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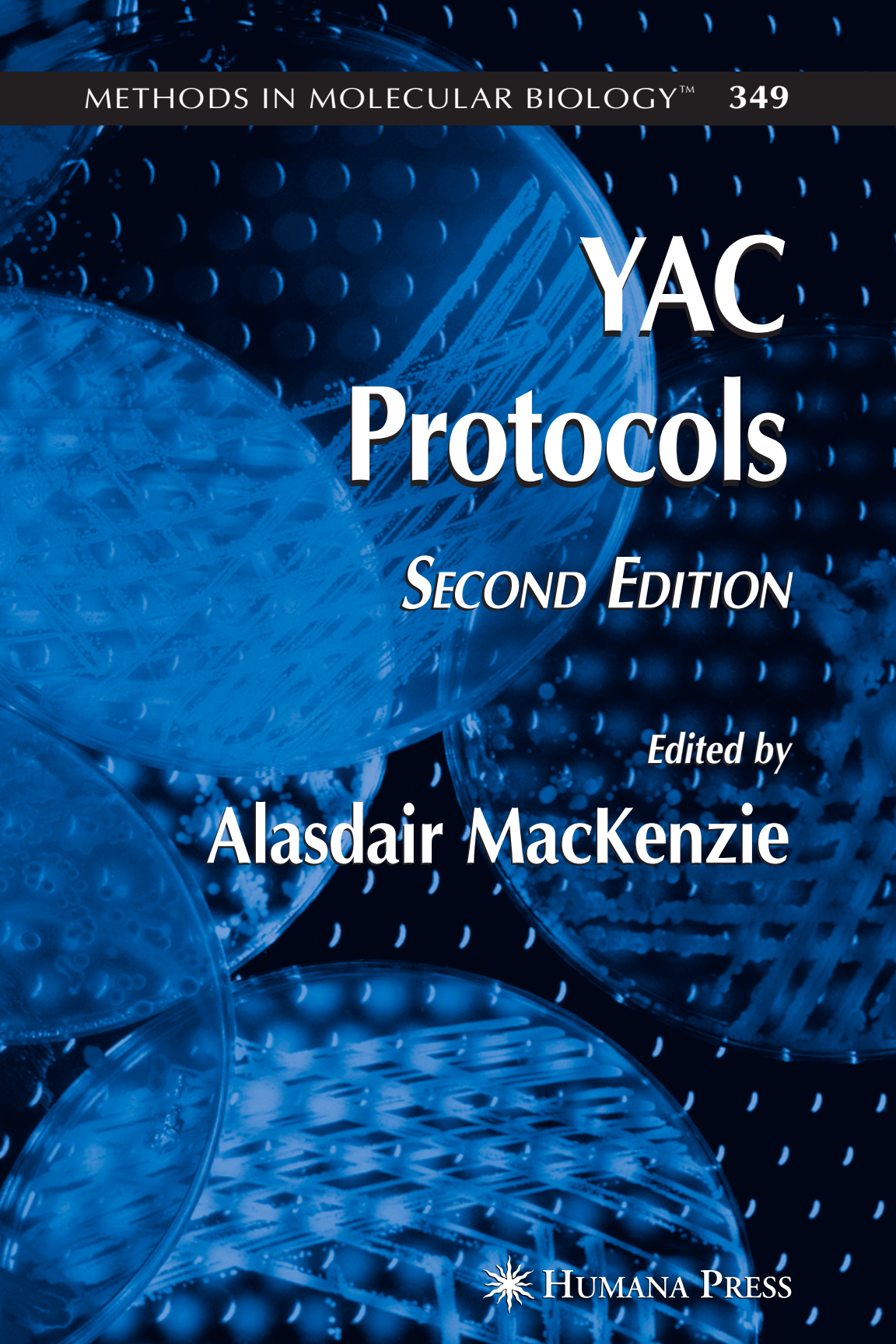

YAC Protocols

Second Edition

Edited by

Alasdair MacKenzie

Humana Press

YAC Protocols

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Preface

It is now 10 years since the first edition of *YAC Protocols* was published in 1996. *YAC Protocols* was first produced to address the huge demand within the research community for a lab-based text that described in detail the wide range of uses for large insert yeast artificial chromosome (YAC) DNA clones. In doing this, the original editor, David Markie, and the many different contributors who provided descriptions of the protocols they used and developed, did a magnificent job. Indeed many of the techniques described within the first edition require little change and have stood up admirably to the test of time. Since the first edition, the use of YACs has proved invaluable for addressing a wide range of new biological problems ranging from those of basic biochemistry to assisting in the mapping and sequencing of the human genome.

The requirement for a second edition of *YAC Protocols* was prompted by a number of major advances in biology since the publication of the first edition. These advances have included the sequencing of the human genome, and the genomes of a wide variety of other organisms, and the increased use of transgenic animals for understanding the molecular basis of human and animal disease. In addition, since the publication of the first edition, the use of YACs for a variety of different applications have been replaced by the use of other large insert cloning vectors such as P1 and bacterial artificial chromosomes (PACs and BACs). PAC and BAC clones appear to address many of the perceived problems associated with YAC clones, such as the need for pulse-field gel electrophoresis purification of YAC DNA, chimerism, and instability. However, PAC and BAC clones can only harbor inserts of up to 200 kb, whereas YAC clones can hold up to 2 Mb of genomic DNA. Because of new evidence demonstrating the huge size of many genes in higher vertebrates (the term "gene" encapsulates all the exonic sequence and all necessary *cis*-regulatory sequences required for normal expression), the smaller capacity of PAC and BAC clones limits their use in the study of a potentially huge number of important genes. For this reason, YACs are still the cloning vehicle of choice when studying the characteristics of genomic fragments greater than 200 kb in length.

The intention of this second edition of *YAC Protocols* is not to completely replace the first edition, whose protocols, in many cases, are still relevant today, but to provide a much needed update on the new techniques currently being employed and to help redefine and illustrate the important roles still to be played by YAC technologies in the postgenomic age.

The first chapter, written by David Markie the previous editor of *YAC Protocols*, describes the very basics of yeast cell culture and outlines the different auxotrophic markers available and the recipes of basic media allowing for their selection in culture. Both Chapters 2 and 3 have been written by Cecilia Sanchez and Michael Lanzer and describe how YAC libraries can be generated from the genomes of novel species and pathogens whose genome sequences have yet to be sequenced (Chapter 2) and how, in the absence of extensive sequence data required for the design of PCR primers, these libraries can be screened for selected YAC clones by filter hybridization (Chapter 3). Chapter 4, by Sylvia Vasiliou and John Quinn, describes how an isolated YAC clone can be characterized further using restriction digestion, Southern blotting, nucleic acid hybridization, and PCR analysis to confirm the identity of the clone and to determine its integrity. Gaining access to, and analyzing, the huge wealth of mapping and sequence information currently available via the internet was an option almost undreamed of immediately after the publication of the first edition of *YAC Protocols*. In Chapter 5, Kerry Miller and Scott Davidson give a basic overview of how genomic sequence can be accessed and how this sequence can be rapidly analyzed using freely available and user friendly internet tools.

In Chapter 6, Shigeki Kawakami and coworkers describe a novel method of transforming DNA into YAC containing yeast clones using calcium alginate beads. In Chapter 7, Sanbing Shen describes how YAC clones can be altered to change different auxotrophic markers, a process known as retrofitting, and to allow amplification of the copy number of YAC clones within yeast cells. This ability provides advantages when attempting to isolate larger quantities of YAC DNA for a range of different procedures such as the production of YAC transgenic animals. In Chapter 8, Gabriela Loots describes how YAC clones can be conditionally altered by the engineering of *lox*P sites flanking target sequences within the clone. The flanking of these target sequences with *lox*P sites allows for their excision in vivo once the YAC clone has been successfully introduced into the genome of an animal transgenic for an inducible *Cre* gene.

Natalay Kouprina and coworkers describe a method that is likely to be the most important development in YAC techniques since their inception. In Chapter 9, Dr. Kouprina describes how novel YAC clones of a defined size and genomic content can be produced using homologous recombination (TAR cloning) in yeast. This technique has become especially relevant in recent years as a result of unparalleled access to multiple genome sequences via the internet. Furthermore, TAR cloning of YACs will promise to remove the specter of

chimerism that has represented one of the main perceived obstacles to the more widespread use of YAC clones.

In many situations, it may be helpful to manipulate YAC DNA clones either to make them smaller and more manageable or to join them together to produce a single "super-YAC." In Chapter 10, Yeon-Hee Kim and coworkers describe a simple and effective protocol that allows the controlled removal of large segments of YAC clones to produce smaller, more manageable, YACs. In Chapter 11, David Markie, Emma Jones, and Jiannis Ragoussis revisit and update a protocol that allows for the fusing of small YAC clones together into one large clone.

The production of an efficient targeting vector is frequently one of the most problematic steps encountered when attempting to carry out gene targeting (gene "knockout") in mouse embryonic stem cells. One of the main obstacles centers on difficulties encountered in the use of long range hi-fidelity PCR to isolate the long stretches of homologous sequences required to ensure the production of an efficient targeting vector. These problems include the insertion of replication errors that, despite what their name suggest, still affect hi-fidelity polymerases. These replication errors reduce recombination efficiencies and may even mutate the wrong part of the protein (a problem that would compromise the development of a conditional knockout model). Furthermore, the frequent inability to successfully amplify many long sequences owing to the presence of repetitive DNA is common. In Chapter 12, Peter Murray describes a protocol that circumvents these problems by allowing the production of highly efficient targeting vectors using YAC DNA. The ability to develop these vectors will greatly enhance the success of gene targeting in mouse embryonic stem cells.

The use of transgenic animals has become an important tool in understanding the role of genes in supporting health. Our added ability to produce transgenic animals using YACs has enhanced our understand of how large genes, or clusters of genes, and their regulatory elements contribute to the development and normal physiology of organisms and how changes within components of these genes can predispose individuals to disease. In Chapter 13, Alasdair MacKenzie describes a protocol that allows for the efficient recovery of intact YAC DNA and its subsequent microinjection into the pronucleii of one-cell mouse embryos. An alternative method of producing YAC transgenic animals is described in Chapter 14 by Pedro N. Moreira and coworkers, who have devised an efficient method of introducing intact YAC DNA into the mouse genome using intracytoplasmic sperm injection. This ground-breaking method complements, and may eventually supersede,

the use of pronuclear injection in the production of YAC transgenic animals.

The introduction and analysis of large genomic fragments in highly differentiated primary cell types such as those of the nervous system or the immune system has the real potential of facilitating a better understanding of the roles of genes in maintaining the differentiated phenotype and physiology of these cells. Furthermore, the use of large human genomic fragments to essentially humanize these cells will greatly aid in the design of more specific and efficacious drug therapies. However, getting these large genomic fragments into differentiated cell types and maintaining these cell types indefinitely has been problematic. Carl Anthony Blau and Kenneth Peterson have addressed many of the obstacles preventing the analysis of large genomic fragments in differentiated cell types. This has been achieved by first producing transgenic animals with YACs, deriving differentiated transgenic cells from these transgenic lines and immortalizing these lines using a novel strategy based on the introduction of crippled retroviruses. A detailed protocol outlining the procedures involved is described in Chapter 17.

The final two chapters in this updated version of *YAC Protocols* relates to the use of YACs in the mapping of genomes. YACs will continue to play a significant role in genome mapping as, although many genomes have been sequenced, many more important genomes, such as those of the myriad of pests and pathogens that still affect a distressing proportion of the human population, remain to be mapped. An essential aspect of mapping the geography of any genome is to understand the chromosomal location of particular genes and markers within that genome. A technique known as fluorescence *in situ* hybridization has been critical in developing detailed maps of different genomes. The use of YAC clones in FISH analysis is described by Thomas Liehr in Chapter 16. In addition, in Chapter 17, Marcia Santos et al. provide a detailed account of how YAC clones have been used to map the genome of the parasite *Trypanosoma cruzi*.

I am extremely grateful to all the authors who have enthusiastically provided these cutting edge protocols and I would like to acknowledge their help and support in realizing this new edition of *YAC Protocols*. We are in little doubt that any decline in the use of YAC-based techniques has stemmed from misconceptions of the difficulties involved in using YACs, such as the need to purify YACs using PFGE and their higher proportions of chimerism compared to PAC and BAC clones. However, many of the protocols described in this volume specifically address these problems. By gathering these protocols into one volume and demonstrating the enormous potential use of YAC technolo-

gies in a variety of different research situations, we hope that the use of YAC technology will continue to be considered by the wider research community as a viable tool in understanding the role of genetics in maintaining health, promoting pathogenesis, and conferring susceptibility to disease.

Alasdair MacKenzie

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1

Markers, Selection, and Media in Yeast Artificial Chromosome Cloning

David Markie

Summary

Yeast have proven to be a valuable and flexible host for cloning and manipulating large fragments of foreign genetic material, providing valuable reagents for the study of gene function and regulation in a variety of applications. However, this approach requires a familiarity with basic procedures derived from yeast genetics, and lack of experience can be a barrier to researchers with backgrounds limited to bacterial and mammalian culture systems. To address this issue, this chapter introduces and discusses the genetic markers and selection systems used in the genetic manipulation of yeast, with particular reference to yeast artificial chromosome (YAC) cloning and manipulation. Detailed descriptions of culture media are provided, allowing for the growth and selection of standard YAC strains, as well as for specialist applications.

Key Words: Yeast genetic markers; yeast selection; culture media.

1. Introduction

The genetic markers used in yeast, and in yeast artificial chromosome (YAC) cloning, are largely defined by the manipulation of growth media. This chapter, apart from providing recipes for media formulation, also contains a brief introduction to genetic markers in yeast that are relevant to YAC cloning. It is hoped that this will provide an adequate understanding of the media that are used for particular applications, and allow adaptation of media for individual requirements.

1.1. Auxotrophic Markers

In contrast to bacterial cloning systems, where the use of dominant antibiotic resistance determinants is common, selective markers used in YAC manipulation are based mainly on complementation of biosynthetic mutations. Such

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mutations, producing a growth requirement in the yeast strain that can be satisfied by the addition of a nutritional supplement to the media, are termed auxotrophies, whereas the wild-type state is the absence of that requirement (prototrophy). When using biosynthetic genes as selectable markers, choice is therefore limited to those genes that are mutant in the proposed host, and for which there is a source of a functional copy of the gene. To apply selection for a functional gene that complements an auxotrophic mutation in the host, the appropriate supplement is omitted from the media. However, most YAC hosts have complex genotypes, and may have nutritional requirements other than those being considered in a particular selection experiment. These "nonrelevant" requirements must not be forgotten when designing media. For example, to maintain selection for a YAC in the pYAC4/AB1380 vector/host system, with functional *URA3* and *TRP1* genes on the YAC and corresponding *ura3 trp1* mutant alleles in the host (*see* **Note 1** for guidance on yeast genetic nomenclature), it is essential to ensure that the growth medium contains no uracil, but provides a source of histidine, lysine, isoleucine/threonine (*see* **Note 2**), and adenine to cover the remaining nutritional requirements of this strain. The presence or absence of tryptophan is optional (*see* **Note 3**).

Furthermore, most biosynthetic pathways are comprised of several distinct genes, each encoding a unique enzymatic function but capable of producing the same phenotype when mutant. The appropriate gene is required for complementation of a specific mutant locus (for example, the functional *HIS3* gene in the pBP series of fragmentation vectors does not complement the *his5* mutation present in AB1380 and these plasmids cannot be used in this host, but they are very effective in yPH857 containing the *his3-Δ200* allele).

1.2. Color Markers

Wild strains of *Saccharomyces* and many laboratory strains form white colonies when grown on standard media. However, mutations that produce colored colonies are of particular value, as they allow visual screening for the segregation of marked genetic elements. There are two genes in the adenine biosynthetic pathway of yeast (*ADE1* and *ADE2*) that, apart from producing an absolute requirement for adenine when mutant, also produce a change in colony color. This is owing to accumulation of intermediates in the adenine biosynthetic pathway prior to the enzymatic steps encoded by the *ADE1* and *ADE2* genes (*see* **ref.** ***1*** for analysis of purine biosynthesis in *Neurospora crassa*). However, red coloration is conditional and can be manipulated by altering the concentration of adenine available in the media, thereby affecting feedback control of the adenine biosynthetic pathway. High concentrations of adenine repress the pathway, no accumulation of intermediates occurs, and the colonies remain white. If the concentration of adenine is adequate but limiting for

growth, then the adenine biosynthetic pathway is derepressed, pathway intermediates accumulate prior to the enzymatic block, and red pigment is formed. Mutant *ade2* alleles are present in several common YAC hosts, and the *ADE2* gene can be used as a selectable marker for YAC modification. For guidelines on the appropriate use of high and low adenine media, *see* **Note 4**.

1.3. Suppressor Mutations

In yeast, as in other organisms, protein translation may be terminated by any one of three stop codons UAG (amber), UAA (ochre), and UGA (opal), owing to the absence of transfer RNA molecules capable of recognizing them. Accordingly, nonsense mutations will produce truncated (and usually nonfunctional) gene products. However, secondary point mutations in the anti-codons of some transfer RNA genes may allow recognition of stop codons and subsequent read-through of missense mutations with "suppression" of the original phenotype. There are three ochre mutations that are potentially suppressible in the common YAC host AB1380 (*ade2-1*, *lys2-1*, and *can1-100*) ***(2)***. Ochre suppression in this strain will simultaneously produce prototrophy for adenine and lysine, the clones will appear white when grown on limiting adenine (*see* **Subheading 1.2.**), and will be sensitive to canavanine (*see* **Subheading 1.4.**). Insertional inactivation of an ochre suppressor gene (*SUP4*) in the vector pYAC4 has been used as a cloning indicator during library construction in this host ***(2)***. The white, canavanine-sensitive colonies produced by transformation with the pYAC4 vector alone (*SUP4* gene intact) can be readily differentiated from the red, canavanine-resistant colonies (*SUP4* gene disrupted) that contain an insert. Ochre suppressor mutations may also arise spontaneously during growth of AB1380 clones, producing a white, Ade+, Lys+, CanS phenotype. This poses a difficulty for later modification using the *ADE2* or *LYS2* genes as selectable markers. To minimize the risk of selecting for ochre suppression, clones should always be expanded in media containing high concentrations of adenine (*see* **Note 4**).

1.4. Resistance Markers

A number of chemicals are used to reveal resistance markers in yeast. Mostly these are metabolite analogs that are toxic, and resistance is mediated by mutations that abolish uptake or metabolism of these compounds. As a consequence, such mutant resistance alleles are usually recessive (one functional allele is adequate for normal processing of the toxic metabolite even in the presence of a nonfunctioning mutant copy), in contrast to the dominant antibiotic resistance markers used in bacteria. For example, wild-type yeast are sensitive to the arginine analog L-canavanine, whereas mutations in the *CAN1* gene (such as in AB1380) produce resistance because of the absence of a functional arginine

permease ***(3)***. Although selection can be exerted for the presence of a functional *CAN1* gene product on particular strain backgrounds ***(4)***, in the common YAC host strains it can only be applied for its absence. Note that when canavanine selection is applied the media should contain no arginine, as competition for uptake reduces the efficacy.

Two further selective agents, α-amino adipate and 5-fluoro-orotic acid (5-FOA), have potential relevance to YAC manipulation because they interact with markers carried by the common host strain AB1380. 5-FOA is metabolized via the uracil biosynthetic pathway, ultimately producing growth inhibition. Mutations at the *URA3* locus (and to a certain extent at *URA5*) produce resistance ***(5)***, while also producing a requirement for uracil. This provides a mechanism for both forward and reverse selection—absence of uracil in the media can be used to select for the presence of a functional *URA3* gene and, when required, 5-FOA can be used to select for its loss. The value of this strategy is well illustrated by procedures for introducing desired mutations into YACs and for reducing the number of YACs within a clone ***(6)***. The chemical α-amino adipate, an intermediate in lysine biosynthesis, can be used in a similar fashion to select against the functional *LYS2* gene, although this has not yet been widely exploited in YAC manipulation. Wild-type strains are unable to grow when α-amino adipate is provided as the only source of nitrogen, whereas *lys2* strains (and to some extent *lys5* strains also) are able to utilize it for growth ***(7)***.

Resistance to cycloheximide (an inhibitor of protein synthesis) can be produced in yeast by mutations affecting its interaction with ribosomal proteins.

A selection system more closely resembling bacterial antibiotic resistance markers using an exogenous thymidine kinase gene has been developed to provide selection for increasing YAC copy number ***(8)***.

1.5. Miscellaneous Markers

Current knowledge of yeast biology is owing in large part to the enthusiastic use of mutagens by generations of yeast geneticists, and as a result mutations affecting many physiological functions have been described. Although most are of little relevance to YAC cloning, a minority may affect some YAC manipulations. Wild yeast strains are often capable of utilizing galactose (Gal^+), whereas many laboratory strains (including AB1380) are relatively slow growing when galactose is provided as their sole carbon source (Gal^-). This is probably owing to a defective galactose permease (encoded by the *GAL2* gene) reducing galactose uptake and subsequent induction of galactose catabolic pathways. The system described for YAC copy number amplification is dependent on galactose-mediated induction of transcription from the *GAL1* promoter, and

consequently, although this system is of value in AB1380, it may not be as effective as when applied in a Gal+ host.

Mutations in DNA recombination and repair systems also have relevance to YAC cloning. The high rate of chimerism in many currently available YAC libraries probably results from in vivo recombination between repetitive sequence elements during library construction ***(9)***. Internal deletions observed in "unstable" YAC clones during culture may similarly be mediated by recombination between tandemly repeated sequence elements ***(10)***. One approach to overcoming these difficulties has been the use of recombination deficient strains as hosts, and *rad52* mutants have met with some success ***(11)***. However, the absence of recombination in these hosts may be something of a double-edged sword, as it precludes the later manipulation of YACs through yeast recombination systems. Transfer of YACs to alternative host strains may overcome this limitation.

One further marker that has been elegantly exploited for YAC transfer between strains is a mutation in the *KAR1* gene, affecting karyogamy without preventing mating. Examples such as this should stimulate further investigation into aspects of yeast physiology and genetics that may have a bearing on the propagation and manipulation of YACs. The enormous investment in yeast genetics over many decades has provided a wealth of literature, and the application of this knowledge to YACs should be limited only by the ingenuity of those wishing to exploit it.

2. Materials

1. Yeast extract (Difco, Detroit, MI, cat. no. 0127-01-7).
2. Peptone (Difco, cat. no. 0118-01-8).
3. D-glucose (dextrose) (Difco, cat. no. 0155-17-4) (*see* **Note 5**).
4. D-galactose (Sigma, St. Louis, MO, cat. no. G-0750).
5. Yeast nitrogen base without amino acids (*see* **Notes 6** and **7**) (Difco, cat. no. 0919-15-3).
6. Agar (Difco, cat. no. 0140-01-1).
7. Casein, acid hydrolysate (Sigma, cat. no. C-9386) (*see* **Note 8**).
8. Stock solutions for nutritional supplementation of synthetic dextrose (SD) medium (*see* **Table 1**).
9. 10X supplement mix for synthetic complete (SC) and drop-out media (*see* **Table 2**).
10. Sorbitol (Sigma, cat. no. S-6021).
11. Potassium acetate.
12. Tryptone (Difco, cat. no. 0123-01-1).
13. NaCl.
14. Casein hydrolysate, enzymatic (Sigma, cat. no. C-0626).
15. $MgSO_4 \cdot 7H_2O$.
16. Glycerol.
17. 0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4, sterilize by autoclaving.

Table 1
Common Stock Solutions Used for Supplementation of Media (SD and SD+C) During YAC Selection and Manipulation[a]

Supplement	Sigma catalog number	Final media concentration (μg/mL)	Stock solution and strength (mg/mL)
Adenine hemi-sulfate	A-9126	20[b]	5 (250X)
L-arginine.HCl	A-5131	20	2 (100X)
L-histidine.HCl	H-8125	20	2 (100X)
L-isoleucine	I-2752	30	3 (100X)
L-leucine	L-8000	30	3 (100X)
L-lysine.HCl	L-5626	30	3 (100X)
L-threonine[c]	T-8625	200	2 (10X)
L-tryptophan	T-0254	20	10 (500X)
Uracil	U-0750	20	2 (100X)

[a]Concentrated stock solutions are sterilized by autoclaving and stored at room temperature, with the exception of tryptophan which should be filter-sterilized and stored at 4°C, protected from light. Brown discoloration of tryptophan solutions indicates decomposition. The appropriate volume of individual solutions is added to sterile SD medium as required. Final concentrations at which some of the supplements are used may vary according to individual preferences.

[b]*See* **Note 4**.

[c]*See* **Note 2**.

3. Methods

Growth media are produced as broth, or made solid by the addition of agar at 20 g/L prior to autoclaving. Most media can be sterilized by autoclaving, although where noted some components are filter-sterilized separately. The media recipes in this section are each for 1 L unless otherwise stated, but volumes can be scaled up or down as required. We find it most convenient to autoclave media in 500-mL bottles (to allow subsequent remelting in a microwave), with storage at room temperature to allow easy identification of inadvertent contamination.

3.1. Complete Undefined Yeast Media

1. YPD (yeast extract, peptone, dextrose) medium: 10 g yeast extract, 20 g peptone, 20 g D-glucose. Add 20 g agar for solid medium. This is an undefined medium that will support the growth of most yeast strains, irrespective of their auxotrophic requirements. The adenine concentration is limiting for *ade1* and *ade2* mutant strains, and these will demonstrate red pigmentation when grown on this medium (*see* **Note 4**).
2. YPAD (yeast extract, peptone, adenine, dextrose) medium: 10 g yeast extract, 20 g peptone, 20 g D-glucose, 50 mg adenine hemisulfate. Add 20 g agar for solid medium. The adenine concentration of this medium is increased to provide faster

Table 2
Composition of 10X Supplement Mix for SC Medium[a]

Supplement	Sigma catalog number	Final media concentration (mg/mL)	Concentration in 10X supplement mix (g/mL)
Adenine hemi-sulfate	A-9126	50[b]	0.5
L-arginine.HCl	A-5131	20	0.2
L-histidine.HCl	H-8125	20	0.2
L-isoleucine	I-2752	30	0.3
L-leucine	L-8000	60	0.6
L-lysine.HCl	L-5626	30	0.3
L-methionine	M-9625	20	0.2
L-phenylalanine	P-2126	50	0.5
L-threonine	T-8625	200	2.0
L-tryptophan	T-0254	20	0.2
L-tyrosine	T-3754	30	0.3
Uracil	U-0750	20	0.2
L-valine	V-0500	150	1.5

[a]The stock solution is filter-sterilized. When tryptophan is included it should be stored at 4°C and protected from light. Some workers regularly omit some nonrelevant amino acids from their supplement mix; for example, threonine can be omitted without adversely affecting the growth of most current YAC hosts (*see* **Note 2**) and reduces preparation costs. For the construction of specific drop-out media, individual 10X supplement mixes should be prepared lacking the appropriate nutrient(s); for example, SC (-lys) would be made with a 10X supplement mix lacking lysine. As an alternative to aqueous mixes, some workers prepare well-mixed dry powder preparations of supplement mix (and drop-out mixes), and the appropriate amount is then weighed and added to media prior to sterilization.

[b]*See* **Note 4**.

growth, higher saturation densities, and absence of red pigmentation with *ade1* and *ade2* mutant strains (*see* **Note 4**).

3.2. Defined and Semi-Defined Media for Selection in Yeast

There are two general approaches taken to providing the required nutrients in selective media. The first requires knowledge and consideration of all the auxotrophic markers present in the host, and appropriate individual supplements are added as required to minimal medium (SD). In the alternative approach, individual components are omitted as required from a defined complete medium (SC) to produce drop-out media, and only the markers for which selection is being applied need to be considered. The most convenient approach for a particular application depends largely on personal habit, but may also be affected by how much is known about the genotype of the host, and the number of auxotrophic requirements present.

1. SD medium: 6.7 g yeast nitrogen base without amino acids, 20 g D-glucose. Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. SD is a defined minimal medium that will support the growth of wild-type yeast. Any auxotrophic requirements of mutant yeast strains must be considered and appropriate supplements added. For example, for the growth of the host strain AB1380 carrying a YAC constructed with the pYAC4 vector, SD medium would at minimum be supplemented with lysine, adenine, histidine and isoleucine (*see* **Note 2**). Such media is denoted SD (+lys, +ade, +his, +ile). Supplementation of SD media is most conveniently carried out by adding individual supplements from sterile concentrated stock solutions just prior to pouring molten solid media, or just prior to use of broth media. *See* **Table 1** for details of individual stock solutions required, and **Subheadings 1.1.** and **1.2.** for the design of selective media.
2. SC and drop-out media: 6.7 g yeast nitrogen base without amino acids, 20 g D-glucose, 100 mL 10X supplement mix (add after autoclaving). Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. This is a defined medium containing a complex mixture of supplements suitable for the growth of most laboratory yeast strains carrying auxotrophic mutations. Selective (drop-out) media are made by omitting individual requirements from the supplement mix (*see* **Table 2**). For example, omitting uracil and tryptophan from the supplement mix provides selection for a YAC containing *URA3* and *TRP1* genes in the host AB1380. This double drop-out medium would be denoted SC (-ura, -trp).
3. Acid hydrolyzed casein (AHC) medium: 6.7 g yeast nitrogen base without amino acids, 14 g casein acid hydrolysate, 20 g D-glucose, 20 mg adenine hemisulfate. Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. This medium provides selection for uracil and tryptophan prototrophy and is a convenient medium for the growth and maintenance of standard YAC strains. The adenine concentration may be increased as desired (*see* **Note 4**) and the addition of tryptophan will also improve the growth rate of standard YAC strains (*see* **Note 3**), while still maintaining the uracil selection necessary for YAC retention. Some versions of AHC routinely include uracil and tryptophan, as well as adenine in the formulation.
4. Synthetic dextrose + casamino acids (SD+C) medium: 6.7 g yeast nitrogen base without amino acids, 14 g casein acid hydrolysate, 20 g D-glucose. Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. This medium is used in a similar fashion to SD medium, but allows the manipulation of only three supplements (adenine, tryptophan, and uracil). Although similar to AHC in formulation, it is here named SD+C to clearly reflect its content and avoid confusion with the various definitions of AHC in common usage.

3.3. Special Yeast Media

1. Regeneration medium: 6.7 g yeast nitrogen base without amino acids, 20 g D-glucose, 182.2 g sorbitol, appropriate nutritional supplements. Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. This is a solid medium used for recovery following cell wall removal (for protoplast transformation or

protoplast fusion experiments). As well as providing appropriate selection for the particular experiment, it also contains sorbitol for osmotic protection of spheroplasts. The sorbitol is added prior to autoclaving the medium. Yeast are poured in a molten overlay (agar concentration may vary according to individual preferences) onto solid medium in plates. It is important that both the plate medium and the overlay contain sorbitol.

2. Sporulation (SPO) medium: 20 g potassium acetate, 2.5 g yeast extract, 1 g D-glucose, 20 g agar. Adjust pH to 7.0 with potassium hydroxide prior to autoclaving. Supplement at 75 μg/mL with required nutrients. This medium is used to induce sporulation in diploid strains.
3. Media for 5-FOA selection: selection with 5-FOA is usually undertaken in defined solid medium (SD with appropriate supplements, or the desired drop-out medium based on SC). The concentration of 5-FOA necessary for effective selection may vary between strains but is generally in the range of 0.5 to 1 mg/mL. An alternative medium designed to lower the concentration of 5-FOA for efficient selection has been reported to be useful in some strains ***(12)***, although from personal experience this does not seem effective with AB1380. From the expense of 5-FOA, the high concentration at which it is used, and its relatively poor solubility, the media is prepared somewhat differently to standard media. In general, the desired media is prepared in small amounts at 2X concentration, the appropriate amount of 5-FOA added (vigorous vortexing and heating to 65°C may be necessary for complete dissolution), and then filter-sterilized. The media is then mixed with an equal volume of sterile molten agar (at 40 g/L) before pouring.
4. Copy number amplification medium: the media used for copy number amplification substitute galactose for glucose as a carbon source, and specific selective agents are added from concentrated stock solutions.

3.4. Bacterial Media

1. Luria-Bertani medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Add 20 g agar for solid media. Adjust pH to 7.0 with NaOH.
2. NZCYM medium: 10 g casein hydrolysate, enzymatic, 5 g NaCl, 5 g yeast extract, 1 g casein hydrolysate, acid, 2 g $MgSO_4 \cdot 7H_2O$. Add 20 g agar for solid media. Adjust pH to 7.0 prior to autoclaving.
3. Terrific broth: 12 g tryptone, 24 g yeast extract, 4 mL glycerol. Make up to 900 mL and autoclave, then when cool add 100 mL sterile 0.17 *M* KH_2PO_4, 0.72 *M* K_2HPO_4.

4. Notes

1. Yeast genetic nomenclature: yeast genes are generally designated by a three-letter acronym (usually an abbreviation of the phenotype produced by mutants at that locus) and a number (to distinguish between independent genes that can give rise to the same phenotype). Dominant alleles of a gene (in yeast these are usually, but not always, wild-type functional alleles) are denoted in upper case italics (for example *ADE2*) and recessive alleles (usually nonfunctional mutant alleles) in

lower case italics (*ade2*). In some cases, specific alleles are further defined by the addition of an allele number (*ade2-100*) that distinguishes them from other mutations at that locus. This information can be very useful as individual mutant alleles may have specific characters, such as low or high reversion rates, suppressibility (*see* **Subheading 1.3.**), intragenic complementation, and so on. Occasionally, additional symbols are used to convey further information about specific alleles, for example Δ signifying a deletion mutant.

Phenotypic descriptions of yeast generally use the same three letter code (with no gene number required), and to differentiate them from gene descriptions they are not italicized. The symbols + and – are used in superscript to signify functional and defective states respectively (for example Thr^- indicates that a strain is defective in threonine biosynthesis, and Gal^+ indicates that a strain is capable of utilizing galactose as a carbon source), ^{R} and ^{S} are used to specify resistance and sensitivity, respectively.

2. In addition to the original description of the AB1380 genotype ***(2)***, this yeast strain seems to have a mutation in threonine biosynthesis. The resulting growth requirement can be satisfied by the addition of either threonine or isoleucine to the medium.
3. For most purposes it is necessary to maintain selection for only one marker on the YAC, rather than both. In addition, the copy of the *TRP1* gene used in the construction of pYAC4 is poorly functional and although pYAC4/AB1380 clones can be grown in the absence of tryptophan, they will have suboptimal growth rates. It is common practice to supplement media with tryptophan and maintain selection for the *URA3* gene only.
4. Adenine is used at different concentrations depending on the application. When *ade2* mutant clones are being expanded (such as during library growth and duplication, growth for transformation, and for agarose block preparation) the development of red coloration is of no utility, and adenine can be used at a high concentration (50 μg/mL or higher). In this high adenine media growth rates are improved, cells protoplast more easily, and the selective advantage for spontaneous mutants causing reversion (or suppression) of the *ade2* mutation is removed. If such mutants do arise within a clone they will quickly predominate under limiting adenine conditions, as is already the case for a minority of clones in currently available YAC libraries.

   However, when plating on solid media for single clones from an *ade2* strain, low adenine medium (around 20 μg/mL) is used, as the red colony color provides confirmation that a selected clone is not a contaminating yeast, or a revertant/suppressor clone that has arisen in the culture. As a general rule when using standard YAC strains (pYAC4 in AB1380), broth medium should always contain high adenine concentrations, and plate culture should usually contain low concentrations.
5. Autoclaving media containing glucose produces a brown discoloration, which may vary from batch to batch depending on the extent of caramelization. Although this does not seem to adversely affect yeast growth, some workers prefer to filter-sterilize solutions of glucose at 20 or 40% (w/v), and add these as required to autoclaved media.

6. Yeast nitrogen base is a complex, but completely defined, mixture of individual components providing everything necessary for the growth of wild-type yeast, with the exception of a carbon source. However, standard yeast nitrogen base also contains some nonessential amino acids, prohibiting the use of certain selectable markers. For this reason, a variant formulation, yeast nitrogen base without amino acids, is also available to allow complete manipulation of the amino acid content of media, and it is this product that is most useful in the production of defined media for YAC cloning and manipulation. It is generally used at a concentration of 6.7 g/L, of which 5 g is ammonium sulfate. A further variation is also available, yeast nitrogen base without amino acids and ammonium sulfate, which contains no source of nitrogen. It is used at 1.7 g/L, and a nitrogen source is also required, usually 5 g/L ammonium sulfate. It is essential to be clear about which formulation is being used, as this determines the amount added to the media and the need for addition of a nitrogen source.
7. Some workers prefer to filter-sterilize a 10X concentration of yeast nitrogen base (without amino acids) and add this to autoclaved media as necessary. However, media containing yeast nitrogen base (without amino acids) can be sterilized by autoclaving without any apparent ill effects on yeast growth.
8. Casein acid hydrolysate (or casamino acids) is acid hydrolyzed milk protein, and is a convenient supplement providing adequate concentrations of amino acids (with the exception of tryptophan) for the propagation of yeast with multiple auxotrophic requirements. Clearly, it cannot be used when selection is exerted for an amino acid prototrophy (with the exception of tryptophan). It contains no adenine or uracil.

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2

Construction of Yeast Artificial Chromosome Libraries From Pathogens and Nonmodel Organisms

Cecilia P. Sanchez and Michael Lanzer

Summary

Many infectious diseases of global impact are caused by parasites. This includes diseases with protozoan etiology, such as malaria, African sleeping sickness, Chagas disease, toxoplasmosis, and amoebiasis, as well as diseases caused by metazoa, such as river blindness, schistosomiasis, ecchinococcosis, and ascariasis. Combined, parasitic diseases affect more than half the world's human population and are responsible for decreased gross national products and billions of dollars in lost earnings. Although the magnitude of the problem precludes quick solutions, there is reasonable hope that a better understanding of these organisms, especially the host–parasite interactions that underpin virulence and pathogenicity mechanisms, will provide new opportunities for rational intervention strategies. Yeast artificial chromosomes (YAC) have substantially aided in this endeavor by providing an unlimited access to defined parts of a parasite's genome, which, in turn, has facilitated a broad range of molecular studies. For example, YACs have facilitated positional cloning strategies to identify genes involved in antigenic variation and drug resistance mechanisms. Moreover, YACs have been invaluable tools for the many genome sequencing projects examining parasites. In this chapter, we provide a detailed protocol of how to generate representative YAC libraries from parasite genomes. This protocol can be applied to both protozoa and metazoa, and can even be used for YAC library construction of parasite material isolated from a single infected host.

Key Words: Parasite; YAC library; patient material; novel species.

1. Introduction

The discovery that yeast can accept large fragments of heterologous DNA as yeast artificial chromosomes (YAC) marked a breakthrough in the analysis of complex genomes ***(1)***. Although standard prokaryotic cloning systems, such as plasmids and cosmids, have a limited cloning capacity, YACs are able to incorporate large fragments of DNA ranging from between 30 kb and several

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megabases **(2–4)**. Access to large pieces of cloned DNA has facilitated a number of applications, including positional cloning of genes of interest, transcriptional mapping of chromosomal domains, and the physical mapping of chromosomes and entire genomes **(5)**. Last but not least, the numerous genome sequencing projects would not have progressed as rapidly if it were not for YACs to assemble the short reads into long stretches of contiguous sequence information, identify gaps in the sequence, and help close them.

The basis of a YAC are the vector arms, which contain all functions necessary for mitotic segregation in yeast, including a centromeric sequence (CEN), an autonomous replication sequence (ARS), and telomere sequences (TEL). In addition, the vector arms harbor selectable markers (*TRP1* and *URA3*, mediating tryptophan and uracil autotrophy in suitable yeast hosts) and an interruptible marker containing the *Eco*RI cloning site (*SUP4-o*, mediating a red/white color selection for DNA insertion events). The most frequently used YAC vector is pYAC4 **(1),** which, besides the elements previously mentioned, contains the *Col E1* replication origin and the ampicillin selectable marker for propagation in *Escherichia coli*.

YAC libraries have been generated for numerous organisms, including human **(2,3)**, mouse **(2)**, bovine **(6)**, *Arabidopsis* **(7)**, rice **(8,9)**, zebra fish **(10)**, and several human pathogens, such as the human malarial parasites *Plasmodium falciparum* **(11,12)** and *Plasmodium vivax* **(13,14)**, the etiological cause of Chagas disease, *Trypanosome cruzi* **(15)**, and *Schistosoma mansoni*, an agent of schistosomiasis **(16)**. In the case of *P. falciparum*, YAC technology has provided, for the first time, a stable source of DNA from this organism. *P. falciparum* DNA exhibits an unusually high A+T content that averages 82% and approaches 90–95% in intergenic regions. As a consequence of its high A+T content, *P. falciparum* DNA is unstable in *E. coli* and is subject to frequent recombination and deletion events. In addition to DNA fragments with a high A/T content, long inverted repeats or Z-structures also tend to be quite unstable in *E. coli* **(17–19)**, but can be stably maintained in yeast as YACs **(20–22)**. Generally, YACs appear to be more tolerant of DNA fragments with unusual properties than are other cloning systems, including plasmids, cosmids, and bacterial artificial chromosomes. Thus, in cases in which DNA stability causes concerns, YACs offer a viable method to generate representative libraries. An additional advantage of using a yeast cloning system is the opportunity to selectively clone a specific chromosomal region from a complex genome by methods termed transformation-associated recombination (TAR) cloning and radical TAR cloning **(23,24)**. TAR cloning allows a specific region of interest, such as a large gap in a sequence, to be captured from a complex genome and transferred, by recombination, to a specific yeast cloning vector.

YAC technology has substantially aided in the analysis of pathogens and their disease-causing interactions with their respective hosts. A prominent example is the dissection of a 400-kb locus on the *P. falciparum* chromosome 7 containing a determinant of resistance to the formerly first line antimalarial drug, chloroquine ***(25)***. Other examples include the identification and characterization of multicopy gene families, such as the *P. vivax vir* ***(14)*** and the *P. falciparum var* gene family ***(26)***, which are involved in antigenic variation and other immune evasion mechanisms. The physical mapping of entire chromosomes by overlapping YAC clones and the subsequent genome project have revealed that these variant genes comprise large gene families with their members being predominantly located at subtelomeric domains ***(14,26,27)*** where they are subjected to frequent recombination events. Since its first description, YAC technology has been improved and simplified ***(4,28)***, rendering it almost a routine technique for laboratories with a background in molecular biology. The construction of a YAC library consists of the following steps:

1. Isolation of high quality genomic DNA.
2. Partial digestion of the genomic DNA.
3. Preparation of the YAC vector arms.
4. Ligation of the vector arms with the partially digested DNA.
5. Yeast transformation.
6. Library organization and maintenance.

The protocols detailed in this chapter were originally developed for the generation of YAC libraries from malarial parasites; however, the protocols can be adapted to any other pathogen, and may even be used to generate YAC libraries from scarce pathogen material isolated from a single patient or a single infected animal.

2. Materials

2.1. Embedding Chromosomal DNA in Agarose

1. 0.5 *M* Ethylenediaminetetraacetic acid (EDTA), pH 8.0. Autoclave and store at room temperature.
2. TSE buffer: 100 m*M* NaCl, 50 m*M* EDTA, and 20 m*M* Tris-HCl, pH 8.0. Autoclave and store at room temperature.
3. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.
4. 1.25% InCert agarose (Biozym, Oldendorf, Germany) dissolved in TE buffer. Store at 4°C. Melt at 68°C and equilibrate at 42°C prior to use.
5. 2 mg/mL Proteinase K (Roche Applied Science, Mannheim, Germany) dissolved in 0.5 *M* EDTA, 1% *N*-lauryl-sarcosinate, pH 8.0. Prepare fresh.
6. $T_{10}E_{50}$ buffer: 10 m*M* Tris-HCl and 50 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.

7. 10X TBE buffer for pulse-field gel electrophoresis (PFGE): 890 m*M* Tris-base, 890 m*M* boric acid, and 20 m*M* EDTA, pH 8.0.
8. SeaKem LE agarose (Biozym).
9. Casting molds (Bio-Rad, Hercules, CA).
10. PFGE system (Bio-Rad).

2.2. Partial Digestion of Genomic DNA

1. 100 m*M* Phenylmethylsulfonyl fluoride (PMSF) in 2-propanol. Dissolve at 68°C and store at –20°C. (**Caution: very toxic**.) Very unstable in aqueous solution. Prepare fresh shortly before use.
2. $T_{10}E_{50}$ buffer: 10 m*M* Tris-HCl and 50 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.
3. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.
4. 10 mg/mL Bovine serum albumin (Fraction V, Sigma, St. Louis, MO).
5. 10X *Eco*RI methylase buffer: 0.8 m*M S*-adenosyl-methionine, 20 m*M* $MgCl_2$, 1 *M* NaCl, 10 m*M* dithiothreitol (DTT), and 0.5 m*M* Tris-HCl, pH 7.6. Store at –20°C.
6. 100 m*M* Spermidine. Store at –20°C.
7. 20 U/µL *Eco*RI (New England Biolabs, Ipswich, MA).
8. 40 U/µL *Eco*RI methylase (New England Biolabs).
9. 0.5 *M* EDTA, pH 8.0. Autoclave and store at room temperature.
10. 10 mg/mL Proteinase K (Roche Applied Science) dissolved in TE buffer. Prepare fresh.

2.3. Vector Preparation

1. pYAC4 vector (American Type Culture Collection, registration number: 67379).
2. 20 U/µL *Eco*RI (New England Biolabs).
3. 20 U/µL *Bam*HI (New England Biolabs).
4. 1 U/µL Calf intestinal phosphatase (New England Biolabs).
5. 10X Calf intestinal phosphatase buffer (New England Biolabs).
6. TE-saturated phenol/chloroform (1:1 v/v). Prepare fresh. Do not use red phenol.
7. 100% Ethanol.
8. 75% Ethanol.
9. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0.
10. 10 U/µL T_4 polynucleotide kinase (New England Biolabs).

2.4. Ligation

1. 1X ligation buffer: 30 m*M* NaCl, 10 m*M* $MgCl_2$, 0.75 m*M* spermidine, 0.3 m*M* spermine, and 50 m*M* Tris-HCl, pH 7.6. Store at –20°C.
2. 10 U/µL T_4 polynucleotide kinase (New England Biolabs).
3. 100 m*M* Adenosine triphosphate, pH 7.5. Store at –20°C.
4. 1 *M* DTT.
5. 400 U/µL T_4 DNA ligase (New England Biolabs).

2.5. Size Fractionation

1. SeaKem low gelling agarose, molecular biology grade (Biozym).
2. 10X TBE buffer for PFGE: 890 m*M* Tris-base, 890 m*M* boric acid, 20 m*M* EDTA, pH 8.0.
3. Yeast chromosome size marker (New England Biolabs).
4. 10 mg/mL Ethidium bromide solution (**Caution: mutagenic**).
5. 1 U/μL β-agarase I (New England Biolabs).
6. β-agarase I buffer (New England Biolabs).
7. PGFE system (Bio-Rad).

2.6. Yeast Spheroplast Preparation and Transformation

1. *Saccharomyces cerevisiae* strain AB1380 (American Type Culture Collection, Palo Alto, CA; reference number: 20843).
2. YPD medium: 1% Bacto-yeast extract (BD Biosciences), 2% Bacto-peptone (BD Biosciences), pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
3. YPD agar: 1% Bacto-yeast extract (BD Biosciences), 2% Bacto-peptone (BD Biosciences), 2% Bacto-agar (BD Biosciences), pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
4. 0.5% Adenine hemisulfate (Sigma). Sterile filter the solution.
5. 1 *M* Sorbitol. Autoclave and store at 4°C.
6. Na-phosphate buffer, pH 7.6: 84.5 mL 1 *M* Na_2HPO_4 and 15.5 mL 1 *M* NaH_2PO_4 in 100 mL H_2O.
7. SPEM buffer: 1 *M* sorbitol, 0.1 *M* Na-phosphate buffer, pH 7.6, and 10 m*M* EDTA. Sterile filter. Add 30 m*M* of β-mercaptoethanol prior to use.
8. 10 mg/mL of 20T Zymolase (MP Biomedicals, Irvine, CA) dissolved in 10 m*M* NaH_2PO_4, pH 7.5. Store in 100 μL aliquots at –70°C. Use a fresh aliquot for each transformation.
9. STC solution: 1 *M* sorbitol, 10 m*M* $CaCl_2$, and 10 m*M* Tris-HCl, pH 7.5. Sterile filter and store at 4°C.
10. PEG solution: 20% polyethylene glycol (molecular weight 8000), 10 m*M* $CaCl_2$, and 10 m*M* Tris-HCl, pH 7.5. After autoclaving, the appropriate amount of a sterile 1 *M* $CaCl_2$ solution is added. A white precipitate may form in the solution on the addition $CaCl_2$. However, this does not seem to interfere with the transformation efficiency.
11. SOS medium: 1 *M* sorbitol, 6.5 m*M* $CaCl_2$, 0.25% Bacto-yeast extract (BD Biosciences), and 0.5% Bacto-peptone (BD Biosciences). Filter-sterilize and store at room temperature. Before use, add sterile-filtered 20 μg/mL adenine, 20 μg/mL histidine, 30 μg/mL leucine, 30 μg/mL lysine, 20 μg/mL tryptophan, and 20 μg/mL uracil.
12. AHC medium: 1 *M* sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 20 μg/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.

13. AHC-TOP agar: 1 *M* sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 5% Bacto-agar (BD Biosciences), and 20 μg/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
14. AHC agar: 1 *M* sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 2% Bacto-agar (BD Biosciences), and 20 μg/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
15. 96- or 384-Well microtiter plates (Greiner, Frickenhausen, Germany).
16. Hematocytometer (Fisher Scientific, Nidderau, Germany).

2.7. Replication and Long-Term Storage of YAC Library

1. YPD medium: 1% Bacto-yeast extract (BD Biosciences) and 2% Bacto-peptone (BD Biosciences). Adjust pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
2. YPD medium/glycerol (2:3).
3. 96 or 384 replicator (Sigma).

3. Methods

3.1. Preparation of High-Quality DNA

DNA is a fragile polymer that easily breaks when subjected to mechanical stress. It is therefore imperative to handle chromosomal DNA with the uttermost care. Shearing is to be avoided, as is contamination with DNA-digesting nucleases, or exposure to X-rays or ultraviolet (UV) light (*see* **Note 1**). The preparation of high-quality chromosomal DNA begins with enriched or purified pathogens of interest (*see* **Note 2**) being embedded in agarose blocks. All subsequent manipulation steps are carried out using these agarose blocks. For protocols to grow, enrich, and purify the organism of interest the reader is referred to the specialized literature (*see* **Note 2**).

1. Resuspend cells in TSE buffer at a concentration of approx 5×10^8 cells/mL. Use a 50-mL tube and place the cells at 37°C.
2. Add an equal volume of melted 1.25% InCert agarose equilibrated at 37°C and mix gently by tapping against the tube.
3. Using a 1-mL tip, slowly suck up the cell/agarose suspension and fill each casting mold with 100 μL of suspension. Keep the molds on ice for 5 min until the agarose hardens.
4. Push the hardened agarose blocks out of the molds with a flat object, such as a Pasteur pipet bent into an L-shape.
5. Place agarose blocks in proteinase K solution and incubate at 50°C for 48 h. Use 0.5 mL of solution for each block.

6. Wash blocks twice in $T_{10}E_{50}$ buffer for 15 min at room temperature while gently shaking.
7. Store blocks in $T_{10}E_{50}$ buffer at 4°C until use.
8. Check the quality of the DNA by PFGE analysis (0.4% SeaKem LE agarose gel; 0.5X TBE buffer; voltage, 2.7 V/cm; running time, 36 h; initial switching time, 120 s; final switching time, 720 s; temperature, 18°C). Each block should contain between 5 and 10 μg of genomic DNA. *(See* **Subheading 3.5.** for more details on the preparation of the gel.)

3.2. Partial Digestion of Genomic DNA

Prior to digestion with restriction endonuclease, the blocks containing the DNA of interest need to be treated with PMSF in order to inactivate any residual proteinase K from the DNA preparation. Partially digested genomic DNA for YAC cloning is then generated using a mixture of the restriction endonuclease *Eco*RI and *Eco*RI methylase. The proper conditions need to be determined experimentally for each DNA preparation by titrating both enzymes against each other. The ratio of both enzymes determines the average DNA size, which, for YAC cloning, should range between 200 and 500 kb. It is recommended to start initially with 1 U of *Eco*RI and 30 U of *Eco*RI methylase per agarose block (5–10 μg of DNA), and then stepwise increase the concentration of the methylase until a ratio of 1:320 is reached. In some cases it may be also necessary to titrate the concentration of *Eco*RI in order to generate DNA fragments of the desired average size. The size of the digested DNA is monitored by PFGE (*see* **Subheading 3.5.** for details on gel preparation and running conditions).

1. Wash blocks twice in $T_{10}E_{50}$ buffer containing 1 m*M* of fresh PMSF (1 mL per block) for 30 min. Shake gently.
2. Wash blocks three times in $T_{10}E_{50}$ buffer (1 mL per block) for 30 min. Shake gently.
3. Wash blocks three times in $T_{10}E_1$ buffer (1 mL per block) for 30 min. Shake gently.
4. Transfer each block into a sterile 1.5-mL Eppendorf tube and add:
   a. 50 μL of bovine serum albumin (10 mg/mL).
   b. 50 μL of 10X *Eco*RI methylase buffer.
   c. 13 μL of spermidine (100 m*M*).
   d. *Eco*RI restriction endonuclease.
   e. *Eco*RI methylase.
   f. Sterile H_2O to a final volume of 500 μL.
5. Incubate at 37°C for 4 h.
6. Stop the reaction by adding:
   a. 55 μL of 0.5 *M* EDTA, pH 8.0.
   b. 62 μL of proteinase K solution (10 mg/mL).
7. Incubate at 50°C for 30 min.
8. Wash the blocks once in TE buffer at 50°C for 30 min. Shake gently.

9. Incubate the blocks in TE buffer containing 1 m*M* PMSF at 50°C for 30 min to inactive the proteinase K (1 mL of solution per block). Shake gently.
10. Wash the blocks twice in TE buffer at 50°C for 30 min.
11. Check the size of the partially digested DNA by PFGE (*see* **Subheading 3.5.** for details on gel preparation and running conditions).

The genomic DNA is now ready for ligation.

3.3. YAC Vector Preparation

The left and right YAC cloning vector arms are liberated from the plasmid pYAC4 by digestion with the restriction endonucleases *Eco*RI and *Bam*HI. pYAC4 can be propagated in suitable *E. coli* strains, such as DH5α.

1. Cleave purified pYAC4 plasmid DNA with *Eco*RI and *Bam*HI to completion.
2. Denature restriction endonucleases by heat treatment at 68°C for 10 min.
3. Dephosphorylate free 5' ends using a phosphatase (0.05 U/μg DNA) in 1X phosphatase buffer. Incubate at 37°C for 30 min. According to our experience, phosphatases from calf intestine or shrimp work equally well.
4. Add EDTA, pH 8.0 to a final concentration of 5 m*M* and incubate the reaction at 68°C for 10 min to inactivate the phosphatase.
5. Purify the DNA by phenol/chloroform extraction followed by ethanol precipitation.
6. Resuspend the DNA in TE buffer at a final concentration of 2 μg/μL.

Digestion of the vector arms is monitored electrophoretically. Dephosphorylation is verified by ligation in the presence and absence of T_4 polynucleotide kinase. The ligation assays are then transformed into *E. coli*. In the case of the dephosphorylated vector arms, only a few, if any, colonies should grow, whereas a large number of colonies should appear when the vector arms are ligated in the presence of T_4 polynucleotide kinase.

3.4. Ligation

1. Wash agarose blocks three times in 1X ligation buffer at room temperature for 30 min.
2. Remove all liquid and place six agarose blocks into a 1.5-mL Eppendorf tube.
3. Add an equal amount of prepared vector arm DNA. Assuming that each of the six agarose blocks contains 5–10 μg of genomic DNA, approx 60 μg of prepared vector arm DNA are required. The mass ratio of 1:1 between genomic and vector arm DNA translates approximately into a 500-fold molar excess of vector arms vs genomic DNA (*see* **Note 3**).
4. Melt agarose blocks at 68°C for 10 min.
5. Allow genomic and vector arm DNA to equilibrate at 37°C for 2 h (*see* **Note 3**).
6. Add 1/10 vol of premade ligation mix (22 U/μL T_4 DNA ligase, 5 m*M* adenosine triphosphate, and 100 m*M* DTT in 1X ligation buffer). Pipet into the middle of the molten agar. Do not mix (*see* **Note 3**).

7. Remove 10 μL of the sample and add 10 U of T_4 polynucleotide kinase to it in order to verify ligation conditions. Incubate at 20°C for 12 h. Self-ligation of the vector arms will occur and is monitored electrophoretically using a conventional 1% agarose gel. As a control, run unligated vector arms on the same gel.
8. Allow the solution to equilibrate at 37°C for 1 h (**Note 3**).
9. Incubate the ligation reaction at 20°C for 24 h. The agarose will solidify during this time.

3.5. Size Fractionation

A size fractionation of the ligation reaction is highly recommended to remove the excess vector arms, as well as small YACs, which would otherwise be over-represented in the YAC library because of their higher transformation efficiency. Size selection is carried out using PFGE.

1. Prepare a 1% agarose gel in 0.5X TBE using a low gelling agarose, such as SeaKem molecular grade (**Note 4**). Tape up several teeth of the gel comb in order to generate a well large enough for the ligation sample. Load yeast chromosome size markers in the outside wells and seal all wells with molten agarose (1% in TBE buffer).
2. Run PFGE (voltage, 4.5 V/cm; running time, 18 h; initial switching time, 5 s; final switching time, 25 s; temperature, 14°C; field angle 120°, running buffer, 0.5X TBE).
3. After electrophoresis, cut off the gel lanes containing the size markers and a very small part of the lane containing the sample. Stain these pieces in 200 mL of running buffer containing 80 μL of a 10-μg/mL ethidium bromide solution for 30 min and examine them under UV light.
4. Mark the area of the gel that holds DNA fragments ranging from between 100 and 500 kb.
5. Reassemble the gel pieces with the rest of the gel which was kept in running buffer during this time to avoid drying out.
6. Excise the region containing the high molecular weight DNA using a sterile glass cover slip.
7. Place this gel slice into a 15-mL tube. Store at 4°C until use.
8. Stain the entire gel as described in **step 3**, examine by UV light, and take a picture for documentation.
9. Wash the gel slice containing the size-fractionated DNA three times for 20 min each in β-agarase buffer.
10. Melt the gel slice containing the size-fractionated DNA at 68°C for 10 min.
11. Allow the sample to cool to 42°C, add β-agarase I to a final concentration of 50 U/mL, and incubate at 42°C for 3 h. The DNA is now ready for transformation into yeast spheroplasts.

3.6. Yeast Spheroplast Preparation and Transformation

S. cereviseae strain AB1380 is used as the host in YAC ***(1)***. As a selective medium we routinely use AHC-medium. The AHC medium is as selective as synthetic minimal media, allows for red/white color selection of positive transformants, but is much easier to prepare (**Note 5**).

1. For high transformation efficiency, plate AB1380 yeast cells from a frozen glycerol stock onto a YPD plate, and incubate the plate at 30°C for 48 h.
2. Take a single red colony and prepare an overnight culture in YPD medium supplemented with 0.002% adenine hemisulfate.
3. Inoculate 50 mL of YPD medium supplemented with 0.002% adenine hemisulfate with the fresh overnight culture to obtain an OD_{600} of 1.0. Use a 500-mL Erlenmeyer flask with bumps. Incubate the culture at 30°C in a shaking incubator (180 rpm) until an OD_{600} of 4.0 is reached. This corresponds to approx 3×10^7 cells/mL. The cell density should double every 90 min.
4. Collect the cells by centrifugation at 400–600*g* for 10 min at room temperature.
5. Wash the cells once with 20 mL sterile water and centrifuge as described in **step 4**.
6. Wash once with 20 mL of 1 *M* sorbitol and centrifuge as described in **step 4**.
7. Resuspend the cells in 20 mL of 30°C prewarmed SPEM buffer containing fresh β-mercaptoethanol. The cell density should be close to 7.5×10^7 cells/mL. Remove sample, dilute it 10-fold in water, and determine the OD_{600}. The OD_{600} value obtained serves as the prespheroplast reference.
8. Add 45 µL of 20T Zymolase solution. Incubate cells by slowly shaking in a 30°C water bath for about 20 min. The extent of spheroplast formation is determined spectrophotometrically. Every 5 min a 50-µL aliquot is removed, diluted 10-fold in water, and the OD_{600} value determined. As spheroplasts lyse in water owing to the lack of a cell wall, the reduction in the OD_{600} value is directly proportional to the amount of spheroplasts formed. Eighty to 90% of the cells must be spheroplasts within 20 min to achieve optimal transformation efficiency. If not, it is strongly recommended to repeat the preparation.
9. Collect the spheroplasts by centrifugation at 200–300*g* for 4 min at room temperature.
10. Gently resuspend the cells in 20 mL of 1 *M* sorbitol, and centrifuge as described in **step 9**.
11. Wash the spheroplasts once in 20 mL of STC, centrifuge as in **step 9**, and finally resuspend them in 2 mL of STC. At this point, the spheroplasts are stable at room temperature for at least an hour. The cell density is now determined microscopically using a hematocytometer. Adjust the volume to a final cell concentration of 6.5×10^8 cells/mL.
12. To 150 µL of spheroplasts, add 50 µL of β-agarase I treated size fractionated DNA (approx 80 ng) in a 15-mL tube (use a 1-mL tip with a widened opening). Incubate at 20°C for 10 min. Scaling up the reaction significantly reduces transformation efficiency (**Note 6**). It is, therefore, recommended to run parallel assays.

13. Add 1.5 mL of PEG solution and mix by gently inverting the tube. Incubate at 20°C for 10 min.
14. Immediately centrifuge at 200–300*g* for 4 min at room temperature.
15. Carefully decant the supernatant and resuspend cells in 225 µL of SOS solution. Incubate at 30°C for 30 min.
16. Gently resuspend the settled spheroplasts. Add 8 mL of molten AHC-TOP agar (prewarmed to 50°C) and gently invert to mix. Quickly pour onto an AHC plate that has been prewarmed to 37°C (**Note 5**). For yeast transformation, AHC-Top agar and AHC plates need to contain 1 *M* sorbitol to osmotically buffer the spheroplasts. Allow plates to sit for 10 min at room temperature.
17. Incubate plates at 30°C for 7 d. The first yeast clones should be visible after 2–3 d. Those yeast clones that contain artificial chromosomes turn red on AHC-plates, whereas revertants and clones harboring pYAC4 remain white.

Red colonies are picked and transferred to 96- or 384-well microtiter plates containing 150 or 80 µL of YPD medium, respectively. Cells are grown for 2–3 d at 30°C.

3.7. Replication and Long-Term Storage of YAC Library

1. Using a 96- or 384-replicator, transfer YAC clones to a new set of microtiter plates containing an appropriate amount of YPD media in each well. Use a sterile replicator for each microtiter plate to avoid cross-contamination of YAC clones. Metal replicators can be sterilized as follows: dip the pins of the replicator in 80% ethanol, flame, and then cool for several seconds on a sterile YPD agar plate.
2. Grow YAC clones for 2–3 d at 30°C. During incubation approximately one-third of the media will evaporate. The YAC library can be stored at 4°C for up to 3 mo or can be used to make frozen glycerol stocks.
3. To freeze down the YAC clones, add an appropriate volume of YPD media/glycerol (2:3 v/v) to each well to yield a final glycerol concentration of 20%. In the case of 96-well plates approx 50 µL YPD media/glycerol is usually added. Resuspend the cells in the medium, using a multichannel pipet.
4. Cells can now be stored at –80°C.

4. Notes

1. Success greatly depends on the quality and purity of the starting DNA material. DNA that is partially degraded, or enzymatically or chemically modified, will never produce a YAC library. Therefore, shearing and exposure of the DNA to UV light or X-rays are to be avoided. UV light and X-rays can introduce strand breakages, depurination, and cross-linking events into the DNA. Exposure to X-rays becomes a problem when DNA is shipped by air freight as all packages, not only hand luggage and carry-ons, are routinely X-rayed for safety inspections. It is, therefore, best to deliver chromosomal DNA in person and, if a flight is involved in the travel, to keep the DNA on your person.

2. In some cases it may be necessary to collect pathogen material from patients or infected animals, for instance when an in vitro culture system is not available. An example is the malarial parasite *P. vivax*, which propagates within reticulocytes. As reticulocytes cannot be readily obtained in large quantities, in vitro culture conditions for *P. vivax* are difficult to establish. Access to parasite DNA, therefore, relies on material obtained from *P. vivax*-infected patients or monkeys. If pathogen material for library construction is collected from host organisms, great care needs to be taken in order to remove any contaminating nucleated cells from the host prior to DNA preparation. For the construction of a *P. vivax* YAC library, we devised a two-step purification protocol ***(13)***. Host leukocytes were initially removed from the erythrocytes using Plasmodipur filters (Organon Teknika). Erythrocytes infected with *P. vivax* were then concentrated using a single step 16% Nycodenz (Sigma) gradient. Nycodenz is resuspended in phosphate buffered saline and centrifugation is carried out at 900*g* for 30 min at 15°C. The resulting material was found to be free of contaminating human DNA, as verified by both Southern blotting and polymerase chain reaction analyses ***(13)***. Recently, we use a strong magnetic field to purify erythrocytes infected with malarial parasites ***(29)***. Malaria parasites degrade the erythrocyte's hemoglobin, thereby liberating large amounts of iron-containing heme. In the oxidative environment of the parasite's food vacuole, where hemoglobin degradation occurs, ferrous iron (Fe^{2+}) is oxidized to ferric iron (Fe^{3+}), which because of its magnetic properties allows infected erythrocytes to be retained in a magnetic field, whereas uninfected erythrocytes freely pass through this field. Magnetic devices and columns can be purchased from Miltenyi Biotec, Germany.
3. Do not speed up the process by pipetting or vortexing. Sheared DNA will never produce a high-quality YAC library.
4. Be careful when handling low gelling agarose gels, as they are slippery and prone to breakage.
5. It has been observed that YAC transformants often do not grow on synthetic minimal medium selective for both tryptophan and uracil autotrophy. This phenomenon has been attributed to the weak *TRP* promoter, which limits expression of the *TRP* gene product. To overcome this problem selective pressure can be applied sequentially, first for uracil and subsequently for tryptophan autotrophy. Alternatively, transformants can be selected on AHC-medium as described herein. AHC medium is initially rich in amino acids. However, autoclaving breaks down most of the tryptophan. The residual amounts of tryptophan do not support permissive growth of trypophan auxotrophic clones, yet they are sufficient to allow the cells to recuperate after transformation and express the *TRP* gene encoded by the YAC vector arm.
6. It is tempting to scale up the yeast transformation reaction. However, there is ample evidence that scaling up the reaction significantly reduces transformation efficiency. Although processing and handling many reaction tubes at the same time is cumbersome and time consuming, it is still faster than repeating the experiment.

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3

Analysis and Screening of Yeast Artificial Chromosome Libraries by Filter Hybridization

Cecilia P. Sanchez and Michael Lanzer

Summary

Large-scale genome mapping and sequencing projects nowadays rely on robotic systems for yeast artificial chromosome (YAC) library processing, handling, and analysis. The advantages are obvious. Robots are fast, less labor intense, and the data reproducibility is high. However, there may be cases in which automated systems are not available or are unnecessary, for example, if the YAC library is small. In this chapter, we describe a simple method to process YAC libraries manually and screen them by conventional filter hybridization.

Key Words: YAC library; screening; analysis; hybridization; manual; nonautomated.

1. Introduction

Generation of a yeast artificial chromosome (YAC) library is a challenging endeavor, requiring optimized conditions and some luck. However, the bulk of the work is involved in the analysis and screening of the library. The analysis is not only labor intensive, but also repetitive and time consuming. For this reason, with large YAC libraries robotic systems are used for processing and analysis ***(1,2)***. These robots expedite the screening process, but are rarely available outside of core facilities because of high costs. Although core facilities may offer these services, they are frequently overbooked. As an alternative to automated systems in case the YAC library or sublibrary is small (<1500 clones), a manual handling, processing, and screening approach may be recommended. This involves manual replication of the library onto nylon filters using commercially available 96- or 384-prong replicators, followed by the analysis of the filters using conventional hybridization methods.

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2. Materials

2.1. YAC Filter Preparation and Analysis

1. YPD medium: 1% Bacto-yeast extract (BD Biosciences, Palo Alto, CA) and 2% Bacto-peptone (BD Biosciences). Adjust pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
2. YPD agar: YPD medium and 2% Bacto-agar (BD Biosciences).
3. 20% Ca-propionate solution (Sigma, St. Louis, MO). Filter-sterilize.
4. 4 mg/mL Novozyme (Sigma) dissolved in 1 *M* sorbitol, 0.1 *M* Na-citrate, 10 m*M* dithiothreitol, and 10 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0. Prepare fresh.
5. Denaturing solution: 0.5 *M* NaOH and 1.5 *M* NaCl.
6. Neutralizing solution: 1 *M* Tris-HCl, pH 7.4, and 1.5 *M* NaCl.
7. 0.25 mg/mL Proteinase K (Roche Applied Science, Mannheim, Germany) dissolved in 0.15 *M* NaCl and 0.1 *M* Tris-HCl, pH 7.4. Prepare fresh.
8. 23 × 23-cm Culture plates (Griener, Frickenhausen, Germany).
9. 3MM Whatmann paper.
10. 22 × 22-cm Nylon transfer membrane Hybond-N^+ (Amersham Biosciences, Uppsala, Sweden).
11. DNA ultraviolet (UV)-crosslinker.
12. 96- or 384-replicator (Sigma).

2.2. YAC Filter Hybridization

1. 1 *M* Na-phosphate buffer, pH 7.2: 68.4 mL 1 *M* Na_2HPO_4 and 31.6 mL 1 *M* NaH_2PO_4 in 100 mL H_2O.
2. Church buffer: 7% sodium dodecyl sulfate (SDS), 1 m*M* EDTA, and 0.5 *M* Na-phosphate buffer, pH 7.2.
3. 10 mg/mL of yeast tRNA (Sigma) dissolved in TE buffer containing, 1% SDS.
4. DNA labeling kit (e.g., random priming DNA labeling kit from Roche Applied Science).
5. α-[^{32}P]-dATP, 3000 Ci/mmol (Amersham Biosciences).
6. Washing buffer: 0.1% SDS and 40 m*M* Na-phosphate, pH 7.2.
7. Stripping buffer: 0.1% SDS and 5 m*M* Na-phosphate, pH 7.2.
8. Hybridization oven.
9. Radiographic film, X-Omax film (Kodak, New Haven, CT).

2.3. Analysis of YAC Clones by PFGE

1. AHC medium: 1 *M* sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BC Biosciences), 1% casamino acids (BC Biosciences), and 20 μg/mL adenine hemi-sulfate. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
2. AHC agar: AHC medium and 2% Bacto-agar (BC Biosciences).
3. 1 *M* Sorbitol. Autoclave and store at 4°C.
4. SPEM buffer: 1 *M* sorbitol, 0.1 *M* Na-phosphate, and 10 m*M* EDTA, pH 8.0. Sterile filter. Add 30 m*M* of β-mercaptoethanol prior to use.

5. 30 mg/mL of 20T Zymolase (MP Biomedicals, Irvine, CA) dissolved in 10 m*M* NaH_2PO_4, pH 7.5. Prepare fresh.
6. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.
7. 1.25% InCert agarose (Biozym, Oldendorf, Germany) dissolved in TE buffer. Store at 4°C. Melt at 68°C and equilibrate at 42°C prior to use.
8. 2 mg/mL of proteinase K (Roche Applied Science) dissolved in 1% *N*-lauryl-sarcosinate and 0.5 *M* EDTA, pH 8.0. Prepare fresh. Add the proteinase K last.
9. $T_{10}E_{50}$ buffer: 10 m*M* Tris-HCl and 50 m*M* EDTA, pH 8.0.
10. 10X TBE buffer for pulse-field gel electrophoresis (PFGE): 890 m*M* Tris-base, 890 m*M* boric acid, and 2 m*M* EDTA, pH 8.0.
11. SeaKem LE agarose (FMC, Philadelphia, PA).
12. Yeast chromosome size marker (New England Biolabs, Ipswich, MA).
13. 10 mg/mL Ethidium bromide solution (**Caution: mutagenic**).
14. Casting molds (Bio-Rad).
15. PGFE system (Bio-Rad).

3. Methods

3.1. Analysis of YAC Libraries by Filter Hybridization

YAC libraries can be analyzed by polymerase chain reaction ***(3,4)*** or filter hybridization ***(5)***. Although both techniques have been successfully applied to YAC libraries derived from pathogens, filter hybridization is the method of choice because of convenience and speed. The analysis of a YAC library by filter hybridization involves the following steps: transfer of the yeast clones onto a nylon filter, processing of the filters to prepare the DNA, and subsequently screening of the YAC filter by hybridization (*see* **Note 1**).

1. Prepare a fresh replicate of the YAC library in microtiter plates (96- or 384-wells). Grow cells in YPD medium for 2 d at 30°C.
2. Prepare YPD agar plates. We usually use large square plates (23 × 23-cm) because they can hold the clones of up to six microtiter plates.
3. For each 22 × 22-cm plate, prepare four 3MM papers and one nylon filter 22 × 22-cm in size. Although the nylon filters can be bought sterile, 3MM paper needs to be sterilized prior to usage by either autoclaving or exposure to UV light. Most commercially available UV-crosslinkers have preset conditions for sterilization.
4. All subsequent steps are carried out in a sterile bench.
5. Mark the top left corner of the 22 × 22-cm nylon filter using a pencil.
6. Soak two 3MM papers for each nylon filter in 200 mL of YPD media supplemented with 2.5 mL of a 20% Ca-propionate solution.
7. Place the two layers of wet 3MM paper into the plate cover and lay the nylon filter on top. Wait until the nylon filter is moist.
8. Now stamp the YAC clones onto the nylon filter, using the appropriate replicator (*see* **Note 1**).

9. Remove the filter and place it, colonies facing up, onto the YPD agar plate.
10. Incubate the plates inverted at 30°C for 2–3 d.
11. Place the filters with YAC colonies facing up onto two layers of 3MM paper soaked in 50 mL of Novozyme solution. Novozyme digests the cell wall. Use the plate cover as an incubation chamber and the plate bottom as the cover after removal of the YPD agar.
12. Seal the plate with Saran wrap and incubate at 37°C overnight.
13. Place the nylon filter onto two layers of 3MM paper soaked in denaturing solution. Incubate at room temperature for 15 min.
14. Let the nylon filters dry on fresh 3MM paper for 10 min at room temperature.
15. Transfer the nylon filters to two layers of 3MM paper soaked in neutralizing solution. Incubate at room temperature for 10–30 min.
16. Place the nylon filter in a 22 × 22-cm plate containing 250 mL of the proteinase K solution. Incubation at 37°C for 2–3 h. During this time the YAC clones will lose their reddish color.
17. Dry the membranes for 36 h on 3MM paper at room temperature.
18. UV-crosslink the YAC DNA to the nylon filter in a UV-crosslinking chamber, according to the manufacturer's recommendations.
19. Preincubate the YAC filters at 50°C overnight in Church buffer supplemented with 0.2 mg/mL of yeast tRNA. Use approx 0.2 mL of Church buffer per cm^2 of nylon filter.
20. Prepare an α-[^{32}P]- or fluorescently labeled probe (using commercially available labeling kits) and denature the probe by heat treatment at 94°C for 10 min (*see* **Note 2**). If a fluorescently labeled probe is used in the analysis of the YAC filters, follow the instructions of the manufacturer of the fluorescent DNA labeling kit used.
21. In case a radiolabeled probe is used, add the labeled probe to the prehybridized nylon filter. For the best signal to noise ratio, use 750,000–1,000,000 cpm of probe per milliliter of Church buffer. Incubate at 50°C overnight.
22. Wash the nylon filters in washing buffer at room temperature for 5 min.
23. Wash the nylon filters in prewarmed washing buffer at 65°C for 15 min.
24. Wrap the damp filters in Saran wrap and expose them to X-ray imaging film.

Filters can be washed more stringently if necessary and can be reused several times. Remove bound radioactive probes by incubating the filters in stripping buffer at 90°C for 30 min. Do not allow the filters to dry.

3.2. Analysis of Individual YAC Clones by PFGE

1. Streak the YAC clone of interest out on an AHC agar plate. Incubate at 30°C for 3 d.
2. Inoculate 20 mL of AHC media with the YAC clone and incubate at 30°C for 2 d while shaking at 175 rpm.
3. Collect cells by centrifugation at 400–600*g* for 10 min at 4°C.
4. Wash the cell pellet in 5 mL of water and centrifuge as described in **step 3**.
5. Wash the cells in 5 mL of 1 *M* sorbitol and centrifuge as described in **step 3**.

6. Resuspend the cells in 250 µL of SPEM, add 30 µL of 20T Zymolase solution.
7. Add immediately 400 µL of 37°C pre-equilibrated 1.25% InCert agarose, mix, and add 100 µL to each casting mold.
8. Leave the blocks on ice for 5 min to solidify.
9. Remove the hardened agarose blocks from the casting molds and incubate them in 5 mL of SPEM supplemented with 50 µL of 20T Zymolase solution at 37°C for 7–8 h.
10. Replace the SPEM/Zymolase solution with 5 mL of proteinase K solution and digest the blocks at 50°C for 48 h.
11. Place the blocks in $T_{10}E_{50}$ for storage at 4°C. If embedded DNA is to be used for restriction analysis, follow **steps 1–3** of **Subheading 3.2.**
12. For PFGE analysis, prepare a 1.0 % SeaKem LE agarose gel in 0.5X TBE buffer.
13. Carefully place the agarose blocks in the wells using glass cover slips, load a DNA size standard, and seal all wells (including the empty ones) with LE agarose.
14. Run the PFGE using the following conditions: running buffer, 0.5X TBE buffer; voltage, 5.5 V/cm; running time, 24 h; initial switching time, 5 s; final switching time, 25 s; temperature, 14°C; field angle 120°.
15. Stain the gel in 500 mL of running buffer containing 80 µL of 10 µg/mL ethidium bromide for 30 min.

4. Notes

1. YAC clones can be transferred to nylon filters manually, using a replicator as described herein. This technique produces filters of high quality without the need of sophisticated and expensive equipment. However, if a larger number of filters are needed, as may be necessary for a large-scale mapping effort, automated devices may be used for filter production. Besides speed, automated systems offer the advantage of arranging YAC clones at a higher density and in duplicates on the filter. This saves materials and reagents, and simplifies the acquisition and interpretation of the data, which can now proceed using an automated reader.
2. Observe safety regulations for handling, storage, and disposal of radioactive materials.

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4

Analysis of Yeast Artificial Chromosome DNA by Restriction Digestion, Southern Blotting Nucleic Acid Hybridization, and Polymerase Chain Reaction

Sylvia Vasiliou and John Peter Quinn

Summary

Restriction enzyme analysis of yeast artificial chromosome (YAC) clones in combination with Southern blotting and polymerase chain reaction (PCR) is necessary to establish the integrity of the DNA contained within the YAC clone, as well as obtain information on the integrity of the cloned DNA. Restriction analysis is also a useful tool that allows a direct comparison between endogenous genomic DNA and the DNA fragment present within a YAC clone. This chapter summarizes the techniques of enzyme digestion of YAC DNA and the separation of the DNA fragments by pulse-field gel electrophoresis. The protocols of Southern blotting and PCR used to identify the cloned DNA fragments will also be described.

Key Words: Polymerase chain reaction; pulse-field gel electrophoresis (PFGE); restriction digests; Southern blotting; yeast artificial chromosome (YAC).

1. Introduction

Once a yeast artificial chromosome (YAC) clone has been identified by either filter hybridization or polymerase chain reaction (PCR)-based methods, it is important that the identity, extent, and integrity of the genomic fragment carried within the YAC clone be determined. Restriction analysis, Southern blotting, and PCR are cheap, accessible, and reliable diagnostic tools for determining the identity of the DNA contained within the isolated YAC and facilitate the detection of deletions and the assessment of possible chimerism within YAC clones ***(1)***.

Restriction analysis, Southern blotting, and PCR also allow a direct comparison with endogenous genomic DNA. For example, the identification of the position of CpG islands (clusters of unmethylated CpGs) within the DNA frag-

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ment cloned in the YAC provides valuable information on the presence of transcribed genes. This is accomplished by a comparison of restriction digests generated by rare cutting enzymes of two separate classes; those that recognize CpG islands with high frequency (e.g., *Not* I, *BssH*II, and *Eag*I), and those that do so with low frequency (e.g., *Mlu*I, *Nru*I, and *Pvu*I). These enzymes will cut more frequently in YAC-cloned DNA as opposed to uncloned genomic DNA because the former is unmethylated, generating restriction fragments that are relatively large in size. Unmethylated sites in both YAC cloned and uncloned DNA will yield identical restriction patterns.

Because of the availability of multiple genome sequences including that of the human (*see* Chapter 5), PCR analysis of YAC clones can now be employed to assess the integrity of YAC clones that are prone to chimerism, and to determine their genomic content. The same PCR primers can also be used to detect the integrity of YAC clone transgenes following their introduction into the genomes of transgenic animals using either pronuclear injection or ICSI (*see* Chapters 13 and 14) ***(2–4)***.

This chapter describes the following:

1. Preparation of YAC DNA in agarose plugs.
2. Size fractionation of digested YAC DNA by pulse-field gel electrophoresis (PFGE).
3. Restriction digestion of agarose-embedded YAC DNA and Southern blot analysis.
4. PCR analysis of YAC–DNA transformed yeast colonies.

2. Materials

1. SD (synthetic dropout + amino acid supplements) broth and agar (*see* **Note 1**).
2. β-mercaptoethanol (Sigma, St. Louis, MO).
3. SE medium: 1 *M* sorbitol and 20 m*M* ethylenediaminetetraacetic acid (EDTA). Sterilize by autoclaving.
4. SEM medium: freshly prepared prior to use by adding β-mercaptoethanol to SE (add 1 μL of β-mercaptoethanol/mL SE).
5. Low melting point (LMP) agarose (Seaplaque GTG, FMC, Rockland, ME).
6. Standard agarose for PFGE (Bio-Rad, Richmond, CA).
7. Plug molds (Bio-Rad).
8. Zymolyase 100T (ICN Biomedicals, High Wycombe, UK) Prepare a stock of 50 U/μL in sterile SE.
9. Yeast lysis solution (YLS): 1% (w/v) lithium dodecyl sulphate (Sigma) buffer (1% [w/v] lithium dodecyl sulfate, 100 m*M* EDTA, and 10m*M* Tris-HCl, pH 8.0 filter sterile). YLS should be filter-sterilized (0.22-μm micropore filter) and can be stored at room temperature. **Caution: very toxic**.
10. TE buffer: 10 m*M* Tris-HCl, pH 8.0 and 1 m*M* EDTA, pH 8.0.
11. 10X TBE electrophoresis buffer: dissolve 108 g Trizma base and 58 g boric acid in water, and add 40 mL 500 m*M* EDTA. Make up to 1 L with water.

12. $T_{0.1}$ E: 10 m*M* Tris-HCl, pH 8.0 and 0.1 m*M* EDTA, pH 8.0.
13. Contour-clamped homogeneous electric field apparatus (Bio-Rad).
14. PFGE markers: λ concatemers (48.5–1000 kb) and yeast chromosome ladder (220 kb to 2.5 Mb) (New England Biolabs). Also available are the low-range PFGE markers from New England Biolabs (15–400 kb).
15. 10 mg/mL ethidium bromide stock solution (Sigma).
16. Restriction enzymes and buffers (Promega or New England Biolabs).
17. 10X Mg^{+2}-free PCR buffer (Promega).
18. 24 m*M* $MgCl_2$ (Promega).
19. dNTP mix (100 m*M* stock) (Promega).
20. Thermophylic *Taq* DNA polymerase enzyme (Promega).
21. Sterile distilled water.
22. 0.5 *M* EDTA, pH 8.0.
23. Water bath.
24. Shaking incubator (New Brunswick Scientific, Hatfield, UK).
25. PCR machine (Thermo).
26. Hybridization buffer: 7% sodium dodecyl sulfate (SDS), 0.5 *M* phosphate buffer, pH 7.2, and 1 m*M* EDTA.
27. Hybridization wash buffer: 5% SDS, 40 m*M* phosphate buffer, pH 7.2, and 1 m*M* EDTA.

3. Methods

3.1. Preparation of Yeast DNA in Agarose Plugs

1. Streak out yeast clone on a SD agar plate with appropriate amino acid supplements (*see* **Note 1**) and grow for 3–4 d at 30°C. Inoculate a single colony into 20 mL SD broth with supplements and grow for 24 h in a shaking incubator at 30°C. Each milliliter of saturated culture will produce one to two agarose plugs.
2. Pellet cells by centrifugation at 2000*g* for 4 min. Discard supernatant.
3. Wash cells in a 50-mL Falcon tube in 5 mL 50 m*M* EDTA (spin 4 min at 2000*g* rpm and discard supernatant).
4. Prepare LMP agarose to a concentration of 2% in SE medium. Microwave to melt and place in a water bath at 45°C to equilibrate. When the gel has cooled down to 45°C, add 2-mercaptoethanol (1 µL β-mercaptoethanol per mL SE) and shake gently to mix. Always keep agarose inside the water bath because it solidifies very quickly.
5. Prepare 6 mL of SEM and make it to 100 µg/mL zymolyase 100T (ICN Biomedicals).
6. Prepare the plug molds by briefly rinsing in ethanol and drying by inversion.
7. Resuspend yeast pellet in a Falcon tube in 400 µL SEM/zymolyase and add 400 µL of the molten LMP agarose. Cut blue tip at the rim and mix contents of Falcon tube gently by pipetting. Dispense into the slots of the plug mold avoiding any air-bubbles. Place on ice and allow to set for 20 min.
8. Remove the tape from the bottom of the plug mold and expel plugs into a 50-mL Falcon tube containing 5 mL SEM/zymolyase. Incubate at 37°C for 2 h.

9. Decant SEM/zymolyase and replace with 5 mL YLS. Incubate at 37°C in a shaking incubator for 30 min.
10. Remove solution and replace with another 5 mL YLS. Incubate overnight at 37°C with shaking.
11. Agarose plugs can be stored in YLS at room temperature or rinsed in TE and stored in 500 m*M* EDTA at 4°C for 1 mo.

3.2. Digestion of DNA in Agarose Plugs by Restriction Enzymes

3.2.1. Single Digests

1. Wash agarose plugs in TE three times for 1 h at room temperature and then leave overnight in fresh TE. To ensure thorough washing of the plugs, do not incubate more than five plugs in 50 mL of TE buffer.
2. Incubate each plug in 300–500 µL restriction enzyme buffer for 1 h on ice.
3. Transfer each plug into a fresh Eppendorf tube containing 300 µL of restriction enzyme buffer plus enzyme, and incubate at the appropriate temperature for 6–16 h. For single digests, the recommended units of enzyme are as follows: 2U *BssH*II, 20 U *Cla*I, 10 U *Eag*, 10 U *Mlu*I, 10U *Not*I, 25 U *Pvu*I, 20 U *Sal*I, 25 U *Xho*I.
4. To stop the reaction, add 50 µL of 0.5 *M* EDTA, pH 8.0.

3.2.2. Double Digests

1. Perform a single digest as described in **Subheading 3.3.1.** and cut each plug in half.
2. Store one-half of each plug in TE buffer at 4°C.
3. Use the other half of each plug to digest with the second enzyme. If both enzymes use the same restriction buffer, incubate the plug in 250 µL of buffer containing the appropriate enzyme. If a different buffer is necessary for the second enzyme, equilibrate the plug in restriction buffer without enzyme for 1 h first and then carry out the digest as before (**Subheading 3.3.1.**).

3.2.3. Partial Digests

1. Incubate each plug in 300–500 µL restriction enzyme buffer for 1 h on ice.
2. Transfer each plug into a fresh Eppendorf tube containing 300 µL restriction buffer plus enzyme. It is recommended to use at least three different amounts of enzyme, including an undigested and a complete digest control. For suggested units of enzyme that result in partial digest reactions (*see* **Note 2**).
3. Equilibrate on ice for 1 h and then incubate for 15 min at the appropriate activation temperature (e.g., at 37°C for *Not*I, or 50°C for *BssH*II).
4. Stop the reaction by adding 50 µL of 0.5 *M* EDTA, pH 8.0, and leave on ice.
5. Run PFGE and perform a Southern blot (*see* **Subheading 3.3.**). For information on recommended hybridization probes, *see* **Note 3**.

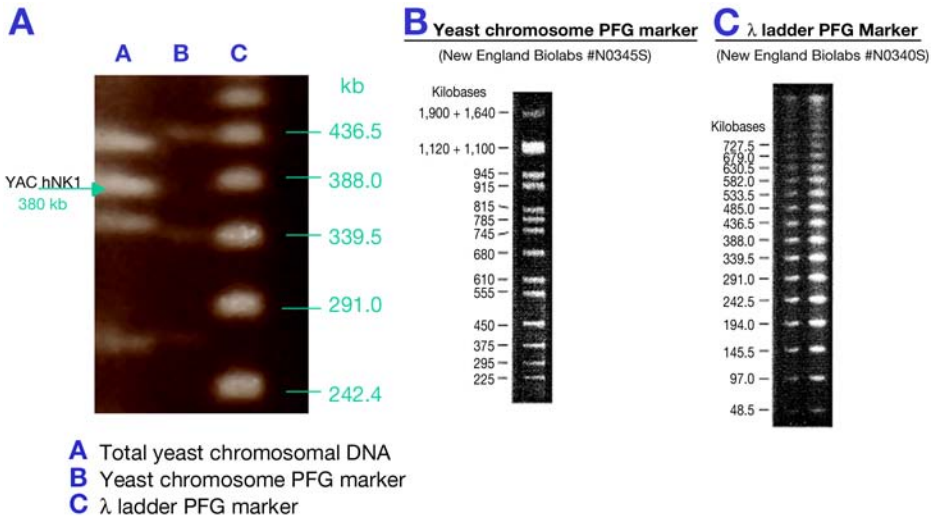

Fig.1. Pulse-field gel (PFG) electrophoresis of a yeast artificial chromosome (YAC) clone containing the human *NK1* (hNK1) gene locus. Endogenous yeast chromosomes (**A**) align with the yeast chromosome marker (**B**). The size of the YAC clone is estimated at 380 kb by reference to the yeast chromosome marker ladder (**B**) and λ ladder marker (**C**). The only yeast chromosome band without a corresponding band in the yeast marker represents the *hNK1*-containing YAC. The λ ladder marker is used to estimate the size of the YAC clone at 380 kb.

3.3. Separation of YAC DNA by PFGE and Southern Blotting Hybridization

3.3.1. Pulse-Field Gel Electrophoresis

1. The plugs should be washed with TE three times for 30 min at 50°C, and subsequently with $T_{0.1}E$ for 30 min (three times) at room temperature.
2. Prepare 1% (w/v) agarose gel in 0.5X TBE.
3. Load plugs in the wells of the gel with reference size markers on each end well and perform PFGE in the following conditions: 40–90 s pulse time at 6 V/cm, 14°C for 24 h (*see* **Fig. 1** and **Note 4**).
4. Stain the gel with ethidium bromide (0.5 μg/mL final concentration in 0.5X TBE) for 15–30 min and photograph gel under UV light using a ruler at the side for reference.

3.3.2. Southern Blotting and Hybridization

1. Digested YAC DNA (typically 10 μg) is run on a 1% agarose gel (**Subheading 3.3.1.**). If membranes suitable for alkaline blotting (e.g., Amersham Hybond N^+ membrane) are used in the hybridization step, the gel should be treated with 0.25 *M* HCl for 20 min.

2. The gel is subsequently denatured in 0.5 *M* NaOH and 1.5 *M* NaCl and neutralized in 0.5 *M* Tris-HCl, pH 7.3, and 3 *M* NaCl.
3. A standard capillary blot is prepared to transfer the DNA from the gel onto the membrane using 0.4 *M* NaOH as buffer (transfer overnight at room temperature).
4. The membrane is neutralized by washing for 2 min in 5X SSC.
5. Prehybridize the membrane in hybridization buffer for 1 h at 65°C in a shaking incubator (or hybridization oven).
6. Denature labeled hybridization probe by boiling for 5 min and dilute to approx 10^6 cpm/mL in hybridization solution. Incubate with the membrane overnight at 65°C with agitation. (*see* **Notes 3** and **5**).
7. Wash membrane three times for 10 min each in hybridization wash buffer at 65°C. Perform one final wash at room temperature.
8. Expose membrane to film autoradiography cassette for 24–48 h.

3.4. PCR Analysis of DNA From Yeast Colonies Carrying a YAC

1. Yeast colony PCR is carried out to determine the presence and integrity of a YAC construct within yeast cells.
2. Primers designed to amplify the left and right "arm" of the YAC are used (for sequences *see* **Note 3**) in a 50-µL PCR reaction containing 5 µL 10X Mg-free buffer (Promega), 3 µL of 25 m*M* $MgCl_2$, 1 µL of 10 m*M* dNTP mix (Promega), 5 µL of 5 µ*M* of each primer, a single yeast colony as template, 0.5 U *Taq* DNA polymerase (Promega), and distilled water up to 50 µL total volume.
3. The reaction consists of 1 cycle at 94°C for 4 min initial denaturation, followed by 35 cycles of 94°C for 1 min, 57°C for 30 s, 72°C for 1 min, and a final extension step of 1 cycle at 72°C for 10 min.
4. The PCR products are separated on a 2% agarose in 0.5X TBE gel. The sizes of the expected products are 330 bp for the left arm primer pair, and 265 bp for the right arm.

4. Notes

1. Laboratory yeast strains carry auxotrophic mutations, i.e., they require amino acid supplements in their growth media in order to grow (*see* Chapter 1). Selective dropout media are prepared by omitting specific amino acids from the supplement mix. Yeast strain AB1380 is deficient for tryptophan and uracil, and by omitting those two amino acids from the growth medium, we are able to select for a YAC clone that contains *TRP1* and *URA3* genes. For most purposes we maintain selection of one auxotrophic marker on the YAC, and given that the *TRP1* gene is poorly functional and results in poor growth rates, it is common to supplement with tryptophan and maintain selection only for uracil. Synthetic dextrose medium contains 6.7 g yeast nitrogen base without amino acids and 20 g D-glucose (for solid medium 20 g of agar should be added). The pH is adjusted to 5.8 and autoclaved prior to addition of the appropriate amino acid supplements. For example, yeast strain AB1380 requires the addition of at least lysine, adenine, histidine, and isoleucine to their growth medium.

2. It is recommended to try 0.2, 1, and 5 U of enzyme in digests in order to establish enzyme activity.
3. Probes specific to the left and right arm of the YAC clone can be produced by PCR using the following primer pairs: YACLAF: 5'- gtgtggtcgccatgatcgcg-3' and YACLAR: 5'-ATGCGGTAGTTTATCACAGTTAA-3' will amplify a 330-bp product of the left arm of the YAC. A 265-bp probe specific for the right arm of the YAC clone is amplified with primers: YACRAF: 5'-GATCATCGTCGCGC TCCAAGCGAAAGC-3' and YACRAR: 5'-CTCGCCACTTCGGGCTCA-3'. The PCR reaction is carried out for 35 cycles at a 57°C annealing temperature in a reaction mixture that contains 1.5 m*M* $MgCl_2$.
   End probes can also be prepared by digesting pBR322 DNA with *Pvu*I and *BamH*I. This digest will give rise to a 2.67-kb fragment that is specific for the "left arm" of the YAC (encompassing the *TRP1* gene and centromere) and a 1.69-kb fragment specific for the *URA3* gene containing the "right arm" of the YAC.
4. To load the agarose plugs onto the gel, make a trough by removing the agarose between consecutive wells and place the plugs inside the trough. Seal the gap with 1% (w/v) agarose in 0.5X TBE (65°C). PFGE run conditions may vary according to the size of the YAC clone. For YACs greater than 900 kb, the following conditions are recommended: 60–120 s pulse time, 6 V/cm, 24 h. To identify any additional YAC constructs in the same clone, it is necessary to hybridize with human cot1 DNA. Cot I DNA is the repetitive sequence fraction of genomic DNA. It is used as a competitor, required to prevent repetitive DNA from binding to the 2target. Hybridization with cot I competitor DNA results in an increase in signal intensity and a decrease in the amount of background noise associated with repetitive DNA.
5. Hybridize the blot using one end-labeled probe at a time and measure the lengths of the detected fragments. The position of the restriction sites on the DNA clone can be determined by adding the extra length of the partial digestion fragments onto the size of the complete digests. A complete map of restriction sites can be constructed by determining the fragment lengths from left and right ends. If the genome sequence of the organism from which the YAC clone has been derived is known, then it will be possible to accurately determine the correct identity of the YAC clone, as well as the extent of the genomic fragment contained within it and its integrity.

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5

Postgenomic Bioinformatic Analysis of Yeast Artificial Chromosome Sequence

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Summary

The free availability of multiple genomic sequences represents one of the greatest advances in biology of the new millennium, and promises to revolutionize our ability to determine and treat the causes of human disease. This chapter highlights a number of basic, freely available, and user-friendly bioinformatic techniques that can be used to predict the functional genetic contents of specific yeast artificial chromosome (YAC) clones. The content of this chapter is written for the level of graduate students, who may be relatively inexperienced with the use of computers for analyzing DNA sequences. The basic instructions that allow the identification of the genomic sequence of interest and to download this sequence onto a personal computer from an online database are presented. Simple instructions are also given on how to perform basic sequence manipulations, how to use online tools to design polymerase chain reaction primers, and how to map restriction sites. Also described are more complicated programs that rapidly and efficiently perform genome alignments that, in addition to predicting the location of protein coding sequences, allow the prediction of functional genomic sequences, such as *cis* regulatory elements and scaffold/matrix attachment sites. The availability of genomic sequences and the rapidly expanding numbers of predictive programs that allow the predictive analysis of these sequences promises to greatly facilitate the use of YAC clones in the search for the causes of disease.

Key Words: Bioinformatics; genome sequence; world wide web; sequence comparison; sequence identification; predictive genetics.

1. Introduction

Yeast artificial chromosome (YAC) clones have been used in the past to characterize a wide variety of biological processes, such as gene expression, protein biochemistry, and disease mechanisms ***(1)***. An individual YAC clone is sufficiently large to hold all the exons, introns, and necessary regulatory ele-

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ments needed to correctly express the relevant gene. With the completion of a number of genome sequencing projects ***(2–4)*** and with the use of freely available bioinformatic tools, it is now possible to determine the identity of gene sequences and perform simple data manipulations or more complex predictions about the functions of specific genomic sequences. This chapter will highlight how freely available bioinformatic programs and DNA databases can be used to identify and analyze a gene or sequence of interest, and will set out many of the steps required to analyze these tools through the internet. This chapter is not aimed at individuals with extensive experience of bioinformatics, but instead at the recent graduate student who may be embarking on a career in science and who requires knowledge for the basics of analyzing DNA sequences *in silico*. This chapter provides a basic explanation of how genomic DNA sequences can be accessed from a database browser through which information from a number of genome sequencing projects can be found. Furthermore, sequence analysis will be described that will allow the prediction of restriction maps, coding sequences, and sequencing/polymerase chain reaction (PCR) primers from downloaded sequences that will greatly facilitate and accelerate basic YAC manipulation. We will further explore the use of programs that predict important functional regions within DNA. These include programs that can highlight evolutionarily conserved sequences between species using pair-wise alignments, in addition to investigating sequences for transcription factor binding sites, and predicting scaffold/matrix attachment sites (S/MARs). A combination of these techniques will simplify the identification and isolation of YAC clones, and will provide a large amount of critical information about the functional contents of these clones.

2. Materials

2.1. System Requirements

The following describes the minimum specification for computers required to efficiently access the web applications to be described in this chapter. Minimum recommended systems are Windows ’98, Internet Explorer 5.0, and 256-Mb RAM.

Access to the internet requires a dial-up line with a modem and an internet service provider (*see* **Note 1**). Some website software requires the use of Java applets, scripts, and pop-up windows in order to function properly. Ensure your security settings are configured to allow Internet Explorer to access the sites (*see* **Note 2**).

Although Internet Explorer 5.0 is the minimal requirement for allowing access to the resources described below, the following methods were all carried out using Windows XP Professional and Internet Explorer 6.0, which provide better access.

3. Methods

3.1. Accessing Genomic Sequence

Accessing genomic sequence via the world wide web has become the most efficient way of isolating, identifying, and analyzing YAC DNA sequences. For example, access to the entire human genome sequence allows the specific and targeted design of human-specific primers and probes that allow isolation of YAC clones either by radioactive screening of YAC libraries (*see* Chapter 3) or by hybridization of YAC pools by PCR (*see* Chapter 4). In addition, once a clone is isolated, having access to the full genomic sequence of that clone allows us an unparalleled ability to predict the location and identity of functional elements, such as gene-coding sequences, restriction sites, and *cis*-regulatory elements.

The Ensembl genome browser is a hugely powerful database that allows full access to the genome sequences of many different organisms (http://www.ensembl.org) ***(4)***. The browser is relatively user-friendly and can be used to rapidly find genomic information from a large number of eukaryotic species. Ensembl presents its genomic information in a variety of different ways, ranging from a chromosomal perspective all the way down to the nucleotide sequence. The following offers a simple guide that allows the identification and procurement of a genomic sequence of almost any size.

1. After clicking on the Internet Explorer icon, input http://www.ensembl.org into the address bar. Choose the eukaryotic genome of interest by clicking on the respective link (**Fig. 1**).
2. At the top right of the page, select **Gene** from the drop-down menu found beside **Search e!**. Type the name of the gene (*see* **Note 3**) in the following box and left click (all instructions to "click" involve the use of the left-hand button on the mouse unless specified otherwise) on **Go**. Alternatively, under the heading Karyotype, a specific chromosome or a specific position within a chromosome can be viewed without a gene name.
3. Scroll down the page and select the highlighted text link (URL or hyperlink) that matches your query. The webpage accessed will provide a great deal of information about your selected query, including the location of the gene, the transcript structure, and orthologous gene sequence (similar gene sequence from other species) prediction. It is from this page that the various viewpoints can be accessed (*see* **Note 4**).
4. Gene sequence information is obtained by clicking **Genomic sequence** from the menu on the left of the screen.
5. Once presented with the screen depicted in **Fig. 2**, scroll down the screen. The coding sequence for the chosen gene can be seen highlighted by a colored box. The number of highlighted boxes is representative of the number of exons of the gene.

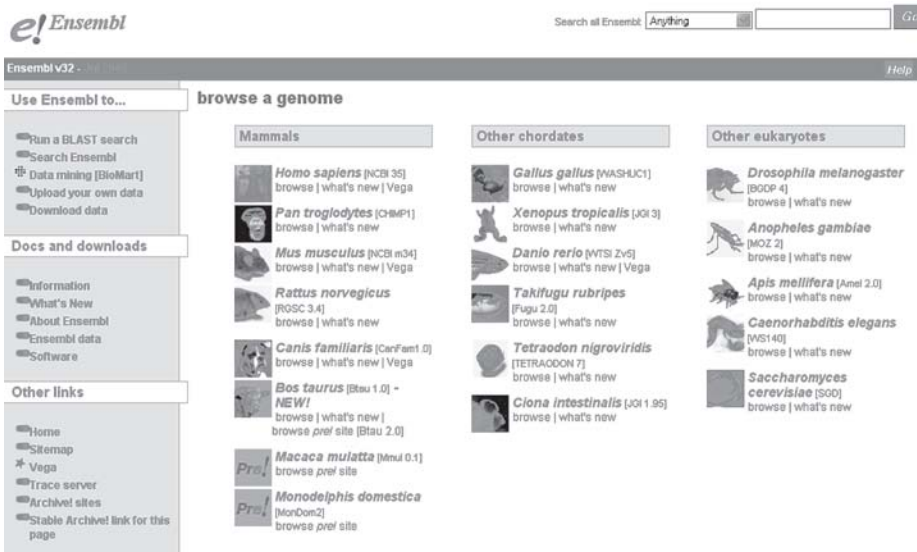

Fig. 1. Ensembl home page showing the various accessible genomes from the browser, the search box at the top right of the window, and the various links and documentation in the left frame (Accessed 3/28/06).

6. To include a larger portion of the surrounding sequence, insert the required numbers for the flanking sequence in the box labeled **Markup options**. There is also the option to vary the output and display of the sequence within this box.
7. To export sequence in Fasta format, select **Export sequence as FASTA** from the menu on the left of the screen (*see* **Note 5**).
8. Select **Text** output and press **Continue**. A new browser window will pop up with the genomic sequence in text format. The sequence obtained can be saved to the hard drive of a computer for further analysis by selecting **Save As** from the **File** drop-down menu on the browser toolbar. After naming the file to be saved, ensure the **Save as type** drop-down menu is showing **Text File**.

There is great demand for newly available genomic information. In some cases, genomes are added to databases before their full completion. This was true of the cow genome with Ensembl at the time of writing. Owing to the speed at which new biological data is being deposited in databases, it is advisable to check them regularly for updates.

3.2. Basic Sequence Data Manipulation

Having access to information about genomic sequences has allowed for the use of simple and freely available predictive tools that facilitate the analysis and manipulation of YAC clone DNA sequences in the lab. For example, pro-

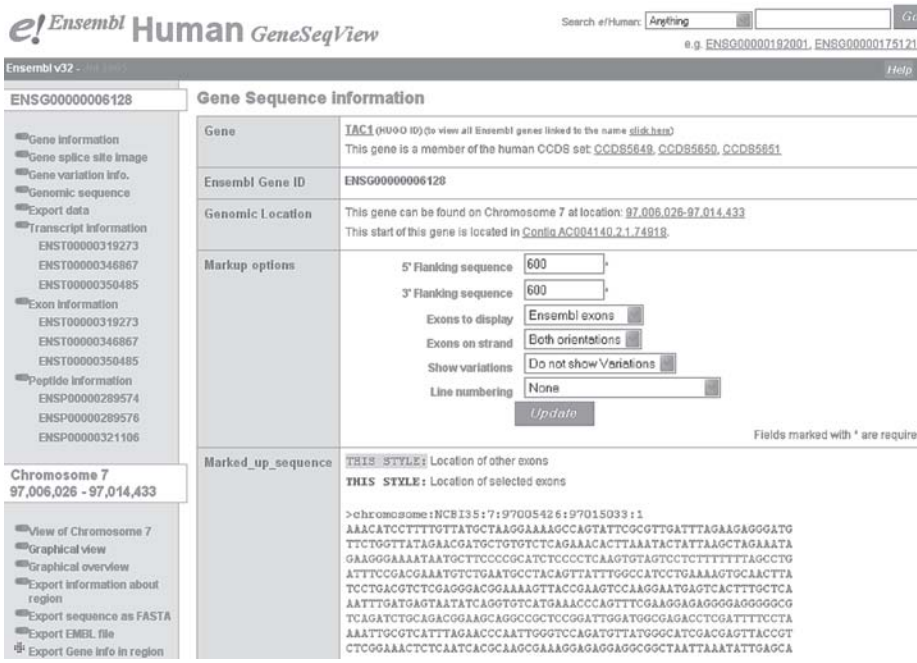

Fig. 2. Example of the genomic sequence page from the Ensembl browser. This page gives the selected genomic sequence with the option of showing more sequence 5' and 3' of that already shown by altering the Markup options.

grams have been available for some time that allow the translation of nucleotide codons to amino acid sequences, simple sequence manipulation, and sequence alignments. The University of Alberta's Sequence Manipulation Suite (http://www.ualberta.ca/~stothard/javascript/index.html) ***(5)*** offers quick and basic tools that can be used to predict a number of different features from DNA sequences downloaded from Ensemble. Each tool offered on the Sequence Manipulation Suite demonstrates an example of an input sequence that can be used to learn how the programs work.

3.2.1. Filtering DNA Data

After retrieving a genetic sequence from a database the sequence may be in a format not usable by other programs and websites. The **Filter DNA** program can be used to remove any blank spaces or numbers from a file containing a genomic or complimentary DNA sequence.

1. From the Sequence Manipulation Suite homepage (http://www.ualberta.ca/~stothard/javascript/index.html) select **Filter DNA** under **Format Conversion**

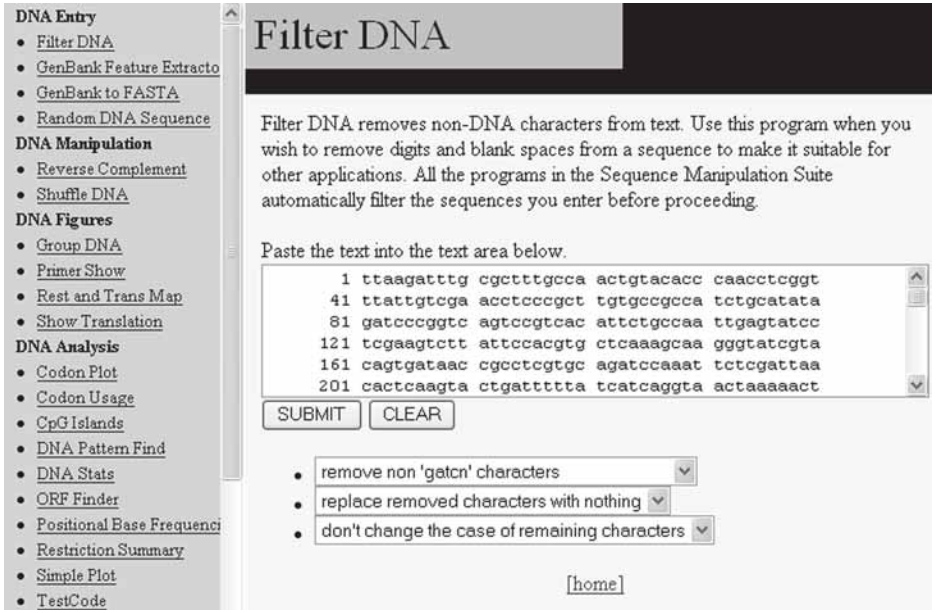

Fig. 3. Example of the filter DNA tool from the Sequence Manipulation Suite website. The example sequence shown at load time, along with three drop-down menus to alter the program's output.

from the options on the left frame; the Filter DNA tool will appear in the right-hand frame (**Fig. 3**).

2. Click on the **CLEAR** button to remove the example data. Paste the sequence of interest into the text box by selecting **Paste** from the **Edit** drop-down menu on the browser toolbar.
3. There are three drop-down menus giving options on how to filter the data, such as removing only digits, removing only white space (blank spaces), what to replace them with, and which case to provide the results in. When the options have been selected click on **SUBMIT**.
4. The filtered DNA sequence will be presented in a new browser window that will pop up automatically.

3.2.2. Reversing a Sequence or Gaining the Complimentary Sequence

Retrieving sequences from a database will usually be in single-stranded notation. However, users may require information contained on the DNA strand that complements the one retrieved. The **Reverse Complement** tool on the

Sequence Manipulation Suite (*see* **Subheading 3.2.**) can be used to gain the complimentary sequence to that entered, switch the sequence from a 5'–3' orientation to the reverse orientation, or carry out both processes on a sequence.

1. From the left-hand frame, select **Reverse Complement** from the list. The Reverse Complement tool will appear in the right-hand frame.
2. Click on the **CLEAR** button to remove the example data. Paste the sequence of interest into the text box.
3. One drop-down menu is presented in order to choose the output required. Select to see the **reverse-complement**, **reverse**, or **complement** of the sequence entered. Click on **SUBMIT**.
4. A new browser window will pop up with the requested sequence.

3.2.3. Translating the Codons of a Coding Sequence From Reading Frames

Coding sequences retrieved from a database may require translation to show the amino acids coded for. Having six reading frames for a double-stranded region of DNA (three forward and three reverse) it makes sense to use the power of computers to quickly and accurately translate genetic sequences. The **Translation Map** tool can be used to view the amino acids coded for by each codon in a reading frame or reading frames of the sequence entered.

1. From the left-hand frame select **Translation Map** from the list. The **Translation Map** tool will appear in the right-hand frame.
2. Click on the **CLEAR** button to remove the example sequence from the text box. Paste the sequence of interest into the text box.
3. Select how many **bases per line** to show on the results page from the drop-down menu. From the **Show the translation for reading frame** drop-down menu, select the reading frame or frames you wish the sequence to be translated into. Reading frames one to three gives the three forward.
4. Select the result sequence to show the sequence entered in **single-stranded** notation or with its complimentary strand in **double-stranded** notation. Click on **SUBMIT**.
5. A new browser window will appear with the reading frames and translations requested. Stop codons are represented by an *.

These are only three programs that we have found to be of most use in our analysis of YAC genomic DNA sequences. However, the Sequence Manipulation Suite has many other programs that can perform different tasks to those described here; however, we do not have the space to cover these in this short chapter. The ease of use and intuitive set up allows for quick understanding of the programs and the uses to which they can be employed.

3.3. Restriction Site Analysis

Manipulation of DNA within the lab undoubtedly requires the use of restriction enzymes at some stage. The advent of sequence availability on the internet allows the use of bioinformatics to efficiently locate restriction sites within a sequence. This permits the more rapid design and production of tools, such as vectors required for the manipulation of YAC DNA (*see* Chapters 9–11). One easily accessible website to use for restriction analysis is Watcut (http://watcut.uwaterloo.ca/watcut/watcut/template.php?act=restriction_new) ***(6)***. Watcut does not only identify restriction sites within a sequence, but can also filter those enzymes searched for by restriction site length, resulting ends, and by enzyme supplier. The filtering options can also be used to exclude nonpalindromic and degenerate recognition sites from the output.

1. On opening the Watcut website the user will be presented with a hyperlink entitled **Restriction Analysis** input screen. Sequence can be input immediately at this stage or, if preferred, a number of hyperlinks at the top of the page will allow tailoring of analysis parameters, e.g., selection of restriction enzyme properties.
2. Because of the huge numbers of different restriction enzymes commercially available, it is often most efficient to only analyze restriction enzyme sites with particular properties. For example, for cloning applications it is often desirable to only select six base pair palindromic restriction sites that have a four base pair 3' or 5' overhang to facilitate ligation. To select particular enzyme properties, click on the **Select enzymes** hyperlink above the input box. This facility allows you to define the minimum length of the restriction site and the resulting ends generated from the digest (blunt end, 5' overhang, or 3' overhang). In addition, the facility also allows the exclusion/inclusion of nonpalindromic and degenerate recognition sites. Preferred supplier can also be selected. After choosing filters required click the **Apply filter; OK** button. The **Restriction Analysis** input screen will reappear.
3. Having defined the restriction site parameters, paste the sequence to be analyzed where indicated in the input box, or click on the **Browse...** button to upload a sequence from your personal computer to the Watcut server.
4. Name the sequence if required. Click on **Submit new sequence**. On the output page of Watcut select **Display results; with sequence** and click the **Update display** button (**Fig. 4**).
5. The restriction sites will be displayed alongside the sequence entered, and each restriction enzyme named along the sequence will also be a link to the Rebase database (http://rebase.neb.com/rebase/rebase.html) ***(6)*** to give information on the individual restriction enzymes (*see* **Notes 6** and **7**).

3.4. DNA Primer Design

PCR is a common method of specifically amplifying a short segment of DNA from a genomic or complimentary DNA sequence. Prior to the availabil-

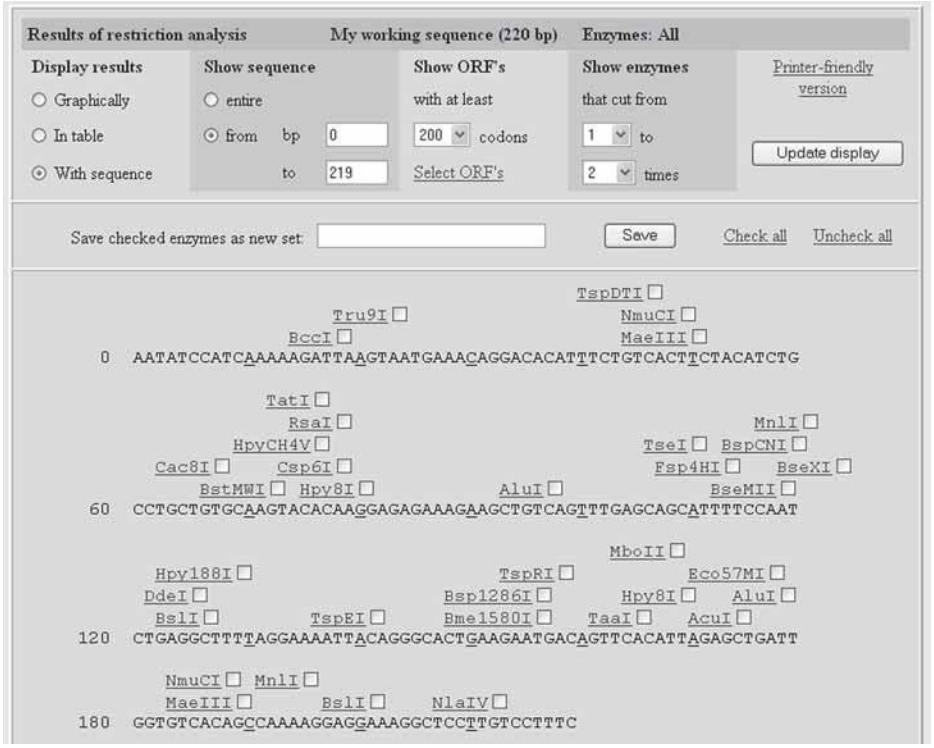

Fig. 4. Output display of Watcut. The display shows the options available at the top of the output box along with an example sequence showing restriction sites.

ity of genome sequencing, the design and use of sequencing and PCR primers was extremely limited. However, in the postgenomic era a number of genomes have been nearly fully sequenced. The availability of this sequence ensures that, theoretically, PCR primers can be designed to amplify sequences from any point within a sequenced genome. Only the vagaries of repetitive DNA sequences and the formation of DNA secondary structures can obstruct the limitless abilities of PCR. However, thanks to bioinformatic tools, even these obstructions can be detected and avoided. Several different examples of web-based software is available to design suitable primers for a given sequence. Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) ***(7)*** is one of the easiest to understand and is intuitive to use.

1. Open the primer 3 website and paste the sequence containing the target sequence where indicated.
2. Surround the target region within the input box with square brackets (e.g., [ATCG]) to indicate that primers must flank this region. Mark any regions you

do not wish primers to be chosen from, i.e., coding sequences, by flanking the sequence with chevrons (e. g., <ATCG>), as they may contain essential restriction sites or functional sequences.

3. Scroll down the page and input any desired primer properties, such as minimum and maximum lengths, primer melting temperature, and primer GC content.
4. Click on **Pick Primers**.
5. The output is presented as a table showing length of the primers, the melting temperature of the primers, and the GC% of the primers. The sequences for the primers are shown on the right of the table (*see* **Note 8**). The chosen primers at the top of the page can be seen adjacent to the sequence below, indicating the position compared with the selected sequence. The forward primer remains the same as the sequence, whereas the reverse primer contains the reverse complement of the sequence on the other side of the target sequence to be amplified.

3.5. Sequence Comparison and Identification of Biologically Important Conserved Sequences

The genome of an organism is subjected to random mutation and rearrangement events, many of which are passed on to following generations. Nonessential regions of the genome will accumulate these mutations over millions of years without reducing the fitness of the organism. However, mutations or rearrangements within regions of functional importance will reduce species fitness and the occurrence of these mutations will, in the vast majority of cases, be selected against. Only if one of these mutations contributes to species fitness will they be retained. Regions of the genome that encode proteins constitute some of the most highly conserved portions of the genome. However, recent comparison of the genomes of numerous species has also shown that large tracts of the genome that do not represent gene-coding sequences, have also been highly conserved and in many cases to a higher degree than coding sequences. Many of these noncoding sequences have been shown to represent *cis*-regulatory elements, such as enhancers and silencers, that are essential to controlling the tissue-specific and temporal expression of coding sequences ***(8–10)***. A number of different programs have been devised that allow long-range comparison of gene sequence for the identification of functional elements. However, we have found that the most powerful and user-friendly of these is the ECR browser jointly developed by one of the contributors of this book (Gabriella Loots). The ECR browser (http://ecrbrowser.dcode.org) ***(11)*** simultaneously compares a number of recently completed genome sequences and automatically screens out regions of human repetitive sequences, in addition to highlighting areas of evolutionary conservation. Evolutionary Conserved Regions (ECRs) are displayed as peaks (or bars) on an *xy*-axis plot (*see* **Fig. 5**). Furthermore, the ECR browser allows for the subsequent prediction of transcription factor binding

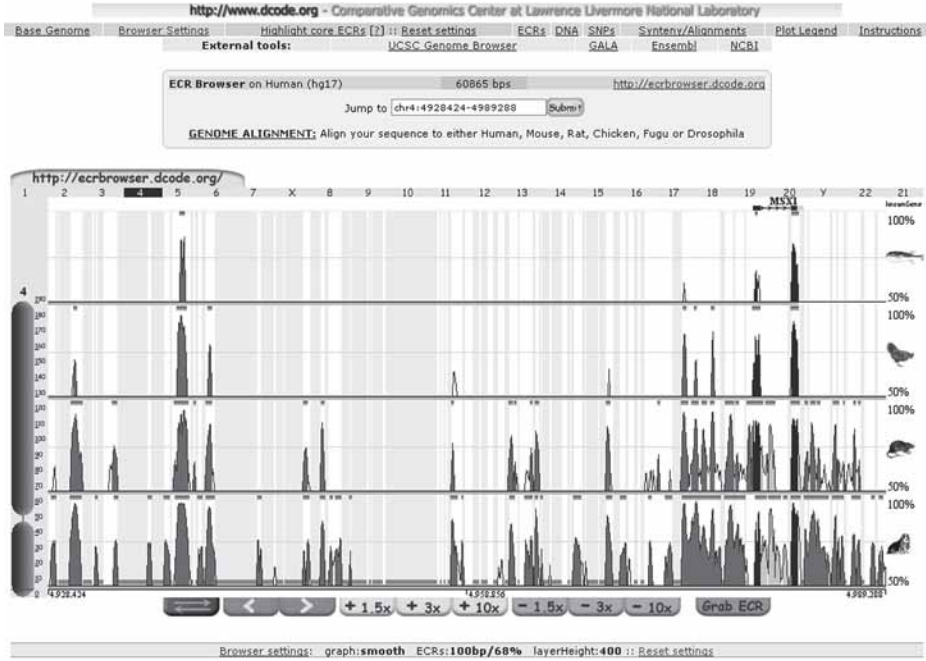

Fig. 5. ECR browser plot of the surrounding region and coding sequence of the *Msx1* locus. Features to note are the title bar at the top allowing the choice of a new base genome and browser settings where optional parameters may be altered to manipulate the plot. The box below the title bar gives the length of sequence displayed, and an input text for jumping to other chromosomal locations or resizing the plot. The icons at the right denote the species used to create the pair-wise alignment with the base genome, in this case human. The peaks on the plot represent regions with a high percentage of conservation. At the top right of the plot the *MSX1* gene is highlighted, identifying the highly conserved exons of the gene (colored blue on the webpage). Peaks surrounding the gene (colored red on the webpage) highlight noncoding conserved sequences between species. The buttons below the plot can be used to manipulate the sequence length viewed.

sites (and their levels of clustering and conservation) by automatically linking to another program called rVISTA 2.0.

1. Open the ECR browser home page by typing the web address above, scroll down the page, and enter the base organism to which other genomes will be compared. Where indicated, enter the name of the gene or the chromosomal location in the format shown (*see* **Note 9**). Click on **Submit**. Note that many genes will be represented by a number of gene names that can all be listed by looking on the National Center for Biotechnology Information website (*see* **Note 3**).

2. A choice of genes or gene isoforms may be presented from the gene name entered. Click on the chromosomal location of the desired gene. The webpage accessed shows a graphical representation of pair-wise alignments to the base genome chosen (**Fig. 5**). Select **Plot Legend** from the right of the title bar found at the top of the browser window for a detailed description of the species, peak color code, and surrounding icons of the plot view (*see* **Note 10**).
3. Select **Browser Settings** on the yellow title bar. Here you can select how to view your plot, for example, which species to display in pair-wise alignment with your selected base genome, graph type (smooth or pip plot), number of layers over which to display the pair-wise alignments, as well as the layer height. Most importantly, the minimum length of ECR and their minimum percentage identity between species can be adjusted. Once selected, click **submit & return**.
4. Below the layered pair-wise alignments there are control buttons to zoom in and out of the genomic location, i.e., +10×. More of the sequence surrounding the locus can be viewed by zooming out from the selected location, therefore identifying any highly conserved regions across the group of species.
5. Having found a peak of interest, the conserved sequence of the selected species can be isolated. This can be done by clicking the **Grab ECR** button found below the plot; the **Grab ECR** button will change color and allow the peak to be selected. Click on the rectangular box (red on the webpage) above the desired peak (*see* **Note 11**).
6. A new web browser window (called rVISTA; http://rvista.dcode.org/) will pop up with detailed information on the selected peak. This includes the length of the alignment, percentage identity, numbered alignment indicating matching bases, and the individual sequences from the two species selected. Analysis of transcription factor binding sites using the link for **rVista 2.0** can be carried out if required. This will be discussed in more detail later in this chapter (*see* **Subheading 3.6.**).

3.6. Transcription Factor Binding Site Analysis

Environmental conditions and cellular interactions are relayed to the genome within the nucleus of a cell by gene regulatory systems that include a combination of cell surface ligand–receptor interactions, signal transduction pathways, and protein–DNA association. Gene transcription is influenced by these gene regulatory systems by promoting the association or disassociation of transcription factors to specific sequences of DNA. These elements in turn help to stabilize the gene transcription machinery to enhance or inhibit gene transcription. The rVISTA search engine assumes that because of their importance in coordinating development and health, many transcription factor binding sites within highly conserved enhancer sequences will themselves be highly conserved. rVista ***(12,13)*** uses the Transfac database to highlight transcription factor binding sites conserved throughout evolution in order to highlight possible DNA targets for these proteins. Sequences submitted to rVista are compared against

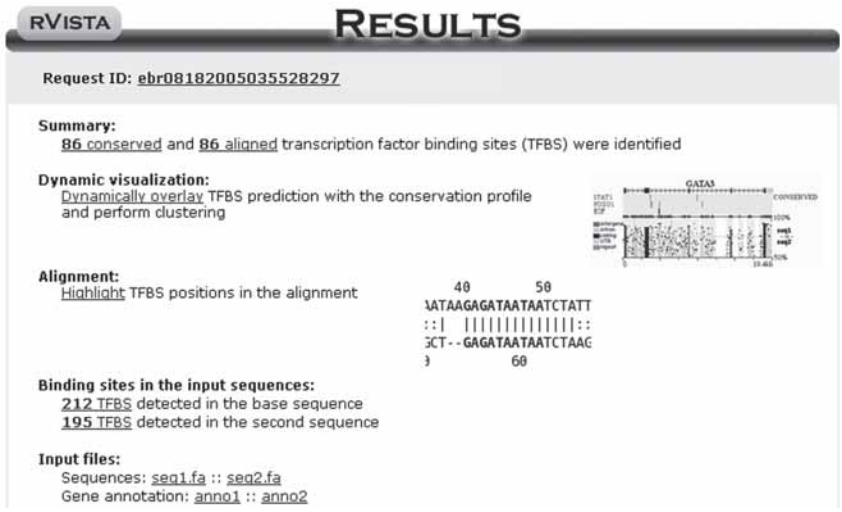

Fig. 6. Results page presenting options for a summary of transcription factor binding sites, dynamic visualization, alignment, lists of binding sites, and input files of the chosen sequences.

matrices of transcription factor binding sites found within the Transfac database. These matrices have all been experimentally elucidated before being entered into the database.

rVista only analyzes sequences which have been prealigned using one of a family of pair-wise alignment tools. The simplest of which is the ECR browser previously described (**Subheading 3.5.**). Using the grab ECR feature of the ECR browser, a short alignment of a conserved region can be dynamically submitted to rVista (*see* **Note 12**).

1. Submit an alignment to rVista from the ECR browser by clicking on the **rVISTA 2.0** link. A page named "Defining transcription factor binding sites" is displayed. Select the relevant library, i.e., vertebrate, plant, fungi.
2. Select the desired matrix similarity from the drop-down box labeled **Predefined as**. Reducing the matrix similarity will provide more transcription factor hits, but may be less accurate than if the chosen similarity was higher. Click the **SUBMIT** button.
3. From the selection of transcription factors presented, select those of interest or scroll down and press the **SELECT ALL** button for a full analysis. Click the **SUBMIT** button to continue.
4. On the next screen click the **CHECK IT** button to receive the analysis.
5. The results page offers a number of options (**Fig. 6**). The **Summary** gives a text list of the transcription factors with binding sites conserved and aligned in both

sequences, with their relative position on the input sequence. The **Dynamic visualization** link presents a graphical view of the conserved alignment with transcription factors overlaid in relative positions. The **Alignment** link allows the individual transcription factors to be highlighted, in turn, within the sequence. The **Binding sites in the input sequences** links give transcription factors found in each sequence entered, however, not all will be featured in both sequences. The **Input files** are a means of retrieving the sequences submitted to rVista.

3.7. Using the Transfac Database to Verify rVista Results

Any highlighted transcription factors of interest should be manually checked against the Transfac database-binding matrix field ***(12)***. A number of transcription factor matrices within the Transfac database are based on low numbers of binding studies and may represent false-positives. The quality of the binding matrix can be seen in the statistical basis table of a matrix field. Use of the Transfac database is free for nonprofit organizations, however, registration to the website is required by new users in order to login and access the database.

1. From the Transfac search page found at the URL (http://www.gene-regulation.com/cgi-bin/pub/databases/transfac/search.cgi) select the **MATRIX** button.
2. Enter the name of a transcription factor into the **Search term** box, the drop-down menu labeled **Table field to search in** should show **All Fields**. Click on **Submit**.
3. If a selection of transcription factors is presented, choose the factor best relating to the species and rVista output received.
4. Scrolling down the page the binding matrix will be in the **PO** row with the statistical information below it in the **BA** row (**Fig. 7**). Good quality binding matrices will have been compiled from large numbers of binding studies.

3.8. Scaffold/Matrix Attachment Region Prediction

S/MARs are regions of DNA that serve as attachment sites for chromatin to the nuclear matrix. Several studies have indicated that these S/MARs may define the regulatory domain of genes, thus acting as insulator sequences ***(14)***. This function can aid in the genes' participation in cellular processes. MAR-Wiz 1.5 (http://www.futuresoft.org/MarFinder/) ***(15)*** is an online tool that predicts S/MAR sites. Although it is free to use, users are required to register in order to login and use the MAR-Wiz website.

1. From the Contents frame on the left of the MAR-Wiz website, click ***MAR Search***; you will be prompted for a login name and password. On first use this registration is free and quick to complete, however, it does require the user have a valid e-mail address.
2. After logging in, the user is presented with the choice of running MAR-Wiz 1.0 or MAR-Wiz 1.5. The selection screen also describes the differences between the two programs. This description will focus on MAR-Wiz 1.5.

PO	A	C	G	T	
01	16.1	20.3	30.7	32.8	N
02	2.4	18	59.1	20.3	G
03	9.8	17.5	39.4	33.3	N
04	5	45.2	27.9	21.9	N
05	77.9	0.8	21.3	0	A
06	12.6	6.8	0	80.6	T
07	0	0	0	100	T
08	0	0	0	100	T
09	0	100	0	0	C
10	0	100	0	0	C
11	0	50	50	0	S
12	0	0	100	0	G
13	0	0	98.2	1.8	G
14	100	0	0	0	A
15	100	0	0	0	A
16	63.3	0	32.7	4	R
17	1.8	13.8	1.8	82.6	T
18	7.7	19.3	70	3	G
19	34.7	18.7	28	18.7	N
20	27.3	18.2	22.4	32.1	N
21	19.6	24.3	16	40.1	N
XX					
BA	55 selected binding sequences				

Fig. 7. A binding matrix for human STAT3 transcription factor. The PO rows show the positions of binding sequences. The columns represent the percentage of times a base was found to bind in the sequence position. The right-hand column gives the consensus binding sequence from the statistical matrix. The BA row at the bottom gives the information on which the statistical matrix is based, in this case 55 binding sequences. Please note that many of the binding matrices are not presented as percentages but as a number of bound sequences containing that nucleotide at that position.

3. Selecting **MAR-Wiz 1.5** presents the user with a choice of pasting a genomic sequence into a text box or uploading the sequence from a text file directly by browsing the user's local personal computer.
4. The MAR-Wiz help link in the contents frame to the left of the screen describes the uses of the optional parameters that the user can alter.
5. Click the **Find MAR** button to run the program.
6. MAR-Wiz 1.5 produces a graphical display of MAR potential along the sequence (**Fig. 8**). Above the graphical display there are buttons for zooming in and out if the sequence entered is longer than the default setting for the display. There is also a link to a **Summary Report** that shows high score regions with the best prediction of being S/MAR sites (*see* **Note 13**).

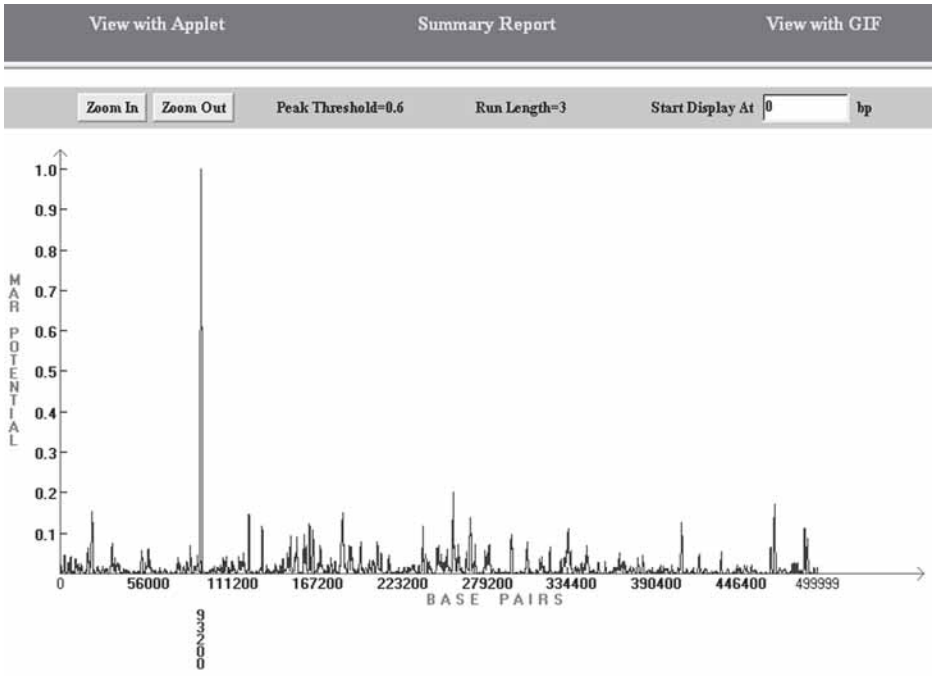

Fig. 8. Example of Applet view output display from MAR-Wiz 1.5, zoomed out to show the whole extent of the sequence entered. The example shows a sequence of 500 kb surrounding the *Tac1* gene. From the display a S/MAR site is predicted 93,200 bases into the sequence. The zoom in and out buttons can be seen at the top left with the summary report link found above.

3.9. Conclusion

We have demonstrated the use of a small sample of programs that are freely available and can be used to find, manipulate, and investigate genomic sequences from the huge body of genomic data available. Although all of the programs are freely available to the public over the internet, a fraction of the sites require users to register some details before being allowed to access the remote servers. Access to the sites referred to above reduces the need for researchers to purchase any of the many commercially available bioinformatic software packages whose services, although excellent, come at inflated prices.

In this postgenomic era, bioinformatic techniques are becoming more and more powerful. From simple programs based on known biological rules, larger, more complicated techniques have been developed using statistical analysis. These systems will be mathematically logical but biology is not always mathematically precise. Therefore, bioinformatic predictions should not be looked

at as 100% accurate, but rather used as useful indicators. Therefore, all bioinformatic predictions should be backed up by rigorous experimental analysis in the lab before being accepted as fact. *In silico* bioinformatic prediction programs have proven to be invaluable facilitators in the search for functional sequences within a genomic sequence and, in combination with increased computer speeds, the increasing availability of ever larger bodies of genome sequences and the ability to biologically analyze that sequence using YAC clones, offer unparalleled possibilities for understanding the molecular basis of pathogenicity and disease.

4. Notes

1. Broadband internet connections will give faster responses to websites, but information being sent to a remote server will be processed at the servers speed rather than your own systems, therefore, patience may be required with some sites that use their own servers.
2. Internet security settings can be altered by clicking on the **Start** button at the bottom left of the windows desktop. Select the **Control Panel** icon from **Settings**. Click on **Network and Internet Connections**, the options available will change. Click on the **Internet Options** icon. A new window will appear, click the **Privacy** tab at the top of the window. The display shows a sliding bar icon that can be used to lower internet settings.
3. There may be more than one name for the gene you are looking for. Therefore, find out any other pseudonyms for the gene in question. The National Center for Biotechnolgy Information at URL http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene will give you all known aliases for the gene if you type in the gene name you know.
4. The term contig derives from the "contiguous sequence" produced when overlaps from sequenced bacterial and yeast artificial chromosome clones that have been used to map and sequence the different genomes available are aligned to produce a larger contiguous sequence. Emsembl also offers a multi-contig view, meaning that a graphical representation of a genomic location is laid out with the corresponding sequence from other species above and below it. Regions of similarity between the different species are highlighted. This offers a basic comparison of evolutionary conservation between species that represent possible regions for further analysis.
5. It is good practice to download genomic code in Fasta format to text files, as this format is widely accepted into most bioinformatic tools for further analysis.
6. The restriction sites within the entered sequence can be displayed in tabular form by updating the page with **Display results**; **In table** selected.
7. There is a link at the top right of the Watcut output display to allow a printer friendly version for a hard copy of the restriction map.
8. The two primers presented on the screen have obtained the highest score from the system used on the website. This suggests they are the best ones to use for ampli-

fication of the chosen sequence. However, other primer sets are presented at the bottom of the page and may be considered as alternate sets.

9. Above the ECR browser text box for entering gene names and chromosomal positions is an example of how to enter the chromosome of choice, along with how to enter the positions on that chromosome to display.
10. The **Instructions** option on the title bar of the ECR browser explains in detail the functions of all the features available; is intuitive to follow and explains the potential of the ECR browser.
11. If a region of interest is found but the entire region cannot be isolated by using the **Grab ECR** feature (one of the ECR browsers minor peccadillos) then it is possible to note the chromosomal position in the information box of the ECR browser. These coordinates can later be entered into the Ensembl browser to gain that sequence.
12. Full instructions for the use of rVista can be found at http://rvista.dcode.org/instr_rVISTA.html including the different methods by which alignments can be submitted for analysis.
13. The S/MAR prediction technologies available, whether these are free online or commercially available, have been described with various accuracies, the higher accuracies being in the 60 to 70% range. It may be useful in determining the functionality of a predicted S/MAR to refer to the ECR browser and determine whether that S/MAR site has been conserved.

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6

Transformation of Yeast Using Bioactive Beads With Surface-Immobilized Yeast Artificial Chromosomes

Shigeki Kawakami, Satoshi Harashima, Akio Kobayashi, and Kiichi Fukui

Summary

Yeast artificial chromosomes (YACs) are useful cloning vectors with the capacity to carry large DNA inserts. The largest barrier using such large DNA molecules in transformation experiments has been their physical instability in a solution. We developed a new method for transforming yeast with chromosome-sized DNA. The method uses bioactive beads composed of calcium alginate to immobilize yeast chromosomal DNAs. Chromosomal DNA immobilized on bioactive beads is physically stable when compared with naked chromosomal DNAs. The bead-mediated transformation performed well, not only with respect to the transformation frequency, but also in successful transformation using split chromosomal DNA that exceeded 450 kb in size. In this chapter we introduce a new method for transforming yeast using bioactive beads. In conjunction with genomic YAC libraries and the yeast chromosome-splitting method, this technique will pave the way to stable and effective transfer of YACs into yeast cells.

Key Words: Yeast artificial chromosome (YAC); transformation; chromosome-sized DNA; bioactive beads; calcium alginate.

1. Introduction

Transformation methods are essential not only to genetic engineering, but also to various fields in molecular biology. Various methods for transforming eukaryotic cells have been developed. Transformation methods using budding yeast, *Saccharomyces cerevisiae*, have been improved since the first successful reports ***(1,2)***. Electroporation ***(3)*** and exposure to lithium acetate ***(3,4)*** have been used to introduce plasmid-sized DNAs. Further development of the spheroplast method has allowed for the introduction of the large DNA constructs ***(5)***.

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Although these improvements allow for efficient transformation using plasmid-sized DNAs, it is still difficult to introduce chromosome-sized DNAs. Chromosome-sized DNAs, such as yeast artificial chromosomes (YACs), can carry large amounts of genetic information; it would be a tremendous advantage if YACs could be efficiently manipulated and introduced into yeast cells. A YAC cloning system has been used to construct genomic libraries by the method that has been previously described ***(5)***. In addition to its usefulness in library construction, the chromosome-splitting technique of fragmenting yeast chromosomes enables us to generate chromosomes of any size and retrofit YACs ***(6,7)***. However, the physical instability of such large DNA molecules in a solution poses a technical problem in transformation experiments. Although promoting the compaction of YAC DNAs with polyamines confers physical stability and reduces the size bias in the transformation frequency to some extent ***(8,9)***, large DNA molecules are inevitably degraded, even when handled with extreme care.

We developed an efficient gene delivery system in yeast ***(10)***, plant ***(11)***, and animal cells ***(12)*** using calcium alginate bioactive beads ***(13)***. Spheroplast cells of yeast were efficiently transformed with alginate bioactive beads containing plasmid DNAs in combination with polyethylene glycol (PEG) treatment. Here we introduce the application of alginate bioactive beads to the transformation of *S. cerevisiae* with large DNA molecules, such as chromosomal DNAs. To take advantage of this transformation system, we evaluated the physical stabilization of yeast chromosomal DNAs immobilized on bioactive beads.

2. Materials

2.1. Buffers

1. Equilibration buffer: 100 m*M* NaCl, 300 μ*M* spermine, and 750 μ*M* spermidine in 0.5X TBE buffer.
2. Polyamine transformation solution: 300 μ*M* spermine, 750 μ*M* spermidine, 50 μg salmon sperm DNA (10 mg/mL; Wako Pure Chemicals Co., Ltd.).
3. TBE buffer: 45 m*M* Tris-borate, pH 8.0 and 1 m*M* ethylenediaminetetraacetic acid (EDTA).
4. TAE buffer: 40 m*M* Tris-acetate, pH 8.0, and 1 m*M* EDTA.
5. STC buffer: 1.2 *M* sorbitol, 10 m*M* Tris-HCl, pH 7.5, and 10 m*M* $CaCl_2$.
6. 100X polyamine solution: 30 m*M* spermine and 75 m*M* spermidine.
7. Yeast lytic enzyme (70,000 U/g; ICN Biochemicals, Irvine, CA).
8. Salmon sperm DNA solution (10 mg/mL).
9. 1 *M* $CaCl_2$.
10. 100 μ*M* $CaCl_2$.
11. 0.5% Sodium alginate.
12. 20% PEG4000 solution (Wako Pure Chemicals Co., Ltd.).
13. Isoamyl alcohol.

14. 1% Low melting point agarose (Sea Plaque® GTG® Cambrex, Rockland, ME).
15. β-agarase I (Wako Pure Chemicals Co., Ltd.).

3. Methods

3.1. Preparation of Chromosomal DNAs

1. The density of yeast cells in agarose plugs was adjusted to approx 5×10^7 cells/plug.
2. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to separate yeast chromosomes. The agarose plugs were loaded onto 1% low melting point agarose gels in 0.5X TBE buffer, and CHEF gel electrophoresis was performed for 15.5 h at 6 V/cm, 14°C with a 60-s switching interval, followed by a 5.5 h run with a 90-s switching interval. Agarose gel plugs were prepared according to the protocol described by McCormick et al. ***(14)***.
3. The CHEF gel was stained with ethidium bromide, exposed to ultraviolet light, and the target bands were excised quickly.
4. Agarose gel plugs or gel slices excised from the CHEF gel were soaked with equilibration buffer, heated to 70°C, and digested with β-agarase I at 30°C.

3.2. Preparation of Chromosomal DNA Immobilized Using Bioactive Beads

1. A solution of chromosomal DNAs, either from melted plugs or gel purified, was gently transferred to a new 1.5-mL tube using truncated microtips.
2. DNA was mixed with polyamine transformation solution, with 50-μg salmon sperm DNA as carrier DNA, and the volume was adjusted to 450 μL.
3. The final concentration of $CaCl_2$ was adjusted to 100 m*M* with 50 μL of 1 *M* $CaCl_2$ solution.
4. To form an emulsion, 100 μL of 0.5% sodium alginate solution was mixed with 900 μL of isoamyl alcohol in a microtube and emulsified with an ultrasonic disrupter.
5. To solidify the sodium alginate solution in the emulsion, the entire $CaCl_2$ solution containing the chromosomal DNAs was added using truncated microtips.
6. To eliminate the isoamyl alcohol, the microtube was centrifuged at 1900*g* for 3 min, and the supernatant was removed.
7. The microtube was filled up with 100 m*M* $CaCl_2$ solution.
8. Resuspended bioactive beads were harvested by centrifugation at 1900*g* for 3 min. This washing step was repeated at least twice. The chromosomal DNA immobilized on the surface of the bioactive beads (*see* **Note 1**).

3.3. Yeast Transformation Via Bioactive Beads

1. Yeast cells were cultured in a 50-mL yeast extract/peptone/dextrose (YPD) culture of cells until the optical density at 660 nm (OD_{660}) was between 6.5 and 8.0. Transformation using spheroplasts was performed as previously described ***(15)***, with some modifications ***(10)***.
2. Then, these cells were centrifuged at 1500*g* for 5 min and were washed once with 25 mL of sterile H_2O, and then with 25 mL of 1 *M* sorbitol solution.

3. The collected cells were suspended in 15 mL of STC buffer.
4. To form spheroplasts, 120 U of yeast lytic enzyme was added into the yeast cell solution, followed by incubation for 40–60 min at 30°C.
5. The spheroplast cells were gently washed twice with 15 mL of STC buffer, and centrifuged at 280*g* for 4 min (*see* **Note 2**).
6. After removal of the supernatant, approx 8×10^8 competent spheroplast cells were gently resuspended in 100 µL of STC buffer.
7. For transformation with naked DNA, 1-µL 100X polyamine solution and 5-µL salmon sperm DNA solution were mixed, and then 12 µL of DNA solution were added with gentle mixing (*see* **Note 3**).
8. After 15 min, 1.9 mL of 20% PEG4000 solution containing polyamine solution were added to the solution containing the yeast spheroplast cells and DNA with gentle mixing.
9. For transformation using the bioactive bead system, the bioactive beads resuspended in 20 µL of 100 m*M* $CaCl_2$ solution were added to the spheroplast suspension, followed by the addition of 20% PEG4000 solution without polyamine.
10. The cells were cultured on selection plates for 6 d after transformation (*see* **Note 4**).

4. Notes

1. To verify the physical stability of chromosomal DNA immobilized on bioactive beads, a solution of gel-purified chromosomal DNA and a suspension of bioactive beads immobilizing gel-purified chromosomal DNA were stirred simultaneously in the microtube mixer. CHEF gel electrophoresis was performed for the DNA solution and bioactive bead suspension. No visible bands were detected in the lane containing naked chromosomal DNA, indicating the complete destruction of the chromosomal DNA. In contrast, distinct chromosomal DNA bands were detected in the lane containing chromosomal DNAs immobilized on the alginate bioactive beads. These results indicate that the chromosomal DNA immobilized on bioactive beads was physically more stable than the naked chromosomal DNA in solution.
2. Do not use mechanical methods to suspend cells, as spheroplasts are fragile.
3. Addition of 50-µg carrier DNA (salmon sperm DNA) was essential for effective transformation using bioactive beads. When the carrier DNA was not added, the transformation efficiency and reproducibility decreased drastically. Carrier DNAs may assist both the introduction of DNA molecules into yeast cells and the effective immobilization of large DNA molecules present in small numbers.
4. To confirm whether gel-purified DNA could be used for transformation, purified chromosomal DNA immobilized on alginate bioactive beads can be introduced into yeast cells in the presence of PEG where it functions as an independent chromosome. Using the alginate bioactive bead method, 14 of the 16 clones obtained from a selection plate carried the 106-kb minichromosome, whereas such transformants were rarely obtained from selection plates with the PEG treatment of naked minichromosome DNAs. Although the 106-kb minichromosome DNA was introduced into yeast cells without bioactive beads, we believed that it would be difficult to introduce the larger chromosomal DNAs. In the case of donor

strain SH4965 that carries three split chromosomes (185-kb *URA3*, 389-kb *ADE2*, and 468-kb *TRP1*) which complement the gene deficiency of recipient strain AB1380, chromosomal DNAs of up to 450 kb was successfully transformed using bioactive beads.

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7

Modification and Amplification of Yeast Artificial Chromosomes

Sanbing Shen, Anthony Harmar, and Nick Hastie

Summary

The development of yeast artificial chromosome (YAC) technology permits cloning of DNA segments that can be thousands of kilobases in size. This facilitates techniques such as the generation of transgenic animals as a YAC clone can, in most cases, carry an entire gene with all its regulatory elements. Similar to the endogenous yeast chromosomes, YAC DNA is replicated in yeast at one copy per cell. This presents problems when attempting to isolate sufficiently concentrated YAC DNA for pronuclear microinjection. To overcome this problem, we have constructed a YAC amplification vector (pYAM4) that concentrates YAC DNA up to three to eight copies per cell, thus ensuring that more concentrated YAC DNA can be purified. To simplify the analyses of transgene expression in transgenic animals, we have also developed a series of YAC modification vectors (pYIV1, 2, 3, and 4) that can be used to introduce a *lacZ* reporter gene into YAC DNA by homologous recombination. The protocols described in this chapter with the use of these vectors have led to generation of up to 10% of YAC transgenic mice born after microinjection.

Key Words: Yeast artificial chromosome (YAC); YAC amplification; lithium acetate transformation; transgenic mice.

1. Introduction

Yeast artificial chromosome (YAC) technology permits the cloning of DNA segments that exceed the capacity of conventional bacterial and P1 artificial chromosome (BAC and PAC) vectors. Development of the homologous recombination system in bacteria with the aid of RecA has made it possible for functional analyses of many genes using BAC and/or PAC clones ***(1)***. However, the cloning capacity of BAC ***(2)*** and PAC ***(3)*** vectors are limited to approx 200 kb. This is an important consideration when choosing a suitable vector for the pro-

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duction of transgenic animals, as human disease-related genes can often exceed the capacity of BAC/PAC vectors. Only YAC clones are able to carry sufficient DNA to effectively replicate the normal biochemistry of the majority of human genes whose exon/intron span alone frequently exceeds 200 kb. In addition, many diseases are not associated with mutations in coding sequences, but are caused by a disruption of long distance regulatory elements ***(4)*** that can be hundreds of kilobases away from the gene and are required for the correct spatiotemporal control of gene expression. Therefore, YAC technology stands as the sole choice for the analysis of such large and complex genes.

A YAC vector is comprised of two arms. The short arm contains a *URA3* auxotrophic marker gene and a telomere sequence. The long arm contains a yeast centromere (CEN4), another telomere sequence, and another auxotrophic marker gene (*TRP1*). In selective medium lacking uracil and tryptophan, YAC DNA is replicated along with the host chromosomes at the rate of one copy per cell, allowing YAC DNA to be isolated at a concentration of approx 1 ng/μL using the protocol described by Schedl ***(5)***. The volume of a pronucleus of a mammalian fertilized egg with a diameter of 10 μm is 0.523pl. If the same volume of 500-kb YAC DNA at such a concentration is injected into a pronucleus, each fertilized egg will receive no more than 0.95 copies of a 500-kb YAC. Therefore, purification of a more concentrated YAC DNA solution is essential for the successful production of YAC transgenic animals using large YAC clones (>500 kb).

Previously, two vectors have been developed that are able to amplify YAC DNA, pCGS966 ***(6)*** and pCGS990 ***(7)***. Both vectors include a centromere conditioned by a galactose-inducible *Gal1* promoter. When glucose is replaced by galactose in the culture medium, the *Gal1* promoter is activated and the centromere function is disrupted by induced transcription. This disruption leads to a biased segregation of the replicated YAC DNA in the daughter cells, and the daughter cell that receives no YAC dies. The presence of a thymidine kinase (*TK*) gene in the pCGS966 and pCGS990 vectors contributes to a further increase of YAC copy number under selection. YAC DNA of less than 600 kb can be amplified 3- to 11-fold with the use of these vectors. However, these vectors have a low retrofitting frequency (0.5–2.5%) ***(7)***. More importantly, they are unsuitable for transgenic studies, because the *TK* gene has been shown to cause male infertility in transgenic mice ***(8,9)***.

We have constructed a YAC amplification vector (pYAM4) ***(10)*** that amplifies YAC DNA three- to eightfold (**Fig. 1B**). This vector is similar to pCGS966 and pCGS990 vectors in that pYAM4 contains a *Gal1*-conditioned CEN4 (**Fig. 1C**). However, unlike pCGS966 and pCGS990, pYAM4 does not contain the *TK* gene, but a *Lys2* auxotrophic selection gene and an antibiotic selection gene (*PGK-Hyg*). The latter also facilitates the transfer of YAC DNA into cultured

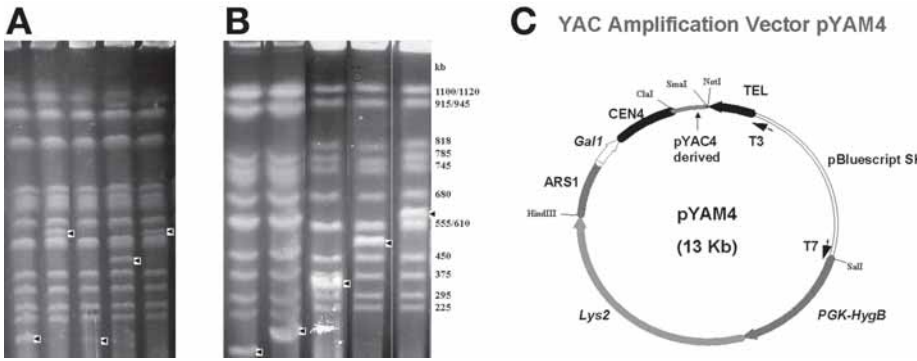

Fig. 1. Yeast artificial chromosome (YAC) amplification. **(A)** Shows unamplified YAC DNA (arrowed). Note that the intensity of YAC DNA staining is similar to or less than that of an endogenous chromosome of a similar size. **(B)** Shows three- to eightfold amplification of YAC DNA (arrowed) by pYAM4 in the presence of galactose. *Not*I-digested amplification vector pYAM4 **(C)** can be transformed into a YAC-containing yeast cell for YAC amplification. If the *Not*I–*Cla*I fragment of pYAM4 is replaced with a genomic DNA fragment isolated by polymerase chain reaction from the YAC clone, the introduction of the modified targeting vector into the YAC containing yeast clone will enable targeted YAC truncation and amplification of the shortened YAC DNA in a single step.

cells. A 13% retrofitting efficiency is achieved with the introduction of a 572-bp *Not*I–*Cla*I fragment derived from pYAC4, which is commonly present in all YAC vectors used for the construction of YAC libraries. Replacement of the *Not*I–*Cla*I fragment with a genomic DNA fragment from a YAC will enable the targeted truncation of the YAC clone and amplification of the shortened YAC DNA in a single step.

To take advantage of the high homologous recombination efficiency in yeast, and to simplify detection of the YAC transgene expression, we have developed a series of YAC modification vectors (pYIV1, 2, 3, and 4; **Fig. 2**). pYIV vectors contain a *lacZ* reporter gene downstream of a viral IRES that allows the expression of the target gene and the *lac*Z marker gene under the same promoter, so that a simple X-gal staining procedure can be used to examine the expression pattern of the YAC transgene in vivo. With the use of the pYIV3 and pYAM4 vectors, we have successfully generated transgenic mice harboring 550-kb YAC DNA ***(10)***.

2. Materials

1. Amino acids (Sigma-Aldrich; *see* **Note 1**): A master mixture of 10X stock solution can be made with 200 μg/mL each of adenine, arginine, histidine, isoleucine,

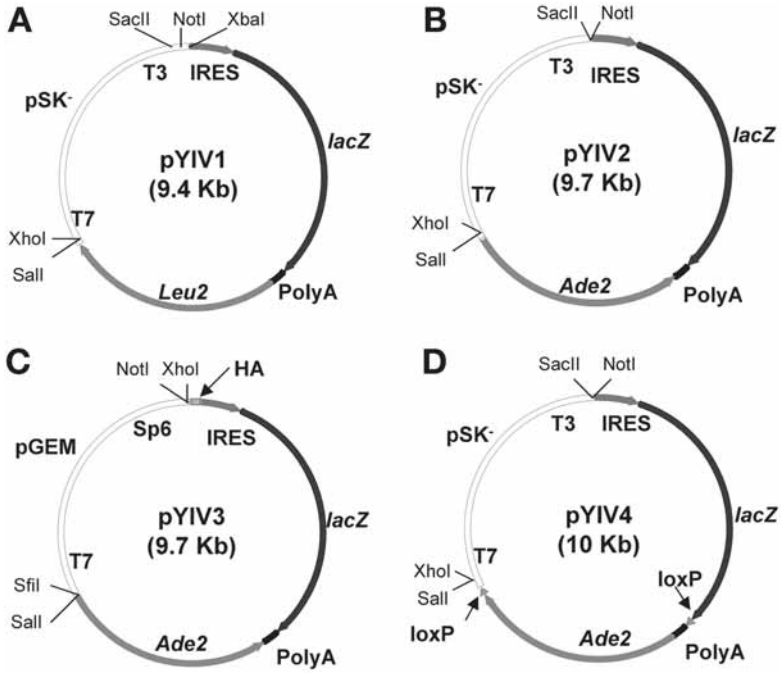

Fig. 2. Yeast artificial chromosome (YAC) modification vectors pYIVs. All pYIV vectors contain an IRES-*lac*Z reporter gene. Selective genes (*Leu*2 or *Ade*2) and unique restriction sites (*Sac*II, *Not*I, *Xba*I, *Xho*I, *Sal*I, *Sfi*I) for introducing two arms of genomic fragments for homologous recombination are indicated. If pYIV3 is chosen, for example, a *Not*I–*Xho*I genomic fragment of approx 500 bp flanking the stop codon of a transgene can be amplified by polymerase chain reaction with a *Not*I site in the forward primer and an XhoI site in the reverse primer, and cloned into the pYIV3. A second genomic fragment (~500 bp) at the 3' UTR can be cloned into the above vector with polymerase chain reaction products containing a *Sal*I site in the forward primer and a *Sfi*I site in the reverse primer. The final construct can then be digested with *Not*I and transformed into yeast. If YAC modification is desirable, it is recommended firstly to modify the YAC with pYIV vectors, and then retrofitted with pYAM4 for amplification, so that every copy of YAC will be modified and amplified.

and methionine, 500 μg/mL phenylalanine, 300 μg/mL tyrosine, and 1.5 mg/mL valine. 100X stock solutions of leucine (6 mg/mL), lysine (2 mg/mL), tryptophan (2 mg/mL, light sensitive), or uracil (2 mg/mL) are prepared individually, and added when needed. The stock solutions are sterilized with Millipore filters and kept at –20°C.

2. Yeast nitrogen base (YNB) (Difco Laboratories, Detroit, MI). For 10X YNB stock solution, dissolve 6.7% of yeast nitrogen base in distilled water, sterilize with Millipore filters, and keep at –20°C.

3. D(+) glucose (BDH Laboratory Suppliers, Poole, UK).
4. D(+) galactose (BDH Laboratory Suppliers).
5. Bacto-agar (Difco Laboratories).
6. Seaplaque GTG low melting point (LMP) agarose (FMC, Rockland, ME).
7. β-mercaptoethanol (14 *M* stock, Sigma-Aldrich).
8. Novozym™ 234 (Novo BioLabs, Bagsvaerd, Denmark).
9. Lithium dodecyl sulphate (Sigma-Aldrich).
10. Proteinase K (Melford Laboratories, Suffolk, UK).
11. Plug molds (Pharmacia, Uppsala, Sweden).
12. Selective media (SD): 2% D(+) glucose, 0.67% YNB and one-tenth volume 10X amino acid stock.
13. SD plates: 2% D(+) glucose, 0.67% YNB, one-tenth volume 10X amino acid stock, and 1.5% bacto-agar.
14. SD/galactose medium: 2% D(+) galactose, 0.67% YNB, and one-tenth volume 10X amino acid stock.
15. SD/galactose plates: 2% D(+) galactose, 0.67% YNB, one-tenth volume 10X amino acid stock, and 1.5% bacto-agar.
16. LTE: 0.1 *M* Lithium acetate (Sigma-Aldrich) in 10 m*M* Tris-HCl, pH 7.5, and 1 m*M* Na_2EDTA.
17. 1 μg plasmid DNA (i.e., pYAM4- or pYIV3-based plasmid prepared using a Qiagen maxiprep kit), and linerarized with *Not*I.
18. 50 μg sheared carrier DNA (10 μL at 5 μg/μL) from calf thymus, chicken blood, or salmon sperm.
19. PEG/LTE (*see* **Note 2**): 40% polyethylene glycol (3500, Sigma-Aldrich), 0.1 *M* lithium acetate, 10 m*M* Tris-HCl, pH 7.5, and 1 m*M* Na_2EDTA.
20. SE buffer: 1 *M* D(–) sorbital (Sigma-Aldrich) and 20 m*M* Na_2EDTA, pH 8.0.
21. NSELMP: melt 1% Seaplaque GTG LMP agarose in SE buffer, keep at 42°C, and add Novozym™ 234 (10 mg/mL).
22. Protein digestion buffer: 2 mg/mL of proteinase K, 0.5 *M* NaCl, 125 m*M* Tris-HCl, pH 8.0, 250 m*M* Na_2EDTA, 1% (w/v) lithium dodecyl sulphate, and 0.5 *M* β-mercaptoethanol.

3. Methods

3.1. Yeast Transformation by Lithium Acetate

1. Inoculate yeast in 10 mL of culture per transformation, and grow the cells to a density of approx 2×10^7 cells/mL.
2. Spin down the cells at 3000*g* for 5 min in a bench-top centrifuge.
3. Wash the cells in one-half volume of the LTE culture.
4. Resuspend cells in one-hundredth volume of the LTE culture.
5. Aliquot the cells at 100 μL per Eppendorf tube, and place on a roller drum at 30°C for 1 h.
6. Add 1 μg of linearized plasmid DNA together with 50 μg of sheared carrier DNA.
7. Mix well and incubate at 30°C for 30 min without shaking.
8. Add 0.7 mL of the PEG/LTE solution, and mix well by inverting the Eppendorf tube.

9. Incubate at 30°C for 1 h without shaking.
10. Heat shock the yeast cells in a water bath at 42°C for 5 min.
11. Spin down the cells in a microfuge at top speed for 5 s.
12. Wash the cells in 1 mL TE.
13. Resuspend the cells in 0.4 mL TE, pH 7.5.
14. Plate out the cells (0.2 mL of cells per selective plate).
15. Incubate plates at 30°C, and transformed colonies should appear after 2–3 d of incubation, depending on the strain, the selective marker, and the vector.

3.2. Mini-Preparation of YAC DNA Amplified With pYAM4 (see Note 3)

1. Inoculate a single colony of yeast transformant in 15 mL of SD/galactose medium (Ura–/Lys–), and shake at 30°C (200 rpm). The cultures are expected to reach late log phase in 2–4 d (*see* **Note 4**).
2. Freshly prepare SE/LMP by melting 1% Seaplaque GTG LMP agarose in the SE buffer, and keep at 42°C (*see* **Note 5**).
3. Pellet cells at 3000*g* for 5 min (*see* **Note 6**).
4. Wash cells twice with one-tenth volume of SE buffer with cut-off tips (*see* **Note 6**).
5. Resuspend cells in 100 µL of the SE, and keep at 37°C.
6. Seal the plug molds with autoclave tape and keep on ice.
7. Prepare NSELMP by dissolving Novozym 234 at 10 mg/mL in the SE/LMP agarose. Keep at 42°C (*see* **Note 7**).
8. Add 100 µL of NSELMP per 100 µL of cells, mix well by pipetting, and quickly transfer 100 µL of mixture per well to presealed plug molds.
9. Leave it to set on ice for 10 min.
10. Transfer plugs to SE buffer containing Novozym 234 (10 mg/mL), and incubate at 37°C for 4 h.
11. Wash plugs twice in 50 m*M* Na_2EDTA, pH 8.0 for 30 min.
12. Replace with the protein digestion buffer solution containing 2 mg/mL of proteinase K, 0.5 *M* NaCl, 125 m*M* Tris, pH 8.0, 250 m*M* Na_2EDTA, 1% (w/v) dodecyl sulphate, and 0.5 *M* β-mercaptoethanol.
13. Incubate at 55°C overnight.
14. Wash plugs with TE (3 × 30 min), run on a pulsed-field gel to examine the YAC DNA, and store the remaining plugs at 4°C in 0.5 *M* EDTA.

4. Notes

1. For growing original YAC clones (i.e., pYAC4-derived clones grown in host AB1380 yeast cells), tryptophan and uracil are not added (*see* Chapter 1). If an original YAC clone is modified with a pYIV3-based construct, the transformants will grow on a plate/medium lacking adenine, tryptophan, and uracil. For amplification of clones modified with the amplification vector pYAM4, transformants will grow on plates/medium without lysine and uracil, but with addition of tryptophan. If a YAC is first modified with pYIV3 and then again modified for amplification with pYAM4, the final transformants must be selected on plates/medium without adenine, lysine, and uracil, but with the inclusion of tryptophan.

2. PEG/LTE solution: first autoclave 44% polyethylene glycol (3500) in distilled water and 10X LTE (1 *M* lithium acetate, 0.1 *M* Tris, pH 7.5, and 10 m*M* Na_2 EDTA) separately, then add one-tenth volume 10X LTE to 44% PEG.
3. For concentrating YAC DNA from a large culture, it is recommended to follow the Schedl protocol ***(5)***.
4. It is best if yeast cells are harvested at late log phase for YAC DNA preparation. The late log phase cultures should be quite cloudy, but not too pink. The pinkness of the culture represents the severity of adenine starvation. In starved cells, the cell wall can be very thick and difficult to digest during the DNA preparation, in addition to cell death and DNA degradation.
5. 1% Seaplaque GTG LMP agarose must be freshly prepared in autoclaved SE buffer.
6. It is essential to avoid mechanical shearing of YAC DNA. Therefore, centrifugation is carried out at a low speed (3000*g*), and cut-off tips used for resuspension and transfer of yeast cells. No vortexing is applied throughout the DNA preparation or transformation procedures.
7. Based on personal experience, Novozym 234 (Novo BioLabs), not Zymolyase (ICN Biomedicals Inc.), is more reliable for producing intact YAC DNA.

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8

Modifying Yeast Artificial Chromosomes to Generate *Cre/Lox*P and FLP/FRT Site-Specific Deletions and Inversions

Gabriela G. Loots

Summary

The ability to efficiently and accurately modify genomic DNA through targeted and tissue-specific mutations is an important goal in animal transgenesis. Here we describe how to exploit two systems of homologous recombination, from yeast and bacteria, to engineer yeast artificial chromosomes (YACs) to generate targeted deletions and inversions in vivo, in transgenic animals, and in the presence of DNA-modifying enzymes known as recombinases. Through homologous recombination in yeast, specific recombinogenic sequences are inserted upstream and downstream of a region in the YAC. The sites of integration of these short sequence elements are chosen carefully, such that the YAC is left functionally intact, and this modified transgene represents the wild-type allele. This YAC is subsequently used to generate transgenic animals, which when bred to animals expressing recombinase proteins result in genetic modifications. By expressing recombinase proteins from different tissue-specific promoters, one can mediate site-specific recombination to generate either ubiquitous or tissue-specific deletions or inversion. These modifications can then be carried through the germline or can be studied somatically. A great advantage of this system is the ability to evaluate subtle genetic effects independent of position-effect variegation, and transgene copy number, eliminating the need to examine several independently generated lines of transgenic animals for each genetic variant.

Key Words: Homologous recombination; *Cre*/*Lox*P recombination; FLP/FRT; site-specific recombination; FRT recombination; YAC transgenics; mutagenesis; recombination-mediated mutagenesis; noncoding deletion.

1. Introduction

To study genetic variants, one greatly benefits from the ability to manipulate cloned DNA constructs and to create transgenic animals carrying genetically modified exogenous DNA. Such modification include the introduction of

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(1) point mutations for the analysis of protein function and transcription factor binding sites, (2) insertions to produce fusion proteins or to introduce reporter genes or motifs, and (3) deletions used to define functional regions of proteins or to remove regulatory elements and evaluate effects on transcription. Cloning in yeast artificial chromosomes (YACs) has many advantages and has proven to be a well-suited system for efficiently manipulating DNA constructs. YAC DNA is easily maintained in the yeast host where one can carry out sophisticated and subtle mutagenesis efficiently ***(1,2)***. The introduction of a selectable marker of mammalian or yeast origin into the arms of a YAC can be carried out with great ease, in a single step integration or replacement. Two rounds of recombination in a process entitled pop-in/pop-out can be used to introduce insertions, deletions, or point mutations into the YAC construct ***(3)***.

In particular, the ability to combine genetic engineering of YACs in yeast, with the *Cre*/*Lox*P recombinase system (*Cre* recombinase is an enzyme product of λ phage in *Escherichia coli*) ***(4,5)***, has powerful applications for the study of deletions in vivo, and eliminates a couple of major problems associated with the study of transgenic constructs in mice, namely position effect and copy number variegation. Also, the large size of YAC inserts is very attractive for the study of intact genomic loci that very often contain intact mammalian genes with all the flanking noncoding DNA, including introns, proximal and distal promoter elements, and long-range enhancers needed to recapitulate the full pattern of gene expression at the appropriate levels. In general, it has been demonstrated that YACs faithfully express their genes independent of the site of integration, and this phenomena has now been demonstrated for several YACs carrying large mammalian genes or gene clusters. A classic example being the human β-globin gene cluster where several founder populations of transgenic mice expressed high levels of the human genes, and the transgenes were correctly controlled in a developmental and tissue-specific manner ***(6)***. Nonetheless, when studying subtle modifications in distant regulatory elements, one has to wonder whether differences between constructs result from the site of integration or individual copy number, rather than the genetic alteration. Therefore, a more elegant approach for studying gene regulation is to flank a putative regulatory element with *Lox*P or FRT sites, create a transgenic animal, and consequently delete the element using *Cre* or FLP recombinase (FLP recombinase target; FLP recombinase is an enzyme native to the 2 μ plasmid of *Saccharmyces cervisiae*) in vivo ***(4,5,7)***. Using this system, one assays for changes strictly associated with the removal of the desired DNA region and the modified transgenes are present in the same genomic context and configuration. Here we describe how YAC DNA can be modified to include *Lox*P/FRT sites flanking a desired sequence using the pop-in/pop-out methods.

1.1. Pop-In/Pop-Out Method

The pop-in/pop-out method was first described by Scherer and Davis ***(3)***, as they applied it to create both a 150-bp deletion and a 2.55-kb insertion in the *HIS3* gene in yeast. A plasmid construct carrying the desired genetic modification, the *URA3* selectable marker and arms of homology to the YAC, are introduced onto the YAC. The plasmid is linearized within the region of homology (cut), transformed into the YAC-containing yeast strain, and the *URA3* gene on the plasmid is selected for. The two most commonly used methods of transforming yeast strains are: (1) lithium acetate (LiAc) and (2) spheroplast transformation (*see* **Note 1**). As the plasmid does not contain an autonomous replicating sequence, the *URA3* gene is only maintained in the yeast through integration. High-frequency integration into the region of homology is directed by the cut ends of the plasmid DNA. Transformants that are shown by polymerase chain reaction (PCR) to have the mutation correctly integrated into the YAC are then plated out onto plates containing 5-fluoroorotic acid (5-FOA) that selects against the *URA3* gene (*see* **Note 2**). Colonies that grow on the 5-FOA plates include those where the *URA3* gene has been excised (pop-out) by homologous recombination between the repeated regions of homology flanking the *URA3* gene. On average, 50% of the pop-out recombination events will remove the wild-type locus, and the rest will remove the mutated locus, leaving the YAC intact.

1.2. Retrofitting the YAC

Most YAC libraries have been constructed with the *URA3* gene as the selectable marker on one of the arms of the YAC. In order to use *URA3* as the selectable marker for the pop-in/pop-out, it is necessary to remove the gene from the YAC and introduce a neomycin marker, if the YAC is intended for mammalian assays (such as tissue culture, embryonic stem cell, or mouse transgenic experiments) ***(8)***. One can select directly for YACs that have mutated the *URA3* gene ***(9)*** (*see* **Note 3**) or use a retrofitting vector that will specifically inactivate the *URA3* gene.

Several retrofitting vectors have been built that replace parts of the *URA3* gene with the yeast selectable marker, *LYS2* or *ADE2*, and potentially insert the neomycin resistance gene (*neo*^{r}) in the process. These vectors include pRV1 ***(10)***, pRAN4 ***(11)***, and pLys2neo ***(7)***. pRV1 can be linearized with *Hin*dIII before transformation into the yeast strain containing the desired YAC. The transformation mix is plated out on –lys plates to select for transformants that have integrated the pRV1 vector. Transformants are subsequently replica plated onto –trp and –ura plates (separately) to identify clones that grow on –trp and fail to grow on –ura plates (*see* **Note 4**).

pRAN4 replaces the right arm of the YAC (including the *URA3* gene) with the yeast selectable marker *ADE2*, and the *neo*^{r} marker driven by the SV40 promoter. Yeast transformed with pRNA4 is selected on –ade plates and then replica plated onto –trp and –ura plates separately. The correctly retrofitted YAC strains should be Ura$^-$, Trp$^+$, and Ade$^+$ (white). As pRAN4 uses the *ADE2* selectable marker, this leaves the strain Lys$^-$ and retrofitting vectors, such as pLUNA ***(12)***, can be used subsequently to retrofit with a *neo*^{r} gene to produce a YAC suitable for use in embryonic stem (ES) cells.

One oddity that has been rarely observed but is worth mentioning is that yeast strains occasionally stably maintain two or more copies of the same YAC. In these instances, transformants will be positive for the introduced marker but will not become Ura$^-$, unless all YAC copies incorporate the mutation by homologous recombination. In a situation where a strain contains two YACs, one could result in loss of *URA3*, whereas the second maintains the *URA3* gene. It is possible to isolate the Ura$^-$ colonies from such strains by screening hundreds of colonies or by separating the YACs by sporulation ***(13)***, kar transfer ***(14)*** or retransformation ***(15)***.

1.3. Making the Pop-In Construct

The starting plasmid for pop-in/pop-out has to be a yeast integration plasmid carrying the *URA3* selectable marker, such as the pRS406 plasmid (Stratagene, La Jolla, CA, cat. no. 217406). The pRS406 plasmid is based on the bluescript vector and has a large polylinker to facilitate cloning ***(16)***. To create a construct containing a desired DNA segment flanked by *Lox*P or FRT sites, one first has to clone a DNA region of sufficient size to include the region to be deleted (RE), plus enough flanking sequence to allow homologous recombination (HA) (**Fig. 1**). Immediately upstream and downstream of the region to be deleted one now needs to insert *Lox*P or FRT sites ***(7)***. If the original DNA construct conveniently harbors unique restriction enzyme sites (uRS) at the borders of the element (RE), then the *Lox*P sites can be obtained by annealing single-stranded oligonucleotides that contain restriction enzyme sticky ends, and be ligated directly into the construct that was originally PCR amplified with primer pair A/F. If that is not the case, there are two alternative options: (1) clone each fragment individually using PCR and uRS linkers (using PCR pairs A/B, C/D, and E/F; **Fig. 1**), or (2) clone the large fragment (PCR amplified with primer pair A/F) and mutagenize the construct to introduce unique restriction enzyme sites in the desired places. In addition, one of the homologous arms requires a uRS to be used so that the final plasmid can be linearized before introduction into the yeast. The HA should be large enough such that there are a few hundred base pairs on either side of the cut site, at a minimum

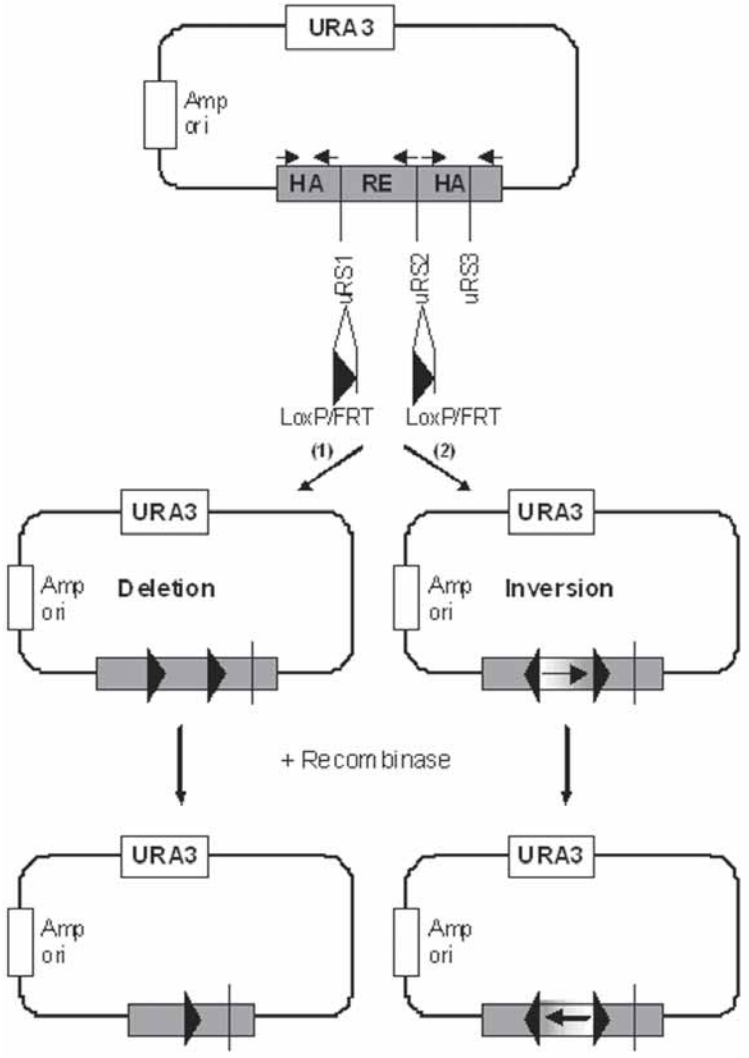

Fig. 1. Generating constructs to introduce *Lox*P/FRT-mediated (1) deletions or (2) inversions into a YAC. The desired genomic segment spanning the DNA region to be deleted (RE) and two homologous arms are cloned into a plasmid carrying the *URA3* gene. To introduce *Lox*P/FRT sites, unique restriction enzymes sites (uRS) are required immediately upstream and downstream of RE, as well as a uRS for linearizing the plasmid before transformation. The orientation of recombinogenic sites determines the recombinase-mediated event. If sites are in the same orientation (1), the element will be deleted, if in opposite orientation (2), the element will invert in the presence of recombinase protein. HA, homologous arm.

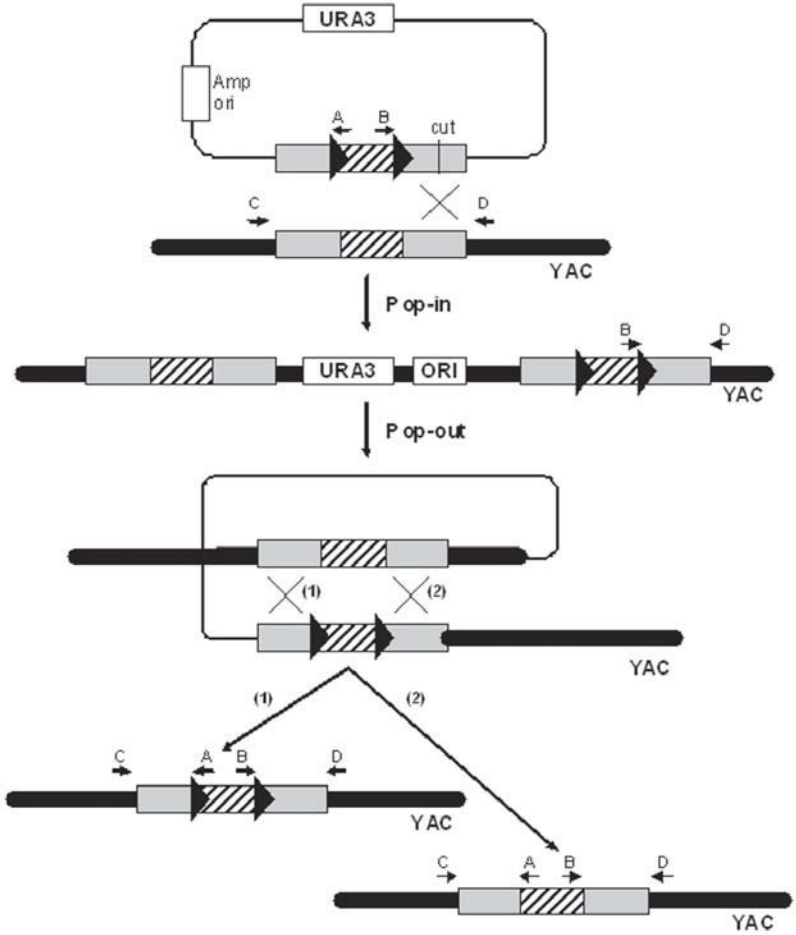

Fig. 2. Overview of the pop-in/pop-out strategy for generating *Cre*/*Lox*P- or FLP/FRT-mediated deletions or inversions. A pop-in plasmid is built that has the *URA3* selectable marker, and *Lox*P/FRT sites inserted upstream and downstream of the desired deletion. The plasmid is linearized at the unique site in one in the homologous arm, and transformed into the yeast strain carrying the yeast artificial chromosome. The reverse recombination event (pop-out) occurs at high frequency when yeast is grown on 5-fluoro-orotic acid media/plates. With relatively equal frequency, the recombination event removes either the wild-type locus ***(2)*** or the engineered locus ***(1)***.

of 50 bp from the proximal *Lox*P site. The frequency of homologous recombination is increased with larger regions of homology; HA 500 bp and larger are preferred. In addition, the ratio of pop-out events that leave the mutation in the YAC to events where the mutation is lost is also primarily determined by the relative size of HA, therefore larger regions or homology are advised.

1.4. Cre/FLP-Mediated Recombination

Transgenic animals carrying a bacterial artificial chromosome modified by *Lox*P or FRT site integration should behave equivalently to transgenic mice carrying the original unaltered YAC. To induce in vivo deletion or inversion of floxed elements (regions flanked by *Lox*P or FRT sites), transgenic animals need to be mated to transgenic mice expressing the appropriate recombinase proteins. To generate germline mutations, one needs to mate the YAC transgenics to transgenic mice that express recombinase proteins either ubiquitously or preferentially in the egg/sperm ***(17)***. To create conditional alleles, the YAC transgenic mice can be mated to transgenic mice that express recombinase molecules in various spatial and temporal patterns ***(18)***.

2. Materials

1. Synethetic complete (SC) and drop-out media: 6.7 g yeast nitrogen base without amino acids, 20 g D-glucose, 100 mL 10X supplement mix (add after autoclaving). Add 20 g agar for solid medium. Adjust pH to 5.8 prior to autoclaving. This is a defined medium containing a complex mixture of supplements suitable for the growth of most laboratory yeast strains carrying auxotrophic mutations. Selective (drop-out) media are made by omitting individual requirements from the supplement mix. For example, omitting uracil and tryptophan from the mix provides selection for YACs containing *URA3* and *TRP1* genes in the host AB1380. This double drop-out media would be denoted synthetic complete (SC) (–ura, –trp).
2. Media for 5-FOA selection: selection with 5-FOA is usually undertaken in defined solid medium (SC plus desired drop-out media). The concentration of 5-FOA can vary among strains of yeast, in general in the range of 0.5–1 mg/mL. For the AB1380 strain, the higher concentration is recommended. Because 5-FOA is highly insoluble, it is suggested to make small amounts at low concentrations, and upon adding 5-FOA to vigorously shake and heat up the solution to 65°C to achieve complete dissolution. To make three 5-FOA/drop-out plates, add the appropriate drop-out medium excluding agar to 50 mL deionized water. Add 0.1 g 5-FOA (Sigma, cat. no. F5013; Sigma-Aldrich, St. Louis, MO, bring into solution, and filter-sterilize. Separately autoclave 2 g agar in 50 mL of water, cool both solutions to 50°C, mix, and pour plates.

3. Methods

3.1. Colony PCR of Yeast Strains

The quickest way to determine whether a specific DNA sequence is present in a YAC strain is to do PCR directly on yeast. Using a toothpick, inoculate 100 μL of water with a single yeast colony and bring to boil (100°C) for 10 min. Add 1–2 μL of yeast dilution to a PCR reaction mix (10–50 μL).

3.2. Pop-In Method

The following procedures are describing for a YAC carrying the *TRP1* marker and lacking a functional *URA3* gene.

1. Transform the YAC-containing yeast strain with linearized plasmid using established protocols (*see* **Note 1**). Plate onto SC (–ura) plates and grow for 3 d at 30°C.
2. Pick colonies onto a SC (–trp) plate that selects for the YAC. This will become the master plate for further analysis of different colonies.
3. To identify colonies with correct recombinants, carry out PCR on individual colonies to determine whether the mutation has been incorporated as expected into the YAC DNA. This could be done with primers pairs A/C or B/D, which will give slightly larger bands for LoxP integrants, as shown in **Fig. 2**. It is suggested to also diagnostic the YAC by restriction digestion and/or Southern blotting to determine whether the mutation has been inserted correctly. Prepare plugs and check YAC on pulse-field gel to determine that no major rearrangements have occurred in the recombinant YAC.

3.3. Pop-Out Method

1. Innoculate 5 mL of SC (–trp) media with a colony of pop-in yeast and grow to confluence overnight at 30°C with shaking. The media will select for maintenance of the YAC, whereas recombination will occur in some of the yeasts during growth without selection of *URA3*.
2. Spread 100 µL of the culture (as well as 100 µL of 1:10 and 1:100 dilutions) of the overnight culture onto to SC (–trp) plates supplemented with 1 mg/mL 5-FOA (*see* **Note 2**). The lack of trp selects for the presence of the YAC, whereas the 5-FOA selects for loss of the *URA3* gene (*see* **Note 3**).
3. Incubate at 30°C for 3 d until the colonies have grown up.
4. Pick about 20 colonies onto a SC (–trp) plate and grow at 30°C (*see* **Note 4**). This is a master plate to which you can return once the correct colonies have been identified. It is necessary to determine at this point whether the mutation is present on the YAC, whether the pop-out has actually occurred, and whether the YAC is otherwise unrearranged.
5. Carry out colony PCR to detect the present of the mutation (primer pairs A/C and B/D, or C/D). This can also be done by diagnostic restriction digests and Southern blotting.
6. Make plugs from single colonies and separate the chromosomes on a pulse-field gel to determine whether the YAC is still the same size as the original. Also, carry out diagnostic digestion to check that no other mutations have occurred to the modified YAC.

4. Notes

1. Yeast transformation methods are discussed in greater detail in Chapters 3 (Spheroblast transformation), 8 (lithium acetate transformation), and 9 (microbead transformation).

2. To make 5-FOA drop-out plates (100 mL/3 plates), add the appropriate drop-out medium, excluding agar, to 50 mL deionized water. Add 0.1 g 5-FOA (Sigma, cat. no. F5013; or the Genetics Society of America), stir well until dissolved, and filter-sterilize. Autoclave 2 g agar in 50-mL water, bring both solutions to 50°C, mix, and pour plates.
3. To get YACs with null *URA3* mutations, a culture of yeast containing the YAC is grown overnight in media lacking the selectable marker present on the left arm of the YAC (usually *TRP1*;–trp drop-out media) while allowing loss of the *URA3* gene. One hundred microliters of the culture and serial dilutions are spread on –trp plates containing 5-FOA. Yeast colonies which grow on 5-FOA plates do not have a functional *URA3* gene. To confirm the *URA3* mutation, colonies from 5-FOA plates should be replica plated onto –ura plates where they should not grow.
4. One of the drawbacks of using YAC DNA constructs, is that YACs are prone to genomic rearrangements, therefore, it is absolutely necessary to screen several colonies for ones that have not been rearranged (a minimum of six colonies should be screened). The integrity of the YAC should also be verified by pulse-field gel electrophoresis and restriction enzyme digest. The *URA3*-null YAC should have the same size and restriction digest map as the original YAC. Nonetheless, one should be wary that additional mutations could have occurred on the YAC, in addition to the loss of *URA3*. Although retrofitting with pRV1 works effectively for the purpose of knocking out the *URA3* gene, it introduces the *LYS2* gene onto the YAC and *LYS2* cannot be subsequently used for retrofitting the YAC. This is not a problem if the *neo*^{r} gene, which is driven by the metallothionein promoter is sufficient for the anticipated experiments, however, if transfer into ES cells requires a stronger promoter driving the *neo*^{r} gene, then pLys2neo is a more suitable retrofitting vector because the *neo*^{r} gene is controlled by the phosphoglycerate kinase (PGK) promoter, which is commonly used for ES targeting selection.

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9

Selective Isolation of Large Chromosomal Regions by Transformation-Associated Recombination Cloning for Structural and Functional Analysis of Mammalian Genomes

Natalay Kouprina, Vladimir N. Noskov, and Vladimir Larionov

Summary

Transformation-associated recombination (TAR) cloning allows selective isolation of full-size genes and genomic loci as circular yeast artificial chromosomes in yeast. The method has a broad application for structural and functional genomics, long-range haplotyping, characterization of chromosomal rearrangements, and evolutionary studies. This chapter describes a basic protocol of gene isolation by TAR, as well as a method of conversion of TAR isolates into bacterial artificial chromosomes.

Key Words: YAC; TAR cloning; gene isolation; complex genomes.

1. Introduction (*see* Notes 1–4)

Transformation-associated recombination (TAR) cloning allows entire genes and large chromosomal regions to be selectively and accurately isolated from total genomic DNA by in vivo recombination in yeast ***(1–5)***. **Figure 1** shows a schematic for isolating a gene as a circular yeast artificial chromosome (YAC) by a TAR cloning vector. The vector contains gene-specific targeting sequences (hooks) at both ends (the hooks can be as small as 60 bp) ***(6)***, a yeast centromeric *CEN* sequence, and a yeast selectable marker. Recombination between the vector and homologous sequences in the cotransformed mammalian DNA can result in a circular YAC that is able to replicate, segregate, and be selected for in yeast. Typically in TAR cloning, the desired gene is produced at frequency between 1 and 20%. The size of the isolated genomic fragment may be as large as 250 kb (summarized in **ref. *5***).

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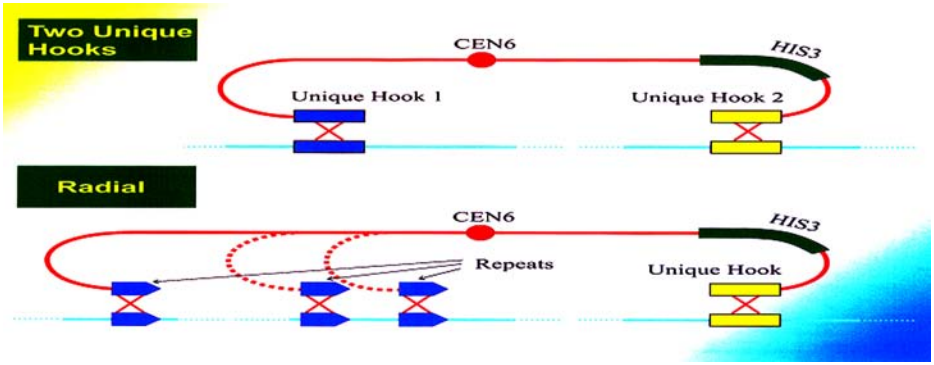

Fig. 1. Isolation of a single copy gene using transformation-associated recombination (TAR) cloning. The upper diagram shows TAR cloning with a vector containing two unique targeting sequences (hooks). Hooks are cloned into the vector in such a way that after linearization of the vector, orientation of the hooks correspond their orientation in genomic DNA as shown. Recombination between sequences in the vector and the locus in genomic DNA creates a circular yeast artificial chromosome (YAC). *CEN6* corresponds to the yeast chromosome VI centromere and *HIS3* is a marker for selection in yeast. The arrows indicate the positions of primers for an internal region that can be used for initial identification of YAC clones containing the gene of interest. This figure is adapted from the paper of Larionov and coauthors ***(23)***. The bottom diagram shows radial TAR cloning when a vector includes a gene-specific targeting sequence (a unique hook) and a repeat sequence (i.e., *Alu* for cloning human DNA) at either end of the linearized vector. Recombination between the vector and genomic DNA can create circular YACs that extend from the unique sequence to upstream *Alu* sequences or to downstream *Alu* sequences. Thus, a set of different size YACs is generated. This figure is adapted from the paper of Kouprina and coauthors ***(11)***.

TAR cloning, as described previously, requires the cloned DNA fragment to carry at least one autonomously replicating sequence *(ARS)*-like sequence that can function as the origin of replication site in yeast. Because each 20 kb of mammalian DNA contains at least one *ARS*-like sequence, the strategy seems applicable for most euchromatic regions ***(7,8)***. In chromosomal regions with multiple repetitive elements, however, such as the centromere and telomere, the *ARS* frequency may be reduced, precluding isolation by the above method. Recently, a modified TAR cloning methodology has been developed that allows isolation of regions regardless of the presence of *ARS* elements ***(9,10)***. This system greatly expands the applicability of TAR cloning for characterization of telomeric and centromeric regions. Moreover, it allows selective cloning of bacterial genome fragments up to several hundred kilobases poor in sequences that can function as *ARS*s in yeast.

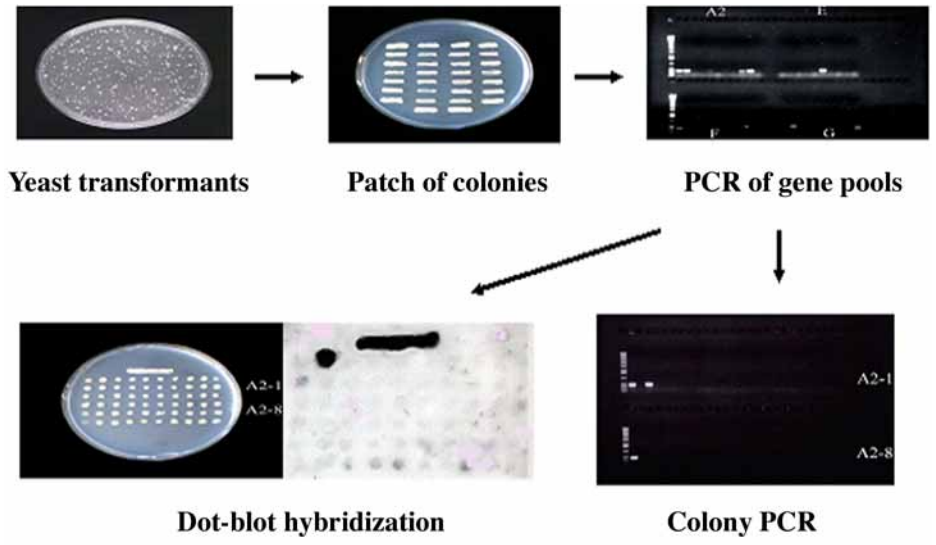

Fig. 2. Detection of gene-positive yeast transformants. To identify gene-positive colonies, primary transformants are combined into pools and examined for the presence of the gene by polymerase chain reaction (PCR). Individual colonies in the pools carrying the gene of interest are usually identified by a second round of PCR using a pair of diagnostic primers or colony hybridization.

When only limited sequence information is available, such as a 3'-flanking expressed sequence tag (EST), TAR cloning with vectors that have two specific targeting sequences is impossible. To circumvent this limitation, we developed another way to isolate a single copy gene directly from genomic DNA ***(11)***. This TAR approach uses a vector that has one unique sequence hook and one repeated sequence hook (*Alu* or *B1* repeats for human or mouse DNA, respectively). The repeated element makes it possible to isolate a set of nested overlapping fragments that extend from the specific hook to different upstream or downstream repeat sequence positions (**Fig. 1**). Because one of the ends is fixed, this approach is called radial TAR cloning. We emphasize the radial nature of this cloning scheme; simply by changing the arrangement of the unique targeting hook, it is possible to clone large regions that extend in both directions along the chromosome from the specific targeting sequence. The size of YACs obtained by radial cloning varies from 40 to 250 kb ***(11–15)***. Clones carrying the gene of interest are usually identified by polymerase chain reaction (PCR) using a pair of diagnostic primers or colony hybridization (**Fig. 2**). Yield of gene-positive clones for radial TAR cloning is approximately the same as that for cloning with two unique targeting hooks.

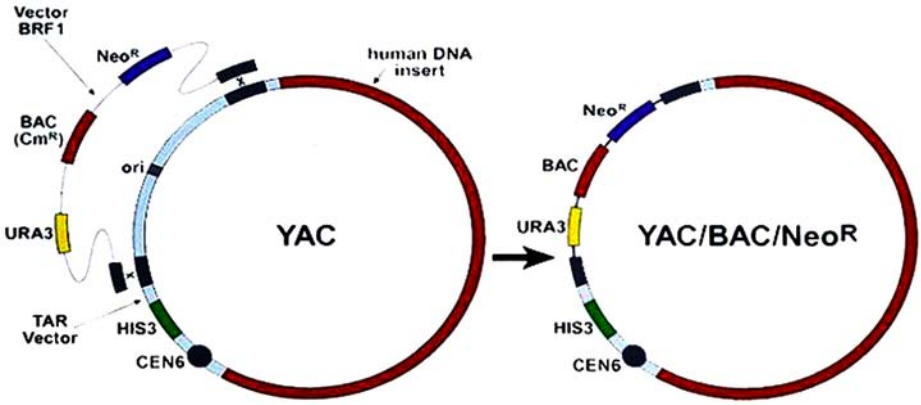

Fig. 3. Schematic representation of retrofitting a circular yeast artificial chromosome (YAC) into a bacterial artificial chromosome using a BRV vector. A linearized retrofitting vector is transformed into yeast cells carrying a circular YAC. Recombination between targeting sequences in the vector and homologous regions in a YAC replaces the *ColE1* origin of replication and the *Ap* gene in the YAC by a cassette containing the *F*-factor origin of replication, the *chloramphenicol acetyltransferase* gene, the *URA3* yeast selectable marker, and a mammalian selectable marker. This figure is adapted from the paper of Kouprina and coauthors ***(11)***.

Introduction of TAR YACs into cultured mammalian cells, including embryonic stem cells, requires a marker on the YAC DNA that can be used to select for the few cells that take up YAC DNA. Moreover, DNA should be purified for transfection. For this purpose a set of BAC retrofitting vector (BRV) yeast-bacteria-mammalian shuttle vectors has been constructed ***(11,16)***. **Figure 3** illustrates conversion of a circular YAC into a bacterial artificial chromosome (BAC) containing a mammalian selectable marker.

At the beginning of the genomic project, TAR cloning has been used to construct chromosome-specific YAC libraries from human-rodent monochromosomal cell lines ***(17–19)*** and for selective isolation of human DNA from radiation hybrids ***(2,20)***. In the postgenomic era, TAR cloning has many other applications. Among them is isolation of full-size genes for functional studies ***(5,12,21–23)*** and isolation of centromeric regions that are poorly cloned by a routine ligation method ***(24)***. TAR cloning provides a tool for the selective isolation of the same specific chromosomal segment or gene from a representative sample of individuals for mutational analysis. Because TAR cloning produces multiple gene isolates, it allows isolation of both parental alleles of a gene that can then be used for haplotype analysis ***(25)***. In many cases, when a gene of interest is represented in multiple copies, physical separation of alleles

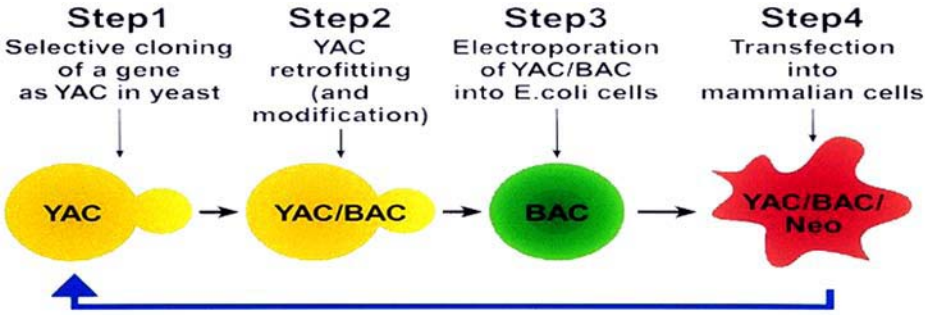

Fig. 4. A scheme illustrating transformation-associated recombination cloning steps to prepare a gene isolate for functional analysis in mammalian cells.

is the only way to determine a haplotype phase. The technique also provides a tool for the isolation of rearranged chromosomal regions, such as translocations from patients and model organisms, without the need to construct a new genomic library of random fragments. The tolerance of TAR cloning to DNA divergence expands the potential applications of the technique for comparative genomics ***(26)***. Given that 15% divergence is characteristic for mammalian gene homolog, most homologous regions of different mammals can be selectively cloned by in vivo recombination in yeast using targeting hooks developed from human exon sequences. This has been demonstrated for several nonhuman primates and mouse genes which were isolated using human-specific targeting sequences ***(27–29)***.

In this chapter, we present updated protocols of TAR cloning. The chapter includes three protocols. The first protocol describes preparation of highly competent yeast spheroplasts and transformation of the spheroplasts by genomic DNA along with a TAR vector (**Subheadings 3.1.** and **3.2.**). The second protocol describes identification of positive clones among primary yeast transformants using PCR (**Subheadings 3.3.** and **3.4.**). The third protocol provides a method for retrofitting of TAR isolated YACs into bacterial BACs with a mammalian-selectable marker and transferring the YAC/BACs into *Escherichia coli* cells (**Subheadings 3.5.** and **3.6.**). Thus, a complete cycle from selective gene isolation in yeast to preparation of a DNA clone for functional analysis is described (**Fig. 4**).

2. Materials

2.1. Strains and Vectors

1. A highly transformable *Saccharomyces cerevisiae* strain VL6-48N (*MAT alpha*, *his3-Δ200*, *trp1-Δ1*, *ura3-Δ1*, *lys2*, *ade2-101*, *met14*) that has *HIS3*, *TRP1*, and

URA3 deleted, is used as a host for TAR cloning experiments. (This strain is available under request from the Laboratory of Biosystems and Cancer, National Cancer Institute [NIH]. The strain is also available from American Type Culture Collection [ATCC] cat. no. MBA-212).

2. The basic TAR cloning vector pVC604 (**Fig. 5**) containing a yeast selectable marker (*HIS3*) and an yeast centromeric sequence (*CEN6*) is also available under request from the Laboratory of Biosystems and Cancer, National Cancer Institute (NIH) the vector is also available from ATCC (cat. no. MYA-3666). Before use, the vector is "activated" by insertion of gene-specific hooks into the polylinker. Before use, the TAR vector should be linearized between the targeting hooks.
3. A set of vectors for retrofitting circular YACs into BACs with different selectable mammalian markers has been developed in the Laboratory of Biosystems and Cancer, National Cancer Institute (NIH) ***(16)***. **Figure 3** shows a schematic representation of retrofitting of a circular YAC into a BAC by homologous recombination in yeast.

2.2. Preparation of Highly Competent Yeast Spheroplasts and Transformation of the Spheroplasts by Genomic DNA Along With a TAR Vector

1. 1 *M* Sorbitol (Sigma, Steinheim, Germany).
2. SPE solution: 1 *M* sorbitol, 10 m*M* Na_2EDTA, and 0.01 *M* Na phosphate, pH 7.5.
3. SOS solution: 1 *M* sorbitol, 6.5 m*M* $CaCl_2$, 0.25% yeast extract (Difco, Kansas City, MO), and 0.5% bactopeptone (Difco).
4. STC solution: 1 *M* sorbitol, 10 m*M* $CaCl_2$, and 10 m*M* Tris-HCl, pH 7.5.
5. Zymolyase solution: 10 mg/mL of zymolyase 20T (ICN Biomedical, Irvine, CA) in 20% glycerol (keep as frozen aliquots at –20°C).
6. PEG 8000 solution: 20% (w/v) polyethylene glycol 8000 (Sigma), 10 m*M* $CaCl_2$, and 10 m*M* Tris-HCl, pH 7.5.
7. YPD medium: 2% D-glucose, 2% Bacto peptone (Difco), 1% yeast extract (Difco), and 0.006 adenine.
8. TOP agar-His: 1 *M* sorbitol, 2% D-glucose, 0.17% yeast nitrogen base (YNB) (Difco), 0.5% $(NH_4)_2SO_4$, 3% Bacto-agar (Difco) containing the following supplements: 0.006% adenine sulfate, 0.006% uracil, 0.005% L-arginine·HCl, 0.008% L-aspartic acid, 0.01% L-glutamic acid, 0.005% L-isoleucine, 0.01% L-leucine, 0.012% L-lysine.HCl, 0.002% L-methionine, 0.005% L-phenylalanine, 0.0375% L-serine, 0.01% L-threonine, 0.005% L-tryptophan, 0.005% L-tyrosine, 0.015% L-valine.
9. SORB-His plates: 1 *M* sorbitol, 2% D-glucose, 0.17% YNB (Difco), 0.5% $(NH_4)_2SO_4$, and 2% bacto-agar (Difco) supplemented as described in **step 8**.
10. Synthetic dextrose (SD)-His plates: 2% D-glucose, 0.17% YNB (Difco), 0.5% $(NH_4)_2SO_4$, 2% bacto-agar (Difco) supplemented as described above in **step 8**.
11. The linearized TAR vector; concentration 0.5–1 μg/μL (keep at –20°C).

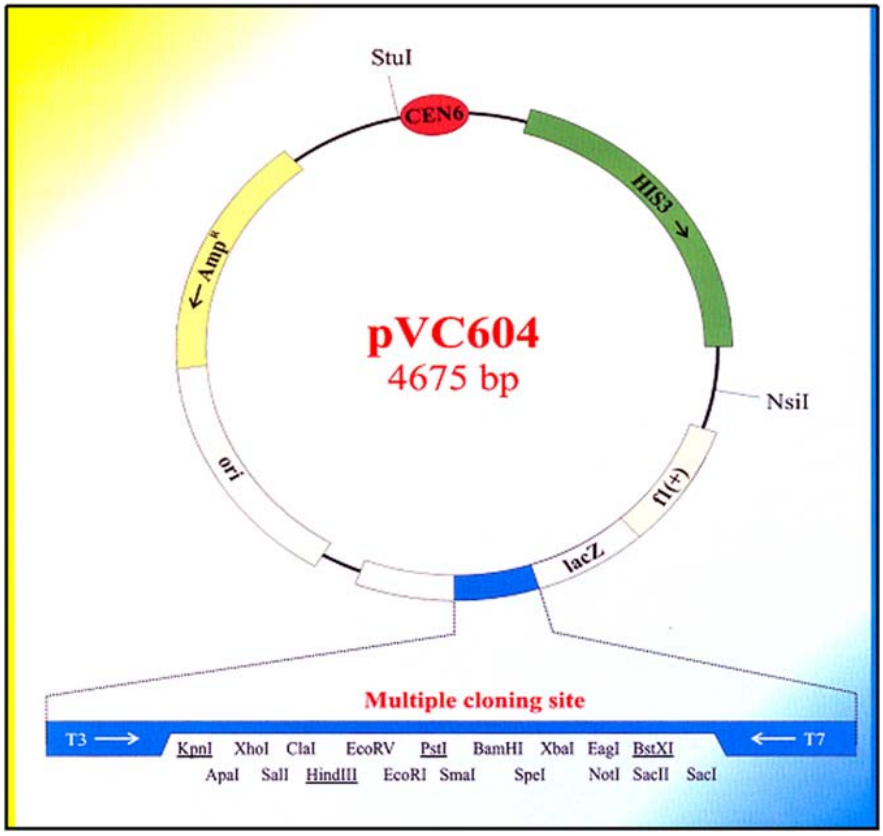

Fig. 5. A scheme of the basic plasmid pVC604 for construction of transformation-associated redcombination (TAR) cloning vectors. pVC604 plasmid is a derivative of the Bluscript-based yeast-*Escherichia coli* shuttle vector pRS313 ***(33)***. pVC604 has an extensive polylinker consisting of 14 restriction endonuclease 6- and 8-bp recognition sites for flexibility in cloning of particular fragments of interest. The functional DNA segments of the plasmid are indicated as follows: *CEN6*, a 196-bp fragment of the yeast centromere VI; *HIS3*, marker for yeast cells; *Ap*, ampicilline-resistance gene. Construction of a TAR vector includes cloning of short specific sequences (hooks) that flank a gene of interest into the plasmid. For TAR cloning experiments, a vector DNA is linearized by an endonuclease digestion to expose targeting sequences.

2.3. Preparation of Genomic DNA for TAR Cloning

Blood and cell culture DNA Maxi kit (Qiagen, cat. no. 13362) (*see* also **Note 5**).

2.4. Isolation of DNA From a Pool of Yeast Transformants

1. 1 *M* Sorbitol (Sigma).
2. SP solution: 1.2 *M* sorbitol and 0.1 *M* Na phosphate, pH 7.5.
3. SPE solution: 1 *M* sorbitol, 10 m*M* Na_2 EDTA, and 0.01 *M* Na phosphate, pH 7.5.
4. Zymolyase solution: 10 mg/mL of zymolyase 20T (ICN Biomedical) in 20% glycerol (keep as frozen aliquots at –20°C).
5. 5 *M* Potassium acetate (KAc).
6. Diethylpyrocarbonate (Sigma).
7. 14 m*M* β-mercaptoethanol (ME; Sigma).
8. 100% Isopropanol (keep at room temperature).

2.5. Identification of Gene-Positive Clones in the Pools

1. Zymolyase solution: 10 mg/mL of zymolyase 20T (ICN Biomedical) in 20% glycerol (keep as frozen aliquots at –20°C).
2. 2% SDS.
3. 5 *M* KAc.
4. 14 m*M* ME.
5. 100% Isopropanol (keep at room temperature).

2.6. Retrofitting of Circular YACs Into BACs With a Mammalian Selectable Marker

1. BRV-based vector linearized at a *Bam*HI and *Aat*II sites; concentration 0.2–1.0 μg/μL (keep at –20°C).
2. Lithium acetate (LiAc) solution: 100 m*M* lithium acetate, 10 m*M* Tris-HCl, and 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.5.
3. PEG 4000 solution: 40% (w/v) polyethylene glycol 4000 (Fluka, Seelze, Germany) aqueous solution.
4. SD-Ura plates: 2% D-glucose, 0.17% YNB (Difco), 0.5% $(NH_4)_2SO_4$, 0.006% adenine sulfate, 0.005% L-arginine·HCl, 0.008% L-aspartic acid, 0.01% L-glutamic acid, 0.004% L-histidine·HCl, 0.005% L-isoleucine, 0.01% L-leucine, 0.012% L-lysine.HCl, 0.002% L-methionine, 0.005% L-phenylalanine, 0.0375% L-serine, 0.01% l-threonine, 0.005% L-tryptophan, 0.005% L-tyrosine, 0.015% L-valine, and 2% Bacto-agar (Difco).
5. SD-His synthetic liquid medium: 2% D-glucose, 0.17% YNB (Difco), 0.5% $(NH_4)_2SO_4$, 0.006% adenine sulfate, 0.006% uracil, 0.005% L-arginine.HCl, 0.008% L-aspartic acid, 0.01% L-glutamic acid, 0.005% L-isoleucine, 0.01% L-leucine, 0.012% L-lysine·HCl, 0.002% L-methionine, 0.005% L-phenylalanine, 0.0375% L-serine, 0.01% L-threonine, 0.005% L-tryptophan, 0.005% L-tyrosine, and 0.015% L-valine.

2.7. Transferring of a YAC/BAC From Yeast to E. coli Cells (see Notes 6–8)

1. DH10B *E. coli* competent cells (Gibco BRL, Carlsbad, CA).
2. LET solution: 0.5 *M* EDTA, 0.01 *M* Tris-HCl, pH 7.5.
3. Carrier salmon DNA: 10 mg/mL sonicated salmon sperm DNA (Stratagene) denaturated by boiling for 10 min every time before the experiment.
4. SOC solution: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgS0_4$, and 20 m*M* D-glucose.
5. LMP agarose (low geling/melting temperature agarose): 1% agarose gel prepared in 0.125 *M* EDTA, pH 7.5.
6. EDTA mix: 0.05 *M* EDTA and 0.01 *M* Tris-HCl, pH 7.5.
7. NDS cell lysis buffer: 0.39 *M* EDTA, 0.01 *M* Tris-HCl, pH 7.5, 1% *N*-lauroyl sarcosine, and 2 mg/mL proteinase K (keep at –20°C).
8. Luria-Bertani chloromphenicol (Cm) plates supplemented with 12.5 μg/mL chloramphenicol.
9. β-agarase, keep at –20°C (BioLabs, cat. no. M0392L).

3. Methods

3.1. Preparation of Competent Yeast Spheroplasts

1. One day before the TAR cloning experiment, inoculate 50-mL aliquots of YPD medium in 500-mL Erlenmeyer flasks with one colony of the host yeast strain VL6-48N freshly grown on a YPD plate, and grow the culture overnight at 30°C with a vigorous shaking to assure good aeration.
2. In the morning, measure optical density (OD) of the culture with 20 min intervals until an OD_{600} of 4.6–4.8 is achieved in the flask. For actual measurement, dilute the culture 1/10 in water; the density should be between 0.46 and 0.48 (*see* **Note 9**). The culture with such an optical density is ready for the preparation of highly competent spheroplasts. This optical density corresponds to approx 2×10^7 cells/mL.
3. Transfer the yeast culture into a 50-mL Falcon conical tube and pellet the cells by centrifugation 5 min at 3000*g*, 5°C. Remove and discard the supernatant.
4. Resuspend a cell pellet in 30 mL of sterile water by vortexing, and centrifuge 5 min at 3000*g*, 5°C. Remove and discard the supernatant.
5. Resuspend a cell pellet in 20 mL of 1 *M* sorbitol by vortexing and centrifuge 5 min at 3000*g*, 5°C. Remove and discard the supernatant. (Yeast cells in 1 *M* sorbitol may be kept overnight.)
6. Resuspend a cell pellet in 20 mL of SPE solution. Add into a tube 20 μL of zymolyase solution, 40 μL of ME, mix well and incubate at 30°C for approx 20 min with a slow shaking. (Note that the treatment time varies depending on the zymolyase stock.)

7. Check the level of spheroplasting by comparison of ODs of the cell suspension in 1 *M* sorbitol vs 2% SDS. To measure the OD_{600} difference, 200-μL aliquots of the zymolyase-treated cell suspension are diluted 10-fold by 1 *M* sorbitol and 2% SDS. The spheroplasts are determined to be ready when the difference between the two OD_{660} readings is three- to fivefold. Both underexposure and overexposure to zymolyase greatly affects transformation efficiency. From this point on, an extreme care must be taken to avoid lysing the delicate spheroplasts: very slow, gentle resuspensions are necessary.
8. Centrifuge spheroplasts for 10 min at 570*g*, 5°C. Decant the supernatant, add 50 mL of 1.0 *M* sorbitol, then rock very gently to resuspend the pellet. Pellet the spheroplasts again by centrifugation for 10 min at 300–600*g*, 5°C.
9. Repeat the wash with 50 mL of 1 *M* sorbitol one more time and gently resuspend the final pellet in 2.0 mL of STC solution. The spheroplasts are ready for transformation and are stable at room temperature for at least 1 h.

3.2. Transformation of Spheroplasts by Genomic DNA Along With a TAR Vector

1. Gently mix 200 μL of spheroplast suspension with 1–2 μg of genomic DNA and 0.5 μg of the linearized TAR vector in 1.5-mL Eppendorf tube. Incubate 10 min at room temperature. (A total number of samples may be 10 for 2.0 mL of spheroplasts in STC solution.)
2. Add 800 μL of PEG 8000 solution into each Eppendorf tube, gently mix by inverting and incubate 10 min at room temperature.
3. Pellet the spheroplasts by centrifugation in the Eppendorf microfuge for 5 min at 300–500*g*, 5°C. Remove the supernatant and gently resuspend the spheroplasts in each tube with 800 μL of SOS solution using Pipetman.
4. Incubate the spheroplasts for 40 min at 30°C without shaking.
5. Transfer the spheroplasts from each tube into a 15-mL Falcon conical tube containing 7.0 mL of melted TOP agar-His (equilibrated at 50°C) using Pipetman, gently mix and quickly pour agar onto a SORB-His plate with selective medium containing 1 *M* sorbitol.
6. Keep the plates at 30°C for 5–7 d until all the transformants become visible. For transformation conditions described above (i.e., with 0.5 μg of a vector, 2 μg of genomic DNA and ~1×10^8 spheroplasts), the yield of transformants varies from 10 to 100 colonies per one plate depending on the hooks used. Typically the higher yield of transformants is observed with the hooks containing nonunique sequences.

3.3. Identification of Gene-Positive Pools

Typically, one to five among 100 primary His^+ transformant colonies contains a gene of interest. To identify positive colonies, primary transformants are combined into pools and examined for the presence of the gene by PCR using a pair of primers specific for its internal sequence (**Fig. 2**). We recommend using pools containing not more than 30 transformants.

1. Transfer 600 primary transformants by toothpicks on SD-His plates lacking histidine. Streak 30 transformants per each master plate.
2. Incubate the plates with pools of transformants at 30°C overnight and replica plate on new plates with SD-His selective medium. Use the master plates for detection of gene-positive pools. (Incubate replica plates at 30°C overnight and later use the positive pools for detection of gene-positive individual colonies by a second round of PCR.)
3. Wash the yeast cells out from the replica plates with 5 mL water into 15-mL Falcon conical tubes, and pellet the cells by centrifugation 5 min at 1000*g*, 5°C. Remove and discard the supernatant.
4. Resuspend each cell pellet in 1 mL of 1 *M* sorbitol by vortex, transfer the suspension to 1.5-mL Eppendorf microfuge tube and spin for 30 s. Remove and discard the supernatant.
5. Resuspend cells in 0.5 mL of SPE solution containing ME (1/1000 dilution), add into each tube 20 μL of zymolyase solution and incubate for 2 h at 30°C.
6. Harvest the spheroplasts by centrifugation for 5 min at 2000*g* on the Eppendorf microfuge and resuspend the pellets in 0.5 mL of 50 m*M* EDTA solution containing 0.2% SDS with Pipetman.
7. Add 1 μL of diethylpyrocarbonate at room temperature and vortex well.
8. Lyse the spheroplasts completely by incubation at 70°C for 15 min.
9. Add 50-μL 5 *M* KAc to lysate, mix well and let the tubes sit on ice for 30 min.
10. Pellet the precipitate by centrifugation for 15 min at maximum Eppendorf minifuge speed (15,000*g*).
11. Transfer the supernatant to fresh microfuge tubes, fill the tubes with room temperature ethanol, mix, and pellet the DNA by centrifugation for 5 min. Remove the supernatant as much as possible and dry the tubes by inverting on blotting paper.
12. Resuspend each damp DNA pellet in 0.4 mL water. (Samples can be incubated at 4°C overnight to completely dissolve.)
13. Add 0.5 mL of isopropanol. Mix well and immediately pellet the DNA precipitate by centrifugation for 5 min at room temperature.
14. Remove the supernatant as much as possible and dry the tubes well.
15. Dissolve he final pellet of DNA in 0.3 mL of water.
16. Use 1 μL of the DNA solution in a 50-μL PCR reaction with appropriate diagnostic primers to identify gene-positive pools.

3.4. Identification of Individual Gene-Positive Clones in Pools

Individual clones from each positive pool are screened by a second round of PCR to identify colonies containing a gene of interest.

1. Touch the streak of each transformant from a replica plate with "a positive pool" with a sterile disposable pipet tip and then rinse the tip thoroughly with 100 μL of zymolyase solution plus 1 μL of ME.
2. Incubate the resulting suspension for 1 h at 30°C.
3. Add 10 μL of 2% SDS. Incubate for 15 min at 70°C.

4. Add 11 µL of 5 *M* KAc and let the tubes sit on ice for 15 min.
5. Spin at 15,000*g* for 2 min.
6. Transfer the supernatant to a new Eppendorf tube and add equal volume of isopropanol. Precipitate at maximum speed.
7. Dissolve the pellets in 30 µL water.
8. Use 1 µL of the DNA solution in a 50-µL PCR reaction with appropriate diagnostic primers to identify gene-positive clones.

3.5. Retrofitting of TAR YACs Into BACs With a Mammalian Selectable Marker

This protocol describes an efficient and accurate procedure for retrofitting circular TAR YACs into BACs using a set of yeast–bacteria–mammalian shuttle BRV vectors (**Fig. 4**) (*see* **Notes 6** and **7**). The retrofitted YAC/BACs can be moved to *E. coli* by electroporation for a standard BAC DNA isolation.

1. Inoculate 5 mL of SD-His synthetic liquid medium without histidine with one individual colony containing a TAR YAC and grow overnight at 30°C with vigorous shaking to assure a good aeration.
2. Transfer the yeast culture into 50-mL YPD and grow for additional 4–5 h at 30°C with vigorous shaking.
3. Pellet the culture by centrifugation for 5 min at 1000*g*, 5°C in the 50-mL Falcon conical tube. Remove and discard the supernatant.
4. Resuspend the cell pellet in 10 mL of sterile water by vortex, transfer into an Eppendorf tube and pellet the cells by centrifugation for 1 min at maximum speed. Remove and discard the supernatant.
5. Resuspend the cells in 10 mL of LiAc solution. Incubate at 30°C for 1 h with a slow shaking. Alternatively, cells can be stored at 5°C during 2–3 d with no effect on transformation efficiency.
6. Collect the cells by centrifugation.
7. Decant the supernatant and resuspend the cells in 100 µL of LiAc solution using Pipetman.
8. Add 1 µg of a linearized BRV vector DNA and 5 µL of carrier salmon DNA to the cells and mix well.
9. Add 0.45 mL of fresh PEG 4000 solution, mix by vortexing and incubate for 1 h at 30°C.
10. Heat shock the cells in a 42°C tempblock for 15 min.
11. Top off the tube with sterile, distilled water and mix by inversion.
12. Collect the cells by centrifugation at a high speed for 1 min.
13. Decant supernatant and resuspend the cells in 1 mL of water using a sterile toothpick.
14. Collect the cells by centrifugation for 1 min.
15. Decant the supernatant and resuspend the cells in 400 µL of water and spread the suspension of 50, 100, and 200 µL on 100-mm SD-Ura plates.

16. Incubate the plates at 30°C. Colonies of Ura^+ transformants should be visible in 2–3 d. With 1 μg of vector, the yield of Ura^+ transformants varies from 50 to 200 colonies. More than 90% of the transformants derive from recombination between a BRV vector and a circular YAC.

3.6. Transferring of YAC/BACs From Yeast Into E. coli *Cells*

1. Replicate Ura^+ transformants on SD-His plates, grow overnight, and inoculate 5–10 His^+Ura^+ transformants separately in 5 mL of YPD medium in 50-mL Falcon conical tubes and grow overnight at 30°C with a vigorous shaking.
2. Pellet the cells by centrifugation. Remove and discard the supernatant.
3. Resuspend the cells in 100-μL EDTA mix (vortex well) and transfer into 1.5-mL Eppendorf tubes. Add 100 μL of zymolyase solution, vortex the cells for 4 s and incubate the suspension for 30 min at 37°C.
4. Melt an appropriate quantity of LMP agarose and place it in a 50°C water bath to cool.
5. Transfer the melted agarose and resuspended cells to a 42°C tempblock and equilibrate for 15 min.
6. Add to the cell suspension an equal volume of the melted agarose and mix well by vortexing. Keep the cell/agarose suspension at 42°C. It is important that the final concentration of agarose should be equal to 0.5%. With a higher concentration of agarose it is impossible to completely melt the plugs for electroporation.
7. Take 50-μL aliquots of the cell/agarose suspension and gently place each into ultra microtips. Keep the tips horizontal for 10 min at 5°C until the agarose is completely solidified.
8. Transfer the agarose plugs into Eppendorf tubes. To do this, take up LET solution in a 6-cc syringe without a needle, place the tip of the ultra microtip into the syringe lure and gently apply pressure. The plug should slide out into the tube. Make three to four 50-μL agarose plugs and incubate them 1 h at 37°C.
9. Remove LET and add enough NDS solution to cover the plugs. Incubate the plugs 1 h at 55°C.
10. Remove the NDS solution carefully and wash the plugs three times with EDTA mix (20 min each time at room temperature). Dialyzed plugs may be stored at 5°C in EDTA mix for several months.
11. Incubate the plugs overnight at room temperature in water before melting and use for electroporation.
12. To electroporate YAC/BACs into *E. coli*, the plugs are melted at 68°C for 15 min, cooled to 42°C for 10 min, treated with 1.5 U of agarase for 1 h at 42°C and chilled on ice for 10 min.
13. Dilute the treated plug twofold with sterile water.
14. Use 1 μL of the mixture to electroporate 20 μL of the *E. coli* DH10B competent cells using a Bio-Rad Gene Pulser with the settings 2.5 kV, 200 oms, and 25 μF.
15. After electroporation, add 1 mL of SOC into a cuvette, mix well by Pipetman, and transfer into a microfuge tube.
16. Incubate the cells for 1 h at 37°C.
17. Spread 30, 100, and 300 μL of the cell suspension onto Luria-Bertani Cm plates.

18. Incubate the plates at 37°C overnight (*see also* **Note 7**).

4. Notes

1. An important step is the selection of specific hook(s) for a TAR vector. Hooks should be unique sequences; no repeated sequences should be present in the hooks. For human and rodent genomes, the uniqueness of hooks can be checked by blasting against genome sequences. We demonstrated that the size of a hook could be as small as 60 bp ***(6)***. A further increase of the length of a targeting sequence had no effect on selectivity of gene isolation. Hooks should be also free of yeast *ARS*-like sequences. Potential *ARS*-like sequences in hooks can be identified based on the presence of a 17-bp *ARS* core consensus, WWWWTTTAYRTTTWGTT, where W = A or T; Y = T or C; R = A or G ***(30)***. The final conclusion about the absence of the yeast origin of replication in a hook(s) can be obtained only by a yeast transformation assay. No or only few His+ transformants should appear when the TAR cloning vector (with its hooks) is transformed into LiAc-treated yeast cells deficient in *HIS3*.
2. One of the potential mistakes that can mislead selective gene isolation by recombination in yeast is incorrect orientation of the hooks in the TAR vector. Hooks should be cloned into the vector in such a way that after linearization of the vector, the orientation of the hooks should correspond to that illustrated in **Fig. 1**. For TAR cloning experiments, the vector DNA should not be contaminated by chromosomal DNA, and the completeness of the vector linearization by endonuclease digestion should be carefully checked by electrophoresis. Nonlinearized vector molecules will be inactive for homologous targeting of chromosomal DNA. In addition, they can induce circularization of linear vector molecules through a gap repair mechanism when the molecules enter the same cell.
3. If a gene (region) is unclonable by a TAR vector with two specific hooks, the radial TAR cloning approach may be exploited. A TAR vector with one unique hook and a common repeat as a second hook, can target a region that is up to 250 kb farther away from a specific sequence, increasing the probability of *ARS*-like sequence capture. In contrast to TAR cloning with two specific hooks, where chimeras are never observed, radial TAR cloning may produce a few chimeras ***(31)***. These cloning artifacts may be because of the presence in genomic DNA of multiple targets for the common repeat hook. Because TAR cloning produces multiple gene isolates, chimera clones can be easily identified by the sequencing of the ends of the inserts. Then they can be eliminated from further analysis.
4. It is notable that isolation of gene homolog may be carried out using the targeting hooks developed from human genome sequences. Examples of TAR cloning of primate gene homologs are presented in several publications ***(27–29)***.
5. Quality of genomic DNA is also critical for TAR cloning. We presently prefer to use DNAs isolated in aqueous solution (Qiagen DNA Maxi kit, cat. no. 13362). Size of genomic DNA should be checked by pulse-field gel electrophoresis before its use. Recently we have discovered that human and mouse DNA purchased from Promega can be also used for TAR cloning when a targeted gene is smaller

than approx 100 kb. Several human genes were successfully cloned by TAR in our laboratory using Promega genomic DNA (60-kb *ASPM*, 50-kb *NBS1*, 50-kb *hTERT*). Size of genomic Promega DNA checked by CHEF was between 50 and 100 kb.

6. Retrofitted YAC/BACs with sizes up to 250 kb can be efficiently and faithfully transferred from yeast cells into *E. coli* cells by electroporation. However, approx 5% of human DNA fragments cloned in YAC/BAC vectors exhibit an abnormally low transformation efficiency during electroporation into *E. coli* cells ***(26,29)***. Clones with such inserts should be analyzed in yeast.
7. Because some YAC/BACs may be deleted during electroporation, it is necessary to compare the size of inserts in yeast and in *E. coli* cells. To estimate the size of circular YACs or YAC/BACs in yeast, and BACs in *E. coli*, they should be linearized by endonuclease digestion (a unique *Not*I site is present in pVC604 vector) separated by pulse-field gel electrophoresis and visualized by ethidium bromide staining for BACs or blot-hybridized with a TAR vector-specific probe for YACs ***(32)***.
8. All plates including TOP agar-His, SORB-His, SD-His, and SD-Ura plates can be purchased in Teknova, Inc (www.teknova.com).
9. Optical density of the culture to make competent spheroplasts may vary depending on: (1) medium for growth, (2) conditions of growing, and (3) a type of spectrophotometer used. Thus optimum optical density should be determined empirically in an OD_{600} interval of 2.0–5.0.

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10

A Polymerase Chain Reaction-Mediated Yeast Artificial Chromosome-Splitting Technology for Generating Targeted Yeast Artificial Chromosomes Subclones

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Summary

The yeast artificial chromosomes (YAC) system makes it possible not only to clone large DNA fragments but also to simplify the physical mapping and functional analysis of chromosomes and genomes through YAC manipulation. To manipulate large DNA fragments cloned into YACs, YAC fragmentation has been developed and also a new method called the YAC-splitting method was developed recently. Although the YAC fragmentation method can be used to delete DNA from one side of a YAC, the YAC-splitting method generates two smaller YACs, both of which are transmitted to daughter cells during mitotic growth. This chapter describes the YAC-splitting method improved by incorporating polymerase chain reaction-mediated chromosome splitting (PCS) technique and by adding yeast autonomously replicating sequence (ARS) to the system. The PCS method combines a streamlined procedure (two-step PCR and one transformation per splitting event) with the Cre/*loxP* system for marker rescue. The improved YAC-splitting method can convert a targeted region of a eukaryotic chromosome within a YAC into a new replicating YAC.

Key Words: PCR-mediated chromosome splitting (PCS); YAC-splitting technology; plant chromosome; autonomously replicating sequence (ARS); *Saccharomyces cerevisiae*.

1. Introduction

Analysis of the complex genomes of higher eukaryotes has been facilitated by the development of methods for cloning large DNA fragments into yeast artificial chromosome (YAC) vectors ***(1)***. The YAC system made it possible

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not only to clone large DNA fragments, but also to simplify the physical mapping and functional analysis of chromosomes and genomes through YAC manipulation. One such manipulation is YAC fragmentation, which deletes either the right or left side of the YAC by using an acentric or centric fragmentation vector ***(2,3)***. A set of fragmentation vectors has been described that can produce a deletion series of smaller YACs from a larger parent YAC, along with the insertion of a eukaryotic selectable marker ***(4)***.

For manipulating YACs, a new method called "YAC-splitting technology" was developed ***(5)***. Although the YAC fragmentation method can be used to delete DNA from one side of a YAC, the YAC-splitting technology generates two smaller YACs, both of which are transmitted to daughter cells during mitotic growth. Although the original YAC-splitting technique extended the means for manipulating YACs, it suffered from two major shortcomings. One was that the procedure was time-consuming because target-specific fragment containing a split site had to be newly cloned into a YAC-splitting vector for each splitting experiment. The other was that the ability of one of the newly generated YACs to replicate was dependent on the fortuitous presence of an autonomously replicating sequence (ARS) on the split fragment, whereas the other fragment still harbors the original ARS from the parental YAC.

To address the first drawback, a polymerase chain reaction (PCR)-mediated chromosome splitting (PCS) method has been developed recently ***(6)***. The PCS method is a simple and highly efficient method originally developed to split natural yeast chromosomes. A schematic diagram of the PCS method is shown in **Fig. 1**. The splitting fragment with a long extension homologous to the target is generated by a two-step PCR (*see* **Fig. 1A**). Two splitting fragments are integrated into the target region of the YAC clone by homologous recombination, resulting in the splitting of the YAC into two monocentric chromosomes (*see* **Fig. 1B**).

To overcome the second shortcoming, the conventional PCS method was further improved by adding an ARS element to the system to generate a new YAC from any targeted region of a plant chromosome that has no endogenous *ARS* elements ***(7)***.

Here, we describe YAC splitting by using the latest PCS method. This efficient method for manipulating YACs provides an important new tool for the structural and functional analysis of eukaryotic chromosomes.

2. Materials

2.1. Strains (see Note 1)

1. *Saccharomyces cerevisiae* SH5679 (*MAT***a** *ura3-52 ade2-1 his5 lys2-1 trp1 can1-100* [*YAC CIC9e2*], AB1380 harboring *YAC CIC9e2*) was used as a source of yeast genomic DNA.

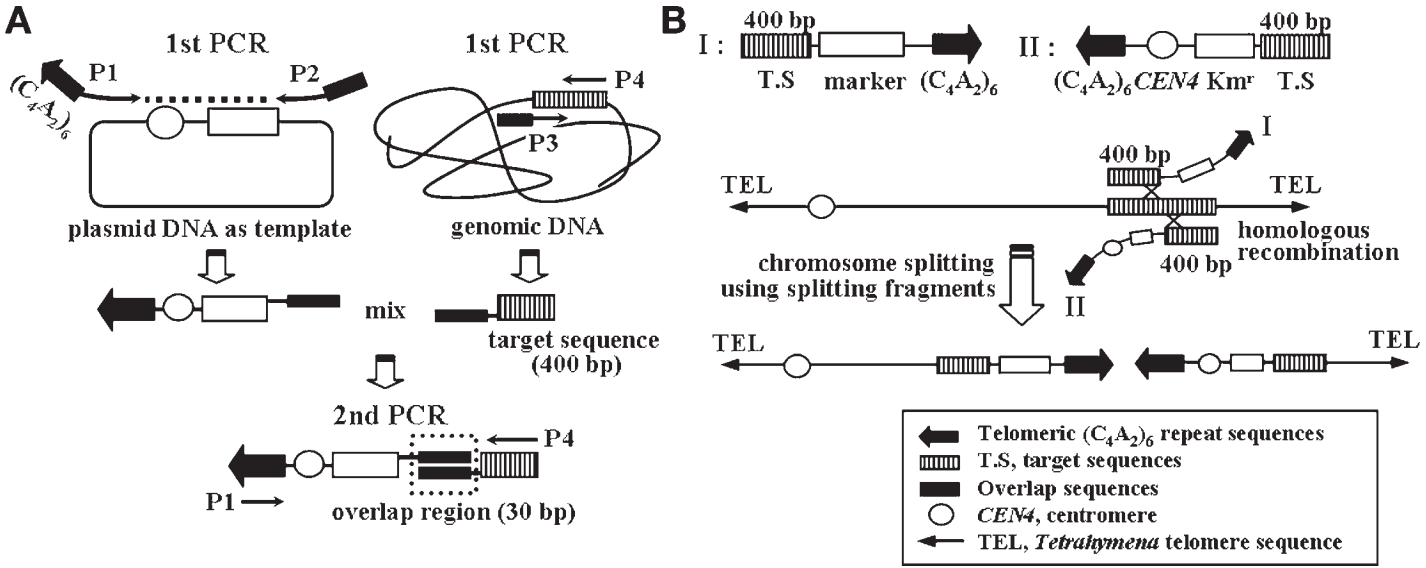

Fig. 1. Schematic diagram of the polymerase chain reaction (PCR)-mediated chromosome splitting (PCS) method. Preparation of a splitting fragment **(A)** Two-step PCR was conducted to generate a splitting fragment with a long homologous extension. One fragment comprising an additional 30-bp sequence, *CEN4*, Km^r and the six copies of the 5'-CCCCAA-3' sequence, and the other fragment comprising the same 30-bp sequence and target sequence were amplified by PCR using two sets of oligonucleotides, P1 and P2, and P3 and P4, respectively. Telomeric 5'-CCCCAA-3' repeat sequences, $(C_4A_2)_6$ repeats, are components of ribosomal DNA (rDNA) telomeres in *Tetrahymena*. The two fragments were then mixed for use as templates for the second PCR using oligonucleotides, P1 and P4. The splitting fragment containing the *loxP-MARKER-loxP* cassette was also amplified by the same procedure. Presumed mechanism of splitting using two splitting fragments **(B)**. Integration of the two splitting fragments at the target region of the YAC clone by homologous recombination results in splitting of CIC9e2 into two monocentric chromosomes. P1~P4, oligonucleotides; I and II, splitting fragments; T. S., target sequences for homologous recombination (*see* **Table 1**).

2. YAC CIC9e2 clone (containing a 590-kb YAC from *Arabidopsis thaliana* chromosome 5) was provided by Ohio State University (Arabidopsis Biological Resource Center, Columbus, OH).
3. *S. cerevisiae* strain SH6173 (*MAT***a** *ura3-52 his3-Δ200 leu2-Δ1 lys2 trp1* [*YAC CIC9e2*]) was used as a model for chromosome manipulation ***(5)*** (*see* **Note 2**).
4. *S. cerevisiae* SH6566 (*MAT***a** *ura3-52 leu2-Δ1 lys2 trp1*) harboring the 490- and 100-kb split YACs generated by splitting of the 590-kb YAC was also used as a model for chromosome manipulation.
5. *Escherichia coli* DH5α was used as a host for plasmid amplification.

2.2. Media

1. Bacto-yeast extract-Bacto-peptone-dextrose-adenine (YPDA) medium: 5% YPD broth (Sigma-Aldrich Co., St. Louis, MO) and 0.04% adenine.
2. Synthetic dextrose (SD) medium supplemented with appropriate amino acids or nucleic acid bases: 0.67% Difco yeast nitrogen base without amino acids (Becton, Dickinson, and Co., Sparks, MD), 2% glucose, and supplement(s) (20 mg/L uracil, 30 mg/L lysine, 20 mg/L tryptophan, 20 mg/L histidine, or 100 mg/L leucine).
3. Luria-Bertani medium containing ampicillin: 2% Luria-Bertani broth (Sigma-Aldrich) and 50 μg/mL ampicillin.

2.3. Template Plasmids for PCR (*see* Note 1)

1. pSK-Km (*see* **Fig. 2A**) plasmid harboring the kanamycin-resistance gene (*Km*^{r}) as a selective marker for plant transformation and centromere of yeast chromosome 4 (*CEN4*).
2. pSKCHY (*see* **Fig. 2B**) plasmid harboring *loxP-CgHIS3-loxP* as a selective marker for yeast transformation.
3. pSKCLY (*see* **Fig. 2C**) plasmid harboring *loxP-CgLEU2-loxP* as a selective marker for yeast transformation.
4. pSK-KCA (*see* **Fig. 2D**) plasmid harboring the Km^r gene, *CEN4*, and *H4ARS* which has an *ARS* located near one of the duplicated nonallelic histone *H4* genes.

2.4. Preparation of Splitting Fragments

1. 0.2-mL PCR tubes.
2. GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster, CA).
3. 100 pmol/μL of each primer.
4. 2.5 m*M* of each dNTP.
5. 5 U/μL of TaKaRa *Ex Taq*® (Takara Bio Inc., Kyoto, Japan).
6. 10X *Ex Taq* polymerase buffer (Takara Bio Inc.).
7. Ethanol precipitation:
   a. TE buffer: 10 m*M* Tris-HCl, pH 8.0 and 1 m*M* ethylenediaminetetraacetic acid (EDTA).
   b. 3 *M* NaOAc: 408.1 g sodium acetate·3H_2O in 800 mL deionized water, pH to 5.2 with glacial acetic acid, and complete to 1 L with deionized water.

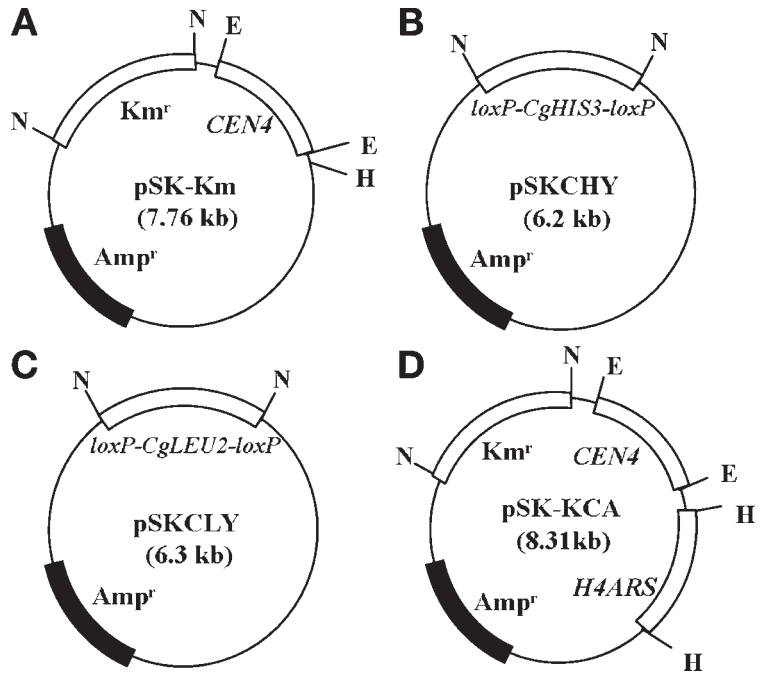

Fig. 2. Structure of template plasmids, pSK-Km **(A)**, pSKCHY **(B)**, pSKCLY **(C)**, and pSK-KCA **(D)** for PCR. Km^r, kanamycin-resistance gene; *CEN4*, centromere of yeast chromosome IV; *loxP-CgHIS3-loxP* and *loxP-CgLEU2-loxP*, *C. glabrata HIS3* and *LEU2* cassette flanked by the *loxP* sequences, respectively; E, *Eco*RI; N, *Not*I; H, *Hin*dIII.

c. 100% Ethanol.
d. 70% Ethanol.

2.5. Clamped Homogeneous Electric Field Gel Electrophoresis and Southern Hybridization

1. 1% Agarose gel (pulsed-field certified agarose, Bio-Rad Laboratories, Richmond, CA).
2. Clamped homogeneous electric field (CHEF) Mapper® XA pulsed-field electrophoresis system (Bio-Rad Laboratories).
3. 0.5X TBE buffer: 45 m*M* Tris-borate buffer, pH 8.3 and 1 m*M* EDTA. A 10X stock solution can be prepared by mixing 108 g of Tris-base, 55 g of boric acid, 7.5 g of EDTA, and deionized water to a final volume of 1 L.
4. 0.5 μg/mL ethidium bromide in deionized water.
5. 20X SSC: dissolve 175.3 g sodium chloride and 88.2 g sodium citrate in 700 mL water and adjust pH to 7.0 with 10 *N* sodium hydroxide. Then bring into final volume of 1 L with deionized water. Autoclave and store at room temperature.

6. Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ).
7. ECL direct™ nucleic acid labeling and detection system (Amersham Biosciences).

3. Methods

3.1. Yeast Transformation and Genomic DNA Preparation

1. For transformation of *E. coli*, a Z-competent *E. coli* transformation kit system (Zymo Research Corp., Orange, CA) was used.
2. Yeast transformation was performed according to the high efficiency transformation protocol ***(8)***. Approximately 5 µg of PCR fragments were used to split the plant chromosome cloned as a YAC.
3. Yeast genomic DNA from strain SH5679 was prepared using the DNA miniprep method ***(9)*** and used as a PCR template (*see* **Note 3**).

3.2. PCR Procedure for Preparation of Splitting Fragments

To manipulate any targeted region of YACs harboring plant DNA by using the PCS method, template plasmids were constructed (*see* **Fig. 2**) and two splitting fragments were generated by using two rounds of PCR, each containing the target sequence; six telomeric copies of the 5'-CCCCAA-3' repeat sequence, 5'-$(C_4A_2)_6$-3', *CEN4*, Km^r (selective marker for plant transformation), or *CgHIS3* (selective marker for yeast transformation) (*see* **Fig. 1**).

To split a 100-kb region from the right end of YAC CIC9e2, two splitting fragments (100-I and 100-II) harboring each target sequence for homologous recombination were amplified as described in **Subheadings 3.2.1.** and **3.2.2.**

3.2.1. Preparation of Splitting Fragment 100-I Harboring CgHIS3, a Telomeric 5'-$(C_4A_2)_6$-3' Repeat Sequence, and a Target Sequence

1. A PCR reaction mix was prepared by mixing: 1 µL of 0.5 µg/µL genomic or plasmid DNA, 1 µL 100 pmol/µL of each primer, 8 µL 2.5 m*M* of each dNTP, 10 µL 10X *Ex Taq* buffer, 78 µL nuclease-free water, and 1 µL of 5 U/µL of TaKaRa *Ex Taq*. Oligonucleotide primers used are listed in **Table 1**.
2. The 1.7-kb *CgHIS3* gene used to select yeast transformants was *first* amplified by PCR using pSKCHY as template, and SK-F and Tr6-1 as forward and reverse primers, respectively. The PCR program consisted of an initial hold at 94°C for 5 min, followed by 25 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Typically, 5 µg of DNA were obtained from a single PCR (*see* **Note 4**).
3. Independently, a 400-bp target sequence corresponding to a sequence from nucleotide position 448,711 to 449,100 of YAC CIC9e2 was amplified by PCR using genomic DNA from yeast strain SH5679 as template and 100kb-1 and 100kb-2 as primers. A 30-bp overlap sequence was added to the 5' end of 100kb-2. The PCR program consisted of an initial hold at 94°C for 5 min, followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s (*see* **Note 4**).

Table 1
List of Oligonucleotides

Oligonucleotides	Sequences
Tr6-1 (P1)	5'-$(CCCCAA)_6$ TCGAGGTCGACGGTATCGAT-3'
SK-F (P2)	5'-**TTACGCCAAGCGCGCAATTA**-3'
100kb-1 (P4)	5'-ATAATTGTAGGCTATGGTCC-3'
100kb-2 (P3)	5'-**TAGTGAGGGTTAATTGCGCGCTTGGCGTAA** ATGATTGATATTAAACAACA-3'
100kb-3 (P3)	5'-**TAGTGAGGGTTAATTGCGCGCTTGGCGTAA** TATAAATTTATCAGAACGGC-3'
100kb-4 (P4)	5'-ACACTTATCTCGAGTCAACC-3'
60kb-F (P3)	5'-**TAGTGAGGGTTAATTGCGCGCTTGGCGTAA** CGGTCAATTGGAGTTTGGCT-3'
60kb-R (P4)	5'-AAGCGAACAAGTTAGCCTAC-3'
Cg60kb (P4)	5'-TTATAGATAAATATAATTATGACTCTCAAGTGGATTTTTTTTTCA TTACGCCAAGCGCGC-3'

Bold letters indicate overlap sequences used for the second polymerase chain reaction. Underlined letters indicate the 45-bp target sequence from nucleotide position 489,156 to 489,200 for splitting YAC CIC9e2 at 60 kb from the right end.

4. The two PCR reactions were pooled, the 1.7-kb *CgHIS3* and 400-bp target fragments were ethanol precipitated (*see* **Note 5**), dissolved in 20 µL TE and 1 µL of the solution was subsequently used as templates in a second PCR with Tr6-1 and 100kb-1 primers to generate the 2.1-kb splitting fragment I (100-I). The PCR program consisted of an initial hold at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min.

3.2.2. Preparation of Splitting Fragment 100-II Harboring CEN4*, Km^r, a Telomeric 5'-$(C_4A_2)_6$-3' Repeat Sequence, and a Target Sequence*

1. A 100-µL PCR reaction mix was prepared as previously described (*see* **Subheading 3.2.1.**, **step 1**).
2. A 3.3-kb PCR product containing *CEN4*, Km^r, and the telomeric 5'-$(C_4A_2)_6$-3' repeat sequence was amplified by a first PCR using pSK-Km as template, and SK-F and Tr6-1 as primers. The PCR program consisted of an initial hold at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min.
3. Separately, a 400-bp target sequence corresponding to the sequence from nucleotide position 449,101 to 449,500 of YAC CIC9e2 clone was amplified using genomic DNA from SH5679 as template, and 100kb-3 and 100kb-4 as primers. The PCR was performed as described in **Subheading 3.2.1.**, **step 3**.
4. The 3.3-kb and the 400-bp PCR fragments were ethanol-precipitated (*see* **Note 5**).
5. A second PCR was performed using the 3.3-kb and the 400-bp PCR fragments as templates, and Tr6-1 and 100kb-4 as primers to generate the second 3.7-kb splitting fragment (100-II). The PCR program consisted of an initial hold at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min.

Splitting fragments 100-I and 100-II were ethanol precipitated and subsequently used to transform strain SH6173 according to the high efficiency yeast transformation protocol ***(8)***. His$^+$ transformants were selected and analyzed (*see* **Fig. 3**) for their karyotype by CHEF gel electrophoresis and Southern hybridization as follows.

3.3. Analysis of Split-YAC by Using CHEF Gel Electrophoresis and Southern Hybridization (see Note 6)

1. Chromosomal DNA from *S. cerevisiae* embedded in agarose plugs was prepared as described in **ref.** ***10*** except that zymolyase was used instead of lyticase.
2. Chromosomal DNA was separated in a 1% agarose gel (pulsed-field certified agarose, Bio-Rad Laboratories) by using the CHEF Mapper® XA pulsed-field electrophoresis system (Bio-Rad Laboratories) in 0.5X TBE buffer at 14°C.
3. CHEF gel electrophoresis was performed for 15 h at 6.0 V/cm with switching intervals of 60 s and a switch angle of 120°, followed by a 9 h run with switching intervals of 90 s and a switch angle of 120°. Following electrophoresis, the gel was stained in 0.5 µg/mL ethidium bromide for 10 min and destained for 30–60 min in distilled water.

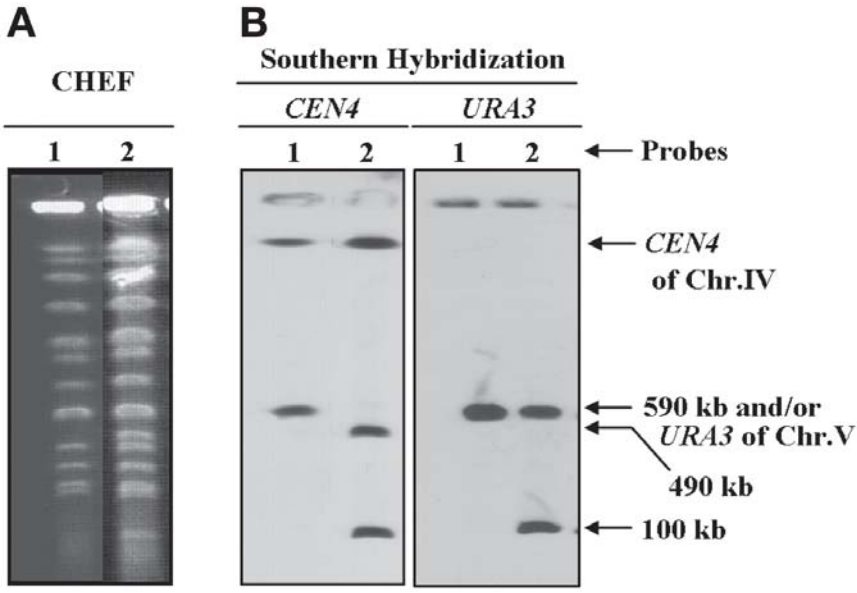

Fig. 3. Splitting of YAC CIC9e2 at 100 kb from the right end. Karyotypic analysis of split-chromosomes by CHEF gel electrophoresis **(A)** and Southern hybridization analysis **(B)** using a labeled 850-bp *Eco*RI fragment of *CEN4* and a labeled 780-bp polymerase chain reaction product containing *URA3* as probes. Lane 1, *S. cerevisiae* strain SH6173; lane 2, His^+ transformant.

4. After staining with ethidium bromide, the chromosomal DNA separated by CHEF gel electrophoresis was transferred to a nylon membrane, Hybond-N^+ (Amersham Biosciences), in 20X SSC by capillary blotting.
5. DNA probes (*CEN4* and *URA3*) were labeled using the ECL direct™ nucleic acid labeling system (Amersham Biosciences).
6. Hybridization, washing, and detection were performed using the ECL system according to the manufacturer's instructions (Amersham Biosciences).

3.4. Improved PCS Method by Incorporating H4ARS

The ability of one of the newly generated YACs to replicate is dependent on the fortuitous presence of an *ARS* on the split fragment, whereas the other fragment still harbors the original *ARS* from the parental YAC (*see* **Note 7**). To overcome this shortcoming, we have improved the conventional PCS method by incorporating an *ARS* element to the system ***(7)***. Hereafter we describe the generation of a new YAC from the desired targeted region of a plant chromosome that has no endogenous ARS element by using new template plasmids (pSKCLY and pSK-KCA).

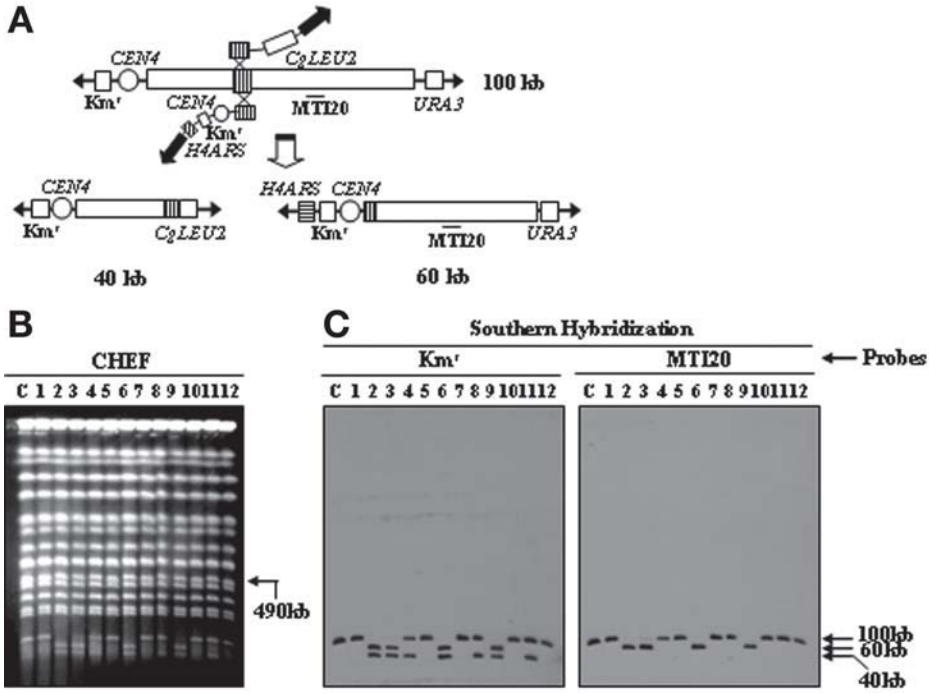

Fig. 4. Splitting of YAC CIC9e2 at 60 kb from the right end using a splitting fragment harboring *H4ARS*. Presumed process of splitting **(A)**. If the 100-kb YAC is split as expected at the target site, two YACs of 40 and 60 kb should be generated. CHEF pattern of split-chromosomes **(B)** and Southern hybridization analysis **(C)** using labeled 2.5-kb *Xho*I fragment of Km^r and labeled 1-kb polymerase chain reaction product from plant chromosome designated MTI20, which corresponds to the sequence from nucleotide position 554,721 to 555,730 of 590-kb YAC. Lane C, *S. cerevisiae* strain SH6566; lanes 1 to12, transformants 1 to 12.

To demonstrate the usefulness of this YAC splitting method, which incorporates a yeast ARS element, we used the 100-kb YAC which was generated by splitting 590-kb YAC into 100-kb and 490-kb YAC subclones in the previous experiment (*see* **Note 6**). The 100-kb YAC was targeted for splitting at the 60-kb position from the right end (*see* **Fig. 4A**). Two splitting fragments (60-I and 60-II) were prepared as described in **Subheading 3.4.1.**

3.4.1. PCR Procedure for Preparation of Splitting Fragments

1. A 100-µL PCR reaction mix was prepared as previously described (*see* **Subheading 3.2.1.**, **step 1**).

2. Fragment 60-I consisting of *CgLEU2*, a telomeric 5'-$(C_4A_2)_6$-3' repeat sequence, and a target sequence was amplified by PCR using pSKCLY (*see* **Fig. 2C**) as template, and Cg60kb and Tr6-1 as primers. The PCR was performed as described in **Subheading 3.2.1.**, **step 2**.
3. Fragment 60-II harboring *CEN4*, Km^r, *H4ARS*, a telomeric 5'-$(C_4A_2)_6$-3' repeat sequence, and a target sequence was prepared as follows.
   A 3.85-kb PCR product containing *CEN4*, Km^r, *H4ARS*, and the telomeric 5'-$(C_4A_2)_6$-3' repeat sequence was amplified by an initial PCR using pSK-KCA (*see* **Fig. 2D**) as template and SK-F and Tr6-1 as primers. The PCR program consisted of an initial hold at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min.
   A 400-bp target sequence corresponding to nucleotide positions 489,201 to 489,600 of YAC clone CIC9e2 was amplified by PCR using 60kb-F and 60kb-R as primers. The PCR was performed as described in **Subheading 3.2.1.**, **step 3**.
   A second PCR was performed using the 3.85-kb and the 400-bp PCR fragments as templates, and Tr6-1 and 60kb-R as primers to generate the second 4.25-kb splitting fragment (60-II). The PCR program consisted of an initial hold at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min 30 s.

Splitting fragments 60-I and 60-II were purified by ethanol precipitation and introduced together into SH6566 according to the high efficiency yeast transformation protocol ***(8)***. Leu^+ transformants were analyzed (*see* **Fig. 4B**,**C**) for their karyotype by CHEF gel electrophoresis and Southern hybridization.

3.4.2. Analysis of Split-YAC by Using CHEF Gel Electrophoresis and Southern Hybridization (see ***Note 8***)

1. Chromosomal DNA from *S. cerevisiae* embedded in agarose plugs was prepared and separated by using CHEF gel electrophoresis as described in **Subheading 3.3.**, **steps 1–3**.
2. Southern hybridization using labeled probes (Km^r and MTI20), washing, and detection were performed as described above (*see* **Subheading 3.3.**, **steps 4–6**).

4. Notes

1. Yeast strains and plasmids used in this chapter are available from Yeast Genetic Resource Center in Japan (http://yeast.lab.nig.ac.jp/nig/english).
2. Most existing YAC libraries were constructed in strain AB1380 ***(11)***. Although AB1380 is transformed efficiently, it is admittedly not an ideal host because of the absence of some frequently used markers (e.g., *leu2* and *his3*) and the presence of point mutations for many of the auxotrophic markers which revert at high frequencies and can cause problems during subsequent YAC manipulations. Therefore, in this study we isolated strain SH6173 from meiotic segregants of the

diploid made by crossing SH5679 to SH5210 (*MATα ura3-52 his3-Δ 200 leu2 Δ1 lys2 Δ202 trp1 Δ63*) as host strain for YAC manipulation.

3. To extract genomic DNA, Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) was used.
4. In the first PCR to amplify the splitting fragment, a 50 μL vol of PCR reaction is sufficient because the PCR product is only used as template for the second PCR.
5. If necessary, the PCR product can be purified using Sephaglas™ BandPrep Kit (Amersham Biosciences) which is designed for the rapid extraction of DNA from agarose gels. This step is effective in removing nonspecific bands in the first PCR reaction, which may interrupt the second PCR.
6. One transformant designated SH6566 harbored the expected 490- and 100-kb split YACs, (*see* **Fig. 3A**, **lane 2**). To prove that these two new YACs were generated from the splitting of the 590-kb YAC, Southern hybridization analysis was performed using *CEN4* and *URA3* as probes. Both probes hybridized as expected with the split YACs (*see* **Fig. 3B**, **lane 2**). These observations confirmed that the 490-kb and 100-kb fragments originated from the 590-kb YAC, indicating that the template plasmids designed for splitting and manipulating the YAC worked well.
7. A 60-kb region from the right end of the 590-kb YAC could not be subcloned by using the original method ***(7)***. We suspected that the 60-kb split YAC might have been generated by splitting but was subsequently lost during mitotic growth due to an inability to replicate in *S. cerevisiae*. This hypothesis was consistent with the fact that no ARS-like sequence was found in the 60-kb region when *ARS*-like sequences in YAC clone CIC9e2 were sought, specifically, an 11-bp conserved sequence element (*ARS* consensus sequence), 5'-(A/T)TTTAT(A/G)TTT(A/T)-3' ***(12,13)*** (http://www. arabidopsis.org/).
8. If the 100-kb YAC had been split at the target site, two YACs of 40 and 60 kb should have been generated (*see* **Fig. 4B**). Among 12 Leu^+ transformants analyzed for karyotype, 4 exhibited the expected split YACs, 40 and 60 kb (*see* **Fig. 4B**, **lanes 2**, **3**, **6**, and **9**). To further verify whether these two new YACs were generated by splitting of the 100-kb YAC, Southern hybridization analysis was performed using the Km^r gene as a probe. The probe hybridized with both split YACs (*see* **Fig. 4C**, **lanes 2**, **3**, **6**, and **9**), indicating that the 40- and 60-kb fragments originated from the 100-kb YAC. In addition, when a 1-kb PCR product, designated MTI20, corresponding to the sequence from nucleotide position 554,721 to 555,730 of the 590-kb *Arabidopsis* YAC was used as a probe, it hybridized to the 100-kb YAC and to the 60-kb YAC subclone, indicating that the 60-kb split YAC was indeed derived from the original YAC (*see* **Fig. 4C**).

Acknowledgments

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11

Genomic Reconstruction by Serial Mitotic Recombination of Yeast Artificial Chromosomes

David Markie, Emma Jones, and Jiannis Ragoussis

Summary

DNA cloned in yeast artificial chromosomes (YACs) is a valuable resource for functional experiments in cell culture as well as whole animal systems. Where the size or chimerism of a YAC clone are limiting factors it may be desirable to generate recombinant YAC clones. One such approach is based on mitotic recombination, and we describe the development of a methodology that allows multiple recombination cycles for serial reconstruction of overlapping YACs. This approach employs retrofitting with standard plasmid vectors, transfer of YACs to a common haploid host by Kar1 mating, and selection for recombination with 5-fluoro-orotic acid.

Key Words: Mitotic recombination; recombinant YAC clone; Kar1 transfer; genomic reconstruction; YAC retrofitting.

1. Introduction

1.1. Approaches to Genomic Reconstruction

Yeast artificial chromosomes (YACs) have been used extensively for the molecular cloning of extended regions of genomic DNA. The large insert size of YACs, reported to average nearly 1 Mb in some libraries ***(1)***, has greatly expedited attempts at producing contiguous clone maps of complex genomes, but YACs also have unique potential for the functional study of genes. The interrupted structure of genes in complex eukaryotes, and the presence of *cis*-acting regulatory elements at some distance from coding sequences, means that functional units of genes, or gene clusters, may extend over very large genomic regions. The development of various techniques for transfer of YACs to mammalian cells in culture ***(2–4)***, and to produce mouse strains transgenic for YACs ***(5)***, offers valuable opportunities for the study of gene function and regulation

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of expression. However, for any particular genomic region, the ability to undertake this type of study will often be limited by the absence in available libraries of an intact clone covering the entire region of interest.

The prevalence of chimerism in most libraries also effectively reduces the contiguous insert size and the presence of extraneous and often uncharacterized material may interfere with analysis of gene function in a chimeric YAC clone. Therefore, it is often desirable to reconstruct contiguous genomic regions from overlapping YACs. Methods for achieving this usually rely on meiotic recombination, utilizing the yeast sexual cycle. This requires mating, sporulation, and spore analysis steps, which is cumbersome but highly effective ***(6)***. An alternative method, utilizing mitotic recombination has also been described ***(7)***. This approach used protoplast fusion to obtain a single yeast strain containing both YACs and twin spot analysis after ultraviolet (UV) irradiation to identify recombinant clones. However, the use of protoplast fusion in this methodology precludes serial reconstruction as the ploidy is increased at each cycle. In addition, the use of UV light to enhance crossing-over rates may also produce unwanted (and unrecognized) mutation events. We have incorporated two further reported techniques into this procedure to overcome these limitations. The use of Kar1-mediated transfer ***(8)*** to produce haploid yeast strains carrying two YACs allows multiple cycles of recombination to take place, and selection of rare spontaneous recombinants with 5-fluoro-orotic acid (5-FOA) in combination with the yeast *URA3* gene ***(9)*** obviates the need for UV exposure and twin spot analysis.

The inclusion of these procedures, in conjunction with standard YAC retrofitting vectors, allows reconstruction of genomic regions contained in overlapping YACs by serial mitotic recombination in haploid yeast strains. In addition, it is possible to obtain recombinants between nonoverlapping YACs from the same species, presumably as a consequence of crossing-over between interspersed repetitive sequences, allowing the construction of single YACs containing desired elements from disparate regions of the genome.

1.2. General Outline of Recombination Cycle

The general strategy is represented schematically in **Fig. 1**. Two or more clones containing single YACs are first selected for recombination. It is essential that the genomic inserts are cloned in the same orientation with respect to the YAC vector arms, otherwise recombination will result in nonviable acentric and dicentric YACs. The YAC that will ultimately form the most centromeric part of the recombined YAC is then targeted with a centromeric retrofitting vector, pCGS990, using selection for the *LYS2* gene. The overlapping second YAC is retrofitted with pRAN4 ***(10)*** using *ADE2* gene selection, providing the alternative selectable markers used for subsequent transfer, recombination, and

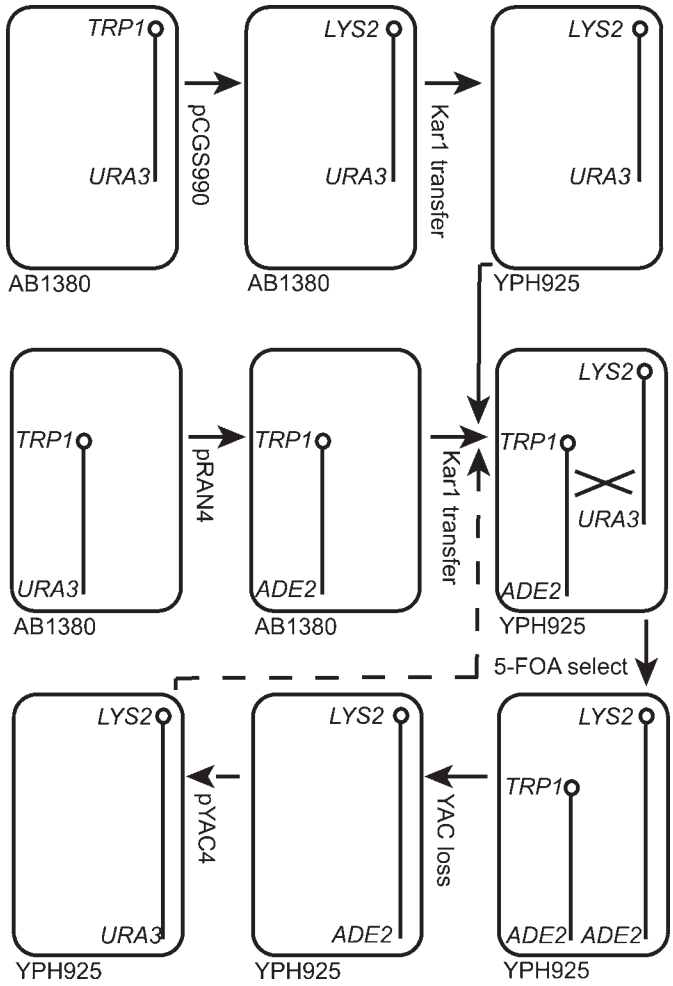

Fig 1. Schematic representation of the procedures involved in the recombination cycle. Yeast artificial chromosomes (YACs) are represented by vertical lines, with the centromeric end indicated by a circle. The yeast markers (*TRP1*, *URA3*, *LYS2*, and *ADE2*) on YAC arms and the yeast host strain (AB1380 or YPH925) are shown for each construct. The first round of the cycle ends with the construct at bottom center of the figure, and if subsequent rounds are required this YAC is first targeted by the pYAC4 vector, and then re-enters the procedure as shown (dashed lines) in conjunction with the next pRAN4-modified YAC.

loss steps. Note that the functional *URA3* gene on the acentic vector arm of the first YAC is preserved and this orientation of YAC modification is essential for 5-FOA selection of recombinants. This also means that in a serial reconstruc-

tion, the step that requires the most screening effort (retrofitting with pCGS990) is used only once. Following retrofitting of the two YACs, they are then transferred one after the other into the same recipient strain (YPH925) using Kar1-mediated transfer producing a single clone containing both YACs in a haploid yeast background. Selection is then applied for loss of the functional *URA3* gene carried on one of the YACs by selection on 5-FOA, enriching for clones in which mitotic recombination between the two YACs has occurred. Clones are then allowed to spontaneously lose the remaining parental YAC. At this point, the first recombination cycle is complete, and the clone contains a single artificial chromosome with material from the first YAC comprising the centomeric end of the insert, and material from the second YAC making up the remainder. Alternative vectors could be used to provide the required markers, but the pCGS990 vector has the advantage of adding a conditional centromere that can be used for amplifying YAC copy number ***(11)***, and the pRAN4 vector provides a marker suitable for selection in mammalian cells ***(10)***. Both of these elements remain in the final reconstructed YAC.

If further cycles of recombination are required, the *ADE2* gene on the right arm of the recombinant YAC must first be exchanged for the *URA3* gene, which can be achieved by "defitting" with the right arm of the original pYAC4 vector. This is necessary to provide the selection for recombination required in the next cycle. A third YAC retrofitted with pRAN4 is then transferred into yeast strain, again by Kar1-mediated transfer with appropriate selection, and the procedure then continues as for the first recombination cycle. We have used this strategy to proceed through two cycles of recombination, but it should be possible to continue this cycling process with further overlapping YACs.

2. Materials

1. 1 *M* Lithium acetate (LiOAc) stock solution: 1 *M* LiOAc (Sigma, St. Louis, MO; cat. no. L-6883) in deionized water, filter-sterilize or autoclave.
2. 100 m*M* LiOAc solution. Make dilutions from the stock solution of **step 1** in sterile water.
3. PEG 50% (w/v) solution in deionized water, filter-sterile or autoclaved. (Sigma, cat. no. P-3640). Store solution tightly capped to avoid evaporation losses.
4. Carrier DNA at 2 mg/mL in sterile water. Salmon testes DNA is suitable (Sigma, cat. no. D-1626) and does not need to be sheared. Stir overnight to ensure it is completely in solution. Heat to 100°C before use and store aliquots frozen.
5. SD medium. SD is a defined yeast minimal medium and consists of 6.7 g/L yeast nitrogen base without amino acids (Difco, Detroit, MI; cat. no. 0919-15-3) and 20 g/L D-glucose. Adjust to pH 7.0 with NaOH and add 15 g/L agar prior to autoclaving if solid medium is required.
6. 100X nutritional supplements for drop-out media. Drop-out media for selection are made by adding sterile 100X solutions lacking specific nutritional supplements

to SD broth before use, or to SD agar while molten. A complete 100X nutritional supplement suitable for the growth of both AB1380 and YPH925 strains would consist of 5 mg/mL adenine (Ade) hemisulfate, 2 mg/mL L-histidine (His), 3 mg/mL L-isoleucine (Ile), 6 mg/mL L-leucine (Leu), 3 mg/mL L-lysine (Lys), 2 mg/mL L-tryptophan (Trp), and 2 mg/mL uracil (Ura) (*see* **Note 1**). Drop-out stock solutions are made by leaving out one or more of the previously mentioned supplements and the specific solutions required for this protocol are:

a. 100X Ade drop-out (contains His, Ile, Leu, Lys, Trp, and Ura only).
b. 100X AdeLeuLysTrpUra drop-out (contains His and Ile only).
c. 100X AdeLys drop-out (contains His, Ile, Leu, Trp, and Ura only).
d. 100X AdeLysTrp drop-out (contains His, Ile, Leu, and Ura only).
e. 100X AdeLysTrpUra drop-out (contains His, Ile, and Leu only).
f. 100X AdeLysUra drop-out (contains His, Ile, Leu, and Trp only).
g. 100X AdeLysTrp drop-out (contains His, Ile, Leu, and Ura only).
h. 100X AdeTrp drop-out (contains His, Ile, Leu, Lys, and Ura only).
i. 100X AdeTrpUra drop-out (contains His, Ile, Leu, and Lys only).
j. 100X LeuLysUra drop-out (contains Ade, His, Ile, and Trp only).
k. 100X LysTrpUra drop-out (contains Ade, His, Ile, and Leu only).
l. 100X LysUra drop-out (contains Ade, His, Ile, Leu, and Trp only).

7. 10 mg/mL Cycloheximide solution. Filter-sterilize and store at 4°C. When required, cycloheximide is added to media just prior to pouring to produce a final concentration of 3 μg/mL.
8. 5-FOA (Toronto Research Chemicals, Canada). 5-FOA is poorly soluble, expensive, and used at a relatively high final concentration in media (1 mg/mL). Make up at 2 mg/mL in 2X SD broth, heat to 65°C, and vortex well to obtain complete dissolution before filter-sterilizing. Store frozen in "single-plate aliquots" of 15 mL. When required, thaw and warm the required number of aliquots to 50°C, mix with an equal volume of molten 30 g/L agar, add any required nutritional supplements, and pour single plates.
9. SD+C broth: SD supplemented with 14 g/L casein acid hydrolysate (Difco) to provide most amino acid requirements (except for Trp). It is used as an alternative to supplemented SD medium on certain occasions when optimal growth of strains is required, but where selection for Ade, Ura, or Trp proficiency needs to be maintained. It is supplemented with 50 μg/mL Ade hemisulfate, 20 μg/mL Trp, or 20 μg/mL Ura as required. Add 15 g/L agar prior to autoclaving if solid medium is required.
10. YPAD broth: 10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose, and 50 mg/L Ade hemisulfate, sterilized by autoclaving. Add 15 g/L agar prior to autoclaving if solid medium is required. This is a complete but undefined medium for the growth of yeast strains.
11. YAC clones for recombination, constructed with pYAC4 in AB1380 host, such as those obtained from the CEPH YAC library.
12. YPH925 yeast strain (ATCC 90834).

13. Plasmid DNA for pCGS990 (ATCC 77417) digested with *Sal*I, pRAN4 (ATCC 77481) digested with *Bam*HI, pYAC4 (ATCC 67379) double-digested with *Bam*HI and *Eco*RI, approx 100 ng/µL. The restriction digests are heat treated at 70°C for 20 min to inactivate the enzymes, but no further purification is required.
14. Replica plating block (Qbiogene, Irvine, CA, cat. no. 5000-001).
15. Cotton velvet squares (20 × 20 cm) for replica plating, sterilize by autoclaving in a large paper envelope, and dry in an oven.

3. Methods

3.1. Retrofitting With pCGS990 or pRAN4

The transformation protocol described here for retrofitting is modified from the original lithium acetate method ***(12)***.

1. Inoculate 0.5 mL of SD+C broth (containing Ade and Trp) with yeast containing the YAC to be modified, and grow overnight with shaking at 30°C.
2. Add 4–6 mL of the same medium to each culture and grow for an additional 4–5 h.
3. Harvest cells in a centrifuge, resuspend each in 1 mL 100 m*M* LiOAc, and transfer to a microfuge tube.
4. Pellet briefly (5–10 s pulse in a microfuge) and wash twice with 100 m*M* LiOAc, then discard the supernatant.
5. To the yeast pellet add the following ingredients without resuspension: 240 µL PEG (50% w/v), 36 µL 1.0 *M* LiOAc, 50 µL carrier DNA (2 mg/mL), and 34 µL water containing the linear vector (0.1–1 µg DNA).
6. Vortex vigorously until the cell pellet is completely mixed, incubate at 30°C for 30 min.
7. Heat shock at 42°C for 20–25 min.
8. Microfuge to pellet cells and remove supernatant.
9. Add 1 mL of YPAD broth and incubate for 30–60 min at 30°C.
10. Pellet and resuspend in 1 mL sterile, deionized water. Plate over three to five plates of selective medium. For pCGS990 modifications this should be LysUra drop-out plates, and for pRAN4 modification this should be AdeTrp drop-out plates. Incubate at 30°C for 3 d until colonies are apparent.
11. To identify retrofitted clones, replica plate to test media using sterile velvet, and allow growth at 30°C for a further 2 d. For the pCGS990 modification, the appropriate test medium is LysTrpUra drop-out medium. The correctly retrofitted clones will fail to grow on this medium and will be rare, but can be identified and recovered from the original transformation plate (*see* **Note 2**). For the pRAN4 modification, the appropriate test medium is AdeTrpUra drop-out and correctly targeted clones will fail to grow in the absence of Ade. These will be much more frequent than the targeted clones for pCGS990.
12. Choose several modified clones from each retrofitting experiment, check that the YACs are the correct size using pulsed-field gel electrophoresis (PFGE) and

Southern analysis (*see* **Note 3**). Select one clone with a YAC of the correct size from each retrofitting experiment for further processing.

3.2. Transfer of a pCGS990-Modified YAC to YPH925

1. Grow the yeast containing the pCGS990-modified YAC in 5-mL selective broth (LysUra drop-out) overnight, and the YPH925 yeast strain in 5-mL YPAD broth overnight.
2. Add 100 µL from each overnight culture (approx 1×10^7 cells) to 1-mL YPAD broth. Incubate at 30°C with gentle shaking for 4–6 h.
3. Plate aliquots of 50, 300, and 700 µL onto plates of LysUra drop-out medium supplemented with cycloheximide. Incubate for 5 d at 30°C.
4. Replica plate to LysUra drop-out medium with cycloheximide and to LeuLysUra drop-out medium with cycloheximide, and grow for 2 d at 30°C.
5. Identify colonies that fail to grow on the medium without Leu and recover them from the plate containing Leu. These probably contain transferred YACs on the YPH925 background and can be confirmed by PFGE and Southern analysis.
6. Select a clone containing the pCGS990-modified YAC of the correct size.

3.3. Transfer of a Second YAC (pRAN4-Modified) to YPH925

1. Culture the AB1380 strain containing the pRAN4-modified YAC overnight in 5-mL Ade drop-out broth, and the YPH925 strain containing the pCGS990-modified YAC in LysUra drop-out broth.
2. Add 100 µL from each overnight culture (approx 1×10^7 cells) to 1-mL YPAD broth. Incubate at 30°C with gentle shaking for 4–6 h.
3. Plate aliquots of 50, 300, and 700 µL onto plates of AdeLysUra drop-out medium supplemented with cycloheximide. Incubate for 5 d at 30°C.
4. Replica plate to AdeLysTrpUra drop-out medium with cycloheximide and to AdeLeuLysTrpUra drop-out medium with cycloheximide, and grow for 2 d at 30°C.
5. Identify colonies that fail to grow on the medium without Leu and recover them from the plate containing Leu. These should now contain both modified YACs in a YPH925 background, and can be confirmed by PFGE and Southern analysis. Select a clone containing both modified YACs of the correct size for further processing.

3.4. Selection for Mitotic Recombination

1. Grow a clone containing both modified YACs under relaxed selection for the *URA3* marker by inoculating into 5 mL of AdeLysTrp drop-out broth and shaking at 30°C overnight.
2. Plate aliquots onto AdeLysTrp drop-out medium containing 5-FOA.
3. After 5 d growth pick multiple individual colonies and analyze by PFGE and Southern analysis. Identify clones containing a novel YAC of the appropriate size (*see* **Note 4**) that also contains one of the parental YACs (*see* **Note 5** and **Fig. 2**).

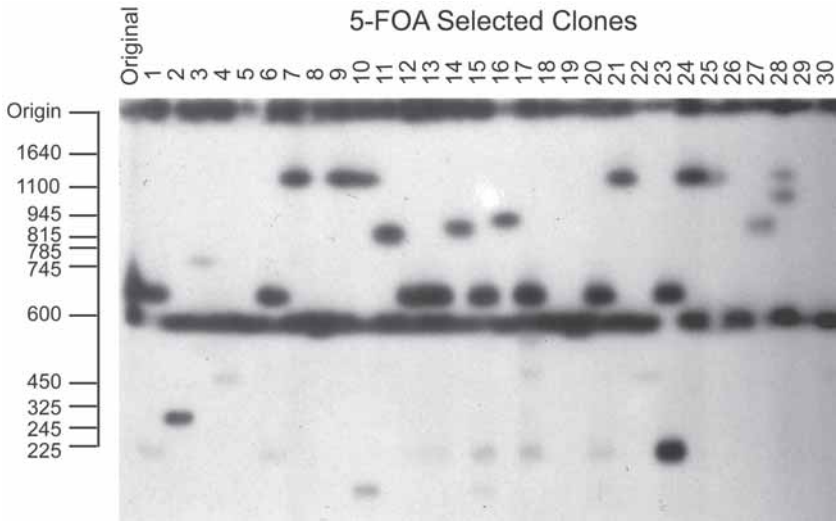

Fig 2. Analysis of yeast artificial chromosomes (YAC) size by pulsed-field gel electrophoresis and Southern analysis after selection for recombination. Two YACs, containing a region of overlap from the human X chromosome, can be seen in the yeast strain prior to selection with sizes of approx 600 and 700 kb, respectively (left lane marked "original"). Among the 30 clones selected with 5-fluoro-otic acid, almost all retain the 600-kb (pRAN4-modified) YAC as expected, but can be classified into three common classes with respect to the size of the second YAC. In the first class (lanes 1, 6, 12, 13, 15, 17, 20, and 23) there is no alteration in YAC size and these clones probably result from events other than recombination that can lead to loss of the functional *URA3* gene, such as mutation. In contrast, lanes 7, 9, 10, 21, 24, and 25 also contain YACs of novel but consistent size, all at around 1100–1200 kb, suggesting that these are the desired clones resulting from recombination within the region of genomic overlap. A third class, represented by lanes 2, 11, 14, 16, and 27 show novel YACs of variable size that probably result from recombination between interspersed repeat sequences. Occasional clones that do not fall into this classification (such as two YACs of novel size in lane 28 and alteration in size of the pRAN4-modifed YAC in lane 23) can be seen and probably result from YAC internal deletions occurring during culture. Conditions for yeast DNA preparation, electrophoresis, and Southern analysis can be found in **ref. *13***. Approximate DNA fragment size is indicated to the left of the image and is based on the size of YPH925 chromosomes. (We thank David Vetrie for the provision of characterized YAC clones from the human X chromosome.)

3.5. Loss of the Parental YAC

1. Grow the selected strain containing the recombinant and parental YAC overnight in 5-mL AdeLys drop-out broth. This step allows the spontaneous loss of the parental YAC from the yeast strain.

2. Make a 10-fold dilution of the culture in sterile water by taking 100 µL of the culture and adding to 900 µL of sterile water (10^{-1} dilution) and repeating this to make a dilution series out to 10^{-6}.
3. Plate 100 µL from each of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions onto plates of AdeLys drop-out medium and grow for 3 d.
4. Select a plate from the series that contains 100–300 colonies and replica plate to AdeLysTrp drop-out medium. Grow for 2 d.
5. Identify the occasional colonies that have lost the parental YAC as demonstrated by failure to grow in the absence of Trp, recover the colonies from the original dilution plate, and confirm by PFGE and Southern analysis. They should contain the recombinant YAC alone. If no further cycles of recombination are required, this is the end point of the procedure.

3.6. Further Cycles

1. If further cycles of recombination are required, it is first necessary to replace the *ADE2* gene on the recombinant YAC with the *URA3* gene. This can be achieved by using the pYAC4 vector (double-digested with *Eco*RI and *Bam*HI) to integratively transform this strain. Grow the recombinant clone overnight in 0.5 mL SD+C broth (containing Trp and Ura supplements), and then continue with the transformation protocol described in **Subheading 3.1.**
2. Plate the transformation on LysUra drop-out plates and grow for 3 d. Identify correctly targeted transformants by replica plating to AdeLysUra drop-out medium, select colonies that fail to grow in the absence of Ade, and confirm they contain a YAC of the correct size by PFGE and Southern analysis.
3. Transfer a new pRAN4-modifed YAC into the recombinant clone using the procedure outlined in **Subheading 3.3.4.** Continue through the procedures outlined in **Subheadings 3.4.** and **3.5.**

4. Notes

1. These are the minimal supplements to cover the nutritional requirements of AB1380 and YPH925. If other yeast strains are used, then alternative supplements may be necessary depending on their particular requirements.
2. The presence of *ARS* and *CEN* sequences in the pCGS990 vector produce a very high background of transformants that are not retrofitted, but result instead from episomal transformation. This necessitates the screening of 100–200 colonies by replica plating to ensure detection of targeted integrants.
3. A suitable probe for detecting all YACs after PFGE and Southern transfer is the pBR322 plasmid.
4. A number of other events can give rise to loss of a functional *URA3* gene apart from mitotic recombination, and there will always be some background of clones without an alteration in YAC size. When the two YACs chosen for recombination contain a significant region of overlap and are well characterized, it should be possible to predict the approximate size of the recombined YAC. However, if the region of overlap is not well characterized, then the recombined YAC size may

not be accurately predicted, but should form the most frequent size class of the 5-FOA resistant clones (*see* **Fig. 2**). In the situation where an attempt is being made to recombine two YACs with no genomic overlap region, then the frequency of clones containing YACs of novel size will be reduced, and the sizes will vary widely. Such novel recombined YACs will need to be characterized to identify one that contains the desired elements from each individual YAC.

5. The parental YAC retained in the recombinant clone should always be the YAC originally modified with the pRAN4 vector.

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12

Targeting Vector Construction by Yeast Artificial Chromosome Modification

Peter J. Murray

Summary

Mouse yeast artificial chromosomes (YACs) are useful platforms for manipulation of targeting vector design and construction, particularly in circumstances where polymerase chain reaction-mediated amplification of targeting arms proves fruitless or the cloned DNA is inherently unstable. This chapter describes a simple procedure where YACs can be modified to produce targeting vectors of various sizes.

Key Words: YAC; ES cell; targeting vector.

1. Introduction

The simplest way to construct a targeting vector for use in mouse embryonic stem cells is to use the sequence information in the mouse genome database as the basis for a long range polymerase chain reaction (PCR) strategy to isolate each arm of the vector. Generally, a targeting vector will contain two arms of lengths 1–6 kb flanking an appropriate selection cassette (e.g., *pgk-neo*). In some circumstances, however, this approach can fail to yield DNA fragments suitable for cloning. Two factors are often the cause: failure of the PCR reaction or an inability to obtain cloned fragments in *Escherichia coli* because of instability. Often these impediments can be overcome by trial and error approaches in PCR primer design and conditions combined with the use of different *E. coli* strains to enhance fragment stability.

An alternative approach is to modify yeast artificial chromosomes (YACs) to obtain a more malleable vector design through the ease of recombination in yeast. YAC modification has a major advantage in targeting vector design: multiple constructs from the same locus can be readily designed and isolated (**Fig. 1**). The YAC containing the mouse gene of interest can be modified to

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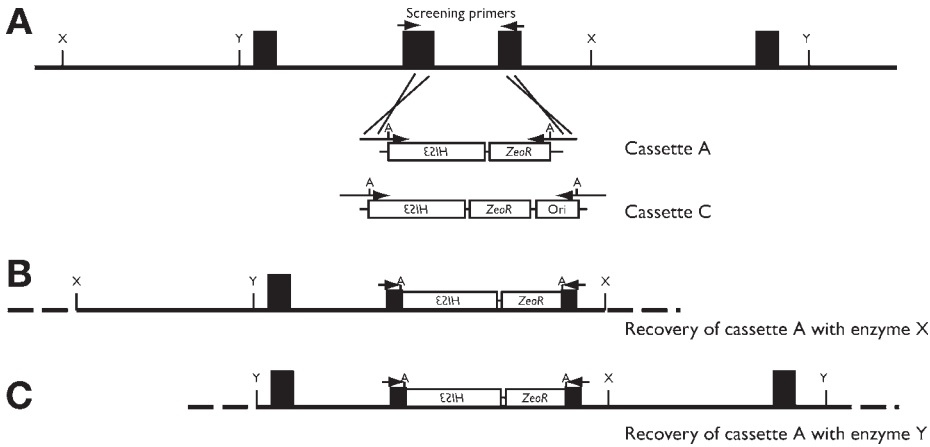

Fig. 1. Insertion of cassettes A or C. Yeast artificial chromosomes are screening using the primers shown; in this case spanning two exons. Cassettes are amplified using primers that contain *Asdc*I sites **(A)** and lie within the screening primers. Following successful insertion of either cassette, the modified locus can be recovered using different restriction enzymes. An example using cassette A and two enzymes (X or Y) is shown. For insertion of cassette C, digestion with X or Y and ligation will lead to a plasmid containing the cassette and two arms for use in targeting experiments.

produce many different arm lengths, and can include additional features, such as *lox*P sites. We have used YACs to create many targeting constructs, particularly for isolating arms that were difficult or impossible to isolate by PCR. The chapter briefly describes the procedure. The main source reference concerning the insertion vectors and overall procedure has been published ***(1)***. Rather than reiterate this information, the focus is instead on the two main methods used to manipulate YACs.

2. Materials

1. YAC libraries: all of our work has used the Whitehead/MIT 820 YAC library constructed from a female C57BL/6 ***(2)***. Originally, this library was arrayed into pools for screening and distributed from Research Genetics. This company was absorbed by Invitrogen Corp. and has discontinued the supply of all components from the library. In principle, however, any YAC library can be used for all procedures described herein.
2. Insertion cassettes: four insertion cassettes were originally described ***(1)***. Here, the focus is on the use of the HIS-zeo (cassette A) and HIS-zeo-ori (cassette C) that are the most useful to obtain arm lengths of various sizes. Modified *pgk-neo* cassettes containing flanking *Asc*I and/or *Mlu*I sites have been described ***(1)***. These cassettes are freely available on request.

3. 1X YPD (used to make a replicate of the YAC plate).
4. 2X YNB lacking uracil (ura), histidine (his), and tryptophan (trp) (the selection media for HIS-zeo insertion).
5. 2X YNB lacking ura and trp. (This media is used for day-to-day growth of the YACs.)
6. 4% Agar (i.e., 2X) to make selection plates with 2X YNB drop-out media.
7. Yeast cells: the genotype of the yeast strain (*Saccharomyces cerevisiae* J57D) carrying the YAC WI/MIT820 library is *MAT a leu3-3,112 ura3-52 trp1 his3-2, -15 ade2 can1*. The YACs are maintained using the *URA* and *TRP* markers encoded on the YAC. We use histidine auxotrophy as the selection marker for the targeting event. For freezing a replicate of the plate, add approx 30 µL yeast to 100 µL YPD and grow overnight at 30°C in an incubator—yeast will grow at the base of the plate. The next day, add 100 µL 30% glycerol (highest quality, made up in Milli-Q water, and filtered) and mix each well gently with a pipet. Cover with adhesive tape and freeze at –70°C.
8. 10X TE: 100 m*M* Tris-HC1, pH 7.5 and 10 m*M* ethylenediaminetetraacetic acid (EDTA).
9. 10X Lithium acetate (LiAc): 1 *M* LiAc, pH 7.5 (adjust pH with acetic acid).
10. 20 mL Lyticase buffer: 3.64 g sorbitol, 50 m*M* Tris-HC1 pH 7.5, approx 10 mg lyticase, and 200 µL β-mercaptoethonol. (Use the cheaper grade Sigma lyticase [L4025] for this application.)
11. Lysis buffer: 1% SDS, 10 m*M* EDTA, and 0.3 *M* sodium acetate, pH 5.5. Add 40 µL proteinase K (20 mg/mL stock).
12. Sheared single-strand (ss)DNA (10 mg/mL herring or salmon sperm DNA).
13. TEL buffer: 50% PEG, 10X TE, and 10X LiAc; freshly prepared.

3. Methods

3.1. YAC Isolation

3.1.1. Primer Design

When preparing screening primers, the major parameters are that they be robust in the PCR reaction and are designed to produce a relatively small size amplicon (~200–500 bp) that can also be applied for screening of the targeting event. An example of a theoretical screening primer pair is shown in **Fig. 1**. In this case, the insertion of the HIS-zeo or HIS-zeo-ori cassettes will occur between primers A and B, but this need not be the case. The insertion of the cassette can span multiple exons if desired. We generally test at least two sets of primers for the screening step. All our PCR reactions are based on an MJ thermocycler with a 96-well format block. We also use TaqGold (Applied Biosystems, Foster City, CA) for all screening procedures.

3.1.2. YAC Pool Screening

There are two ways to screen for the desired YAC clone: first, if the location of the gene is known with some precision, then it is possible to simply order a

set (2–10) of clones that span the region. Select clones that have been doubly linked onto the mouse genetic and physical maps (www-genome.wi.mit.edu/cgi-bin/mouse/index). Order the clones and test them by PCR (*see* **Note 1**). This procedure is very dependent on how well your gene has been mapped relative to the markers in its vicinity. Note that the host yeast strain must be a *his3* mutant for use with the vectors described here. Second, the system of arrayed clones from the WI/MIT genome center allows screening 96-well plates representing frozen yeast clones. The initial screening of the superpool (50 reactions) followed by the plate pools can easily be accomplished in 1 d. There are normally 2–12 positive superpools, each of which gives rise to a further eight PCR reactions to find the plate. There are approx 480 plates in the original library (i.e., 480 × 96 individual YAC clones). Once the plate has been located, order it and follow the instructions on regrowth and freezing a duplicate plate. Make sure you have a frogger for this purpose (a replicator device with 48 pins from Dan-Kar Corp, Woburn, MA).

3.1.3. YAC Isolation

Having frozen a copy of the plates, now grow the yeast on selective media lacking ura and trp. Make a stack of 20 ura–, trp–YNB plates. Using a frogger (Dan-Kar Corp.), plate onto two plates (2 × 48 clones) and allow to grow for approx 48 h at 30°C. Using a multichannel pipet, scrape a small amount of yeast with the tip ends and place in 100 µL water. Screen 2 µL by PCR using the screening assay designed above (*see* **Subheading 3.1.2.**). Note that this assay may require some optimization because whole yeast are the source of template, rather than purified, DNA.

3.2. Cassette Insertion and Targeted Clone Isolation

3.2.1. The HIS-zeo and HIS-zeo-ori Cassettes

A diagram of the cassettes is shown in **Fig. 1**. The HIS-zeo cassette is a fusion of the intact *HIS3* gene from *S. cerevisiae* (Genbank 1420477; Swiss-Prot. P06633), including the endogenous promoter and terminator sequences with the zeocin-resistance gene. The *Zeo* gene was obtained by subcloning from pCMV-zeo (Invitrogen). The *Zeo* gene is driven by the CMV promoter and the EM7 bacterial promoter. The *HIS3* and *Zeo* genes are transcribed in the opposite orientations. It is possible to clone the cassette in either orientation into the region to be targeted, but we generally proceed with the orientation as shown. The HIS-zeo-ori cassette includes an *E. coli* origin of replication to facilitate recovery from targeted YAC-bearing yeast strain and its use is described in **Subheading 3.6.4.**

3.2.2. Amplification of the Cassettes

The central step in the targeting procedure is the amplification of the cassette with overlapping ends homologous to the regions of the gene to be amplified. Generally, the primers should be designed with approx 55–60 bp of homologous sequence; including the regions in the cassette means that the primer length will be approx 80 bp. Primers are resuspended at a final concentration of 20 μ*M* in preparation for PCR. It is important to obtain approx 100 μg of the amplified cassette. Initially, we found it very difficult to obtain PCR product using standard conditions. We found that two variables are critical to the success of the PCR: (1) *Taq* polymerase should be used (e.g., Qiagen) rather than a high fidelity enzyme, and (2) it is crucial to perform a preliminary Mg^{2+} titration. We found that lower Mg^{2+} concentrations allowed the amplification of greater levels of the product (*see* **Note 2**). Generally, Mg^{2+} should be titrated from 0 to 3 m*M*. Examine the products on an agarose gel and chose the best conditions. Then set up a large scale PCR mix and distribute into 10–20 tubes (using a 100-μL reaction volume),

e.g.	1X	10X
10X Buffer	10 μL	100 μL
10X Mg^{2+}	6 μL	60 μL
dNTPs	0.5 μL	5 μL
Cassette A or C	10 ng	1 μL
Primer 1	1 μL	10 μL
Primer 2	1 μL	10 μL
Taq polymerase	2 U	approx 4 μL
H_2O 8	1.5 μL	815 μL

10X Mg^{2+} = 25 m*M*; primers are 20 μ*M*, and dNTPs are 25 m*M* each nucleotide.

Following successful amplification, phenol/chloroform extract the DNA, ethanol precipitate, and resuspended at a final concentration of 1 μg/μL.

3.3. Production of Competent Yeast

Competent yeast are prepared from using a common protocol. Cells are grown overnight and treated with LiAc to induce competence.

1. Grow overnight (~10 mL) culture in YPD at 30°C with shaking or rotation.
2. Dilute to OD_{600}=0.2 in 200 mL of prewarmed YPD and incubate at 30°C until OD_{600} about 0.8. This will take about 4 h.
3. Divide into four 50-mL disposable tubes and spin at room temperature for 5 min on three-quarters speed in small, table top centrifuge.

4. Completely remove supernatant, resuspend each pellet in 5 mL sterile water, and combine.
5. Spin 5 min on three-quarters speed, remove supernatant, resuspend in 10 mL TEL buffer (prepared fresh from 10X TE and 10X LiAc), spin 5 min, and resuspend to a total volume of 2 mL TEL.

The cells are now competent for transformation. It is possible to freeze them for later use by incubating in TEL for 1 h at 30°C in rollerdrum, then add glycerol to 15% (from an 80% stock) before freezing. The efficiency of transformation will drop approximately three- to fivefold after freezing. In practice this should be avoided and we recommend using fresh cells each time.

3.4. Yeast Transformation

1. Add 100 µg of well-sheared ssDNA (10 mg/mL herring or salmon sperm DNA) and amplified cassette DNA to an Eppendorf tube.
2. Add 100 µL of competent cells and mix by pipetting.
3. Add 0.8 mL of 40% PEG-3350 in TEL buffer (*see* **Subheading 2.**, **item 13**) and mix well but gently.
4. Incubate 30–120 min at room temperature (transformation is improved if the cells can be shaken gently).
5. Heat shock for 15 min at 42°C.
6. Spin for 5 s and completely remove supernatant.
7. Wash with 1 mL TE.
8. Spin 5 s, remove supernatant, resuspend in 100 µL TE and plate onto ura–, trp, his– plates and incubate at 30°C for 2–3 d.

For the transformation of the cassette, use 2, 5, or 10 µg of the PCR-amplified DNA from **Subheading 3.2.2.** mixed with 100 µg salmon sperm DNA. The controls that should be incorporated into the transformation include a no DNA control (that includes salmon sperm DNA because this can be mutagenic) and a positive control, such as the intact *HIS3* gene on a high-copy (2 µ) plasmid where approx 1 µg of this plasmid should give approx 100–1000 colonies.

3.5. Isolation of Correctly Targeted Yeast Clones

After 2–3 d of growth at 30°C, there should be between 1 and 20 colonies per plate, depending on the amount of DNA added to the transformation reaction. It is now necessary to characterize these clones to determine if the cassette has been correctly incorporated into the locus of interest. We recommend a two-step procedure at this stage: (1) PCR of the yeast with the HIS or zeo primers ***(1)***, as well as the original screening primers, and (2) Southern blot using a *HIS3* probe and a probe from the region of interest. The first step in screening the his+ yeast clones is to patch each clone onto a fresh ura–, trp–, his– plate and allow the patch to grow for approx 1 d at 30°C. We recommend

picking every colony that grows out, up to about 50 clones. From the patch, place a small amount of yeast using a toothpick into 100 µL of water. Dilute 1:10 and use 3 µL per PCR reaction (as in screening the YAC plates). Set this up in an array in a 96-well plate. Set-up the following three PCR mixes:

1. Primer A + His primer (oCW240).
2. Primer B + zeo primer (oCW357).
3. Primer A + Primer B.

Putative correct clones will give positive signals for the PCR reactions with the His and zeo primers. In contrast, nontargeted clones will still have the original PCR product generated by primers A + B. The locations of the His and zeo primers have been designed to give products of approx 500 bp when primers A and B are close to the site of the cassette insertion.

3.6. Construction of the Vector: Moving From YAC to Bacteria

Transfer of the correctly targeted locus from the YAC to an *E. coli* vector is the critical and potentially most technically demanding part of this protocol. We recommend that particular attention is paid to the restriction sites that you will use to remove the targeted locus. From **Subheading 3.4.**, we normally perform Southern blots with several enzymes that are often useful for locus isolation, with sufficient overhang on either end for good targeting in embryonic stem cells. In practice, many restriction sites can be accurately determined from the current genomic sequence, and thus a wide variety of arm lengths can be selected, increasing flexibility in construct design.

3.6.1. Yeast Genomic DNA Preparation

1. Grow a 50-mL overnight culture of each YAC clone in YNB media lacking ura, trp, and his at 30°C with gentle shaking (~120 rpm).
2. Spin 25 mL of the cells (retaining the other half at 4°C in case DNA isolation fails) in 50-mL tubes (e.g., Falcon).
3. Wash cells with 20 mL water, spin again, and resuspend in 3 mL lyticase buffer. The lyticase will digest away the yeast cell wall leaving vulnerable spheroplasts that can be easily lysed.
4. Incubate at 30°C for approx 1–1.5 h.
5. Spin down spheroplasts using a slow setting on a bench top centrifuge (e.g., ~500 rpm). Note that spheroplasts are delicate and it is important to isolate them intact prior to lysing.
6. Tip off supernatant or aspirate, and resupend in 1 mL lysis buffer. Do this in a 15-mL Falcon tube.
7. Incubate at 55°C for 1 h, preferably with gentle shaking or rocking.
8. Add 3 mL of water. Add 4 mL phenol/chlorform/isoamylalcohol and extract. Spin and take aqueous phase and repeat extraction.

9. Extract once with chloroform.
10. At this stage, the aqueous phase may be cloudy. Add another 2 mL water and this may clarify the solution.
11. Add 5–6 mL isopropanol and mix gently. You should get an impressive precipitate.
12. Spin DNA and wash once with 70% ethanol.
13. Resuspend DNA in 500 µL TE. DNA will take some time to dissolve. Add 5 µL of 10 mg/mL RNase.
14. Quantitate DNA amounts.

3.6.2. Digestion and Conventional Ligation to Recover the Targeted Region

If using Cassette A (His-zeo cassette), the targeted region can be recovered by ligation into a plasmid backbone and recovery of zeocin-resistant clones. If using Cassette C (His-zeo-ori), **Subheading 3.6.4.** describes how to recover the targeted region. Have decided on the restriction enzyme to use, prepare the following:

1. 20–50 µg YAC clone total genomic DNA digested with an appropriate restriction enzyme, phenol/chloroform extracted (also heat inactivate the enzyme), ethanol precipitated, and resuspended to a final concentration of 0.5 µg/µL.
2. Vector DNA digested with the appropriate enzyme. We normally use pbluescript or pGEM vectors that have been digested, treated with shrimp alkaline phosphatase (or calf intestinal phosphatase [CIP]), and gel purified. Resuspend DNA to a concentration of 100 ng/mL.
3. Set up ligations using different ratios of vectors to cut genomic DNA:

e.g.	Vector	g DNA
	100 ng	–
	100 ng	100 ng
	100 ng	200 ng
	100 ng	500 ng
	100 ng	1000 ng
	200 ng	–
	200 ng	100 ng
	200 ng	200 ng
	200 ng	500 ng
	200 ng	1000 ng

4. Perform ligations overnight at 15°C. Use a 10 µL vol if possible.
5. Use electrocompetent *E. coli*, such as DH10B (Invitrogen) because they are prepared in such a way to facilitate the uptake of larger plasmids. The cell should be more than 10^9/µg competent. Zeocin is purchased from Invitrogen. Note that regular LB media inhibits the drug activity because the high salt conditions cause the

copper atom to dissociate from the organic moiety of the drug. Therefore, use the low salt LB formulation recommended by the manufacturer.

3.6.3. Transformation of E. coli and Selection for amp and zeo

Prepare low salt LB amp (100 μg/mL) +zeo (25 μg/mL) plates, approximately five per transformation, as well as regular LB amp plates. Dilute the ligation mixture with water to 90 μL. Electroporate 1 μL of the diluted ligation and allow cells to recover in SOC media and plate 100 μL onto an LB amp plate (this gives the overall efficiency) and the remainder onto four to five low salt LB amp+zeo plates. It is advisable to grow the *E. coli* at all steps from now on at 30°C to reduce the incidence of rearrangements in the large clones. You should see approx 100–2000 colonies on the LB amp plate. The amp+zeo selection will yield 1 to 25 colonies. You can calculate your ligation conditions as follows:

Yeast genome	~13.1×10^6 bp (not including the ~0.8 Mbp YAC)
Average insert	~2.0 kb
Onefold coverage	~6500 colonies
Fivefold coverage	~32,000 colonies

Therefore, in order to cover the genome five times, you would need to see approx 3200 colonies on the LB amp plate (1/10 of the entire transformation). Thus, for more difficult fragments, it may be necessary to perform 5–10 separate transformations. In our experience, we normally always get one or two colonies, even with somewhat poor ligation reactions.

3.6.4. Self-Circularization to Recover the Targeted Region

If Cassette C was used then an origin of replication has been included within the construct.

1. Digest 5 μg total yeast genomic DNA where the insertion site of the HIS-zeo-ori (Cassette C) has been verified by PCR or Southern blotting. Cut DNA overnight in 100 μL total volume. It is important that this reaction goes to completion.
2. Add 200 μL water and 300 μL phenol/chloroform and extract using a phase-lock light gel (Eppendorf).
3. Precipitate DNA with ethanol, spin, and resuspend to 100 μL final volume in TE and quantitate a 1:200 dilution.
4. Ligations: 300 ng digested DNA in 100 μl final volume with T4 ligase overnight at 16°C.
5. Dilute ligation to 200 μL final volume with water and phenol/chloroform extract with a phase-lock gel.

6. Precipitate DNA with ethanol and 1/10 vol 3 *M* Na acetate, centrifuge for 20 min at 14,000 rpm and wash tiny pellets twice with 70% ethanol—this is important to remove as much salt as possible.
7. Resuspend DNA to 10 µL with water. This will be approx 30 ng/µL assuming complete recovery.
8. Transform 1 µL into electrocompetant DH10B and recover at 30°C. Plate 250 µL of the transformation on low salt zeo plates and grow at 30°C. There should be 10–3000 small colonies per plate the next morning.

3.6.5. Swapping in a Neo Cassette for Selection in Embryonic Stem Cells

An original intention of this procedure was to use zeocin selection for both *E. coli* and for embryonic stem (ES) cells. Subsequent experiments showed, however, that zeocin-resistant ES colonies could not be obtained, most likely from the radiomimetic actions of the drug that cannot be overcome by expression of significant enough levels of the zeocin-binding protein, encoded by the *Zeo* gene. As such, we built in a simple system to trade the cassette for a traditional, drug-resistance marker for use in ES cells: the *pgk-neo* cassette (*see* **Note 3**). To do this it is crucial to incorporate *Asc*I sites into the primers that amplify the cassettes. Digestion of the recovered, targeted region in *E. coli* with *Asc*I allows the replacement with *pgk-neo* cassettes (with or without an *E. coli* origins of replication) that were created with *Asc*I or *Mlu*I ends (*Asc*I and *Mlu*I are rare enzymes that have compatible ends).

An alternative approach is to use the recovered, targeted region as the basis for further subcloning. Because the regions recovered depend on the restriction sites used for digestion of the YAC, a tremendous degree of flexibility in terms of arm length can be utilized.

3.7. Insertion of LoxP *Sites*

*Lox*P sites can be easily integrated into this procedure by utilizing the vectors created by Hegemann et al. ***(3)***. These vectors contain the *KanR* gene (selectable with G418 in yeast) flanked by *lox*P sites. The cassette is amplified as previously described for integration into a precise position. A *Cre*-expressing 2-µm plasmid is then introduced to delete the *KanR* gene with subsequent selection on media lacking G418. The YAC-bearing yeast can then be cured of the *Cre*-expressing plasmid by growth in rich media. Additional *lox*P sites can then be introduced in an iterative procedure. We have used this method to create a conditional allele of the *Arginase I* gene, an essential gene in mice.

4. Notes

1. The PCR reaction to find the YAC (**Subheading 3.1.2.**) must work on both genomic DNA and on whole yeast. To date, we have not had many problems with this as long as TaqGold is used.

2. The PCR reaction on the cassettes (**Subheading 3.2.2.**) was difficult to optimize. The PCR reaction is very primer and Mg^{2+} dependent. Different primers will require different Mg^{2+} concentrations that are nearly always in the lower range.
3. Use a YAC that is isogenic for the ES cell to be targeted. In our case we used a YAC library from C57BL/6 and subsequently targeted C57BL/6 ES cells as described **ref.** ***1***. Maintaining an isogenic background of YAC and ES cell is crucial for efficient targeting.
4. In some cases, it may be necessary to perform all manipulations in *E. coli* at temperatures from 16 to 30°C (**Subheading 3.5.3.**). Lower temperatures may be crucial when handling very large recovered fragments.

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13

Production of Yeast Artificial Chromosome Transgenic Mice by Pronuclear Injection of One-Cell Embryos

Alasdair MacKenzie

Summary

The generation of transgenic animals has become an important tool in helping to understand the roles of genes in maintaining health and the roles of human gene variants in contributing to disease susceptibility. In order to model the subtleties of gene expression, which include tissue-specific transcriptional regulation and alternative gene splicing, it is useful to include entire genes that comprise all of the exons, introns, and regulatory sequences required for proper gene expression. A significant proportion of the genes contained in the human genome have been found to span areas of chromosomes that frequently exceed 200 kb. This limits the use of many large cloning vectors, such as P1 artificial chromosomes or bacterial artificial chromosomes, whose upper capacity is around 200 kb of genomic DNA, to be used as transgenes that efficiently model the mechanisms that determine the role of complete genes and their variants in the development of disease. The only cloning vector that can be used to model the mechanisms controlling the expression and functioning of these genes are yeast artificial chromosomes (YACs). In this chapter, a detailed account is given of how YAC DNA can be prepared and purified using pulse-field gel electrophoresis, how this DNA can be introduced into the mouse genome by pronuclear microinjection into one-cell mouse embryos, and how the transgene can be rapidly detected in the resulting transgenic animal by polymerase chain reaction. A brief account of a method of assessing YAC transgene copy number is also outlined.

Key Words: Yeast artificial chromosome; pronuclear microinjection; transgenic mouse; pulse-field gel electrophoresis.

1. Introduction

Natural genetic variation within human populations guarantees that many individuals within these populations will inherit a greater susceptibility to disease. In order to understand how variation within the human population leads to disease susceptibility, we must first understand how the genetic systems

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encoded by the genome operate and how variance within these systems affect disease susceptibility. The components of these genetic systems include protein-coding sequences and the proximal sequences required for proper transcription, splicing, and translation. These components include 3' and 5' untranslated regions (3' and 5' UTRs), splicing donor/acceptor sites, and core promoter sequences ***(1)***. In addition to these proximal elements, proper gene expression in vivo also relies on the presence of essential, but often remote, *cis*-regulatory sequences, such as enhancer, silencers, insulator elements, and scaffold/matrix attachment regions (S/MAR) sequences ***(2)***.

Many genes within the human genome fall into a loose category known as housekeeper genes that are required by every cell in the body and are therefore expressed constitutively. The genomic structure of these genes appears to be comparatively simple with few exons and introns, and with comparatively little requirement for remote or complex regulatory systems ***(3)***. However, a large proportion of genes within the human genome that are also essential for health often contain large numbers of exons that are separated by huge intronic sequences, which often stretch the coding regions of these genes greater than hundreds of kilobases of the genome. In addition, new evidence has shown that many genes that display a complex tissue-specific or inducible expression distribution are controlled by multiple long-range *cis*-regulatory elements often located many hundreds of kilobases away from the transcriptional start sites of these genes ***(4)***. Plasmid-based analysis of these genes can be carried out in cell lines using isolated complementary DNA sequences driven by known promoters, often of viral origin. Although this approach is often very effective in determining the role of a particular splice variant of a gene within an isolated cell line, plasmid-based studies are not able to model critical subtleties in the roles of tissue-specific alternative splicing or transcriptional regulation to the normal function of these genes in living, four-dimensional systems. The limitations of in vitro plasmid-based analyses of gene function and regulation have been addressed by technologies that have allowed the introduction of artificial chromosome constructs into the genomes of transgenic animals ***(5)***. The artificial chromosome constructs that are currently most popular include P1 artificial chromosomes (PACs) and bacterial artificial chromosomes (BACS) that can be isolated and purified with relative ease. However, the manipulation of these PAC/BAC clones is not without problems and the capacity of these clones is limited to around 200 kb ***(6,7)***. The limited capacity of PAC/BAC clones precludes their use for the analysis of a large proportion of the genes in the human genomes whose sizes (including introns, exons, and all essential regulatory regions) frequently greatly exceed 200 kb ***(6,7)***.

Yeast artificial chromosomes (YACs) are currently the largest cloning vectors available and provide researchers with the capacity to amplify well over 1

Mb of foreign genomic DNA within their yeast hosts ***(6,7)***. In addition, YACs can be easily modified using homologous recombination that occurs with high frequency in yeast cells. Furthermore, protocols have been developed that allow YAC DNA to be rapidly purified and microinjected into the pronucleii of one-cell embryos to produce YAC transgenic animals. The ability to reliably manipulate YAC DNA and to produce YAC transgenic animals has opened up huge possibilities for the analysis of gene function and disease progression in living, four-dimensional systems. The following chapter is based on a protocol previously described in the first edition of YAC protocols ***(5)***, but with modifications based on YAC protocols regularly used in our own lab ***(8–10)***. I will also refer to other protocols published elsewhere ***(11)***. This revised protocol allows for the efficient purification and microinjection of YAC DNA into the pronucleii of one-cell mouse embryos to produce YAC transgenic mice.

2. Materials

All of the following solutions should be made up using the highest purity water. We either buy water (Sigma, cat. no. W4502) or use water produced by a well-maintained ultrapure reverse osmosis system that produces 18.2 MΩ purity water (Millipore Milli-Q system). Naked YAC DNA in solution is very susceptible to degradation by heavy metal ions and commonly used laboratory glassware is known to leach ions into solution over time. Consequently, all solutions should be made up and stored using autoclavable plastic measuring apparatus and containers.

2.1. Extraction and Purification of YAC DNA

1. SD medium: for growth of pYAC4 clones in the host AB 1380 this should be supplemented with 10 mg/L adenine, 20 mg/L histidine, 50 mg/L lysine, 50 mg/L isoleucine, and 50 mg/L tryptophan (maintaining selection for the *URA3* gene). For other YAC vectors, hosts, or retrofitted YACs, supplements will have to be altered accordingly (*see* Chapter 1).
2. 50-well disposable plug molds (Bio-Rad, Richmond, CA, cat. no. 170-3713XTU).
3. Bellis buffer: 0.5 *M* NaCl, 0.25 *M* ethylenediaminetetraacetic acid (EDTA), 0.125 *M* Tris-HCl, pH 7.5, 1% sodium dodecyl sulphate (SDS), 2 mg/mL-1 proteinase K (Roche), 0.5 *M* β-mercaptoethanol ***(12)***.
4. Zymolyase-100T (store at 4°C) (ICN Biomedicals Inc).
5. Nusieve GTG low-melting point (LMP) agarose (FMC).
6. β-mercaptoethanol (Sigma, 14 *M* stock).

2.2. Purification of YAC DNA by Pulse-Field Gel Electrophoresis

1. SE: 1 *M* sorbitol and 20 m*M* EDTA, pH 8.0.
2. TENPA: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 100 m*M* NaCl.
3. Microinjection buffer: 10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA, and 100 m*M* NaCl.

4. Nusieve GTG LMP agarose (FMC).
5. GELase™ with buffer (Epicentre Biotechnologies, cat. no. G09200).
6. Dialysis filters: 0.05-μm pore size (Millipore, cat. no.VMWP01300).
7. Pulse-field gel electrophoresis (PFGE) system with refrigerated buffer circulation system (CHEF-DR II) (Bio-Rad).
8. 1X TAE: 40 m*M* Tris-acetate and 1 m*M* EDTA, pH 8.5.
9. Yeast chromosome and λ ladder PFGE markers (NEB, cat. no. N0345S and N0340S, respectively).

2.3. Pronuclear Microinjection of YAC DNA Into One-Cell Embryos

1. Automatic injection system (Narushigi IM-300 Microinjector) and compressor/compressed air source.
2. Zeiss Axiovert inverted microscope with differential interference contrast optics (DIC) and Narashigi micromanipulators. If Femtotips are to be used, a specific adaptor will have to be obtained from Eppendorf.
3. CO_2 water jacket incubator (6% CO_2, 37°C, 100% humidity).
4. M2 (Sigma, cat. no. M7167) and M16 (Sigma, cat. no. M7292) embryo culture media containing penicillin G (100 U/mL) and streptomycin sulfate (50 mg/mL). Prepare 1000X stocks of streptomycin and penicillin in M16 medium (*see* **Note 1**).
5. Femptotips (Eppendorf) (*see* **Note 2)**.
6. Holding pipets (Eppendorf).
7. Microloader pipets (Eppendorf).
8. Light mineral oil (Sigma, cat. no. M8410).
9. 10 mg/mL Hyaluronidase (Sigma, cat. no. H3884) (*see* **Note 3)**.
10. Microinjection buffer: 10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA, and 100 m*M* NaCl.
11. 3-cm Tissue culture dishes (Gibco BRL).
12. 200–300 one-cell mouse embryos (*see* **Note 4**).

2.4. Rapid Recovery and Analysis of Ear Punch Biopsy DNA

1. Three small metal ear punches.
2. Tail tip buffer (TTB): 1% SDS, 0.3 *M* sodium acetate (*see* **Note 5**), 10 m*M* Tris, pH 8.0, 1 m*M* EDTA, 200 μg/mL proteinase K (to be added just prior to digest) (*see* **Note 6**).
3. *Taq* polymerase, *Taq* buffer, primers, and dNTPs.
4. Thermal cycler (Thermo-Hybaid).

3. Methods

3.1. Preparation of YAC DNA Plugs for PFGE

1. Inoculate 500 mL SD medium (containing appropriate supplements depending on auxotrophic requirement of yeast strain to be cultured; *see* Chapter 1) with one yeast colony and incubate for 2–3 d at 30°C with agitation.
2. Prepare a solution of 1% LMP agarose in SE buffer containing 14 m*M* β-mercaptoethanol and keep in water bath at 42°C until required.

3. Harvest cells at 2000*g* for 6 min and wash three times with 50 mL SE buffer.
4. Resuspend final pellet in SE buffer (add 200–300 mL of SE medium) to a final volume of 1.5 mL. The yeast suspension at this stage will be very viscous so a wide-mouthed pipet (2–4 mm) is recommended.
5. Place several disposable Bio-Rad plug formers on ice.
6. Aliquot the cell suspension into 0.5-mL aliquots and keep at 37°C.
7. Dissolve 10 mg of Zymolase 100T in 2.5 mL of the warm LMP agarose solution (*see* **Note 7**).
8. Add 0.5 mL of molten LMP agarose solution (with or without 1 mg/mL Zymolase) to a warm aliquot of the cell suspension and pipet up and down gently several times with a wide-mouth pipet to mix. Keep this mixture at 42°C.
9. Pipet the mixture into the Bio-Rad plug formers and allow plugs to set on ice for 10 min.
10. Transfer the plugs into SE buffer containing 14 m*M* β-mercaptoethanol and 1 mg/mL Zymolase. Incubate for 2–4 h at 37°C. (If Zymolase has not been added this step can be avoided.)
11. Transfer the plugs to 1-mL per plug of Bellis buffer and incubate overnight at 42°C with gentle agitation. Following incubation plugs should become transparent. Plugs can be stored in Bellis buffer in the dark at room temperature for up to 1 yr.

3.2. Isolation of Intact YAC DNA by PFGE for Microinjection

1. Dissolve 1 g of agarose in 100 mL of 0.25X TAE and cast a gel around a gel comb using a Biorad gel-casting apparatus. Tape the middle comb teeth together leaving two wells at each side for loading of PFGE marker plugs (**Fig. 1A**).
2. Wash several plugs (10–12) between four and six times in 50 mL of 0.25X TAE until no further foam is generated by agitation.
3. Load the washed plugs into the large middle lane on the PFGE gel and seal gaps around the plugs with 1% LMP agarose in 0.25X TAE.
4. Run the PFGE gel under conditions optimized to resolve the YAC DNA from the yeast chromosomes. For example, to separate a 380-kb YAC from endogenous chromosomes we ran a PFGE gel at 6 V/cm for 24 h with a pulse ramp of 0.3–30 s (*see* **Note 8**).
5. After the PFGE run, cut away the marker lanes and about 3 mm of the sample lane from the edge of the gel (**Fig 1A**; *see* **Note 9**).
6. Stain the marker lanes with ethidium bromide (EtBr) for 30 min. Destain for a further hour in three changes of water (*see* **Note 10**).
7. View the stained marker lanes under ultraviolet light and mark the edge of the YAC band and the two endogenous chromosome bands immediately above and below with a hypodermic syringe needle dipped in "Quink" ink (**Fig. 1B**).
8. Wrap up the stained lanes with cling film/Saran wrap and reassemble the gel.
9. Locate the YAC bands in the unstained middle portion of the gel and flanking chromosomal bands and excise the three bands using a nonmetal cutting edge (**Fig. 1B**).

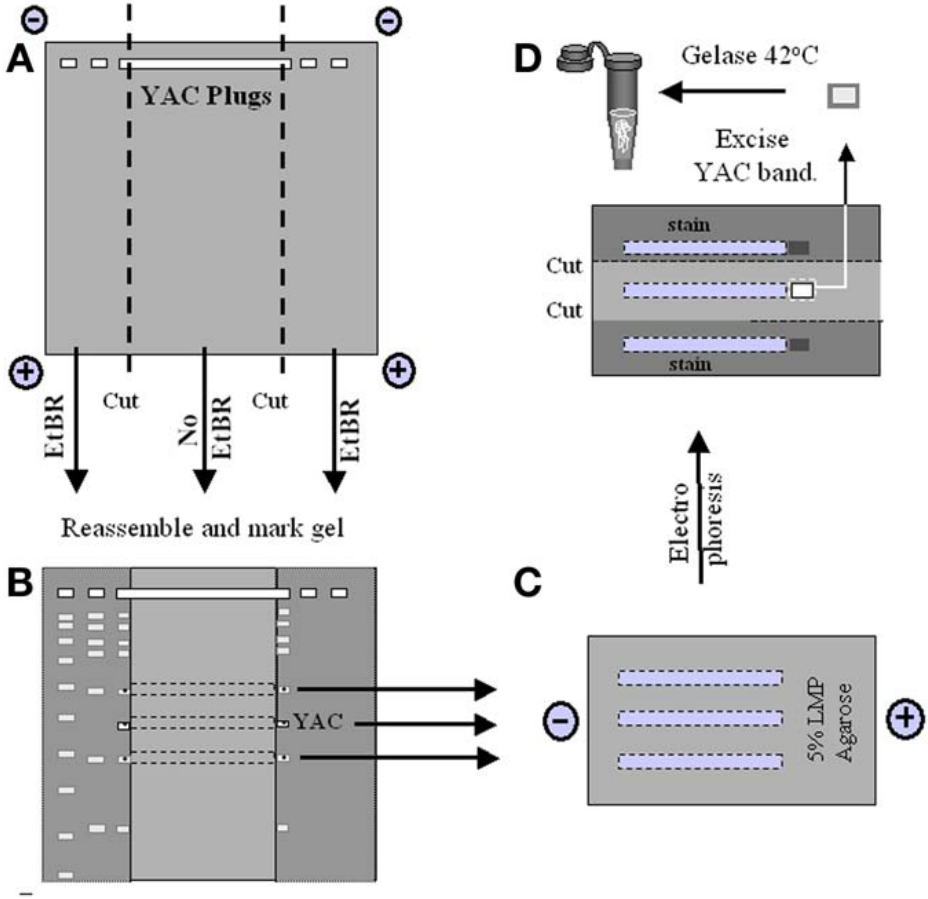

Fig. 1. A diagrammatical representation of the process used to recover yeast artificial chromosome (YAC) DNA from yeast plugs using electrophoresis and Gelase digestion. **(A)** Once pulse-field gel electrophoresis has been carried out on yeast plug marker lanes, a 3-mm portion of the YAC plug lane is excised and stained with ethidium bromide (EtBr). **(B)** The EtBr-stained portions of the gel are washed several times in water and blotted dry. Under ultraviolet light, the positions of the YAC and the endogenous yeast chromosomes below and above the YAC are marked (black dots) and the gel slices are wrapped in clingfilm/saranwrap. The marked and wrapped gel slices are then repositioned with respect to the unstained portion of the gel. Gel slices containing the YAC and endogenous chromosomes are located with respect to the markings in the stained gel slices and excised. **(C)** The gel strips are placed into a taped electrophoresis gel tray with their longest edges parallel to the direction of the current to be applied. A 5% low-melting point (LMP) molten agarose solution is poured around the slices. The slices are then subjected to electrophoresis to concentrate the YAC DNA into the 5% LMP agarose. **(D)** The portions of the gel containing the endogenous

10. Position the gel slices on a minigel tray with the YAC slice in the middle and cast a 4% LMP 0.25X TAE agarose gel around the slices (**Fig. 1C**).
11. Carry out electrophoresis on the YAC DNA at 90°C to the PFGE gel for 6–8 h at 4 V/cm in 0.25X TAE in a cold room if possible (**Fig. 1C**; *see* **Note 11**).
12. Excise and stain the two marker slice lanes with EtBr to assess migration of YAC DNA (**Fig. 1D**).
13. Cut out the concentrated YAC DNA from the corresponding position of the YAC DNA lane (**Fig. 1D**).
14. Equilibrate the gel slice with agitation in 20 mL of TENPA buffer for 2 h.
15. Transfer the agarose block to an Eppendorf tube and melt at 68°C for 10 min with occasional gentle agitation with a wide-mouth pipet.
16. Cool to 42°C, add 2 U of GELase per 100 µL of final gel volume and incubate for 3 h at 42°C with occasional gentle pipeting with a wide-mouth pipet (**Fig. 1D**). Once the supporting gel is digested it is important that the YAC DNA solution receives minimal stirring, centrifugation, or pipeting (except with a wide-mouth pipet) to prevent shearing of the DNA (*see* **Note 12**).
17. Immediately prior to microinjection, the YAC DNA is dialyzed against 40 mL of microinjection buffer for 1 h. This is done by floating a Millipore VM disc on the surface of a Petri dish (glossy side up) half filled with microinjection buffer. The YAC DNA solution is then dropped onto the center of the floating filter using a wide-mouth pipet, and the Petri dish lid is replaced to reduce dust and evaporation. The YAC DNA is left to dialyze for 1–3 h at 4°C.
18. The YAC DNA is now ready for microinjection into the pronucleus of a one-cell embryo (*see* **Note 13)**.

3.3. Microinjection of YAC DNA Into One-Cell Embryo Pronucleii

The following description briefly summarizes the methods used in our lab to microinject YAC DNA into the pronucleii of one-cell mouse embryos. The derivation of mouse one-cell embryos and host females is better described in other excellent texts devoted to the generation of transgenic animals, such as the latest addition of *Manipulating the Mouse Embryo* by Andreas Nagy et al. ***(11)***. The microinjection of YAC DNA is slightly trickier than that of plasmid DNA so we would recommend that the individual carrying out the following procedure has previous experience of pronuclear injection and embryo manipulation. It is strongly advised that licensing and ethical protocols, which vary greatly between different countries, be scrupulously adhered to. In addition, it is also strongly advised that considerations such as animal housing, mouse strain preference, and preferred anaesthetic and analgesic regimes be discussed with a

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Fig. 1. (*continued from opposite page*) chromosomes are then excised and stained with EtBr to assess the degree of migration of the DNA. Again, the YAC DNA should not be exposed to EtBr. If all of the DNA has migrated into the 5% LMP agarose then the area of agarose containing the YAC is excised, melted, and digested with Gelase.

qualified veterinarian or mouse house technician prior to embarking on the following protocols.

1. Recover 200–300 fertilized one-cell embryos from superovulated and mated C57BL6xCBA F1 females into preincubated M16 medium previously equilibrated overnight under oil in a CO_2 incubator (many transgenic facilities have specific mouse strain preferences that differ from those described here).
2. Add 4 µL of hyluronidase solution to 100 µL of M2 medium containing the embryos, and digest away adherent cumulus cells at 37°C for 5 min.
3. Wash embryos by transferring the eggs by mouth pipet between six and eight drops of preincubated M16 under oil.
4. Introduce the YAC DNA solution into an Eppendorf Femtotip (*see* **Note 14)**.
5. Screw the Femtotip into the holder on the side of the microscope stage and set the pressure constant on the Narushige microinjection system to between four and 10 bars (*see* **Note 15**).
6. Transfer 10–20 embryos into a drop of M2 medium contained under oil on a depression microscope slide and position onto the stage of the DIC-inverted microscope.
7. Pick up one of the embryos with the holding pipet and locate a likely pronucleus (*see* **Note 16**).
8. If the pronucleii are large and clearly visible insert the injection needle into the most accessible pronucleus and observe its rate of swelling (*see* **Note 17**).
9. If mortality of the injected embryos exceeds 50% following injection of the first 10 embryos, it is likely that the concentration of the YAC DNA is too high. Dilute the YAC DNA solution by half with injection buffer and repeat **steps 7** and **8**. Keep repeating until embryo mortality is reduced to at least 20–30%.
10. Inject all of the embryos. Oviduct transfer surviving embryos in batches of 16 into both sides of pseudopregnant CD1/MF1/Swiss female mice either on the same day or the following morning following overnight incubation of the embryos to the two-cell stage (*see* **Note 18**).

3.4. Rapid Analysis of Ear Punch Biopsy DNA

Many governing bodies around the world have recently taken steps to phase out the genotyping of live rodents using either toe or tail tip biopsies. As a result, we have recently developed a rapid and efficient protocol to genotype YAC transgenic animals using ear punch biopsies. Ear punching is a standard method used in many countries to visually identify animals. Therefore, in addition to being less stressful to the animal, ear punch biopsy also has the added benefit of not constituting a licensed procedure in countries such as the United Kingdom.

1. Following weaning of pups (6–8 wk after YAC injection) mark each pup using an ear punch and transfer the piece of ear tissue to a clean 0.5-mL Eppendorf tube (*see* **Note 19**).

2. Add 100 μL of TTB with proteinase K to the biopsy and incubate overnight at 52°C.
3. The next day, vortex the digest and freeze at –20°C for 30 min.
4. Spin the sample at 13,000*g* for 10 min at 4°C. The white precipitate at the bottom of the tube consists of precipitated SDS crystals and undigested biopsy material (*see* **Note 20**).
5. Remove 5 μL of the supernatant and dilute in 500 μL of TE or water. Heat the DNA solution to 95°C for 10 min to ensure inactivation of any residual proteinase K.
6. Carry out standard polymerase chain reaction (PCR) procedure on 1 μL of this diluted DNA solution.

In addition to allowing the identification of YAC transgenic animals, analysis by PCR also allows a detailed characterization of the integrity of the YAC DNA within the transgenic mouse genome. Thanks to the accessibility of the genome sequence of different species, PCR primers can be designed to amplify specific sections of YAC DNA (*see* Chapter 5). It is also important that the transgenic status of the F1 and F2 generation be analyzed also to ensure that the YAC transgene is transmitted appropriately.

Fluorescent *in situ* hybridization analysis on blood samples derived from transgenic animals can be carried out as described in Chapter 16 using the original YAC clone DNA as probe to determine the numbers of YAC integrants and their genomic position.

The number of copies of the YAC can be assessed by Southern blotting as described in Chapter 4 with the following modifications. Briefly, once a YAC transgenic line has been established, spleen samples are recovered from several individuals, homogenized, and genomic DNA extracted (Qiagen DNeasy kit, cat. no. 69504). If the genome sequence of the organism from which the YAC is derived is known, then restriction enzymes flanking the area of the YAC of most interest (i.e., a coding sequence) can be chosen as described in Chapter 5 and used to digest 10 μg of genomic DNA. It is also useful if the chosen enzymes flank an endogenous mouse gene (not necessarily the homolog) to give a recognizable restriction fragment of a slightly different size. Prior to undertaking electrophoresis of the digested samples it is useful to load in parallel dilute aliquots of known concentration of the DNA samples to be used as probes. These probes should include one specific for an endogenous mouse gene and one specific for a sequence within the YAC. Ideally, both probes should be a similar size. Ten micrograms of digested YAC transgenic mouse spleen DNA are then separated out on a standard electrophoresis gel and Southern blotted as described in Chapter 4. Once the blot is hybridized to a mixture of both probes (endogenous mouse gene and the YAC-labeled probes can be labeled in the same reaction using radioactivity or nonradioactive methods) and washed, signal intensities on the blot are detected using a phosphoimager

and densitometry is carried out on signals on the blot. Differences in labeling of both the endogenous and YAC-specific probes can be normalized by comparing the different intensities of labeling from the diluted probe sample on the blot. Normalization must also take into account the different sizes of each probe, i.e., an equimolar solution of a 4-kb probe will give twice the signal intensity of a 2-kb probe if hybridized to target DNA fragments of the same size. Labeling intensities of probe directed at the endogenous target sequences (of which two copies exist) and the YAC sequence from the digested DNA can then be compared in order to deduce transgene copy number.

4. Notes

1. Following addition of penicillin and streptomycin mix, filter-sterilize M2 and M16 media using Millipore filter units into sterile plastic bijous. Discard the first 5 mL filtered of each medium, as many filter units contain filter preservatives that may be toxic to embryos.
2. Injection pipet tips can be made for a few pennies in house using a Browning pipet puller commonly found in many laboratories. Although expensive, we obtain consistently better embryo survival rates following pronuclear injection using Femtotips.
3. Make up in 50-µL aliquots in M2 medium and freeze.
4. Most often derived from superovulated 4–6 wk old C57BL6xCBA F1 mice, although embryos from other strains are frequently used ***(11)***.
5. It is critical that the sodium acetate is adjusted to pH 7.0 with 5 *N* NaOH.
6. Keep TTB in small aliquots to reduce the chances of accidental DNA contamination.
7. Zymolase is very expensive and if costs are a concern then addition of zymolase can be dispensed with.
8. We also recommend using a refrigerated buffer circulation system that maintains the temperature of the gel at 14°C.
9. We use plastic edges (i.e., a sharpened piece of a broken Petri dish lid) to avoid the possibility of contaminating the solutions with metal ions from scalpel blades.
10. It is vital that the YAC DNA to be recovered does not come in contact with EtBr or ultraviolet light, as this will cause unacceptable shearing of the clone.
11. It is best to have a dedicated minigel apparatus for this procedure that has not been exposed to EtBr.
12. Prior to **step 17**, some researchers undertake a stage of purification and concentration that involves the concentration of the YAC DNA using a Millipore or Microcon 30 microconcentration column ***(11)***. This stage may be useful because it removes the products of the agarose digestion that may render the YAC solution more viscous and compromise embryo survival. However, it is advised that the integrity of the YAC DNA be assessed by analysing an aliquot of YAC DNA solution by PFGE prior to microinjection.

13. The YAC DNA must ideally be injected within 1 wk of purification because the solution cannot be frozen and must be kept in the dark at 4°C to reduce fragmentation.
14. Two methods have been tried successfully to introduce DNA into Femtotips. The first involves the transfer of YAC DNA solution to the needle using an Eppendorf microloader pipet. When using these pipets it is often useful to blow a blast of compressed air into the pipet to dislodge any dust. Between 5 and 10 μL of YAC DNA are gently drawn up into the microloader. The end of the microloader is then inserted into the blunt end of an Eppendorf Femtotip and gently deposited at the end of the needle. This method may cause shearing of the YAC. Alternatively the YAC DNA solution can be introduced by inversion of the Femtotip into the DNA solution to allow capillary action to draw up the YAC DNA solution. Pronuclear injection can proceed once 2–4 mm of solution has been drawn to the top of the needle.
15. A constant low-pressure flow of DNA solution reduces the chances of accidental "suck back" of medium into the Femtotip and subsequent injection of culture medium as opposed to DNA solution.
16. Ideally, pronucleii should be roughly a fifth to a quarter of the diameter of the embryo prior to injection. If the pronucleii are only just visible or are less than one-tenth of the embryo diameter, then it is advised to return the embryos to the incubator for another couple of hours because microinjection will be difficult and largely ineffective.
17. If swelling of the pronucleus is slow, then withdraw the injection needle and gently jab its tip against the holding pipet until a piece breaks off, thus, widening the needle and increasing the flow of YAC DNA. Reintroduce the needle tip and again observe the swelling. The DNA solution should cause the pronucleus to swell to two times its normal size within 10 s of the introduction of the needle.
18. Although more demanding in terms of time, we have found that higher numbers of pups are born following same-day transfer.
19. To reduce the chances of cross-contamination, flame the ear punch between pups by dipping in 70% ethanol and holding the punch in a naked Bunsen flame with long forceps until all the alcohol has burned off. We use three ear punches to ensure one is cooled sufficiently following flaming.
20. If the digest is spun at room temperature, the SDS will not precipitate and the supernatant may not be sufficiently pure to allow the PCR process.

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14

Generation of Yeast Artificial Chromosome Transgenic Mice by Intracytoplasmic Sperm Injection

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Summary

Genomic-type transgenes are usually expressed in appropriate spatial- and temporal-specific manners. The largest genomic transgenes can be prepared using yeast artificial chromosomes (YACs). Normally, YAC transgenic mice are produced by standard pronuclear microinjection, although other methods, involving the use of embryonic stem (ES) cells, have been also devised. To overcome the difficulty and time extension of ES cell-type approaches and to improve the rather usual low efficiency of YAC DNA transgenesis by pronuclear microinjection, that is mostly dependent on the YAC DNA quality of samples, we have devised an updated intracytoplasmic sperm injection (ICSI) method for the stable incorporation of YACs into the germ line of mice. DNA transgenesis efficiencies achieved are often 10 times greater than those usually obtained by standard microinjection, thus enabling the identification of either more transgenic founder animals and the use of reduced numbers of individuals in animal experimentation.

Key Words: Artificial chromosomes; intracytoplasmic sperm injection (ICSI); in vitro fertilization; sperm-mediated gene transfer (SMGT); transgene integrity; artificial chromosomes; assisted reproductive technology (ART).

1. Introduction

The use of yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and P1-derived artificial chromosomes (PACs) in animal transgenesis has allowed the identification of new genes by complementation of mutations, the systematic characterization of fundamental regulatory sequences within expression domains that are crucial for faithful expression of genes and, more importantly, the design and generation of improved genetically modified animal models for a vast number of human genetic diseases ***(1)***.

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Because of their extraordinary cloning capacity (in excess of 1 Mb), YACs have been the most preferred eukaryotic cloning vector for manipulating large genomic DNA segments. The use of large genomic-type transgenes (such as YACs) has proven useful to reproduce the expression pattern of the gene of interest, because their size normally guarantees the inclusion of most, if not all, of the regulatory elements that are relevant for the correct expression of the gene ***(1,2)***.

Several methods have been described to generate transgenic mice carrying YACs, most notably standard pronuclear injection of fertilized-oocytes ***(3,4)***, but also yeast sphaeroplast fusion with mouse embryonic stem (ES) cells ***(5)***, or lipofection of YAC DNA lipid-coated molecules (liposomes) into ES cells ***(6)***. The two ES cell-mediated methods can be difficult and time-consuming ***(1)***, whereas the microinjection of YAC DNA into fertilized oocytes is also not free of problems, because low transgenic efficiencies can be observed.

The use of DNA-coated sperm as a vehicle for animal transgenesis was a matter of debate ***(7–9)*** until recently, when a reproducible method involving sperm cells with damaged membranes by freeze–thaw cycles or exposure to detergents was eventually reported ***(10)***, and the interest in intracytoplasmic sperm injection (ICSI) techniques was rediscovered. This method was called "metaphase II transgenesis (MII)" because it involved the use of nonfertilized metaphase II oocytes as recipients for the injection of membrane-damaged sperm cells combined with DNA and resulted in the generation of transgenic mice at high efficiency ***(10)***. To explore the limits of this method, several transgene archetypes were assayed and it was initially possible to generate transgenic mice carrying BACs or small mammalian artificial chromosome (MAC) (ranging from 12 to 170 kb), although the integrity of these first genomic constructs could not be directly demonstrated ***(11)***. Recently, we have improved the ICSI method with the modifications that are detailed below, in **Subheading 3.** (methods section), thus, allowing the generation of YAC transgenic mice by ICSI ***(12)***, at higher efficiencies (35%) than those reported with pronuclear injection methods (<5%) ***(1,2)***. We have also proven the integrity and functionality of a YAC genomic construct of 250 kb in size, carrying the mouse tyrosinase locus and, hence, supporting the rescue of the albino phenotype of recipient mice ***(12)***. Furthermore, we have been able to expand the limits of this technique with the efficient generation of transgenic mice carrying a 520-kb YAC by ICSI ***(13)***.

We call our improved technique ICSI-mediated YAC transfer IMYT ***(12)***. With the use of IMYT we believe that the use of large genomic constructs, such as YACs, in standard mouse transgenic experiments can be further expanded and popularized. In addition, this technique should allow the use of large genomic transgenes in other species, vertebrates and invertebrates, where pronuclear injection has been historically difficult or less efficient.

The generation of transgenic mice by IMYT involves several steps:

1. Initial isolation and purification of YAC DNA.
2. Gamete collection.
3. Sperm freezing.
4. Sperm/YAC DNA transgene mixing.
5. ICSI and embryo micromanipulation, culture, and transfer into recipient pseudopregnant females.

A detailed protocol describing all these different steps is provided next, in **Subheading 3.**

2. Materials

1. Pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet, Boxmeer, Holland). This hormone comes in a lyophilized form, in ampullae of 500 IU or 1000 IU. For its preparation, it is dissolved in 10 mL of saline physiological serum (0.9% NaCl), for a final concentration of 5 IU per 0.1 mL. Aliquots can be stored in Eppendorf tubes at –80°C for up to 3 mo (*see* **Notes 1** and **2**).
2. Hormone chorionic gonadotropic (HCG Leporin, Farma-Lepori, S.A., Barcelona, Spain). This hormone comes in a lyophilized form, in ampullae of 1500 IU. For its preparation it is dissolved in 20 mL of saline physiological serum (0.9% NaCl), for a final concentration of 7.5 IU per 0.1 mL. Aliquots can be stored in Eppendorf tubes at –80°C for up to 1 mo (*see* **Notes 1** and **2**).
3. M2 medium (Sigma, Madrid, Spain; cat. no. M7167) does not require preparation. It should be warmed to 37°C before use. A 50-mL bottle can be stored at 4°C for up to 15 d. In order to avoid protein precipitation from repeated warming and cooling only the required volume should be warmed.
4. KSOM Medium (potassium simplex optimized media, Specialty Media, Piddington Northampton, UK; cat. no. MR-020P-D) does not require preparation. It should be equilibrated at least for 2 h before use at 37°C in a 5% CO_2 air atmosphere. A 50-mL bottle can be stored at 4°C (or at –20°C if aliquoted) for up to 3 wk. Repeated freezing and thawing causes salt precipitation. A thawed aliquot should not be stored again.
5. Hyaluronidase type IV: from bovine testes (embryo tested) (Sigma, Madrid, Spain; cat. no. H4272). One vial of hyaluronidase in powder should be reconstituted with 3 mL of M2 medium to obtain a 10 mg/mL stock solution. The stock solution should be diluted with M2 medium to obtain a final working concentration of approx 300 μg/mL. This enzymatic solution can be stored in aliquots of 500 μL at –20°C until use (*see* **Note 3**).
6. Polyvinyl-pirrolidone (PVP; Sigma; cat. no. PVP-360). In order to prepare a 10% working solution, 200 μg of PVP should be layered on top of 2 mL of M2 medium and, placed in a 35-mm Ø Petri dish without agitation for 2 d at 4°C. The final solution can be used for up to 15 d if stored at 4°C. Sterility should be a concern throughout the procedure. In order to avoid evaporation and salt concentration, the Petri dish used to dissolve PVP should be sealed with Parafilm.

7. Mineral oil (Sigma, cat. no. M8410A) It should be stored at room temperature. The mineral oil should have minimal exposure to light because it induces a toxic molecular breakdown.
8. Mercury (Merck, Briare Le Canal, France; cat. no. 25 359.188). This product is highly toxic. Skin contact and inhalation should be avoided. It vaporizes.
9. Borosilicate glass capillaries (Kwik-Fil, Sarasota, FL; cat. no. TW100-4). The hardness of these glass capillaries and their inner and outer diameter (0.75 and 1.0 mm, respectively) are crucial features for the elaboration of efficient microinjection tools.
10. Stereoscope (Nikon SMZ 1500, Kanagawa, Japan).
11. Pipet Puller (Model P87, Sutter Instrument Co., Novato, CA).
12. Microforge (MF-900, Narishige Scientific Instruments Lab, Tokyo, Japan).
13. Inverted microscope (Nikon TE Eclipse 2000S) with Hoffman modulation contrast. Please note that microinjection is usually done on a plastic lid that does not allow the use of polarized light and consequently DIC (direct interference contrast) lenses. Direct interference contrast lenses can be used with a glass microinjection dish.
14. Micromanipulators (Eppendorf TransferMan NK 2, Hamburg, Germany).
15. Microinjectors (Eppendorf Cell Tram Vario). These microinjectors are extremely efficient for all microinjection work requiring the use of mercury-loaded injection glass capillaries. They also allow extreme but careful control as well.
16. Piezo impact unit (PMM150FU, Prime Tech, Ibaraki, Japan). Although this equipment is relatively expensive, this microinjection instrument made easy several embryo manipulation procedures that were only at reach of few gifted microinjectionists. It is a fundamental piece of equipment, especially for mouse embryo manipulation.

3. Methods

3.1. Initial Isolation and Purification of YAC DNA

A number of different methods have been described to prepare high-quality YAC DNA suitable for the generation of transgenic mice ***(4,13–18)***. In the first edition of *"YAC Protocols,"* a standard method was described ***(14)***. Subsequently, this method, has been updated and slightly modified ***(15,16)***, eventually resulting in a collection of protocols for the preparation of YAC DNA for pronuclear microinjection that are used routinely in the laboratory and have been included in the third edition of the *Manipulating the Mouse Embryo* book ***(17–19)***. A detailed YAC DNA purification protocol can be directly downloaded from the following web site: http://www.cnb.uam.es/~montoliu/prot.html.

The generation of YAC transgenic mice by ICSI ***(12)*** has strictly followed protocols in the previously mentioned web site using ultrafiltration units (Millipore Ultrafree MC 30,000) instead of the classical two-gel approach ***(4,13)***. Ideally, the YAC DNA preparation should be within the range of a

DNA concentration between 5 and 40 ng/mL (*see* **Note 4**), once the YAC DNA sample has been purified and dialyzed in microinjection buffer containing polyamines ***(16–18)***. It is also highly recommended the use of freshly made YAC DNA preparations, within 1–2 wk (in any case less than 1 mo old), stored at 4°C. Older preparations could show DNA precipitates and aggregates that will interfere with further manipulation of DNA samples, especially microinjection or proper mixing/adherence with sperm cells.

3.2. Gamete Collection

1. Collect unfertilized MII oocytes 14 h post-hCG administration, from 6 to 8-wk-old female mice (*see* **Note 5**) superovulated with 5 IU of equine chorionic gonadotropin, followed 48 h later, by an equivalent dose of hCG. These hormones are usually diluted with saline solution to the concentration of 5 IU per 0.1 mL (the volume injected with a 26-gauge needle into the peritoneal cavity of each oocyte donor) (*see* **Note 1**). Aliquoted hormones are stored at –20°C, and thawed at room temperature when needed (*see* **Note 2**).
2. Sacrifice superovulated females by cervical distension, remove their oviducts and place them on a 35-mm Ø Petri dish containing 2 mL of M2 medium prewarmed at 37°C.
3. Swirl the oviducts gently to remove blood, adipose cells, and other contaminating particles and placed them into fresh M2 medium. Cumulus masses are released in clean M2 medium by inducing the rupture of the oviductal ampula with sterile forceps (Dumont, no. 3).
4. Cumulus masses are gently picked up with a glass pipet (*see* **Note 6**) and placed into a 300-µL dispersion drop. Cumulus cells are dispersed by a 3- to 5-min incubation (*see* **Note 3**) at 37°C in M2 medium containing 350 IU/mL hyaluronidase.
5. Dispersed oocytes are picked up with a glass pipet of sufficient diameter, washed twice in 2 mL of warm M2 medium (*see* **Note 7**), and placed in KSOM medium at 37°C in a 5% CO_2 air atmosphere until use (*see* **Note 8**).

3.3. Sperm Freezing

Freeze–thawing sperm is prepared essentially as described in **ref. *20*** with minor differences.

1. Epididymal sperm from mature (3–6 mo old) males (*see* **Note 9**) is collected at room temperature in M2 medium by excising cauda epidydimis and vas deferens with a pair of fine scissors, and removing as much excess fat as possible. Gently squeeze out the sperm using forceps (Dumont, no. 5) (*see* **Note 10**).
2. The collected sperm cells, in a minimal volume, are placed with a sterile pipet tip in the bottom of a 1.5-mL polypropylene centrifuge tube and overlaid with the volume of fresh M2 medium necessary to obtain a final concentration of 2.5×10^6 sperm cells/mL. The sperm extender used (M2 medium) does not contain Ca^{2+} chelating agents, such as ethylenediaminetetraacetic acid (EDTA) or ethyleneglycol *bis* (β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA; *see* **Note 11**).

3. After gentle mixing, 70–100-µL aliquots of the sperm suspension are transferred to cryogenic 1.5-mL vials (NUNC), tightly capped, and directly placed into liquid nitrogen (*see* **Note 12**).
4. Sperm samples are stored for periods ranging from 1 d to 4 wk at –75°C (*see* **Note 13**). Asepsis should be maintained throughout the procedure.

3.4. Sperm/YAC DNA Transgene Mixing

1. Thaw an aliquot of the sperm sample at room temperature.
2. Carefully mix equal volumes (usually 4 mL) of thawed sperm in M2 and approx 4 ng/mL YAC DNA and keep the mixture on ice for 2 min before being mixing it with 40–50 mL of a 10% polyvinyl-pyrrolidone (PVP; (Mr 360,000) in M2 solution. Finally, place a drop of the final solution on the culture dish for microinjection (*see* **Note 14**).

3.5. ICSI and Embryo Manipulation

1. ICSI-mediated YAC transfer (IMYT) with frozen–thawed spermatozoa is performed in M2 medium at room temperature (*see* **Note 15**). As previously mentioned, 1 vol of sperm-YAC solution should be mixed with five of M2 medium containing 10% PVP to decrease stickiness of YAC DNA-coated sperm cells within the injection pipet. The microinjection dish used for ICSI should contain, under mineral oil, a manipulation drop (M2 medium) (*see* **Note 16**), a sperm-YAC drop (sperm-YAC solution in M2/10% PVP), and a M2/10% PVP needle-cleaning drop.
2. Injections are performed with a piezo drill-impact unit using a blunt-ended mercury-containing pipet with 6–7 µm of inner diameter (*see* **Note 17**). Mercury can be placed inside the injection pipet with the help of a small gauge plunger. After placing the needle in the injection arm, the mercury should be pushed with the microinjector to the tip of the injection pipet until small mercury drops are released into the cleaning drop. The tip of the injection needle should be filled with M2/10% PVP needle-cleaning medium, while maintaining the mercury meniscus visible at ×100 (*see* **Note 18**).
3. Individual sperm heads decapitated by the freeze/thaw procedure or mechanically separated with the piezo drill are co-injected with adherent YACs into oocytes. When tail removal is necessary, frozen–thawed spermatozoa are aspirated, tail first, into the injection pipet and decapitated by the application of piezo-pulses in its neck region with strong intensity (setting 13 or higher). The tail is released into the injection drop, and the isolated YAC-coated sperm head, is picked to be injected into the oocyte (*see* **Note 19**). Zona-drilling prior to ICSI is accomplished by piezo pulses of strong intensity (setting 13 or higher) at the 3 o'clock position, after rotating the metaphase plate (*see* **Note 20**), or the polar body (when the first is not clear), to the 12 or 6 o'clock position (*see* **Fig. 1**). A piece of the zona pellucida is usually expelled into the perivitelline space before bringing the sperm head to the tip of the injection pipet. The injection pipet is then deeply inserted

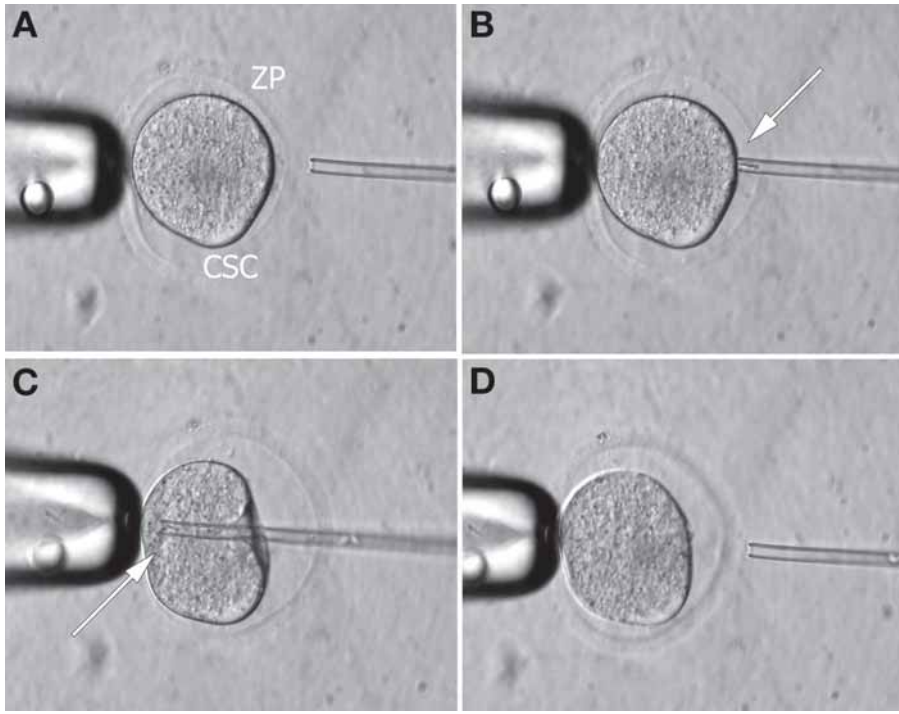

Fig. 1. Mouse intracytoplasmic sperm injection with frozen–thawed spermatozoa. Four to five sperm heads released during sperm freeze–thawing are picked to be injected into (MII) metaphase II oocytes. Zona-drilling is accomplished by piezo pulses of stronger intensity at the 3 o'clock position, after rotating the metaphase plate, or the polar body (when the first was not clear), to the 12 or 6 o'clock position **(A)**. A piece of the zona pellucida is usually expelled into the perivitelline space before bringing the sperm head to the tip of the injection pipet **(B)**. The injection pipet is then deeply inserted up to three-quarters of the oocyte, after which, the oolemma is broken by the application of a single low-intensity piezo-pulse **(C)**. The sperm head is released with the minimal amount of medium possible, before withdrawal of the injection pipet **(D)**. Arrows indicate a sperm head. CSC, chromosome–spindle complex; ZP, zona pellucida.

into the oocyte (around three-quarters of its total diameter), after which, the oolemma is broken by the application of a single low-intensity piezo-pulse (usually setting two or three). The sperm head is released with the minimal amount of medium possible, before withdrawal of the injection pipet (*see* **Note 21**). Oocytes are usually injected in groups of 10 in order to reduce the period of suboptimal conditioning. After 15 min of recovery at room temperature (*see* **Note 22**) in M2 medium, surviving oocytes are returned to mineral oil-covered KSOM

and cultured at 37°C in a 5% CO_2 air atmosphere. For full term development, 24 h later, both one- and two-cell embryos are transferred to the oviducts of recipient females (*see* **Note 23**).

Details on the embryo transfer procedure are described elsewhere ***(20)***. CD1 females mated with vasectomized CD1 males, are normally used for surrogate mothers for embryo-transfer experiments. Lactating CD1 foster mothers are occasionally used to raise pups. The presence of YAC DNA among founder mice (F_0) can be subsequently investigated by (PCR) polymerase chain reaction, Southern blot, and slot analyses. It is very important to use a number of DNA probes spanning the entire YAC transgene to identify all the entire length of our YAC construct within the mouse host's genome.

For all IMYT experiments, mice are fed *ad libitum* with a standard diet and maintained in a temperature and light-controlled room (23°C, 14 h light: 10 h dark). All these animal experiments should be performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines, and in adherence with the established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

4. Notes

1. The injection of a volume greater than to 0.2 mL per female mouse is not recommended.
2. Hormones should not be thawed at temperatures higher than 37°C because they can loose activity. Thawing at room temperature ensures safety of the procedure.
3. Repeated freezing and thawing of the hyaluronidase solution causes activity loss. Long exposures to this concentration of hyaluronidase can induce oocyte activation, poor development after microinjection and, in extreme situations, can result in oocyte death. For these reasons, extensive oocyte washing from hyaluronidase is highly recommended.
4. Starting from highly concentrated YAC DNA solutions (20–40 ng/μL) will allow subsequent dilution steps of YAC DNA in buffer, thus titrating out co-purifying toxic agents that may interfere with embryo survival.
5. MII oocytes from outbred CD1 mice and hybrid mouse strains (i.e., B6D2F1) give usually better results than those obtained from inbred mouse strains (i.e., C57BL/6J).
6. Cumulus masses should not be forced through the opening of the glass pipet used. Its diameter should allow easy flow of these masses to avoid any oocyte damage.
7. For the motives previously explained (risk of oocyte activation upon long hyaluronidase exposure) an extensive oocyte wash in clean M2 is highly recommended.
8. Oocytes should be transported/kept in KSOM medium with minimal amounts of M2 medium in order to avoid pH alteration.

9. Sperm from outbred CD1 mice and some hybrid mouse strains (i.e., B6D2F1) give usually better results than those obtained from inbred mouse strains (i.e., C57BL/6J) or other hybrid mouse strains (i.e., C57CBAF1) ***(12,20)***.
10. Tissue-free epididymal caudae should be washed at least twice in 2 mL of M2 medium in order to extensively remove adipose cells, which are the main contaminators of the sperm cell-collection medium.
11. It has been demonstrated that calcium chelating agents protect against DNA fragmentation ***(21)***. To a certain extent, IMYT relies on DNA fragmentation for the integration of such large YAC DNA molecules into the host genome.
12. Complete immersion of cryogenic vials should be avoided because internalization of liquid nitrogen has been shown to compromise embryo development ***(20)***, possibly because it induces extremely high levels of sperm DNA fragmentation.
13. When frozen sperm cells are stored for longer periods, a gradual decrease in embryo development should be expected as result of the gradual deterioration of stored sperm samples.
14. It is recommended that at least a 2-min incubation is conducted for proper contact and adherence between YAC DNA molecules and sperm heads. It is of crucial importance to work on ice and that pipets of wide or cut-off tips are used to manipulate and mix YAC DNA molecules with sperm cells to avoid shearing of these large DNA molecules.
15. Fertilization should be done preferably within 120 min of sperm–DNA mixing, since because sperm freezing and thawing releases endonucleases that will degrade sperm DNA, as well as, any surrounding DNA, such as the YAC DNA used in the procedure.
16. An extra M2 drop is recommended as a backup microinjection drop if for some reason in the first M2 drop it becomes difficult to work (contamination with mercury, PVP, sperm cells, and so on).
17. The tip of the injection pipet should be as sharp as possible. The sharpness of the injection pipet can only be defined on a microforge with a magnification of at least ×400.
18. Air bubbles should be avoided in the injection needle and in the hydraulic system attached to the microinjector because they considerably reduce the efficiency of the piezo drill, and the microinjector.
19. For best results, sperm heads should not rest inside the injection pipet for more than 5 min.
20. The metaphase plate can be identified in mouse oocytes by a translucid and peripheral area, some times prominent as well. Injection should be avoided in this area as it can induce damage to the metaphase plate and, in addition, it is usually an area where membrane healing is poor.
21. The intensity of the piezo pulse used to penetrate the oolema and the volume of injected fluid should be the smallest possible in order to reduce oocyte damage and increase the possibility of oocyte survival and development after microinjection.
22. Recovery at higher temperatures reduces oocyte survival.

23. Microinjection of sperm heads coated with such large DNA molecules (such as YACs) results in some cases in a slower transition through the first mitosis (the same happens for high DNA concentrations), for which it is recommended to transfer all embryos (including undivided ones) if this is done on the day following microinjection (into day 1 recipients).

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15

Establishment of Cell Lines That Exhibit Correct Ontogenic Stage-Specific Gene Expression Profiles From Tissues of Yeast Artificial Chromosome Transgenic Mice Using Chemically Induced Growth Signals

C. Anthony Blau and Kenneth R. Peterson

Summary

Transgenic mice produced with human yeast artificial chromosomes (YACs) generally display transgene expression patterns that reflect those of the normal human host. Because mice are expensive and time-consuming to generate and maintain, extensive mutation–phenotype correlation studies cannot be readily carried out. Cell lines are better suited for analysis of a plethora of mutations. However, these types of gene regulatory studies have been complicated by the lack of suitable cell lines, most of which do not exactly replicate the gene expression patterns observed in vivo. We reasoned that cells established from tissues of YAC transgenic mice might express the transgenes in the correct tissue and developmental stage-specific pattern from which they were derived because YAC transgenic mice display correct regulation of gene expression during ontogeny. We used our human β-globin locus YAC (β-YAC) transgenic mice to demonstrate this approach. All existing erythroid cell lines coexpress β-like globins from different developmental stages or express them inappropriately based on the developmental stage from which they were obtained. Cell populations were established from the adult bone marrow (BM) of β-YAC transgenic mice, which express exclusively adult β-globin, using dimerizer technology. A derivative of the thrombopoietin receptor (mpl) was used to bring the proliferative status of primary BM marrow cells under the control of a small molecule drug called a chemical inducer of dimerization (CID). Cells generated in this manner can be expanded to extremely large numbers, remain strictly CID-dependent, and retain megakaryocytic, erythroid, and granulocytic potential. Marrow cells transduced with a retrovirus vector encoding the mpl derivative proliferated extensively in the presence of the CID, AP20187. RNAse protection assays demonstrated that the transcripts for human β-globin and mouse α-globin were present, while γ-globin transcripts were absent, thus, these cells had the predicted expression phenotype. Exposure to 5-azacytidine or introduction of a hereditary persistence of fetal hemoglobin mutation activated γ-globin, which was expressed in addi-

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tion to β-globin, again consistent with the predicted expression profile of these cells. This approach extends the usefulness of YAC transgenic mice for the generation of cell lines amenable to more detailed studies regarding gene regulation.

Key Words: YAC; yeast artificial chromosome; globin; dimerizer; CID; bone marrow cells; gene regulation; growth switch; development; transgenic mice.

1. Introduction

The use of yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), or bacteriophage P1 artificial chromosomes (PACs) encompassing large genes or whole loci as transgenes has become a well-established practice during the last decade ***(1–3)***. Experimental methods exist for modification of the inserts contained within these vectors by homologous recombination, which avoids the inclusion of foreign DNA and selectable marker cassettes, leaving only the desired mutational change. YACs still retain one advantage over BACs and PACs, the ability to harbor human DNA sequences into the megabase size range. BACs and PACs are limited to approx 350 kb. Transgenic mice generated with human YAC constructs more faithfully recapitulate the developmentally and tissue-specific gene expression patterns observed in humans, likely because of the presence of both gene proximal and distant regulatory elements, as well as the maintenance of normal genome organization surrounding the locus ***(3)***. Mutant versions of these YACs, including point mutations, insertions, deletions, and rearrangements of multigene loci have been enormously helpful in identifying *cis*-regulatory motifs and deciphering their function in transgenics. However, the production of YAC transgenic lines is time-consuming and expensive and, thus, limits the number of mutant YAC mice that can reasonably be generated to those that test fundamental hypotheses regarding gene regulation. Methods that extend the usefulness of existing or future YAC transgenic mice and allow more comprehensive studies of control of gene expression beyond the production of mice will be valuable to the scientific community.

Direct introduction of YACs (microinjection, lipofection, spheroplast–protoplast fusion) into existing long-established cell lines does not always result in proper expression of the transgene. For example, human β-*globin* locus YACs require passage through a nonerythroid cell line prior to transfer by protoplast fusion to erythroid cells to obtain nearly proper gene expression patterns, perhaps because establishment of the globin chromatin domain occurs in stem cells or progenitors prior to erythroid differentiation ***(4)***. Because YAC transgenics, in general, accurately reflect the ontogenic expression patterns of the endogenous human genes they carry, it follows then that cell populations or cell lines established from the organs of these transgenic mice might be “locked” into the expression phenotype of that particular tissue and developmental stage.

Such cells would represent an improvement over most existing cell lines because they usually exhibit inappropriate gene expression. To demonstrate this concept, human β-*globin* locus YAC (β-YAC) transgenic mice were used to derive and maintain bone marrow cells (BMCs) using artificial growth receptors under control of chemical inducers of dimerization (CIDs ***[5–14]***).The human β-*globin* locus consists of five functional genes arrayed in their order of expression during development, 5'-ε-^{G}γ-^{A}γ-δ-β-3', and an upstream master control sequence, the locus control region ***(15)***. There are two switches of globin gene expression, from ε-globin synthesis in the yolk sac during primitive erythropoiesis to the γ-*globin* genes in the fetal liver during fetal definitive erythropoiesis, and then from γ-*globin* gene expression to β-globin expression (and to a much lesser extent δ-globin expression) in the bone marrow (BM) during adult definitive erythropoiesis. β-YAC transgenic mice mirror this temporal and spatial pattern during their approx 21-d gestation period. Correct human hemoglobin switching can be largely mimicked in transgenic mice containing a human β-*globin* locus YAC ***(16,17)***. An improved understanding of globin gene regulation is clinically relevant because the effect of elevated γ-globin levels in patients with sickle cell anemia and β thalassemia results in amelioration of symptoms. Many investigations have focused on identifying new compounds capable of inducing γ-*globin*. However, the identification of γ-globin inducers has been hampered by the lack of suitable in vitro model systems for selection of activators or for screening chemical compounds. None of the established cell systems completely mirror adult erythropoiesis; that is, γ-globin expression is markedly higher than normal adult human physiological levels, even in those cell lines where β-globin is the major species synthesized. Thus, these models can only be used to screen for a further increase of already existent γ-*globin* gene expression, rather than select for activation of a silent γ-*globin* gene. We reasoned that immortalized cells derived from the BM of β-YAC transgenic mice might express exclusively β-globin because the human pattern of globin transgene synthesis was recapitulated in these mice.

β-YAC BMCs were established from our wild-type β-YAC transgenic mice using an artificial proliferation signal comprised of the thrombopoietin (mpl) signaling domain fused to FKBP12 ligand-binding domains responsive to a synthetic CID, AP20187 (**Figs. 1** and **2** ***[13,14]***). The fusion protein was transfected as a retroviral construct into BMCs. In the presence of the CID, homodimers are generated and the resultant growth signal maintains the BMC population indefinitely, as long as the CID is present. These cells exclusively expressed human β-globin, but γ-globin expression could be reactivated by various known chemical inducers or through the presence of a hereditary persistence of fetal hemoglobin (HPFH) mutation in the ^{A}γ-*globin* gene (**Fig. 3**). Therefore, these CID-dependent, multi-potential β-YAC BMCs provide a

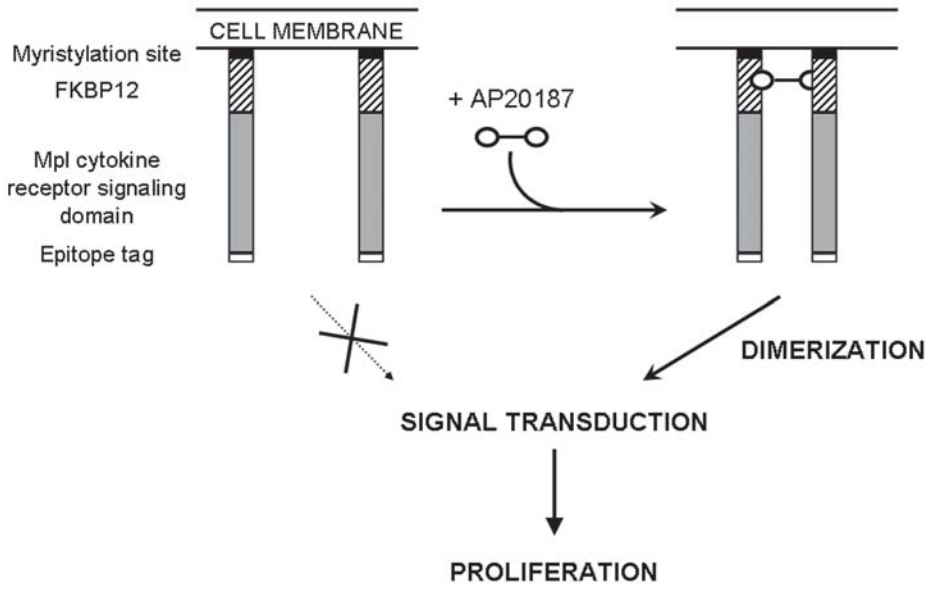

Fig. 1. Dimerizer technology used to establish drug-dependent proliferating cell populations. A chimeric growth switch consists of receptor sequences and a dimerization domain. The growth switch is activated following addition of a chemical inducer of dimerization (CID AP20187). The CID AP20187 catalyzes dimerization by binding dimerization domains (FKBP12) on neighboring molecules. Dimerization produces a functional growth signal from the homodimerized receptor sequences (Mpl cytokine receptor signaling domain). The molecule also carries a myristylation domain derived from c-src for targeting the molecules to the inner cell membrane and an epitope tag for expression and cellular localization studies using an epitope-specific antibody.

model system in which γ-globin protein transactivators can be selected and pharmacological inducers of hemoglobin F definitively screened.

Our approach provides a model for establishing cell lines from any tissue of YAC transgenic mice. The caveat is that the growth signal, specifically the signaling domain fused to the FKBP-binding domains, must function in the tissue of choice to achieve immortalization. This method should extend studies beyond those in the mouse into less-expensive and less time-consuming cell experiments in cells that more accurately reflect the ontogenic stage from which they were derived. The YAC transgene expression pattern should mirror that which is observed in vivo, providing readouts useful in screening compounds or treatments that affect gene expression. Investigators are cautioned that variations on the themes presented in the method described next likely need to be determined empirically to account for differences in tissue source, available retroviral vectors, tissue-specific growth switch moieties, and others.

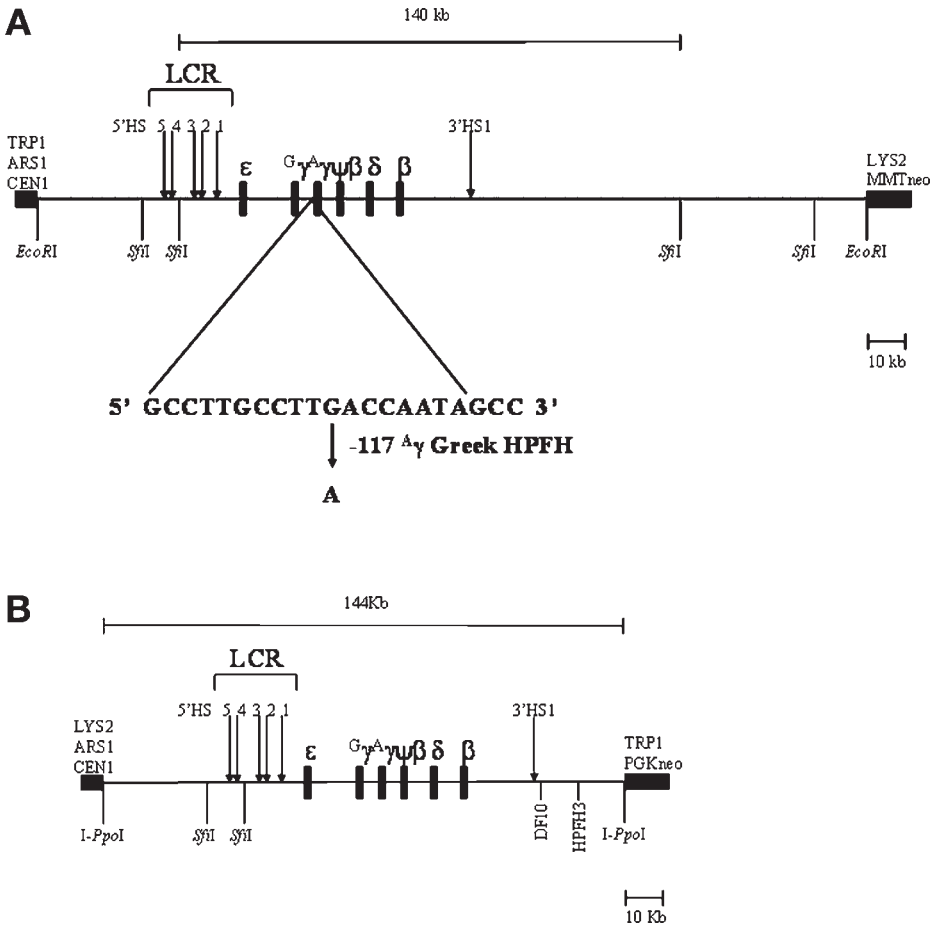

Fig. 2. Schematic maps of human β-*globin* locus yeast artificial chromosomes (β-YACs) used in this study. **(A)** 213-kb -117 hereditary persistence of fetal hemoglobin (HPFH) β-YAC ***(20)***; **(B)** 155-kb wild-type β-YAC ***(16,21)***. CID-dependent bone marrow cells were derived from transgenic mice bearing these YACs as described in the text (*see* **Subheading 3.2.–3.3.**). The point mutation at position –117 relative to the messenger RNA start site of the $^{A}\gamma$-*globin* gene results in a condition in humans called HPFH, in which fetal hemoglobin is produced in adults at significantly higher than normal levels. Transgenic mice produced with this β-YAC recapitulate this phenotype.

2. Materials

2.1. Culture Media

1. D8 medium: 500 mL Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, cat. no. 15-017-CV), 50 mL 8% Nova-Tech fetal bovine serum (FBS) (ISC BioExpress, Kaysville, UT, cat. no. 15-167-P50), 6 mL 100 m*M* sodium pyruvate

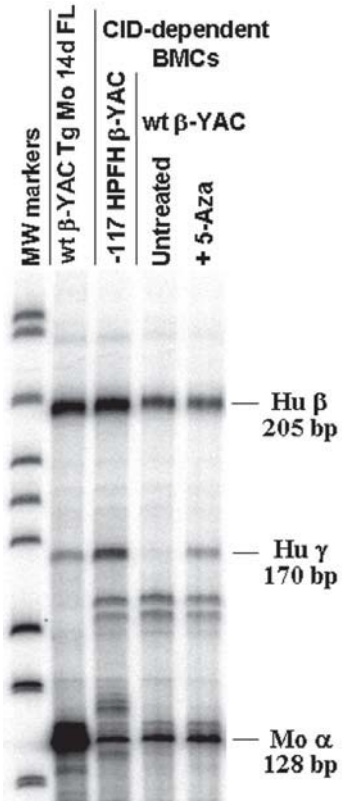

Fig. 3. Demonstration that γ-globin can be reactivated in globin locus chemical inducer of dimerization (CID)-dependent β-yeast artificial chromosome (β-YAC) bone marrow cells (BMCs). RNAse protection assays were performed on total RNA isolated from CID-dependent BMCs established from –117 hereditary persistence of fetal hemoglobin (HPFH) and wild-type β-YAC transgenic mouse bone marrows ***(18)***. Sample sources are indicated at the top of the autoradiograph; protected fragments and sizes in basepairs (bp) are shown to the right. Hu β, human β-globin protected fragment; Hu γ, human γ-globin protected fragment; Mo α, mouse α-globin protected fragment. The left lane is radiolabeled pBR322 *Msp*I molecular weight markers (MW markers). Lane 2 shows a RPA of d 14 (postconception) fetal liver from a wild-type β-YAC mouse. At this stage γ-globin expression is on the decline as β-globin expression is switched on to eventually become the predominant β-like globin species synthesized in the adult. Consistent with the adult β-YAC transgenic mouse phenotype, CID-dependent BMCs established from adult wild-type β-YAC mice express exclusively human β-globin, as predicted (lane 4). In the presence of 5-azacytidine, a compound known to reactivate silent γ-globin expression in human adults, γ-globin is switched on in CID-dependent wild-type β-YAC BMCs (lane 5). In addition, the Greek HPFH mutation, shown to cause continued ^{A}γ-globin synthesis in adults, results in constitutive ^{A}γ-*globin* gene expression

(Mediatech, cat. no. 25-000-CI), 6 mL 100X MEM nonessential amino acids (Mediatech, cat. no. 25-025-CI), 6 mL 200 m*M* L-glutamine solution (Mediatech, cat. no. 25-005-CI), 6 mL 50X penicillin-streptomycin solution (Mediatech, cat. no. 30-001-CI).

2. D16 medium: same as D8, but contains double the amount of the FBS (16%), 5% murine interleukin-3-conditioned medium (BD Biosciences, Bedford, MA, cat. no. 354040), 100 ng/mL human interleukin-6 (Chemicon International, Temecula, CA, cat. no. IL006), and 50 ng/mL murine stem cell factor (Chemicon International, cat. no. GF049).

2.2. Mobilization and Collection of Transgenic Mouse BMCs

1. 5-fluorouracil (5-FU): dissolve 1 g 5-FU (Sigma-Aldrich, St. Louis, MO, cat. no. F6627) in 10 mL dimethylsulfoxide, and add 10 mL sterile distilled H_2O for a final concentration of 50 mg/mL. Just prior to injection, it should be freshly diluted 1:4 in sterile phosphate-buffered saline (PBS) to a working concentration of 12.5 mg/mL.
2. PBS: sterile 10X PBS (HyClone, Logan, UT, cat. no. SH30258.01) diluted 1/10 in sterile distilled water.
3. Turk's counting fluid: 1% Genetian violet and 1% acetic acid; filter before use.

2.3. Maintenance of CID-Dependent Cells

1. Iscove's modified dulbecco's medium (IMDM): IMDM (HyClone, cat. no. SH30005.03) 10% FBS (Mediatech), 50 U/mL penicillin-50 μg/mL streptomycin (Mediatech).
2. CID, AP20187: available exclusively from Ariad Pharmaceuticals (Cambridge, MA). Small quantities may be obtained free with completion of a materials transfer agreement (MTA) and online registration/justification. The lyophilized compound is solubilized in 100% ethanol to produce a 5 mg/mL stock that is stored at –20°C. A 100X working stock is prepared by diluting to 10 μ*M* AP20187 in IMDM. This solution is stable at 4°C for 2 mo.
3. Retroviral producer line: methodology describing generation of retrovirus producer lines is beyond the scope of this chapter. Each line must be designed to bear the tissue-appropriate growth signal motif fused to FKBP12-binding domains. For our BMCs, the growth switch, F36Vmpl, consisted of a CID-binding domain (F36V) fused to the intracellular part of mouse mpl (thrombopoietin receptor). This fusion was inserted into two MSCV-based retroviral vectors containing reporters for either G418 resistance (neo) or green fluorescent protein (GFP). Expression of either F36Vmpl^{neo} or F36Vmpl^{GFP} in transduced BMCs allowed for sustained growth in suspension cultures supplemented with the CID, AP20187.

Fig. 3. (*continued from opposite page*) in CID-dependent BMCs derived from adult –117 HPFH β-YAC transgenic mice (lane 3). Thus, these cell populations produced from YAC transgenic mice mirror the ontogenic stage from which they were obtained.

Producer lines containing these clones were made and characterized as previously described in **ref. *14***.

3. Methods

We utilized a system that allows multipotential cells to be established from YAC transgenic mouse BM (**Fig. 2 *[13,14]***). This system uses a retroviral vector to introduce a gene encoding a conditional signaling molecule into mouse BMCs, followed by activation of the signaling molecule using a small molecule drug called a CID (**Fig. 1**). A CID-activated derivative of the thrombopoietin receptor, mpl, induced transfected mouse BMCs to expand dramatically in culture. Cells generated in this manner adopt predominantly megakaryocytic features, but also include multipotential progenitors capable of generating monocytes, neutrophils, and erythroid cells, but not B or T lymphoid cells on addition of the appropriate growth factors. Cultures can be maintained for longer than a year, and cell growth remains strictly dependent on the continued presence of the CID.

3.1. Day 1

Trypsinize confluent retroviral producer clone(s) and plate out cells at dilutions of 1:10 and 1:20 in fresh D8 medium (*see* **Note 1**).

3.2. Day 2

Inject mice interperitoneally with 5-FU at a dosage of 150 mg/kg. Mice have a mass of approx 20 g (0.02 kg). Therefore, 3 mg/mouse is required and 0.24 mL should be injected ip using a 27-gauge needle and a 1-cc syringe. The mice should be sacrificed 48 h postinjection.

3.3. Day 4

Sacrifice mice, harvest BM, and prestimulate BMCs. Aliquot 3–5 mL of PBS with 2.5% FBS and 50 U/mL penicillin-50 µg/mL streptomycin into a 30-mm tissue culture dish and place on ice. Anesthetize mouse by placing in covered beaker containing ether-soaked tissue paper or using Avertin. After mouse is unconscious, sacrifice and clean with generous amounts of 70% ethanol. Dissect femurs out, minimizing connective tissue contamination. Place femurs into tissue culture dish (*see* **Note 2**). Use a 23-gauge needle and a 3-mL syringe to bore out each end of the femurs. Rinse marrow cavity multiple times from each end of the femur to extract marrow (*see* **Note 3**). Draw the PBS/BM mixture up into the syringe and dispense into a sterile tube. Disperse cells by trituration. Count the total number of cells using a nuclear specific dye, such as Turk's counting fluid, to avoid counting mature erythrocytes. Dilute the BM to a final concentration of 1×10^6 cells/mL in D16 medium (prestimulation). Incubate culture at 37°C, 5% CO_2, and 100% humidity for 48 h.

3.4. Day 6

Irradiate retroviral producer cell clone and establish cocultivation of producer cell clone and prestimulated BMCs. Use producer cells that are 50–70% confluent. Irradiate cells with 1500 cGy. Harvest BMCs from the prestimulation plate using a sterile cell lifter. Gently pipet cells up and down and transfer to a 50-mL conical tube. Count cells in Turk's counting solution (*see* **Note 4**). Resuspend cells in D16 medium containing 8 μg/mL polybrene (Sigma-Aldrich, cat. no. S-2667) at a density of approx 1×10^6 cells/mL. Aspirate supernatant from irradiated producer clones, replace with BMC culture using 10 mL BMC suspension per 10-cm plate of producers. Incubate for 48 h at 37°C, 5% CO_2, and 100% humidity. Cells are now transduced and ready for experiments.

3.5. Day 8

Cultures are subsequently maintained in IMDM containing 100 n*M* AP20187 dimerizer at 37°C under 5% CO_2.

3.6. Analysis of Gene Expression

Detailed methods for measuring gene expression are beyond the scope of this chapter. Standard protocols may be employed to isolate RNA or proteins for analysis. RNAse protection assays and semi-quantitative reverse transcriptase-polymerase chain reaction were employed to measure messenger RNA levels in β-YAC BMCs (**Fig. 3** ***[18,19]***). Antibody staining for globin chains was performed on cells fixed to microscope slides.

4. Notes

1. Culture these cells, then irradiate and freeze in aliquots. Thaw them and culture on gelatin-coated plates 2–3 d prior to use.
2. Consider using tibias and hips to obtain maximal numbers of BMCs.
3. The middle of the femur should appear white after thorough extraction of BM.
4. Cell number will have decreased owing to cell death, this is normal.

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16

Application of Yeast Artificial Chromosomes in Fluorescence *In Situ* Hybridization

Thomas Liehr

Summary

In addition to the well-known applications of yeast artificial chromosomes (YACs) in classical molecular genetics, they also are used for molecular cytogenetic studies. YACs, as well as other locus-specific probes like DNA, plasmids, cosmids, P1-clones, or bacterial artificial chromosomes can be labeled with fluorochromes and applied in fluorescence *in situ* hybridization (FISH) experiments. Various applications are possible, such as gene mapping, FISH banding, determination of chromosomal breakpoints, characterization of derivative chromosomes, studies on the interphase architecture, or karyotypic evolution studies. This chapter outlines the basic principle of how YACs can be hybridized *in situ* on chromosome preparations. Moreover, an overview is given on possible questions to be processed using YACs as FISH probes.

Key Words: Molecular cytogenetics; fluorescence *in situ* hybridization (FISH); gene mapping; FISH banding; chromosomal breakpoints; derivative chromosomes; interphase architecture; karyotypic evolution.

1. Introduction

The hybridizing of nucleic acid sequences that are immobilized on a nitrocellulose membrane is a well-known approach for everybody working in the field of molecular genetics; Southern blotting is designed to locate a particular sequence of DNA within a complex mixture, e.g., to locate a particular gene within an entire genome ***(1)***.

On the other hand, hybridizing and visualizing nucleic acid sequences directly within a tissue or on chromosomes is a technique known and applied in specialized, mainly or even exclusively, molecular cytogenetic working labs. This is somehow surprising because *in situ* hybridization was already introduced in 1969 in a radioactive ***(2)*** and 1986 in a nonradioactive variant (fluores-

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cence *in situ* hybridization [FISH] ***[3]***) and is a straightforward method for the direct localization of DNA sequences within any genome (e.g., **ref. *4***). Moreover, FISH provides the option to use two or more DNA sequences at the same time, and distinguishes them by labeling in different colors or color combinations. Multicolor FISH (mFISH) was first described by Nederlof et al. in 1989 ***(5)***. The multiple possibilities of mFISH applications were reviewed by **ref. *6***.

Many kinds of probes can be used for FISH and mFISH: whole genomic DNA in comparative genomic hybridization ***(7)***, whole chromosome painting probes ***(3)***, partial chromosome painting probes ***(8)***, repetitive centromeric ***(9)*** or telomeric probes ***(10)***, and locus-specific probes ***(4,11–13)***. FISH probes can either be generated by chromosome flow sorting ***(3)*** or by microdissection ***(8)***. Additionally, probes generated in molecular genetics can be used for FISH-like cDNA ***(11)***, plasmids ***(12)***, cosmids ***(11,12)***, P1-clones ***(13)***, bacterial artificial chromosomes (BACs) ***(14)***, and yeast artifical chromosomes (YACs) ***(4)***.

YAC probes have already been used successfully in FISH for the following applications: (1) gene mapping ***(4)***, (2) creation of FISH-banding probe sets (reviewed in **ref. *15***), (3) determination of chromosomal breakpoints ***(16)***, (4) characterization of derivative chromosomes ***(17)***, (5) three-dimensional structure of the interphase nucleus ***(18)***, and (6) interspecies comparative studies, called ZOO-FISH, aimed to find out more about (karyotypic) evolution ***(19)*** (*see* **Table 1**).

The most important advantage of YAC probes in FISH is their big size, which leads in general to very bright, intense, and evaluable FISH results. Thus, they will be of use in future studies for the aforementioned fields of three-dimensional structure of the interphase nucleus and ZOO-FISH. However, it has to be stated that the size of YAC probes also causes some problems for molecular cytogenetics: i.e., (1) the larger a YAC probe, the higher the probability of creating undesired cross-hybridizations, (2) moreover, a large YAC may create a too large FISH signal, e.g., for FISH diagnostics or to narrow down a gene region exactly enough. Additionally, there is (3) the well-known problem of chimeric YACs producing FISH signals in two or more distinct loci spread over one chromosome or over the whole genome ***(20)***. Because of these problems, during the last years human-derived YACs are applied less frequently for FISH approaches in the fields of gene mapping, creation of FISH-banding probe sets, the determination of chromosomal breakpoints, and the characterization of derivative chromosomes, and they were replaced by smaller single BAC probes or BAC contigues instead. Nonetheless, in other species besides the human, YACs are often the only available source of probes covering the whole genome. To find more about the genomic structure of a species, the possibility to apply YACs in FISH experiments should not be neglected.

Table 1
Overview of the Application That YACs Were Used For in Different Species: Exemplary References are Given

Application of YACs	Human	Mouse	Other species
Gene mapping	+ (4)	+ (24)	+ rat (25) cattle (26) rice (27)
FISH banding	+ (20,28) review in **ref.** 15	+ (29)	–
Determination of chromosomal breakpoints	+ clinical genetics (16) tumor genetics (30)	+ (31)	+ rat (32)
Characterization of derivative chromosomes	+ (17)	+ (31)	–
Three-dimensional structure of the interphase nucleus	+ (18,33)	–	–
ZOO-FISH	human YAC probes in hominoide + (19,34)	Murine YAC probes in other species –	YAC probes of other species –

Summary of the published applications for yeast artificial chromosomes in FISH experiments. Six possible research fields are listed and examples are given for human, mouse, and other species, like rat, cattle, and even plants.

The basic principle of using YACs in any kind of FISH study is outlined in this chapter. For YAC cultivation and DNA extraction, please refer to Chapters 1–4.

2. Materials

2.1. Chromosome Preparation

1. Cell culture medium: RPMI 1640 medium with Glutamax (Gibco BRL, Gaithersburg, MD cat. no. 72400-021), 20% fetal calf serum (Seromed, Berlin, Germany, cat. no. 50113), 1 U/mL penicillin together with 1 µg/mL streptomycin (Seromed, cat. no. A 2212), and 0.1 mL phytohemagglutinine (Seromed, cat. no. M 5030).

2. Colcemid (Seromed L, cat. no. 6221).
3. 0.075 *M* KCl (Merck, Dormstadt, Germany, cat. no. 1.04936.1000).
4. Carnoy's fixative: methanol (Merck, cat. no. 1.0600.96025) and glacial acetic acid (3:1) (Merck, cat. no. 1.00063.2500).

2.2. Probe Labeling

1. Digoxigenin (DIG)-nick translation mix for *in situ* probes (Roche, Mannaheim, Germany, cat. no. 11745816910-1745816).
2. Biotin-nick translation mix for generation of highly sensitive probes for *in situ* hybridization labeled with biotin-16-dUTP (Roche, cat. no. 11745824910-1745824).
3. Double-distilled water (Braun, Tuttlingenn, Germany, cat. no. 235 1544).
4. COT1 DNA (Gibco BRL, cat. no. 15279-001).
5. 5 *M* Sodium acetate (Merck, cat. no. 106268). Hybridization buffer: dissolve 2 g dextran sulphate in 10 mL 50% deionized formamide, 2X standard saline citrate (SSC), and 50 m*M* phosphate buffer for 3 h at 70°C. Aliquot and store at –20°C.

2.3. Slide Pretreatment

1. 20X SSC stock solution: 3.0 *M* NaCl and 0.3 *M* Na-citrate. Prepare with double-distilled water, adjust to pH 7.0, autoclave, and store at room temperature.
2. RNase stock solution: 5 μg/μL of RNase type A (Roche, cat. no. 109142). Prepare with filtered, double-distilled water. Aliquot and store at –20°C.
3. RNase solution: 100 μL 2X SSC plus 1 μL of RNase stock solution are necessary per slide; make fresh as required.
4. Pepsin stock solution (10% w/v): dissolve 100 mg pepsin (Serva, cat. no. 31855) in 1 mL of filtered, double-distilled water at 37°C. Aliquot and store at –20°C.
5. Pepsin buffer: add 1 mL of 1 *M* HCl to 99 mL of distilled water and incubate at 37°C for about 20 min, then add 50 μL of the 10% (w/v) pepsin stock solution and leave the coplin jar at 37°C, make fresh as required.
6. 1X phosphate-buffered saline (PBS)/ $MgCl_2$: 1 *M* $MgCl_2$ (5% v/v) in 1X PBS.

2.4. *Fluorescence* In Situ *Hybridization*

2.4.1. Slide Denaturation

1. Denaturation buffer: 70% (v/v) deionized formamide, 10% (v/v) filtered, double-distilled water, 10% (v/v) 20X SSC, 10% (v/v) phosphate buffer; make fresh as required.
2. Deionized formamide: add 5 g of ion exchanger Amberlite MB1 (Serva, cat. no. 40701) to 100 mL of formamide (Merck, cat. no. 1.09684). Stir for 2 h (room temperature) and filter twice through Whatmann no. 1 filter paper. Aliquot and store at –20°C.
3. Phosphate buffer: prepare 0.5 *M* Na_2HPO_4 and 0.5 *M* NaH_2PO_4, mix these two solutions (1:1) to get pH 7.0, then aliquot and store at –20°C.

2.4.2. Probe Denaturation

1. Hybridization buffer: *see* **Subheading 2.2.**, **item 6**.

2.4.3. Posthybridization and Detection Washing

1. Solution I: FITC-avidin (CAMON Vector Laboratories, Wiesbaden, Germany, cat. no. A2011), 4X SSC, 0.2% Tween-20, 5% bovine serum albumin (1:300 both Sigma); make fresh as required.
2. Solution II: biotinylated antiavidin (CAMON Vector Laboratories, cat. no. BA0300), antidigoxinenin-rhodamine (Roche, cat. no. 1207750), 4X SSC, 0.2% Tween-20, 5% bovine serum albumin (1:20:100); make fresh as required.
3. 4,6-diamidino-2-phenylindol.2HCl (DAPI) solution: dissolve 5 µL of DAPI stock-solution (Serva, cat. no. 18860) in 100 mL 4X SSC and 0.2% Tween-20; make fresh as required.

3. Methods

This section describes how human chromosomes are prepared from peripheral blood (**Subheading 3.1.**), how YAC DNA can be labeled (**Subheading 3.2.**), how the target of the hybridization, i.e., cytogenetic slides with spread chromosomes have to be pretreated (**Subheading 3.3.**), and how FISH itself is performed (**Subheading 3.4.**).

3.1. Preparation of Peripheral Blood Lymphocyte Suspension

Here, only the preparation of human peripheral blood metaphase spreads is described; for more specific preparation protocols (e.g., for bone marrow or solid tumors) see more specialized handbooks (*see* **ref. *21***).

1. Add 1 mL of heparinized blood to 9 mL of cell culture medium (*see* **Note 1**). Mix the suspension carefully and incubate for 72 h at 37°C/5% CO_2 (*see* **Notes 2** and **3**).
2. 30 min before harvesting of the cells, 1 µg of colcemid is added to a 10-mL culture flask, the flask is mixed gently and incubated at 37°C/5% CO_2.
3. The fluid is put into a 15-mL tube. Sterile conditions are no longer to be observed.
4. Centrifuge the solution at room temperature (RT) for 8 min at 800*g* and discard the supernatant by sucking it of carefully with a glass pipet (1 mL of supernatant are left in the tube to avoid loss of cell material).
5. For hypotonic treatment the pellet is resuspended in 10 mL 0.075 *M* KCl (37°C) and incubated at 37°C for 20 min.
6. Slowly add 0.6 mL of 4°C Carnoy's fixative (*see* **Subheading 2.1.**, **item 4**) and mix the solution carefully.
7. Repeat **step 4**.
8. Resuspend the pellet in 10 mL of 4°C fixative and incubate at 4°C for 20 min.

9. Repeat **step 4**.
10. Resuspend the pellet in 5 mL of 4°C fixative and repeat **step 4**.
11. Repeat **step 10** twice.
12. According to the density of the suspension, the pellet is finally resuspended in 0.3–1.0 mL of fixative (suck off as much of the suspension as necessary after **step 11**).
13. Bring one to two drops of the suspension each on a clean and humid slide by a glass pipet, and let the slide dry at RT.
14. After an overnight incubation at RT the slides can be subjected to the pretreatment (*see* **Subheading 3.3.**), stored dust free at RT for several weeks, or frozen at –20°C for several months.

3.2. Probe Labeling

YAC probes are most easily labeled by nick-translation ***(22)***. Here, labeling in biotin and DIG is described. Using these two haptens, a detection with fluorescence-labeled antibodies is necessary. A probe labeling with dye-labeled dUTP can be done accordingly.

1. Add 1 μg YAC DNA to sterile, double-distilled water to end up with a final volume of 16 μL.
2. Add 4 μL DIG- or biotin-nick translation mix (provided in the mentioned kits [*see* **Subheading 2.2.**]), mix by pipet tip, and centrifuge briefly.
3. Incubate for 90 min at 15°C.
4. Stop the reaction by heating to 65°C for 10 min.
5. Precipitate the labeled YAC DNA together with 2 μL COT1 DNA (*see* **Note 3**), 5 μL 5 *M* sodium acetate, and 125 μL 100% ethanol for 20 min at –80°C or overnight at –20°C.
6. Sediment the pellet by centrifugation (12,000*g*, 5 min), remove the supernatant, and dry the DNA pellet in a speed vac.
7. Dissolve the pellet in 20 μL of hybridization buffer. Use immediately as mentioned in **Subheading 3.4.** or store at –20°C for up to 1 yr.

3.3. Slide Pretreatment

Pretreatment of the slides with RNase and pepsin followed by a postfixation with formalin buffer is required to reduce background in the FISH experiment ***(23)***.

1. Slides are incubated in 2X SSC for 5 min at RT (in a 100-mL Coplin jar on a shaker).
2. Remove slides from the coplin jar, add 100 μL of RNase solution per slide and cover with a 24 × 50-mm cover slip.
3. Incubate the slides in a humid chamber for 15 min at 37°C (*see* **Note 4**).
4. Put slides back into the Coplin jar with 100 mL 2X SSC (at RT) and remove the cover slips by forceps. Leave slides in 2X SSC solution for 3 min with gentle agitation.
5. Discard 2X SSC and replace it with 100 mL 1X PBS (at RT) for 5 min (shaker).

6. Replace 1X PBS with 100 mL prewarmed pepsin buffer (37°C) and incubate the slides for 10 min at 37°C, without agitation (*see* **Note 4**).
7. Replace fluid with 100 mL 1X PBS/$MgCl_2$, incubate at RT for 5 min with gentle agitation. $MgCl_2$ will block the enzymatic activity of pepsin.
8. Postfix nuclei on the slide surfaces by replacing 1X PBS/$MgCl_2$ with 100 mL of formalin buffer for 10 min (RT, with gentle agitation).
9. Formalin buffer is replaced by 100 mL 1X PBS for 2 min (RT, with gentle agitation).
10. Finally, slides are dehydrated by an ethanol series (70, 90, and 100%, 3 min each) and air-dried (*see* **Note 5**).

3.4. Fluorescence In Situ *Hybridization*

FISH procedure itself is divided in several steps: denaturation (**Subheadings 3.4.1.** and **3.4.2.**), hybridization (**Subheading 3.4.3.**), and posthybridization together with detection washing (**Subheading 3.4.4.**).

3.4.1. Denaturation of Target DNA

1. Add 100 µL denaturation buffer to the slides and cover with (24 × 50-mm) cover slips.
2. Incubate slides on a warming plate for 2–3 min at 75°C (*see* **Note 4**).
3. Remove the cover slips immediately by forceps and place slides in a coplin jar filled with 70% ethanol (4°C) to conserve target DNA as single strands.
4. Dehydrate slides in ethanol (70, 90, and 100%, 4°C, 3 min each) and air-dry.

3.4.2. Denaturation of Probe DNA

1. For each slide to be hybridized, denature 20 µL of the probe solution. In case only one YAC probe is hybridized (e.g., biotin labeled), mix 10 µL of labeled probe mentioned in **Subheading 3.2.**, **step 7** with 10 µL of hybridization buffer. In case a biotin labeled together with a DIG-labeled YAC shall be used, mix 10 µL each of the labeled probes mentioned in **Subheading 3.2.**, **step 7** (**Fig. 1**).
2. Denature 20 µL of probe mix from **step 1** at 75°C for 5 min and cool immediately on ice to conserve probe DNA in single strands.

3.4.3. Hybridization

1. Add 20 µL of probe solution onto each denatured slide, put 24 × 50-mm cover slips on the drops, and seal with rubber cement (Fixogum; Marabuwerke GmbH and Co. KG, Tamm, Germany).
2. Incubate slides for one night at 37°C in a humid chamber (*see* **Note 6**).

3.4.4. Posthybridization and Detection Washing

1. Take the slides out of the 37°C humid chamber, remove rubber cement with forceps, and remove the cover slips by letting them swim off in 4X SSC/0.2% Tween-20 (RT, 100-mL coplin jar) (*see* **Note 7**).
2. Postwash the slides three times for 5 min each in formamide solution (45°C) followed by three times for 5 min in 2X SSC (37°C) in a 100-mL Coplin jar with gentle agitation.

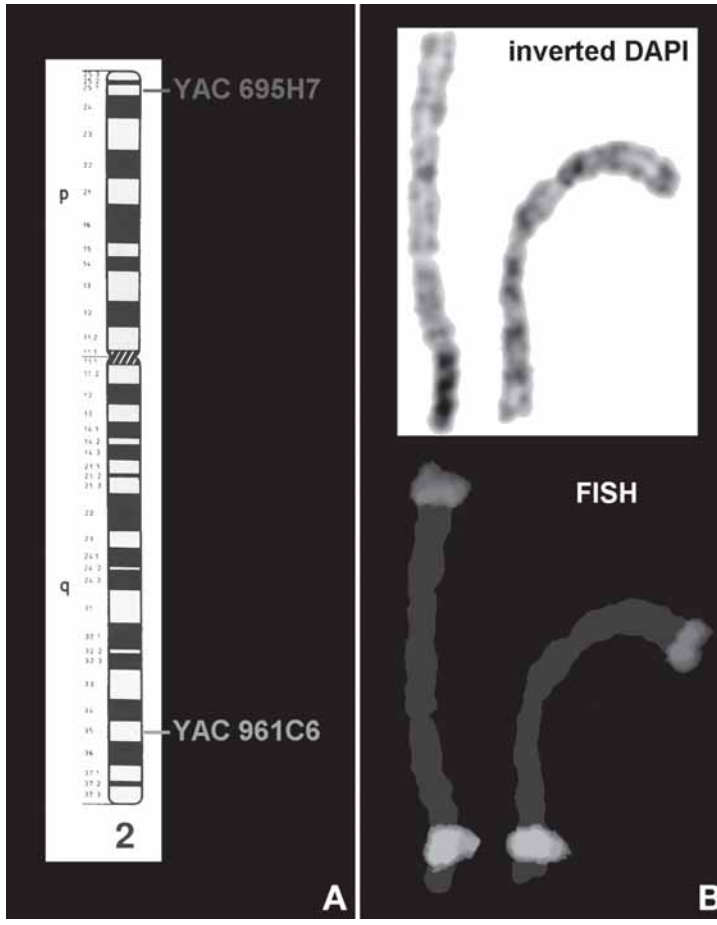

Fig. 1. Two-color fluorescence *in situ* hybridization (FISH) using two chromosome 2-specific yeast artificial chromosomes (YACs) as probes: YAC 695H7 is located in 2p25.1 and YAC 961C6 hybridizes to 2q35. **(A)** An ideogram of chromosome 2 together with the localization of the two YAC probes is shown. **(B)** The FISH result and the inverted DAPI banding result are shown. DAPI produces a banding pattern quite similar to that banding shown in the ideogram of **(A)**. Note that YACs do not produce one spot-like signal on each chromosomal chromatid but, because of their size (~1.5 Mbp), lead to larger, outlapping, painting-like signals. (Figure kindly provided by Dr. Anja Weise.)

3. Put the slides in 4X SSC/0.2% Tween-20 (100 mL, RT), for a few seconds.
4. Add 50 µL of solution I to each slide, cover with 24 × 50-mm cover slips, and incubate at 37°C for 30 min in a humid chamber.

5. Remove the cover slips and wash three times, 3 min each in 4X SSC/0.2% Tween-20 (RT, with gentle agitation).
6. Add 50 µL of solution II to each slide, cover with 24 × 50-mm cover slips, and incubate at 37°C for 75 min in a humid chamber.
7. Repeat **step 5**.
8. Repeat **step 4**.
9. Repeat **step 5**.
10. Counterstain the slides with DAPI solution (100 mL in a Coplin jar, RT) for 8 min.
11. Wash slides several times in water for a few seconds and air-dry.
12. Add 15 µL of antifade Vectashield (CAMON Vector Laboratories H1000), cover with cover slips, and look at the results in a fluorescence microscope.

4. Notes

1. It is a well-known fact that EDTA- or sodium acetate-treated blood samples can not be successfully cultured in short term culture.
2. Sterile cell culture conditions have to be kept to not contaminate the culture.
3. COT1 DNA is added to the hybridization mix when nonrepetative sequences are to be detected, like YAC probes. If no COT1 DNA would be added, the YAC probes would not only label "their" specific site on a chromosome, but undesired cross-hybridization all over the genome would occur as well. Two to 10 µg COT1 DNA may be necessary for YAC probes, depending on the amount of repetitive DNA within the individual YAC. General rule: the more cross-hybridizations a YAC probe produces in FISH, the more COT1 can be used to block them.
4. RNase and pepsin pretreatment conditions, as well as the denaturation time of the target DNA, should be tested in each laboratory on a single slide first. Both RNase and pepsin concentrations can be too stringent, resulting in clean slides without any remaining nuclei.
5. The pretreated slides can be hybridized immediately or stored at RT for up to 3 wk. If longer storage is necessary, slides are stable at –20°C for several months.
6. Incubation can be stopped, if necessary, after 48 or 72 h as well. If after approx 12 h the signals are too weak, prolongation of hybridization time can enhance signal intensity; however, some cross-hybridization problems may arise in parallel.
7. During the washing steps it is important to prevent the slide surfaces from drying out, otherwise background problems may arise.

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17

Contribution of Yeast Artificial Chromosome-Based Physical Maps to the Final Assembly of the *Trypanosoma cruzi* Genome

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Summary

This chapter describes the methodology used both in performing the electrophoretic karyotype of the protozoan parasite *Trypanosoma cruzi* and mapping the genetic markers of the chromosomal bands, the construction of chromosome-specific YAC contigs, and their use to assign a chromosomal location to whole genome shotgun sequences.

Key Words: *Trypanosoma cruzi*; protozoan parasite; molecular karyotype; chromosome-specific marker; YAC; whole genome shotgun (WGS) sequences; integrated physical and WGS map.

1. Introduction

Trypanosoma cruzi is a parasitic protozoan that causes Chagas' disease, a disease without effective drug treatment affecting 16–18 million people on the American continent. Classical genetic studies on this parasite have been hampered owing to the existence of many parasite strains with distinct biological and immunological characteristics, lack of sexual stages, and a high variability in both number and size of chromosomes ***(1,2)***. For this reason, a first goal of the *T. cruzi* Genome Initiative was to construct a physical map encompassing the whole parasite nuclear genome ***(3,4)***. The initial step in this construction was the production of partial physical maps of different chromosomes, and/or chromosomal regions ***(5,6)***. Yeast artificial chromosomes (YACs) were chosen because large DNA fragments can be accommodated. Reliance on YACs is justified because previous works have shown that YACs containing *T. cruzi*

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genomic fragments are stable, and the cloned sequences are colinear with the genomic sites from which they are derived ***(5,6)***.

Two major advantages for this approach are the facts that *T. cruzi* chromosomes can be defined and many of them can be isolated by pulsed-field gel electrophoresis (PFGE) ***(1,2)***, and that simple Southern blots of electrophoretic karyotypes can be used to map genetic markers for chromosomal bands ***(5,6)*** (**Fig. 1A**). Chromosome-specific genetic markers would be then useful in selecting chromosome-specific YACs, prompting construction of YAC-based contigs (**Fig. 1B,C**). In turn, they may provide an additional scaffold to complete the assembly of the whole genome shotgun (WGS) sequence ***(7)*** (**Fig. 2**). The *T. cruzi* genome is rich in repeated sequences (at least 50% of the genome) that may confound shotgun approaches to complete its sequencing ***(7)***. The methods presented in this chapter provide a suitable framework for further ordering of *T. cruzi* genomic megafragments cloned in YAC and bacterial artificial chromosome vectors; in addition, they will also aid in the assembly of genomic data from high-throughput DNA sequencing ***(7)***.

2. Materials

2.1. Separation of T. cruzi *Chromosomal Bands and Identification of Chromosome-Specific Markers*

1. PSG: phosphate buffered saline plus 0.3 *M* glucose.
2. Trypanosome lysis solution: 0.5 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, 1% sodium lauroylsarcosinate, and 1 mg/mL proteinase K.
3. 50 m*M* EDTA, pH 8.0 and 0.5 *M* EDTA, pH 8.0.
4. 1X TBE (Tris-Borate-EDTA): 0.089 *M* Tris-borate, pH 8.3 and 0.025 *M* EDTA.
5. 0.5 μg/mL Ethidium bromide (EtBr).
6. PFGE size markers: chromosomes of *Saccharomyces cerevisiae* and *Hansenula wingei*.
7. Depurination solution: 0.25 *M* HCl.
8. Denaturation solution: 0.5 *M* NaOH and 1 *M* NaCl.
9. Neutralization solution: 1 *M* Tris-HCl, pH 8.0, and 0.6 *M* NaCl.

Fig. 1. Identification of YAC clones harboring *Trypanosoma cruzi* chromosome-specific markers. **(A)** Separation of *T. cruzi* (clone CL Brener) chromosomal bands by pulsed-field gel electrophoresis (PFGE) and staining with ethidium bromide. Roman numerals indicate the numbers of chromosomal bands (*see* **ref. *1***). Identification of chromosome-specific markers by Southern blot hybridization (markers 1 and 2 are specific for band XX). Screening of the *T. cruzi* YAC library with chromosome-specific markers. Probes 1 and 2 hybridized with three and two recombinant yeast artificial chromosomes (YAC) clones, respectively. **(B)** Confirmation of YAC identity by PFGE and dot blot hybridizations. YAC clones were separated by PFGE, blotted onto nylon filters, and hybridized with markers 1 and 2. YAC clones 2 and 3 hybridized with both

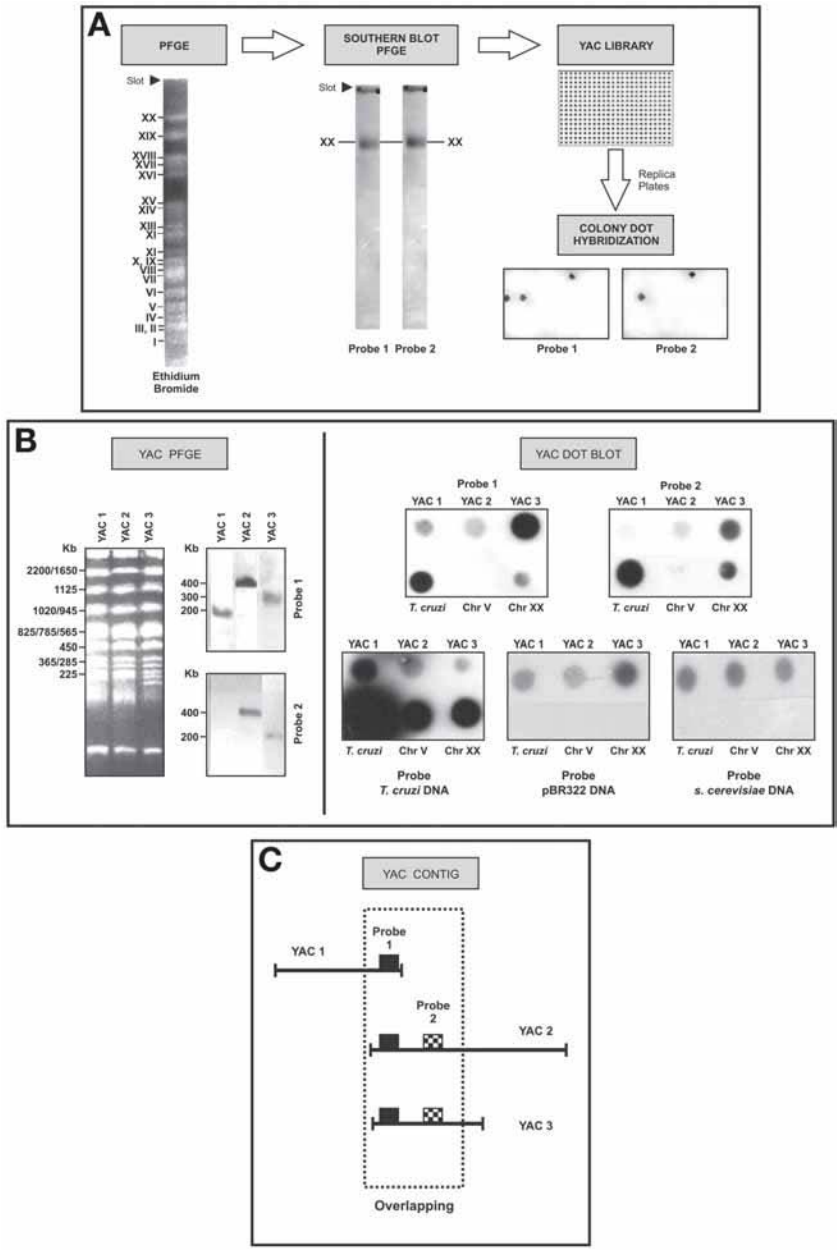

Fig. 1. (*continued from opposite page*) markers, whereas clone 1 reacted only with marker 1. Dot blots carrying DNA from both YAC clones and *T. cruzi* chromosomal bands (Chr V and Chr XX) excised from preparative PFGs were hybridized with the probes indicated in the figure. Note that probes 1 and 2 only hybridized with DNA from chromosomal band XX. **(C)** Result of YAC contiging based on clone marker content.

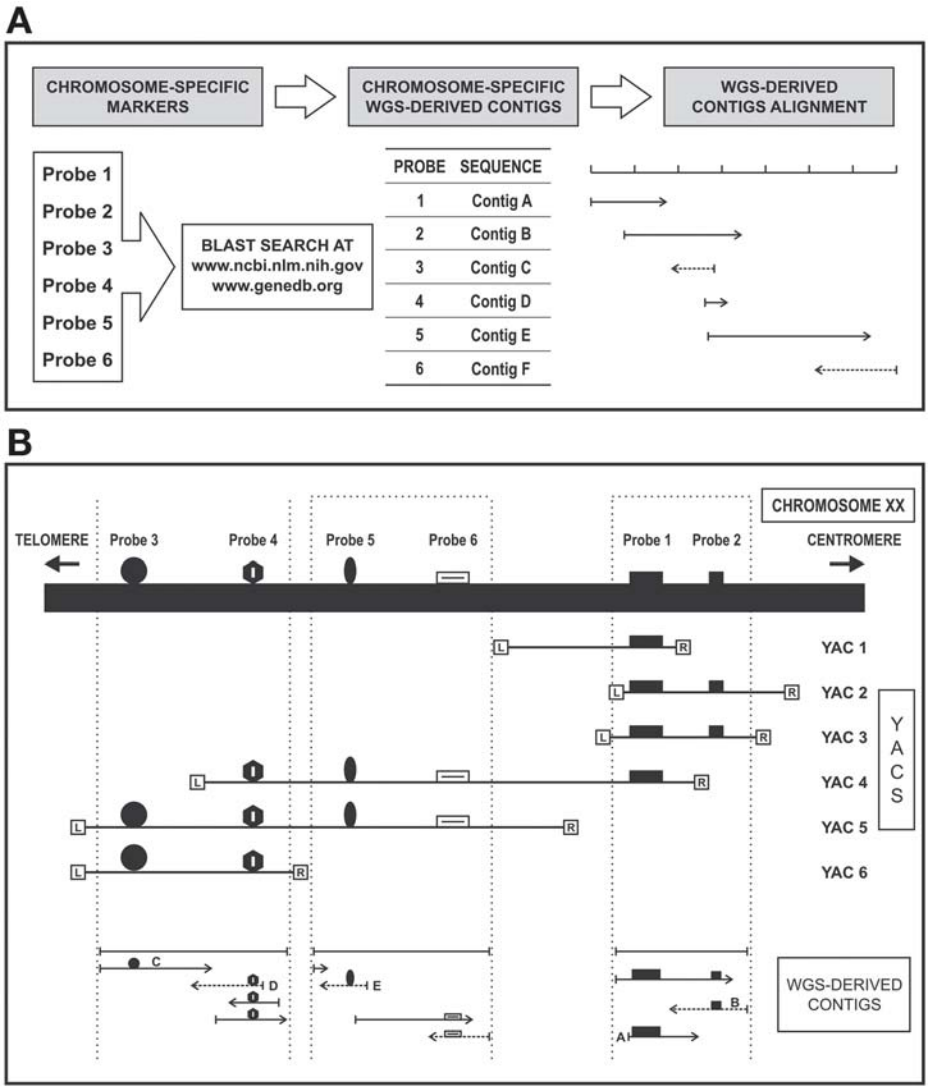

Fig. 2. Anchoring of *Trypanosoma cruzi* genome sequencing data (WGS-derived contigs) into yeast artificial chromosomes (YAC) physical map. **(A)** Identification of WGS-derived contigs harboring chromosome-specific markers by BLASTN algorithm in *T. cruzi* databases at htpp://www.genedb.org and www.ncbi.nlm.nih.gov. Assembly of WGS-derived contigs by alignment using SeqMan (DNASTAR). **(B)** An integrated physical and WGS-derived contig map of *T. cruzi* chromosome XX (top). A subset of six YAC clones is shown. The YAC ends (L, left end; R, right end) were determined by inverse polymerase chain reaction. WGS-derived contigs localized on the basis of chromosome-specific markers are indicated (bottom).

10. 20X standard saline citrate (SSC): 3 *M* NaCl and 0.3 *M* Tri-sodium citrate, pH 7.0.
11. Nylon filter (15 × 15 cm) (Hybond N, Amersham, Little Chalfont, Bucks, UK).
12. Hybridization solution: 50% formamide, 5X Denhardt's solution (100X Denhardt's solution: 20 mg/mL Ficoll 400-DL, 20 mg/mL polyvinylpyrrolidone 40, 20 mg/mL BSA pentax fraction V), 5X SSC, 50 μg/mL yeast transfer RNA, 100 μg/mL sonicated herring sperm DNA, 0.1% SDS.
13. Wash solutions: 1.0X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS.
14. T-MAT G/RA film (Kodak, Sao Jose dos Campos, S. Paulo, Brazil).

2.2. Screening of a T. cruzi *YAC Library and Characterization of YAC Clones*

1. AHC medium and plates: prepare 1 L of medium as follows: dissolve 1.7 g yeast nitrogen base without amino acids and without ammonium sulfate, 5.0 g ammonium sulfate, 10 g casein-hydrolysate-acid, 100 mg adenine hemi-sulfate. Adjust to pH 5.8 with about 50–60 μL of HCl (12 *N*). For plates add 20 g of bacto agar. Autoclave for 20 min. Cool to 65°C, add 50 mL of sterile 40% glucose.
2. 11.9 × 7.8-cm Nylon filters (Hybond N, Amersham).
3. 384-Pin replicating tool.
4. SCE: 1 *M* D-sorbitol, 100 m*M* trisodium citrate, pH 5.8, and 10 m*M* EDTA, pH 8.0. Just before use, add zymolyase to the final concentration of 1 mg/mL.
5. Denaturation solution: 0.5 *M* NaOH and 1.5 *M* NaCl.
6. Neutralization solution: 1.5 *M* NaCl and 1 *M* Tris-HCl, pH 7.5.
7. Proteinase K solution: 0.15 *M* NaCl and 0.1 *M* Tris-HCl, pH 8.0. Just before use, add proteinase K to the final concentration of 250 μg/mL.
8. 50 m*M* NaH_2PO_4, pH 7.2.
9. Whatman paper.
10. YPD liquid medium: prepare 1 L by dissolving 10 g yeast extract and 20 g bacto tryptone, adjust to pH 5.8 with HCl. Autoclave for 20 min. Cool to 65°C, add 50 mL of sterile 40% glucose.
11. SCEM buffer: 1 *M* sorbitol, 50 m*M* EDTA, 0.1 *M* sodium citrate, and 7 m*M* β-mercaptoethanol. Just before use, add zymolyase to the final concentration of 10 U/plug.
12. PKB: 0.1 *M* NaCl, 50 m*M* EDTA, pH 8.0, 1% *N*-lauroylsarcosine. Just before use, add proteinase K to a final concentration of 1 mg/mL.
13. TE: 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA.

3. Methods

3.1. Separation of T. cruzi *Chromosomal Bands and Identification of Chromosome-Specific Markers*

Size fractionation of chromosomal bands by PFGE and hybridization to different DNA probes have been used to establish the molecular karyotype of several strains and clones of *T. cruzi* (**1**,**2**). The single-run protocol here described allows

a good separation of the *T. cruzi* chromosomal bands ranging from 0.45 to 4.0 Mb ***(1)*** (**Fig. 1A**; *see* **Note 1**). Chromosome-specific markers can be identified from the *T. cruzi* expressed sequence tag (EST) and genomic libraries, and from the screening of parasite complementary DNA libraries with ^{32}P-labeled chromosomal bands ***(5,6)***. Markers can be hybridized to *T. cruzi* YAC libraries, thereby assigning individual genomic clones to the specific parasite chromosomes (**Fig. 1A,B**). The physical map of a given *T. cruzi* chromosome can be constructed by overlapping the YAC clones identified by hybridization with chromosome-specific markers and probes generated from the YAC ends (**Fig. 1C**).

3.1.1. Preparation of Agarose-Embedded T. cruzi Chromosomal DNA

1. Grow epimastigote cells from *T. cruzi* (clone CL Brener) to late logarithmic phase (5–10 × 10^6 cells/mL) in liver infusion tryptose (LIT) medium at 28°C ***(8)***.
2. Harvest the cells by centrifugation (5 min, 4°C, 2500*g*), and wash them three times with cold PSG (5 min, 4°C, 2500*g*).
3. Prepare a 2% low-melting point (LMP) agarose in water and melt it by using a microwave oven. Equilibrate the solution temperature at 50°C in a water bath.
4. Resuspend the cells in one-half the final volume of plugs to be made by using PSG at room temperature. Approximately 1 × 10^8 epimastigotes/100 µL are used for each gel plug (*see* **Note 2**).
5. Equilibrate the cell suspension at room temperature, add an equal volume of 2% LMP agarose at 50°C, and mix gently and thoroughly.
6. Warm the cell/agarose suspension to 50°C for 1 min, and immediately transfer the suspension to plug molds using a pipet.
7. Allow the agarose to solidify by placing the molds at 4–8°C in a refrigerator for 60 min.
8. Remove the agarose plugs from the molds, and transfer them into a 50-mL conical centrifuge tube. Add 5.0 mL of trypanosome lysis solution for each mL of agarose plugs.
9. Incubate the plugs at 56°C for 24 h without stirring.
10. Wash the plugs three times at 37°C for 1 h in 10 mL of 50 m*M* EDTA, pH 8.0. Use 1.0 mL of solution per mL of agarose plugs.
11. Store at 4°C in 0.5 *M* EDTA, pH 8.0.

3.1.2. Separation of T. cruzi Chromosomal DNA by PFGE

1. Running conditions:
   a. Apparatus: Gene Navigator (Pharmacia, Biotech, Uppsala, Sweden) using a hexagonal electrode array ***(1)***.
   b. Agarose concentration: 1.2% agarose gels in 0.5X TBE.
   c. Run time: 132 h at 13°C using 0.5X TBE as running buffer.
   d. Voltage and pulse time: 5 phases of homogeneous pulses with interpolation at 80 V: phase 1: pulse time 90 s (run time 30 h), phase 2: 200 s (30 h), phase 3: 350 s (24 h), phase 4: 500 s (24 h), and phase 5: 800 s (24 h).
   e. PFGE size markers: chromosomes of *S. cerevisiae* and *H. wingei*.

2. Stain the gel for 30 min with EtBr (0.5 μg/mL) and photograph the gel over a 302-nm ultraviolet (UV) transilluminator.
3. Transfer the DNA from the gel to a nylon filter (15 × 15 cm) (Hybond N, Amersham) by applying a low-pressure vacuum (*see* **Note 3**). Load the gel on the VacuGene XL (Pharmacia Biotech), cover the gel with depurination solution, and stabilize the vacuum at 50 mbar. Pour about 15 mL each of the following solutions onto the gel with a pipet and leave it for the recommended time: depurination solution, 30 min, denaturation solution, 20 min, and neutralization solution, 30 min. Make sure that the gel remains immersed all the time. If necessary pour more solution onto the gel. Pour about 15 mL of 20X SSC onto the gel with a pipet. Leave the gel for 2 h. Make sure it remains immersed all the time.
4. Fix the DNA onto the membrane by crosslinking with UV light (150 mJ) in a UV oven.

3.1.3. Hybridization of DNA Probes to Southern Blots of PFGs

1. Prepare labeled probes using standard techniques. Separate labeled probe from unincorporated labels by ethanol precipitation or by size exclusion chromatography (Sephadex G-50 columns, Pharmacia, Biotech).
2. Prehybridize the nylon filter for 1 h at 42°C with 25 mL/filter of hybridization solution.
3. Hybridize the filter overnight with radiolabeled probes at 42°C in a fresh batch of hybridization solution (20 mL/filter).
4. Pour off the hybridization solution and wash the filter two times (30 min each) with wash solution 1 (50 mL/filter) at 42°C, and three times (30 min each) with wash solution 2 (50 mL/filter) at 56°C. In general, a signal can be detected by exposing the blot to a T-MAT G/RA film (Kodak) for 24–96 h.
5. Assign the markers to the chromosomal bands by comparing the autoradiograms to actual-size photographs of the original EtBr-stained gels (*see* **Note 4**). The assignment of markers to the chromosomal bands must be confirmed in two independent hybridizations using different PFG blots.

3.2. Screening of a T. cruzi *YAC Library and Characterization of YAC Clones*

The YAC library was constructed by using DNA from epimastigotes of clone CL Brener in pYAC4 ***(4)***. The library consists of 2770 individual YAC clones (mean insert size of 365 kb) that represent about 10 genome equivalents. The library can be screened by direct hybridization of DNA probes labeled with ^{32}P to YAC library grid blots ***(4–6)***.

The screening is carried out by using specific markers from a given chromosome (*see* **Fig. 1A**,**B**). Usually, the filters are hybridized with a pool of five chromosome-specific markers labeled with ^{32}P. This strategy involved the selection of seed YAC clones for mapping along the length of the chromosome of interest and extending outward from those to develop YAC contigs.

Once a positive YAC clone is identified, a secondary screening method is used to confirm the presence of genetic markers in the YAC (**Fig. 1B**). Two approaches will be described: (1) separation of the YAC by PFGE and hybridization with the probes, and (2) polymerase chain reaction (PCR) amplification using specific primers based on the genetic marker sequences.

3.2.1. Hybridization-Based Screening With Chromosome-Specific Markers

3.2.1.1. Transfer of YAC Clones to Nylon Filters for Screening With Nucleic Acid Probes

1. Store the recombinant YAC clones in 384-well microtiter plates with AHC liquid medium at –70°C in the presence of 20% glycerol.
2. Grid directly the YAC clones from these plates onto 11.9 × 7.8-cm nylon filters (Hybond N, Amersham) using a 384-pin replicating tool.
3. Grow YAC clones for 24 h at 30°C on 2% agar in AHC.
4. Label the filter with water-resistant ink. Dry the filter at room temperature for 5 min.
5. Soak one piece of Whatman 3MM (Whatman, Brentford, Middlesex, UK) paper in a tray with SCE containing 1 mg/mL of zymolyase. Roll out bubbles, and transfer the filters (colony up side) to the tray. Wrap in cling film, and incubate overnight at 37°C.
6. Set up three trays, each containing a sheet of Whatman 3MM paper saturated with: tray 1, denaturation solution, tray 2, neutralization solution, and tray 3, proteinase K solution. During incubation the Whatman paper should be quite wet.
7. Using forceps, place the filter (colony side up) in tray 1 and incubate for 20 min at room temperature. Transfer the filter to a dry Whatman paper and leave it for 1 min at room temperature (*see* **Note 5**).
8. Transfer the filter (colony side up) to tray 2 and incubate for 20 min at room temperature. Dry the filter on Whatman paper.
9. Place the filter in tray 3 (colony up side) and incubate for 2 h at 42°C.
10. Submerge the filters and shake them very slowly in 50 m*M* NaH_2PO_4, pH 7.2 for 2–5 min at room temperature. Dry the filters on Whatman paper.
11. Fix the DNA onto the membrane by crosslinking with UV light (150 mJ) in a UV oven.

3.2.1.2. Hybridization of Nucleic Acid Probes to Nylon Filters

1. Prepare labeled probes using standard techniques. Separate labeled probe from unbound label by precipitation with ethanol or chromatography.
2. Prehybridize the nylon filter for 2–3 h at 42°C (usually 25 mL/filter) with the hybridization solution (*see* **Subheading 2.1.**, **item 12**).
3. Hybridize the filter overnight with radiolabeled probes at 42°C in a fresh batch of hybridization solution (20 mL/filter).
4. Pour off the hybridization solution and wash the filter two times (30 min each) with wash solution 1 (50 mL/filter) at 42°C, and three times (30 min each) with

wash solution 2 (50 mL/filter) at 56°C. In general, a signal can be detected by exposing the blot to a T-MAT G/RA film (Kodak) for 24–96 h.
5. Identify the positive clones in the YAC master plates. It is essential to maintain throughout the screening process a correct correspondence between the YAC master plates and the samples derived from them.

3.2.2. Confirmation of YAC Identity

3.2.2.1. Confirmation of YAC Identity by Hybridization With Chromosome-Specific Markers

1. Streak yeast cells taken from the positive well onto agar plates to isolate single colonies. Use plates containing yeast synthetic minimal medium without tryptophan or uracil.
2. Incubate the plates for 2 d at 30°C.
3. Pick four colonies and grow them in overnight cultures at 30°C in YPD liquid medium.
4. Process these cultures in order to generate agarose-embedded yeast DNA. Prepare blocks of 100 µL (3×10^8 yeast cells) in 1% LMP agarose.
5. Incubate the plugs for 3 h in SCEM buffer with zymolyase (10 U/plug) at 37°C.
6. Wash the plugs in SCE buffer and incubate overnight with 1 mL PKB containing 1 mg/mL proteinase K at 50°C.
7. Wash the plugs five times with TE (5 mL/plug) in order to remove proteinase K. Store the plugs in 50 m*M* EDTA, pH 8.0, at 4°C.
8. Separate yeast chromosomes by electrophoresis on 1% LMP agarose gels in 0.5X TBE using a contour-clamped homogeneous electric field (CHEF DRIII, Bio-Rad, Hercules, CA) apparatus with pulse times ranging from 60 to 90 s (6 V/cm, 220 V) for 18 h at 12°C.
9. Stain the gel for 30 min with EtBr (0.5 µg/mL) and photograph the gel over a 302-nm UV transilluminator.
10. Transfer the DNA from the gel to a nylon filter (15 × 15 cm) (Hybond N, Amersham) by low pressure vacuum (*see* **Subheading 3.2.**, **step 3**).
11. Fix the DNA onto the membrane by crosslinking with UV light (150 mJ) in a UV oven.
12. Hybridize the filter with the probe used to identify the positive YAC originally (*see* **Notes 6** and **7**). The hybridization details are in **Subheading 3.2.1.2.**
13. Store samples of each confirmed positive YAC clone at –70°C in 15% glycerol.

3.2.2.2. Confirmation of YAC Identity by PCR Amplification Using Primers Derived From the Chromosome-Specific Markers

1. Incubate plugs containing YAC DNA with agarase (Epicentre Technol., Le Perrayen Yvelines, France). Dilute DNA in water, boil for 10 min, and store at –70°C (*see* **Note 8**).
2. PCR conditions: 50-µL reaction volume mixtures containing 200 µ*M* of each deoxyribonucleotide triphosphate, 1X *Taq* polymerase buffer (Perkin Elmer), and 1.25 U of *Taq* polymerase. Denature YAC DNA (usually 50 ng) at 94°C for 4

min. Perform a 30-cycle amplification with denaturation at 94°C for 1 min, annealing temperatures specific to each primer for 1 min, and extension at 72°C for 30 s. Perform the final extension at 72°C for 6 min. Separate the products by electrophoresis on agarose gels and visualize by EtBr staining. Transfer the DNA fragments to nylon membranes and hybridize with specific probes.

3.3. Integration of T. cruzi *Genome Sequencing Data (WGS-Derived Contigs) Into the YAC Physical Map*

Chromosome-specific markers are used to find out homologous sequences in contigs of *T. cruzi* databases (**Fig. 2**). WGS-derived contigs will be anchored in both YAC clones and chromosomes generating an integrated map of megabase regions of the parasite genome. This strategy involves the selection of seed clones for mapping along the length of the chromosomes, and extending outward from those to develop large sequence contigs.

1. Search for WGS-derived contigs carrying chromosome-specific markers in the *T. cruzi* databases at htpp://www.genedb.org/ or www.ncbi.nlm.nih.gov/. Use BLASTN algorithm.
2. Align and assemble contigs using SeqMan II (DNASTAR) (*see* **Note 9**).
3. Anchor the WGS-derived contigs carrying chromosome-specific markers into the YAC clones.

4. Notes

1. The fluorescence distribution in EtBr spots is not the same for all *T. cruzi* chromosomal bands, this indicates that most of them contain two or more comigrating chromosomes which are not necessarily homologous. For this reason, we refer to chromosomal bands as those separated by PFGE and visible after staining with EtBr. A chromosome is a single DNA molecule.
2. The *T. cruzi* genome size (0.12–0.33 pg of DNA per cell) varies among different strains, isolates, and clones derived from the same strain. The concentration of 1×10^8 cells per plug was established for the clone CL Brener.
3. Several procedures for transferring DNA fragments onto membranes have been developed. We have obtained consistent results with vacuum transfer.
4. In order to visualize all chromosomal bands, the blots can be also hybridized with a probe containing the *T. cruzi* telomeric hexameric repeat (5'-GGGTTA-3').
5. To avoid transferring excess solution from one tray to another, dry the filter for 1 min on Whatman paper 3MM.
6. In order to check whether more than one YAC recombinant clone is present in a single yeast colony, YAC clones are usually separated by PFGE and hybridized with ^{32}P-labeled pBR322 sequences (present in the YAC cloning vector).
7. Usually, markers are also hybridized with both dot blots carrying DNA from YAC clones and *T. cruzi* chromosomal bands excised from preparative pulsed field gels (*see* **Fig. 1B**).
8. Colony PCR can be also employed to confirm the YAC identity. Pick up a yeast

colony with a sterilized toothpick, suspend it in a tube containing 50 μL of PBS, and mix. Directly administer 5 μL in the PCR reaction.

9. Manual editing improves the sequencing assembly by both identifying potential joins between contigs and performing merges, while increasing the overall contiguity of the resource.

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