with a deletion of *ESR* had a ER expression level similar to those without a deletion; the ER expression level of tumor cells with 3–4 copies of *ESR* is at the same level as that of cells with two regular copies of *ESR*. Thus, the copy number of the *ESR* gene is not directly correlated with the expression of the ER, and a gene dosage effect attributable to loss of the *ESR* gene seems not to be the major cause of negative ER expression in breast carcinoma. Other mechanisms may be involved in determining the ER expression level.

### 1.1.5. Detecting the Proliferation Level of Tumor Cells

In general, tumor cells have a proliferation advantage over normal cells and thus, are very active in cell division. The increased proliferation level of tumor cells may be

a part of the causes leading to an aggressive clinical course in some patients (28). Simultaneous immunophenotyping and FISH detect the proliferation level of tumor cells that have certain gene or chromosome aberrations. Using the Ki-67 antibody as a cell proliferation marker, we applied this combined technique to a natural killer cell leukemia. We found that the tumor cells with trisomy 7 as a tumor clone marker had a very high intensity of fluorescence staining with Ki-67 in immunophenotyping, whereas normal cells with two regular copies of chromosome 7 showed very weak or negative staining with Ki-67 (Fig. 1B). Thus, tumor cells in natural killer cell leukemia have a much higher proliferation ability than do normal cells.

## 2. Materials

### 2.1. Reagents

1. Monoclonal mouse anti-human antibodies, such as CD4, CD8, and CD56.
2. Human COT-1 DNA.
3. Human placental DNA.
4. Dextran sulfate.
5. Sephadex G-50 minicolumn.
6. Sonicated salmon sperm DNA.
7. 70, 85, and 100% ethanol.
8. Rubber cement.
9. Deionized formamide.
10. DAPI (4,6-diamidino-2-phenylindole).
11. DABCO (1,4-diazabicyclo[2,2,2]octane).
12. Normal mouse serum.
13. Mouse monoclonal anti-digoxigenin antibody.
14. Mouse anti-FITC antibody.
15. AMCA-conjugated avidin.
16. FITC-conjugated avidin.
17. Biotinylated goat-anti-avidin antibody.
18. Cy3-conjugated avidin.
19. Cy3-conjugated goat anti-mouse antibody.
20. Cy3-conjugated rabbit anti-goat antibody.
21. Cy3-conjugated donkey anti-rabbit antibody.
22. Cy3-conjugated rabbit anti-mouse antibody.
23. Digoxigenin-conjugated sheep anti-mouse antibody.
24. FITC-conjugated donkey anti-mouse antibody.
25. FITC-conjugated sheep anti-digoxigenin antibody.

### 2.2. Buffers and Solutions

1. PN buffer: 0.1 M  $\text{NaH}_2\text{PO}_4$ , 0.1 M  $\text{Na}_2\text{HPO}_4$ , 0.03%  $\text{NaN}_3$  (poisonous), pH 8.0.
2. PNM buffer: Add 5 g dry milk powder to 100 mL PN buffer; heat to 50–60°C and stir overnight until completely dissolved. It can be stored at 4°C for several months or at –20°C for years.
3. 20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
4. Antifade solution: Add 0.23 g DABCO and 10 mL PN-buffer to 90 mL glycerol; mix well.
5. DAPI solution: Dilute DAPI at 0.2 mg/mL in 2X SSC, pH 7.0, protect it from light at 4°C.
6. Carnoy's fixative: Freshly prepare 3:1 methanol:glacial acetic acid, and keep at 4°C.

7. 1% Paraformaldehyde: Add 1 g paraformaldehyde (CAUTION!!) to 100 mL distilled water, adjust to pH 7.0. This buffer may be stored at 4°C for 2 wk.
8. Hybridization master mix I: 60% formamide, 10% dextrane sulfate, 1X SSC.
9. Hybridization master mix II: 50% formamide, 10% dextrane sulfate, 2X SSC.

### 3. Methods

#### 3.1. Slide Preparation

For simultaneous immunophenotyping and FISH, cytospin preparations, smears, imprints, or cryostat sections may be used. Appropriate handling and preservation of the cells are critical for successful performance of the procedure. In general, freshly prepared slides should be air dried overnight at room temperature prior to being stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . Slides may be kept in this way for several weeks or even up to several years. After being taken out from freezers, slides should be thawed at room temperature and air dried for several minutes prior to use. Also, the morphology and location of cells should be checked under a phase contrast microscope before use. The following is a protocol for cytospin preparation.

1. Cut biopsies such as lymph node or solid tumor tissue in PBS buffer into small pieces with needles and knives.
2. Collect the obtained cell suspension from the sample.
3. Spin at 200g for 10 min.
4. Discard the supernant and resuspend the cells in PBS buffer.
5. Determine the cell number.
6. Dilute or concentrate cells to  $1 \times 10^4/\text{mL}$ .
7. Add 200  $\mu\text{L}$  of cells in a cytospin centrifuge and spin at 200g (800 rpm) for 5 min.
8. Air dry slides over night or for at least 2 h at room temperature.
9. Keep slides at  $-20^{\circ}\text{C}$ .

#### 3.2. Immunophenotyping

Depending on the questions to be answered, single or double immunophenotyping may be performed. The following are two protocols for single and double immunophenotyping.

##### 3.2.1. Immunophenotyping with a Single Antigen (such as CD4)

1. Take preserved slides out from  $-20^{\circ}\text{C}$  freezer and air-dry for 10 min at room temperature.
2. Put slides in fresh acetone for 10 min at room temperature, followed by air-drying for a few minutes.
3. Rinse slides in PN buffer.
4. Incubate with primary monoclonal mouse antibody such as CD4: Add 100  $\mu\text{L}$  mouse anti-human CD4 antibody diluted in PNM buffer to slides and incubate for 25 min at room temperature. Dilution of mouse anti-human antibody varies from 1:50 to 1:100, depending on quality of antibodies and antigen expression level of cells (see **Note 1**).
5. Wash with PN buffer three times, 5 min each, at room temperature.
6. Incubate with 100  $\mu\text{L}$  Cy3-conjugated goat anti-mouse antibody diluted 1:200 in PNM buffer at room temperature for 25 min.
7. Wash with PN buffer three times, 5 min each, at room temperature.
8. Incubate with 100  $\mu\text{L}$  Cy3-conjugated rabbit anti-goat antibody diluted 1:200 in PNM buffer at room temperature for 25 min.

9. Wash with PN buffer three times, 5 min each, at room temperature.
10. Incubate with 100  $\mu$ L Cy3-conjugated donkey anti-rabbit antibody diluted 1:200 in PNM buffer at room temperature for 25 min.
11. Wash with PN buffer three times, 5 min each, at room temperature.
12. Fix the cells in 1% ice-cold paraformaldehyde solution for 1 min (*see Note 2*).
13. Wash in A. bidest once at room temperature.
14. Put slides in fresh Carnoy's fixative at 4°C for 10 min (*see Note 2*).
15. Wash slides in A. bidest for 2 min at room temperature.

At this point, immunophenotyping may be checked under a fluorescence microscope. Mount the slides with PN buffer and cover with a coverslip and check whether certain cells show definitive fluorescence. If not, stop here and perform another control test with various concentrations of primary and secondary antibodies. Please note that the general fluorescence intensity of cells is relatively low when the slides are covered with PN buffer. Do not take long time for checking under microscope.

16. Dehydrate cells in a series of 70, 85, and 100% ethanol at room temperature for 3 min each, followed by air-drying at room temperature for at least 10 min. The cells are ready for FISH study (*see Subheading 3.3.*).

### 3.2.2. Double Immunophenotyping (such as CD4 and CD8)

#### 3.2.2.1. IMMUNOPHENOTYPING FOR FIRST ANTIGEN (E.G., CD4)

1. Perform **steps 1–3** in **Subheading 3.2.1**.
2. Incubate with 100  $\mu$ L monoclonal mouse antibody against human CD4 1:50 to 1:100 diluted in PNM buffer at room temperature for 25 min (*see Note 3*).
3. Wash with PN buffer three times for 5 min each at room temperature.
4. Incubate with Cy3-conjugated rabbit anti-mouse antibody diluted 1:200 in PNM buffer at room temperature for 25 min.
5. Wash with PN buffer three times for 5 min each at room temperature.
6. Incubate with Cy3-conjugated donkey anti-rabbit antibody diluted 1:200 in PNM buffer at room temperature for 25 min.
7. Wash with PN buffer three times for 5 min each at room temperature.
8. Incubate with 20% normal mouse serum diluted in PNM buffer at room temperature for 15 min (do not wash the slides after incubation and go directly to **Subheading 3.2.2.2.**) (*see Note 4*).

#### 3.2.2.2. IMMUNOPHENOTYPING FOR SECOND ANTIGEN (E.G., CD8)

1. Incubate with biotinylated monoclonal mouse antibody against human CD8 1:100 diluted in PNM buffer containing 20% mouse serum at (room temperature) RT for 25 min.
2. Wash with PN buffer three times, 5 min each, at room temperature.
3. Incubate with AMCA-conjugated avidin diluted 1:50 in PNM buffer at room temperature for 25 min.
4. Wash with PN buffer three times, 5 min each, at room temperature.
5. Incubate with biotinylated goat anti-avidin diluted 1:100 in PNM buffer at room temperature for 20 min.
6. Wash with PN buffer three times, 5 min each, at room temperature.
7. Incubate with AMCA-conjugated avidin diluted 1:50 in PNM buffer at room temperature for 25 min. To enhance the signal intensity, **steps 5–7** may be repeated.
8. Put slides in 1% ice-cold paraformaldehyde solution to fix the cells.

9. Wash in A. bidest once at room temperature.
10. Put slides in fresh Carnoy's fixative at 4°C for 10 min.
11. Wash slides in A. bidest for 2 min at room temperature.
12. Mount the slides with PN butter and check immunophenotyping under a fluorescence microscope as described in **Subheading 3.2.1**.
13. Dehydrate slides in a series of 70, 85, and 100% ethanol at room temperature for 3 min each, followed by air drying at room temperature for at least 10 min.

### **3.3. FISH**

#### **3.3.1. DNA Probe Preparation**

##### **3.3.1.1. REPETITIVE PROBES**

Many probes of this kind are commercially available from several biological companies, such as Vysis. In general, hybridization master mix I is used. However, if commercial probes are used, it is recommended to follow the manufacturer's instructions for probe preparation.

##### **3.3.1.2. SINGLE COPY OR LOCUS SPECIFIC DNA PROBES**

Such probes are cloned in different vectors from phage to PAC, BAC, or YAC. After DNA labeling with biotin, digoxigenin, or fluorescence dyes such as Spectrum green or orange, DNA probes have to be precipitated to reduce the volume. Hybridization master mix II can be used for dissolving the probes. For some commercial probes, such as painting probes, this protocol is also applicable. It is necessary to follow the manufacturer's suggestions for probe preparation. For example, for preparation of DNA probes purchased from the Vysis, 1  $\mu\text{L}$  of directly labeled probes should be premixed with 2  $\mu\text{L}$  deionized water and 7  $\mu\text{L}$  supplied CEP hybridization buffer.

##### **3.2.1.3. DNA LABELING**

We recommend the use of commercial DNA nick-labeling kits from Life Technologies/Gibco for DNA labeling with biotin or digoxigenin. For each reaction, 1  $\mu\text{g}$  of DNA probe is labeled at 15°C for 1 h. After labeling, the reaction is spun through a Sephadex G-50 minicolumn (Amersham Biotechnologies). Load 5  $\mu\text{L}$  of the Nick-translated probe on a 1% Agarose gel to check the size of the labeled DNA probe fragments. The fragments should be between 200–800 bp in length, with the majority between 300–600 bp.

##### **3.3.1.4. PRECIPITATION OF LABELED DNA PROBE**

1. Add the following in a 1.5-mL Eppendorf tube and keep at  $-70^{\circ}\text{C}$  for 30 min: 5–10  $\mu\text{L}$  labeled DNA, 5  $\mu\text{g}$  human COT-1 DNA, 1  $\mu\text{g}$  human placental DNA, 3  $\mu\text{g}$  salmon sperm DNA, 1/10 vol 3 M NaOAc (pH 5.2), 2.5 vol 100% ethanol.
2. Centrifuge at 13000g for 30 min at 4°C.
3. Discard the supernatant, add 2 vol 70% ethanol to dissolve the remaining salt and spin again at the same speed for 10 min.
4. Air-dry at room temperature or by a Speed-vac.
5. Add 10  $\mu\text{L}$  hybridization master mixture I or II and vortex to dissolve probes.

### 3.3.2. Hybridization

If a single immunophenotyping is done, dual-color FISH with biotinylated and digoxigenin-labeled probes may be performed. In dual-color FISH, equal amounts of biotin- and digoxigenin-labeled probes are precipitated together and hybridized to cells. When double immunophenotyping is done, only a digoxigenin-labeled probe can be used in the FISH study (*see Note 5*).

1. Pipet 2–3  $\mu\text{L}$  of hybridization mixture containing DNA probes to the cell-rich areas of the slides.
2. Cover with a 12-mm round coverslip.
3. Seal with rubber cement and let it dry.
4. Place the slides at the bottom of a metal box with a wet paper towel. After covering, put the metal box in a waterbath at 76°C for 5 min (*see Note 6*).
5. Transfer the box to a 37°C incubator. Hybridization takes from a few hours to overnight or even two days, depending on the DNA probe types. For repetitive DNA probes, hybridization may be just for 2 h, whereas for cDNA probes or chromosome painting probes, hybridization for 2 d are recommended.

### 3.3.3. Post-Hybridization Washes

1. Remove rubber cement.
2. Put the slides in pre-warmed 0.1X SSC buffer, pH 7.0, at 60°C in a waterbath and let coverslips fall down (*see Note 7*).
3. Wash slides in 0.1X SSC three times, 5 min each, with shaking.
4. Rinse slides in PN buffer for 3 min at room temperature.

### 3.3.4. Detecting Hybridization Signals

The following protocol is used for detecting both biotin- and digoxigenin-labeled probes. In simultaneous double immunophenotyping and FISH, only digoxigenin-labeled probe should be used. The antibodies for detecting the biotinylated probe, i.e., AMCA-avidin and biotinylated goat anti-avidin, should be omitted in the following steps (*see Note 5*).

1. Incubate with a mixture of AMCA-conjugated avidin and monoclonal mouse anti-digoxigenin antibody, both diluted 1:100 in PNM buffer, at room temperature for 25 min.
2. Wash with PN buffer three times, 5 min each, at room temperature.
3. Incubate with a mixture of biotinylated goat anti-avidin antibody and digoxigenin-conjugated sheep anti-mouse antibody, both diluted 1:100 in PNM buffer, at room temperature for 25 min.
4. Wash with PN buffer three times, 5 min each, at room temperature.
5. Incubate with a mixture of AMCA-conjugated avidin and FITC-conjugated sheep anti-digoxigenin antibody, both diluted 1:100 in PNM buffer, at room temperature for 25 min.
6. Wash with PN buffer three times, 5 min each, at room temperature.
7. Mount the slides with antifade medium. If only red and green fluorescence dyes are used in the study, cells may be stained with DAPI prior to mounting by putting slides in DAPI solution for 2 min followed by rinsing in 2X SSC. This counterstaining may help in defining the location of hybridization signals in certain cell nuclei.

### **3.4. Image Recording and Evaluation of Immunophenotyping and FISH Results**

The results of immunophenotyping and FISH may be evaluated simultaneously or sequentially under a fluorescence microscope, depending on the use of single-, dual- or triple-dye fluorescence filter sets. The results may be photographed with a high-speed film (ASA 400) or recorded with a digital imaging system, such as ISIS from Metasystems. For evaluation of the results of immunophenotyping, it is important to distinguish true- from false-positive reactions that may result from background staining or cross-reaction of the antibodies used. Generally, the former shows a bright and fresh fluorescence, whereas the latter has a dim and weak or extremely bright fluorescence. For FISH analysis of immunophenotyped cells, only intact nonoverlapping cells should be evaluated. We generally analyze at least 50 positive cells and more than 100 negative cells per slide. More important, a blinded evaluation of these results should be performed by another independent observer.

## **4. Notes**

1. For immunophenotyping, monoclonal mouse anti-human CD antibodies as well as polyclonal Cy3- or AMCA-conjugated antibodies are diluted from 1:50 to 1:200 in PNM buffer. The appropriate concentration depends on the quality of the antibodies and on the expression level of the antigen. For example, the strongly expressed CD3 on T lymphocytes may easily be detected by incubation of only the first or the first and the second antibodies. Thus, a control study should be done for each newly purchased antibody for determining its optimal concentration. In general, Cy3 and FITC are very strong and durable fluorescence dyes, whereas AMCA is weak and unstable. Immunophenotypes detected with Cy3 can be reviewed even after several weeks or months, whereas those detected with AMCA will disappear very soon. It is not possible to review slides detected with FITC later than after a few days, because of the background due to the auto-fluorescence of the cells.
2. For refixation after immunophenotyping, either 1 or 4% paraformaldehyde may be used. It can be stored for up to 2 wk at 4°C, whereas Carnoy's fixative must be freshly prepared each time. The times of refixation of these buffers are very critical for successful combination of immunophenotyping and FISH. The shorter the time that cells are fixed, the more fluorescence intensity is lost. However, too long fixation will result in a dramatically low hybridization efficiency in FISH study.
3. For immunophenotyping, a negative control must be set up each time for exclusion of any false-positive results. Any fluorescence from this procedure represents nonspecific staining (noise). Replacing the monoclonal or primary antibody with water, PBS, or irrelevant isotype-matched antibodies could be used as negative control. In order to examine cross-reactions or interruption of polyclonal antibody's chain derived from different animals, a control slide should be used in the double immunophenotyping study (23).
4. In double immunophenotyping experiments, incubation with 20% normal mouse serum containing high concentrations of mouse immunoglobulins is very important for successful second immunophenotyping. The free binding sites of the rabbit anti-mouse antibody are blocked and will not be able to bind with a second primary mouse anti-human antibodies (here, CD8 is used as an example). Do not wash slides after this incubation. Also, in FISH on double-immunophenotyped cells, do not use biotinylated probes, because they will bind nonspecifically to the antibody cascade of the second monoclonal mouse anti-human antibody, i.e., CD8.

5. Because of color fluorescence limitations, it is difficult in practice to combine three fluorescence colors, i.e., green (FITC), red (Cy3), and blue (AMCA), in one experiment. In fact, the blue fluorescence of AMCA is very weak and unstable, and it can be used only in detecting repetitive probes in FISH. On the other hand, FITC will decrease intensity faster than Cy3 after detection steps, and the contrast to background becomes less. Therefore, we suggest the use of the Cy3-conjugated antibodies for detecting weakly expressed antigens or small locus-specific DNA probes.
6. We do not find any difference between separate and combined denaturing of cellular DNA and probes. In many laboratories, targeted cellular DNA and probe DNA are denatured separately, followed by preannealing of the DNA probe with human Cot-1 DNA. We find that combined denaturing is faster, easier, and results in similar FISH results as separate denaturing procedures.
7. In the FISH study, we found that post-hybridization washing in 0.1X SSC three times at 60°C results in a washing efficiency equal to that of washing in 50% formamide/2X SSC at 42°C, whereas the former solution is much cheaper and not toxic.

## Acknowledgment

This work was supported by European Community grant QLG1-2000-00687 and by German Cancer Aid grant 10-1556-Schl4.

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## BAC Resource for Molecular Cytogenetics

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### 1. Introduction

Bacterial artificial chromosomes (BACs) are ideal materials to use for the purpose of integrating DNA sequence with cytogenetic markers. They have been the major vectors used in genome sequencing. BACs are also well suited for fluorescent *in situ* hybridization (FISH) in that they represent a stable and easily manipulated form of cloned DNA that produces bright, well-defined signals on metaphase and interphase chromosome preparations (1). To link chromosomal position with DNA sequence throughout the human genome, we have developed an integrated BAC resource (1) by using FISH, PCR, and sequencing. The Resource contains a total of 6000 randomly mapped BAC clones, out of which, 1021 are BAC-sequence-tagged sites (STS) pairs representing 957 BACs (Fig. 1). This tool can be now used to rapidly identify genes affected by chromosomal rearrangements seen in genetic disorders and cancers. After this initial development, an international effort has assembled a collection of BAC clones that are both sequence-tagged and mapped relative to cytogenetic bands using FISH, resulting in a collection of 7600 clones (2; see Note 1).

Over the past decade, FISH has evolved into a method that is used in clinical and research laboratories around the world. FISH is capable of simultaneously detecting regions of homology among chromosomes and providing a window into the evolutionary history of exchanges, duplications, and rearrangements in the genome (1,2). In particular, the ability to hybridize complex DNA probes to specific metaphase chromosomes and interphase nuclei has made FISH an indispensable tool for both clinical diagnostics and basic research (3–6). However, the applications of FISH techniques were limited owing to lack of high-quality DNA probes that cover the whole genome. This situation has now been remedied by the generation of the BAC resource (1,2).

We have described here a detailed protocol and examples of the applications using the Integrated BAC Resource for solving chromosomal puzzles that are beyond the resolution of standard cytogenetics. These include application to cancer genetics and analysis of germline chromosome aneuploidy (see Notes 2–4).





4. Slide warmer (Precision).
5. Spectrophotometer (Beckman) or Fluorometry (Turner Designs).
6. Shaking waterbath (Precision).
7. Incubator oven (Fisher Science) set to 37°C.
8. S/P Brand superfrost slides (25 × 75 mm) (Allegiance)
9. S/P Brand coverglass (22 × 50 mm; 22 × 40 mm; 22 × 22 mm) (Allegiance).

## 2.2. Chemicals

### 2.2.1. BAC DNA Extraction

1. Terrific Broth (Life Technologies): Add 48.2 g/L of purified water. Mix well to dissolve, add 8 mL glycerol and then autoclave at 121°C for 15 min. Cool to 50°C, and add chloramphenicol (Sigma) to 12.5 µg/mL.
2. RNase solution: 50 mM Tris-HCl, pH 8, 10 mM EDTA and RNase A (100 µg/mL) (Life Technologies).
3. DNA extraction kit: NucleoBond Nucleic Acid Kit (ClonTech); QIAGEN Plasmid Midi/Maxi kit (Qiagen).
4. Phenol: Chloroform (Life Technologies).
5. Agarose (Life Technologies).
6. 1 kb DNA marker (Gibco-BRL).

### 2.2.2. Chromosome Preparation

1. Cell culture medium: RPMI 1640 (Life Technologies) supplemented with L-glutamine (2 mM) (Sigma), 15% fetal calf serum (Sigma), penicillin (100 Iu/mL) (Life Technologies), streptomycin (0.05 mg/mL) (Life Technologies) and 0.02% phytohemagglutinin (Life Technologies).
2. Hank's BSS (Life Technologies).
3. 5-Bromode oxyuridine (Sigma).
4. Thymidine (Life Technologies).
5. Colcemid (Life Technologies), 10 mg/mL. Stored at 4°C.
6. Hypotonic solution (0.075 M KCl): 5.6g KCl/L of purified water.
7. Fixative: acetic acid/methanol: 1/3 (vol/vol). Make fresh right before use and keep on ice.

### 2.2.3. Probe DNA Labeling

1. Nick-translation kit and Bionicktranslation kit (Life Technologies) Store at -20°C.
2. Digoxigenin-11-dUTP (Boehringer Mannheim), 1 mM. Store at -20°C.
3. Fluorolink Cy3-dCTP (Amersham Pharmacia Biotech). Store at -20°C.
4. Fluorolink Cy5-dCTP (Amersham Pharmacia Biotech). Store at -20°C.
5. DNase I (Gibco-BRL).
6. G 50 Sephadex Quick spin column (Life Technologies).

### 2.2.4. FISH and Post-Hybridization Detection

1. Human COT1™ DNA (Life Technologies), 1 mg/mL. Store at -20°C.
2. Salmon sperm DNA (3'-5') Store at -20°C.
3. 3 M Sodium acetate (Sigma).
4. Bovine serum albumin (Sigma). Store at 4°C.
5. FITC (avidin-conjugated fluorescein isothiocyanate) (Vecter Labs).
6. Sheep-anti-digoxigenin-Rhodamine (Boehringer Mannheim).
7. Chromomycin A3 (Sigma) 0.5 mg/mL (in 50% McIlvane's buffer).

8. Distamycin A (Sigma) 0.1 mg/mL (in 50% McIlvane's buffer).
9. Denaturing solution (70% formamide [EM Science]/2X SSC): add 35 mL formamide, 10 mL distilled water, 5 mL 20X SSC, pH 7.0. Store at 4°C. Prepare fresh every 2–4 wk.
10. Ethanol series: prepare 70, 80, and 100% ethanol in distilled water and keep on ice.
11. Hybridization Master Mix: 10% v/v dextran sulfate (Sigma), 50% formamide (EM Science), 2X SSC, pH 7.0. Store at –20°C.
12. Formamide (Fisher) and 2X SSC wash solution: add 15 mL 20X SSC, 60 mL distilled water, 75 mL formamide, pH 7.0. Store at 4°C.
13. 20X SSC: 3 M NaCl, 0.3 M Na<sub>3</sub> citrate, pH 7.0.
14. 4X SSC/0.1% Tween-20 (Sigma): add 100 mL 20X SSC, 400 mL distilled water and 500 µL Tween-20. Mix well.
15. TE (Tris/EDTA) buffer: 1X TE is 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, 7.5, or 8.0.
16. 1 M Tris-HCl, pH 7.5 or 8.0.
17. 0.5 M EDTA, pH 8.0.
18. McIlvane's buffer (pH 9.0): citric acid 0.63 g, sodium phosphate dibasic 6.19 g, 500 mL of purified water.
19. Antifade solution (7): Dissolve 100 mg *p*-phenylenediamine dihydrochloride (Sigma) in 10 mL PBS. Adjust to pH 8 with 0.5 M bicarbonate buffer (0.42 g NaHCO<sub>3</sub> in 10 mL water, adjust pH 9.0 with NaOH). Add to 90 mL glycerol. Store in aliquotes at –20°C. The solution darkens with time, but remains effective even after a few years.

### 3. Methods (8)

#### 3.1. Metaphase Preparation and Slide Selection (see Note 5)

1. Human peripheral lymphocytes are grown in cell culture medium for 72 h at 37°C.
2. The cells are blocked in S-phase by adding 5-bromodeoxyuridine (0.8 mg/mL) for 16 h.
3. The cells are then washed once with HBSS (Hanks' Balanced Salt Solution) to remove the synchronizing agent and are released by additional incubation for 6 h in cell culture medium with 2.5 µg/mL of thymidine.
4. Cultures are harvested by addition of 0.1 µg/mL of colcemid for 10 min followed by treatment with 10 mL 0.075 M KCl for 15 min at 37°C and fixation for 4× in a freshly made solution of methanol and glacial acid (3:1/v).
5. To obtain high quality chromosome preparations, metaphase spreads are prepared by letting one drop of cell suspension fall from 15 in on alcohol-cleaned slides, then placing above a container filled with heated (close to boiling) water for 20–40 s. This time varies with the ambient humidity and with the individual cell preparations. It must be determined for each cell preparation and checked using a phase contrast microscope. If there is cytoplasmic residue visible around the metaphase spread, wash the remaining cells with fixative several times before dropping. Ideally, the chromosomes in metaphase spreads should appear dark black (see Note 6). If chromosomes appear glassy or refractile, this suggests that the cells have dried too slowly. The slides are then kept in the dark for at least 2–3 wk at room temperature, and stored at –70°C until use.
6. Slides are reviewed before using for FISH. Look at the slide under phase contrast (×10), select a region of interest containing at least five metaphase spreads per field and mark the area at the edges of the cell spreads with a diamond tip pen.
7. The RNase treatment step is usually omitted if the slides are aged more than 2 wk. In general, it does not affect the ratio of signal to noise. If RNase treatment is used, 100 µg/mL RNase is used for 30 min at 37°C and is then followed by dehydration through an ethanol series (70, 90, and 100%).

### 3.2. BAC DNA Extraction (see Note 7)

The following protocol is from Qiagen, good for preparing 20–50  $\mu\text{g}$  of DNA (QIAGEN plasmid Midi/Maxi kit). Alternative protocols employ the NucleoBond BAC Miniprep kit or the NucleoBond BAC Maxi kit (ClonTech) according to the directions provided by the manufacturers.

1. Streak the bacterial culture on an LB agar plate containing chloramphenicol (12.5  $\mu\text{g}/\text{mL}$ ) for overnight at 37°C.
2. Pick up single colonies and grow in 100 mL of LB containing chloramphenicol (12.5  $\mu\text{g}/\text{mL}$ ) for overnight at 37°C. Try to achieve an OD<sub>600 nm</sub> of 1.4–1.6.
3. Spin culture in 250 mL buckets using a GSA rotor/Sorval at 3000g for 15 min.
4. Resuspend bacterial pellet in 10 mL of RNAse solution. Pipet up and down using a 10 mL pipet and resuspend completely. Transfer cells to Oak Ridge tubes (Allegiance).
5. Add 10 mL of 0.2 M NaOH and 1% SDS. Mix tubes gently by inverting tubes repeatedly but slowly 10 $\times$ . Incubate at room temperature for 5–10 min and make sure the solution turns from turbid to translucent.
6. Add 8 mL of chilled 3 M KAc, pH 5.5. Mix tubes gently by inverting slowly 10 $\times$ . Excessive violent mixing will increase contamination with *E. coli* genomic DNA. Allow to stand on ice for 15 min.
7. Centrifuge at 4°C in a Sorvall RC 28S centrifuge using an SS34 rotor for eight tubes or an SA-600 (12 tubes). Spin at  $\sim$ 33,000g or more (16,000 rpm for the SA-600) for 30 min and remove the supernatant promptly.
8. Remove supernatant carefully using 25 mL pipet while avoiding debris. Place supernatant in a clean autoclaved Oak Ridge tube.
9. Centrifuge again as in **step 6**. Even though your supernatant may appear clear, you must centrifuge again since skipping this step will cause the column to clog. Transfer supernatant to a fresh 50 mL tubes and keep on ice.
10. Equilibrate “Qiagen-tip 100” columns with 4 mL of QBT buffer.
11. Apply the supernatant from **step 8** to a Qiagen-tip 100 column.
12. Wash the Qiagen-tip 100 with 10 mL of QC buffer twice.
13. Elute the DNA from the column with 5 mL of 60°C-QF buffer. Collect the eluant in a 14-mL culture tube containing 5 mL of isopropanol. Use culture tubes from the VWR cat no. 60818-725 since these can withstand high G-forces. Thinner walled tubes can break. Oak Ridge tubes can be substituted, but the pellet is more difficult to observe.
14. Centrifuge tubes at 11,700g for 30 min at 4°C. For the Sorval SA-600 rotor, use 9000 rpm  $\sim$  11,700g.
15. Wash pellet with 5 mL of 70% EtOH at room temperature. Spin again for 10 min. Carefully pour off supernatant. Air dry 10 min. This step removes excess salt from the BAC DNA preparation.
16. Resuspend pellets in 400  $\mu\text{L}$  of TE buffer using pipet tips with the ends cut off. Because BAC DNAs are large, it may take some time to totally resuspend the DNA. You may want to let the tubes sit overnight at room temperature until the pellets are dissolved into TE buffer. Transfer DNA to a clean 1.5 mL Eppendorf tube.
17. Add 400  $\mu\text{L}$  of phenol/chloroform (1:1) and vortex. Spin for 5 min at a maximum speed of any microcentrifuger (see **Note 7**).
18. Remove and transfer aqueous phase (top) to clean 1.5-mL tubes.
19. To precipitate the DNAs, add 40  $\mu\text{L}$  of the 3 M NaOAc and 1100  $\mu\text{L}$  of cold EtOH and keep at  $-20^\circ\text{C}$  for 2 h .

20. Centrifuge at maximum speed for 30 min and wash pellet with 750  $\mu\text{L}$  of cold 70% EtOH twice.
21. Dissolve pellet in 50  $\mu\text{L}$  TE buffer and leave the tube at 4°C for overnight or at 37°C for overnight if the pellets are large.
22. Expect a total of 20–50  $\mu\text{g}$  yield of a concentrations of about 0.5–1  $\mu\text{g}/\mu\text{L}$  when resuspending in 50  $\mu\text{L}$ .
23. Determine BAC DNA yield by UV spectrophotometry or fluorometry. Confirm BAC integrity by Agarose gel electrophoresis.

### 3.3. Probe DNA Labeling (see Note 8)

The procedures below follow the protocol provided in the Nick-translation kits (Gibco-BRL Life Technologies).

1. Select the nucleotide to be used from one of the five mixes, which contain all dNTPs except those to be used for tagging with biotin or digoxigenin, and thaw it on ice. Keep the DNase I/Polymerase I mix on ice or at –20°C before use.
2. Add the following reagents to a 1.5-mL microcentrifuge tube placed on ice and then mix briefly: 5  $\mu\text{L}$  dNTP Mix,  $x$   $\mu\text{L}$  solution containing 1  $\mu\text{g}$  test DNA, 1  $\mu\text{L}$  of digoxigenin-11-dUTP (for digoxigenin labeling) and  $y$   $\mu\text{L}$  distilled water to make a total of 42  $\mu\text{L}$  volume. The volumes of  $x$  and  $y$  may vary depending on the concentration of DNA.
3. Add 5  $\mu\text{L}$  of DNase I/Pol mix and 3  $\mu\text{L}$  of a 1/1000 dilution of DNase I (3 mg/mL). Mix gently but thoroughly. Centrifuge for 5 s in a microcentrifuge to bring down the solution from the cap.
4. Incubate at 15°C for 60 min.
5. Take 5  $\mu\text{L}$  of the labeling solution and run on a 1.2% nondenaturing agarose gel to check the fragment size by comparing with 1 kb DNA marker.
6. If the fragment size is within the size range of 100–500 bp, add 5  $\mu\text{L}$  of Stop buffer. If the size is bigger than 500 bp, add 1/100 diluted DNase I (3 mg/mL) to the tube and incubate for 15–40 min (The time length will depend on the measured size of the DNA fragments).
7. Unincorporated nucleotides are separated by chromatography (Sephadex G-50) or by using ethanol precipitation.

### 3.4. Fluorescence In Situ Hybridization (FISH)

#### 3.4.1. Hybridization

1. Denature chromosome slides in denature solution at 67–70°C for 10 s–2 min (see Note 6).
2. Precipitate 100–200 ng probe DNA, 3  $\mu\text{g}$  of Cot-1 DNA and 7  $\mu\text{g}$  of sonicated salmon sperm DNA by using 3 *M* sodium acetate (10:1/v) and cold 100% Ethanol (2.5 X/v). Resuspend the pellet in 10  $\mu\text{L}$  hybridization mixture.
3. Denature hybridization solution at 75°C in a waterbath for 5 min, move to 37°C waterbath for 30 min for preannealing.
4. Apply 10  $\mu\text{L}$  of denatured and preannealed solution from **step 3** to denatured chromosome slides. Place a coverslip on top of the solution, squeeze bubbles out and seal it with rubber cement.
5. Incubate the slides in a humidified chamber at 37°C overnight.

#### 3.4.2. Post-Hybridization Washes and Detection (see Note 9)

1. Wash slides 4 $\times$  in Coplin jars placed in a buffer containing 2X SSC and 50% formamide for 5 minutes each at 44°C. For directly labeled probes, go to the **Subheading 3.5.** after **step 1.**

2. Wash slides 3× at 50°C shaking waterbath for 5 min each in 0.1X with a gentle shaking.
3. Block sites of specific hybridization on slide with 100 µL of 4X SSC, 3% BSA and 0.1% Tween-20 covered with a 22 × 50 cm coverslip and incubate at 37°C for 20 min.
4. Remove the coverslip and drain the blocking solution very briefly. Then replace the blocking solution with a detection solution. For biotinylated probes, avidin-conjugated fluorescein isothiocyanate (FITC) is used (5 µg/mL in 0.4 µg/mL in 4X SSC, 1% BSA and 0.1% Tween-20), and for digoxigenin-labeled probes, sheep-anti-digoxigenin antibody is used (0.4 µg/mL in 4X SSC, 1% BSA and 0.1% Tween-20).
5. Cover chromosomal area with coverslips and incubate slides in a chamber (or a large Petridish) at 37°C for 30 min.
6. Remove coverslips and wash slides 3× in 2X SSC, 0.1% Tween-20 at 42°C for 5 min each.

### **3.5. Chromosome Counterstain (R Banding) (see Note 10)**

To view chromosome bands and fluorescence signals simultaneously, Chromomycin A3 and Distamycin A are used as a counterstain (8). This reverse banding pattern generates a more reproducible and higher resolution banding pattern than that the Q type pattern revealed by DAPI (9). Although the emission spectrum of chromomycin overlaps that of FITC, it can be separate by using the appropriate filter combination (8).

1. Rinse slides briefly in 1/2 McIlavane's buffer, pH 9.0 prior to staining, shake off the excess fluid.
2. Place 100 µL of Chromomycin A3 (0.5 mg/mL in 50% McIlavane's buffer, pH 9.0) onto slides for 40–60 min at room temperature.
3. Rinse slides in 50% McIlavane's buffer for 1 min at room temperature and shake off the excess fluid.
4. Place 50 µL of 0.1 mg/mL distamycin A on the slide for 1–2 min at room temperature.
5. Rinse slides in 1/2 McIlavane's buffer very briefly (10–20 s).
6. Place a very thin layer of antifade solution on slides and cover with a coverslip (20 × 50 cm).

### **3.6. Microscopy and Image Analysis (see Note 11)**

1. Analysis of *in situ* hybridization preparations may be performed by visual inspection, photography, or electronic image capture combined with digital image processing, using two different types of Zeiss fluorescence microscopes. The Zeiss Axiophot 100 microscope was used for generating black and white photographs from experiments using single color FISH. Kodak Technical pan ASA100 black and white films were used. For capturing color images from single, dual or multicolor FISH experiments, the Zeiss Axiovert 135 microscope was equipped with a 200-W mercury lamp and combined with a Photometrics Cooled-CCD camera employing BDS (Biological Detection System) image software was used.
2. To view multiple-labeled probes that have been simultaneously hybridized to chromosome slides, images are viewed sequentially with single-bandpass filter sets or with multiple bandpass filter sets. In our hands, the images were acquired using a ×63, 1.2 N.A. objective and filter sets for excitation and observation of Texas Red or Rhodamine and FITC or Cy3 and CY5 fluorescence, respectively (Chroma Technology, Brattleboro, VT). Chromomycin A3 and Distamycin A reverse banded chromosomes are captured by using Quinacrine filter sets (ex. 440 nm; em. 495 nm) (8).

3. To detect chromosomal aneuploidy or rearrangement, a total of 20 metaphase cells are chosen in the best area to be evaluated. That area is represented by a higher signal to noise ratio and best metaphase spreads. The representation of the images are acquired and are stored in an image database. Only the raw images are saved. No enhancement, correction or modification should be performed at this stage.

#### 4. Notes

1. When combined with a well-characterized reagent resource such as BACs, FISH is one of the most rapid and accurate methods for mapping human genes and novel chromosomal breakpoints. Moreover, the production of a reproducible high-resolution banding pattern has been highly useful for the FISH assignment of DNA fragments to human metaphase chromosomal bands essential for the *sis* and identification of chromosome rearrangements. Through sequence-linked BACs, a researcher can link the cytogenetic findings to a sequence rapidly, and further apply the information to a broad spectrum of molecular cytogenetic analyses.
2. We illustrate two applications that employ this integrated BAC resource for molecular cytogenetics: cancer and germline aneuploidy detection. Other applications discussed elsewhere include chromosome 21 analysis (*10*), cancer (*11*), Gene isolation (*12*) and genome organization and construction in Williams syndrome (*13*), and chromosomal aneuploidy analysis (*14*).
3. In the first example (**Fig. 2A**), the BACs from the Resource are used in FISH analysis on chromosomes made from a thyroid follicular carcinoma. This assay was used to determine specific regions amplified on chromosome 2p21 previously defined by using Comparative Genomic Hybridization (CGH). This step was essential to link the low resolution of the amplification observed by CGH to the DNA sequence. This work led to the definition of a candidate gene, PKC $\epsilon$ , that was then implicated for a role in thyroid tumorigenesis (*11,15*). The BACs used in this study demonstrated that chromosome band-specific BACs can facilitate identifying novel candidate gene(s) for tumorigenesis and determining the molecular structure of an amplified region.
4. In the second example of a chromosomal aneuploidy analysis (**Fig. 2B**), FISH was used to narrow a chromosomal breakpoint associated with a birth defect by using BACs linking to chromosome 21q22.1–22.3 and to chromosome band 8p23.3. In contrast to results with standard cytogenetics, we showed that translocation of the telomeric markers D21S348 through S100B was accompanied by the deletion of markers D21S65–D21S55. Clinically these results revealed deletion for the Down syndrome markers D21S55 and D21S65, but diploidy for the markers from this region to the telomere. For 8p, of six BACs mapping in the distal band, only the most telomeric was deleted, suggesting little contribution of the usual 8p deletion to the phenotypic defects of the child. This STS definition of the breakpoint region also provides links to *in silico* analyses of candidate genes for features defined in the child.
5. In order to produce consistent FISH mapping results, the following points should be taken into consideration. These involve each step, from chromosome preparation to image capture. One of the most important elements is to begin with high quality chromosome preparations. The DNA denaturation, hybridization, and probe detection parameters appear to be less important. We will describe these issues in sequential order as we go through the protocol.
6. The quality and the pretreatment of metaphase preparations are both critical to the success of signal production. A high quality chromosome preparation should meet the fol-

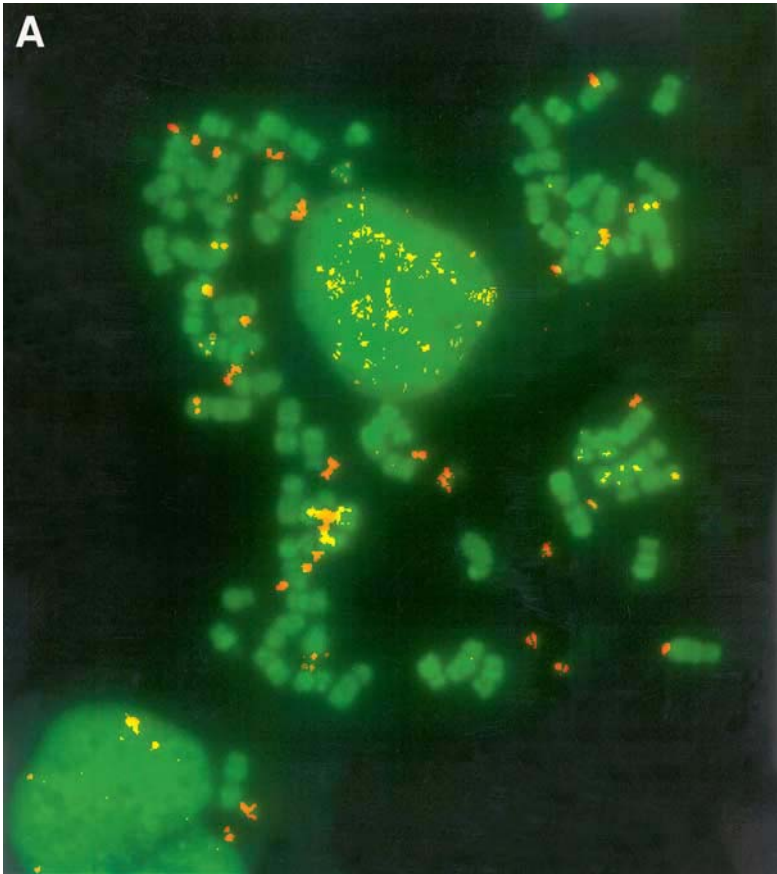
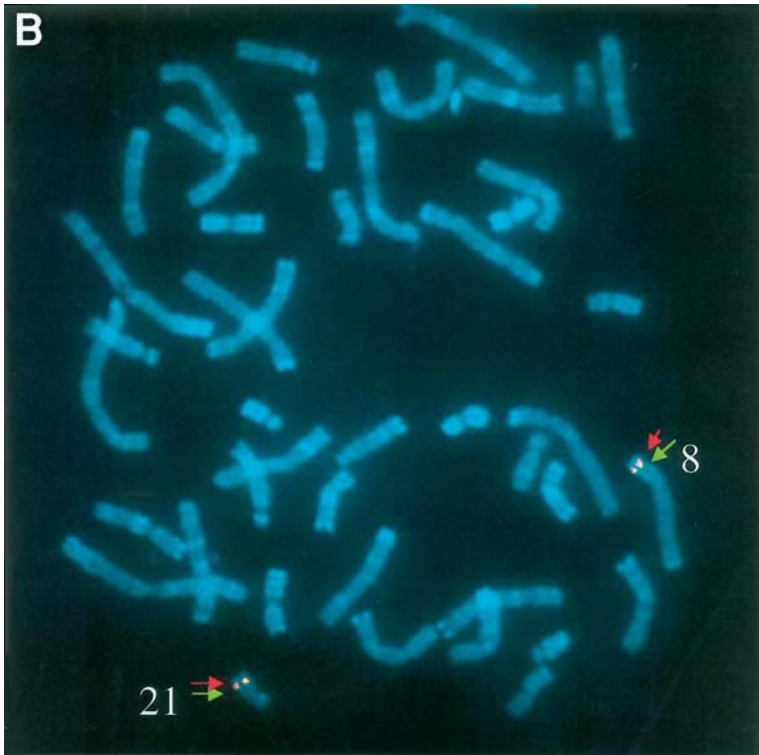


Fig. 2. (A) The FISH hybridization of BAC 1D9 on chromosomes derived from WRO, a cell line derived from a thyroid follicular carcinoma. In addition to the FITC signals (yellow dots) detected on band 2p21 of the three apparently normal copies of chromosome 2, distinct clusters of signals are seen, representing amplifications of band 2p21 shown both on double minute chromosomes and in the adjacent interphase nuclei. (B) Dual color FISH analysis of a translocation between chromosome 21 and 8 using STS linked BACs from the Integrated BAC Resource (*I*). BACs for D21S348 (labeled with biotin-14-dATP) and D21S231 (labeled with digoxigenin-11-dUTP) were cohybridized on metaphase chromosomes and detected with FITC (green signals) and Rhodamine (red signals) respectively. Both markers revealed a clear translocation from chromosome 21q22.3 to chromosome 8p23.3.

lowing criteria: (1) Metaphase spreads are evenly distributed on the slides with as little as possible residual cytoplasm; (2) few overlapping chromosomes; (3) metaphase spreads appearing dark black. To achieve this quality, the following parameters must be adjusted: hypotonic treatment time and fixation of cell pellets; dropping cell suspension and steaming slide during preparation; chromosome slide baking and finally; the length of chromo-



some denaturation. Fresh chromosome preparations need a shorter time of denaturation. The appropriate adjustments should result in chromosomes that are dark and not refractile. These should provide enhanced DNA target accessibility to the probe without undesirable loss of DNA from the target.

7. The best preparation of BAC DNAs is performed with kits that can be obtained from a number of companies. **Steps 17–21 (Subheading 3.2.)** are necessary for higher quality DNA extraction. The DNA should be free of contaminants that might inhibit DNA polymerases such as metals, detergents etc. In many cases, an alkaline lysis DNA isolation protocol followed by phenol-chloroform extraction and isopropanol precipitation is sufficient.
8. There are a number of different labeling methods that can be used to map BACs using FISH. These include Nick-translation or random priming, each employing either direct- or indirect-labeling reagents. The probes labeled with Nick-translation give much higher hybridization signal to background noise ratio than that labeled with random priming, however, they use 10× more DNA. The commercially available reagents used for direct labeling, i.e., fluorochrome-labeled nucleotides, are more expensive than the reagents used for indirect labeling, i.e., biotin or digoxigenin. However, both labeling methods give equally bright signals when using BACs as probes. In our hands, Nick-translation was often used in the presence of either biotin-14-dATP or digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals) for indirect-labeling and fluorolink Cy5-dCTP and

fluorolink Cy3-dCTP (Amersham Pharmacia Biotech) was used for direct labeling employing a Nick-translation Kit (Gibco-BRL). The procedures are performed according to the instructions provided with the kits, except for the DNase concentration and the length of the incubation of Nick-translation (as described in this chapter). The fragment size after labeling is important and should be in the range of 300–500 bp. A fragment size of 1000 bp or above, results in high background and less intense signals, contributing to failure of experiments.

9. The blocking and detection steps are important, but straightforward as long as the correct solutions and procedures are used. However, a word of *caution* is in order for the step involving immunocytochemical signal detection. The slide should *never* be allowed to dry, not even a part of it. Although it is important to drain the liquid from the slides to prevent overdilution of the antibodies, the next solution, such as the blocking solution or antibodies, should be applied immediately. If the slides are allowed to dry, the signal to noise ratio can decrease significantly owing to highly increased background.

The antibodies should be kept at  $-20^{\circ}\text{C}$  in small aliquots. If stored for more than a year, the antibody concentration should be increased twofold. If it yields a high background, a Sephadex G-50 column can be used to purify the antibody. Usually, this will restore the signal to noise ratio. If it does not give sufficient signal, a fresh antibody should be obtained.

10. As we described previously (8), the best banding is obtained by using the most aged chromosomes, i.e. from months to as many years as 10. However, aged chromosomes do not produce the strongest signals. Therefore, to obtain optimal signals with clear banding, freshly prepared chromosomes need to be baked at  $50\text{--}55^{\circ}\text{C}$  for about 4 h (can be left for 15 h with no change in signal). Aging chromosomes at room temperature ( $20\text{--}25^{\circ}\text{C}$ ) for 2–4 wk will yield the best banding results, plus reasonably bright hybridization signals. After one month, the slides can be stored at  $-70^{\circ}\text{C}$  until use. For fresh slides, the duration of staining with chromomycin A3 should be extended to 1.5 h, and for aged slides, the staining time may be as little as 30 min. The antifade solution is crucial to prevent fading, but only a thin layer should be used.
11. The metaphase selected for image capture and storage should meet the following criteria: (1) Strong but homogeneous hybridization signals; (2) low background; (3) little or no overlap of chromosomes; (4) relatively even condensation along the length of the chromosomes. Most fluorochromes fade quickly, including the reverse banding obtained with chromomycin. Thus, expose the slides to any excitation light only for the minimum amount of time. To map each BAC DNA, count at least 10–20 cells and save a minimum of two images for analysis and data basing. If needed, signals should be enhanced to identify additional sites that may have been missed due to low signal intensity. If BAC DNAs are used for cytogenetic analysis, a standard quality control protocol should be developed. This is crucial to the success of experiments.

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