

Differential Display Methods and Protocols

SECOND EDITION



Edited by

Peng Liang
Jonathan D. Meade
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METHODS IN MOLECULAR BIOLOGY™

Differential Display Methods and Protocols

Second Edition

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Preface

Since the first edition of this book dedicated to differential display (DD) technology was published in 1997, we have witnessed an explosive interest in studying differential gene expression. The gene-hunting euphoria was initially powered by the invention of DD, which was gradually overtaken by DNA microarray technology in recent years. Then why is there still the need for second edition of this DD book? First of all, DD still enjoys a substantial lead over DNA microarrays in the ISI citation data (*see* Table 1), despite the hundreds of millions of dollars spent each year on arrays. This may come as a surprise to many, but to us it implies that many of the DNA microarray studies went unpublished owing to their unfulfilled promises (*1*). Second, unlike DNA microarrays, DD is an “open”-ended gene discovery method that does not depend on prior genome sequence information of the organism being studied. As such, DD is applicable to the study of all living organisms—from bacteria, fungi, insects, fish, plants, to mammals—even when their genomes are not sequenced. Second, DD is more accessible technically and financially to most cost-conscious “cottage-industry” academic laboratories. So clearly DD still has its unique place in the modern molecular biological toolbox for gene expression analysis.

The second edition of *Differential Display Methods and Protocols* consists of a varying collection of chapters that highlight both recent methodological refinements (Chapters 1–8) and some of the fine examples of DD applications in recent years. Most of the published DD screenings in the past took a shotgun approach, by using only a limited number of primer combinations, in which only one gene was identified and characterized. This gave DD an image of low tech, low throughput, and low gene coverage. With the mathematical model for DD now solved (Chapter 1), a genome-wide comprehensive DD screening has become possible. The key to the success of high-throughput and high coverage of DD platform lies in a transition from radioactive labeling to fluorescence digital readout, as well as automated liquid handling of hundreds of DD PCR reaction setups combined with capillary electrophoresis (Chapters 2 and 4). A prototype computer program has been developed to automatically allow positive band identification from an FDD image (Chapter 7). Restriction fragment-based DD screenings offer an alternative to traditional DD and has a potential of linking any cDNA fragment directly to a given gene once the se-

Table 1
ISI Citation List ^a

Methods	Citation No.	Original publication
Differential display	3597	SCIENCE 257 (5072): 967-971, 1992
DNA microarrays	2269	SCIENCE 270 (5235): 467-470, 1995
Oligo arrays	740	SCIENCE 274 (5287): 610-614, 1996

^aISI Search (2).

quence information of all transcripts becomes available (Chapters 3, 4, and 8). Efforts to combine DD and DNA microarrays by reducing the complexity of cDNA probes while increasing the sensitivity of detection has been made successfully (Chapter 6). A DD approach has also been formulated to detect prokaryotic mRNA expression (Chapter 5).

Obviously, no matter which gene discovery methodologies one chooses to use, ultimately it will be the functional characterizations of each isolated gene, by genetic, cell biological and biochemical methods, that will likely provide the real proof (or disproof) of the relevance of the genes to a biological system under investigation. In a preface to a methods book on protein purification, Dr. Arthur Kornberg had once quoted an admonition of Efraim Racker, “Don’t waste clean thinking on dirty enzymes,” to illustrate the importance of good biochemical practice at the core of enzymology. A similar doctrine, “Don’t waste clear thinking on dirty data,” will certainly continue to help to produce better quality of science in the field of gene expression analysis over the next ten years. With this principle as a guiding light, we are extremely pleased to be able to demonstrate to our readers the power of DD technology, which is best substantiated by the genes it uncovered. Given the limit in scope and space of this book, here we can only showcase a few out of the thousands of successful DD applications published. The elegant studies described in Part II of this book have led to the discovery of many important genes involved in viral infection, Prion disease, cancer, ovulation, circadian clock, floral color, transcription repression, gene silencing, mRNA polymorphism, and protein–RNA interaction. The hallmark of a successful gene hunting expedition common to all of these DD applications is that these studies did not end with gene listing, but rather that finding the gene(s) by DD only served as a beginning of a long and often difficult scientific pursuit. We certainly hope that their footsteps will

be followed as by all those seriously contemplating a fruitful gene-hunting expedition in the future. Happy gene hunting!

Peng Liang
Jonathan D. Meade
Arthur B. Pardee

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2. ISI Search conducted on Dec 30, 2004 at Thompson Scientific, ISI Web of Knowledge. www.isinet.com.

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Global Analysis of Gene Expression by Differential Display

A Mathematical Model

Shitao Yang and Peng Liang

Summary

Differential display (DD) is one of the most commonly used approaches for identifying differentially expressed genes. However, there has been lack of an accurate guidance on how many DD polymerase chain reaction (PCR) primer combinations are needed to display most of the genes expressed in a eukaryotic cell. This study critically evaluated the gene coverage by DD as a function of the number of arbitrary primers, the number of 3' bases of an arbitrary primer required to completely match an mRNA target sequence, the additional 5' base match(s) of arbitrary primers in first-strand cDNA recognition, and the length of mRNA tails being analyzed. The resulting new DD mathematical model predicts that 80–160 arbitrary 13mers, when used in combinations with three one-base anchored oligo-dT primers, would allow any given mRNA within a eukaryotic cell to be detected with a 74–93% probability, respectively. The prediction was supported by both computer simulation of the DD process and experimental data from a comprehensive fluorescent DD screening for target genes of tumor-suppressor p53. Thus, this work provides a theoretical foundation upon which global analysis of gene expression by DD can be pursued.

Key Words: Differential display; gene expression; microarrays; p53 target genes.

1. Introduction

Prior to human genome sequencing, there were estimated to be 50,000–100,000 genes embedded in our genome. However, only around 30,000 genes could be predicted from the drafts of finished human genome sequences from

both Celera and the National Institutes of Health (NIH)-founded Human Genome Project. But more recently it was shown that there was little overlap between the genes predicted by Celera and NIH, suggesting that the gene number could be significantly higher than 30,000, which is more in agreement with earlier prediction (1). No matter exactly how many genes there are in our genome, only a fraction of them are “turned on” (expressed as mRNAs for protein synthesis) at any given time in each one of our cells. Thus, interpretation of the genomic instruction in the post-genome era will have to rely, at least in large part, on tools that can allow us to determine when and where a gene is to be turned on or off in a cell as it divides, differentiates, and ages. Obviously, such tools are also important for the detection of when and where a seemingly precise interpretation of genomic instruction goes awry, which underlies many disease states such as cancer.

Some of the major technologies developed for gene expression studies include differential display (DD) (2), serial analysis of gene expression (SAGE) (3), and DNA microarrays (4). Although, DD technology has been validated by over 4000 publications, which is more than those by any other technology based on a recent Medline search, the method was rarely considered high throughput with the possibility for global analysis of gene expression. In contrast, DNA microarray technology, while clearly high throughput, is a “closed” system in which one can only sample known gene sequences, including expressed sequence tags (ESTs). In addition, because gene “chips” are species-specific, microarray applications are for now confined to limited biological systems, such as yeast, mouse, and human, where the collection of cDNA and genomic sequences are more extensive. A true global analysis by microarrays, thus, will have to wait until all the genes in a genome are identified and annotated from genomic sequences, which is a challenging task (1). Besides, there are many pitfalls and technical problems that are being more recently realized with microarrays (5–14), despite widespread enthusiasm for the technique. Another potential technology for comprehensive gene expression analysis is SAGE (3), which is a similar, but much more clever EST sequencing strategy. However, a recent study by computer simulation has revealed that several previously published SAGE studies had overestimated the gene coverage, in part owing to errors in sampling and sequencing, as well as nonuniqueness and nonrandomness of the SAGE Tags (15).

The concept of DD is to use a limited number of short arbitrary primers in combination with the anchored oligo-dT primers to systematically amplify and visualize the 3' termini from most of the mRNA in a cell (2). Because differential display, like the SAGE technique, does not require any prior knowledge in genes to be detected, the method is an “open” system with a potential to detect most of the genes expressed in a cell. However, previous calculation of the

number of arbitrary primers required for gene coverage by DD was a rather rough approximation, without the consideration of 5' mismatches of arbitrary primers, and lack of validation by computer simulation (16). The optimal length of arbitrary primers for DD was determined by statistical consideration that each primer would recognize 50–100 mRNA species (2). To do so, these primers had to hybridize as 7mers (17). In practice, however, primers shorter than nine bases failed to be used for polymerase chain reaction (PCR) amplification (18), probably owing to the minimum contact surface between the Taq polymerase with a double-stranded DNA template. The optimal length of arbitrary primers for DD has been determined to be 13 bases, with 7 bases at the 3' end providing most, but not all, the selectivity for mRNAs (19,20) (Table 1).

Here we describe two versions of a mathematical model for gene coverage by DD, one without and the other with the consideration of arbitrary primer mismatches to the cDNA template sequences. Our new models take into account all major parameters that will affect DD gene coverage, including the number of arbitrary primers (n), the number of 3' bases from an arbitrary primer required to complete match the mRNA template (m), the length (h) and additional 5' base(s) of an arbitrary primer (x) in providing additional recognition of an mRNA template, and the length of mRNA tails being analyzed (k). Our theoretical calculation agrees well with both computer simulation and actual experimental results of DD process, which provides a foundation for global analysis of gene expression by the method.

2. Materials

1. RNApure® (GenHunter Corporation, Nashville, TN).
2. MessageClean® kit (GenHunter).
3. RNAspectra™ Red kits (GenHunter).
4. Biomek 2000 automatic liquid dispensing workstation (Beckman Coulter, Fullerton, CA).
5. FMBIO® II fluorescent laser scanner (Hitachi, Alameda, CA).
6. Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, Germany).

3. Methods

3.1. Cell Culture

The inducible p53 colon cancer cell line, A2, was cultured as described previously (21). For the induction of p53 expression, cells were grown to 80% confluence. After removing the old media, the cells were washed three times and incubated with fresh media either with (no p53 induction) or without (p53 induction) 1 µg/mL of tetracycline (*see Note 1*). The cells were continued in culture for an additional 4 h and 8 h before being analyzed.

Table 1
Priming Characteristics of Arbitrary Primers in Differential Display

DNA cloned	Arbitrary 13mer ^a	Mismatches (bp)
SP100	AAGCTtGCACCAT	2
Mob-1	AAGcttCGACTGT	4
Pai-2	aagCttGCACCAT	5
Mob-5	AagcttTGATTGCC	4
Osteopontin	aagcttTAGAGGCA	5
Mob-40	aagcTTTCATATG	4
TK	aagCTTGATTGCC	3
HoxB5	aAGCttTTGAGGT	3
Ect2	aagctTTTCATATG	5
No. of mismatches	6685640000000	4

^aThe mismatches between the arbitrary primers and the target mRNAs amplified are denoted by lower-case bases.

3.2. RNA Isolation

Total RNA was isolated with a one-step acid-phenol extraction method using RNApure reagent (GenHunter) according to the manufacturer’s instructions. To remove all chromosomal DNA contamination, total RNA was treated with DNase I using MessageClean Kit (GenHunter) as instructed.

3.3. Fluorescent Differential Display

Fluorescent differential display (FDD) was carried out with the RNAspectra Red kits (GenHunter) essentially as described (22). All FDD PCR reactions were set up automatically on a Biomek 2000 automatic liquid dispensing workstation (Beckman) (see Note 2). PCR reactions were carried out on a MasterCycler gradient thermal-cycler. For fluorescently labeled PCR products, gels were scanned without drying using the Hitachi FMBIO II fluorescent laser scanner. The digital FDD images were analyzed using Hitachi FMBIO data analysis software (v8.0) according to manufacturer’s instruction before differentially expressed genes were further analyzed (see Note 3).

3.4. Mathematical Models for Gene Coverage by DD

Given an mRNA with k bases for recognition and an arbitrary primer consists of h bases at the 5' end and m bases at the 3' end. The primer “hits” the mRNA if m bases at the 3' end of the primer and at least x bases among the h bases at the 5' end of the primer match the mRNA target sequences. Note that not all h bases at the 5' end have to match with the mRNA target sequences.

When this happens, it is referred to as the primer degeneracy. It is assumed that all four bases “G,” “A,” “T” (U for mRNA), and “C” occur at each base in the primers as well as mRNA targets independently with an equal probability of 25%.

3.4.1. Gene Coverage Without the Consideration of Primer Mismatches at the 5'Ends

To model the gene coverage by DD, we first consider the simplest case where we ignore the h bases at the 5' end of the primer where mismatches to first stand cDNAs occur. In other words, we consider an arbitrary primer only consists of m bases at the 3' end. The primer “hits” the mRNA if the m bases of the primer completely match the mRNA target sequences.

Before proceeding, we define some notations. Let:

k = the upper limit of the number of bases from an mRNA 3' tail to which the arbitrary primers hybridize.

n = the number of arbitrary primers randomly selected without replacement.

m = the number of bases an arbitrary primer contains at the 3' end.

H_i = the event that i^{th} arbitrary primer “hits” one of the mRNA recognition site; that is, there is a match between m bases at the 3' end of i^{th} arbitrary primer and the mRNA target sequences, where $i = 1, 2, \dots, n$.

H_{ij} = the event that the i^{th} arbitrary primer “hit” the mRNA's j^{th} recognition site, where. (Given the mRNA with k bases for recognition, there are $k - m + 1$ recognition sites. This number can be easily obtained by overlaying succeeding m base primers with one base difference along a target mRNA with k bases, see **Fig. 1**).

$P(k, m, n)$ = the probability that n randomly selected m -base arbitrary primers “hit” one of the k -base mRNA's recognition sites (at least once).

Given an mRNA with k bases for recognition, we randomly choose an arbitrary primer with m bases ($m < k$) at its 3' end to scan the mRNA starting from the first recognition site (i.e., 1 to m) to the last recognition site (i.e., $k - m + 1$ to k). The probability that the first arbitrary primer hits any one of the mRNA recognition sites j is:

(1)

$$P(H_{1j}) = \frac{1}{4^m}$$

Thus, the probability of not hitting the recognition site j is:

(2)

$$P(H_{1j}^c) = 1 - P(H_{1j}) = 1 - \frac{1}{4^m}$$

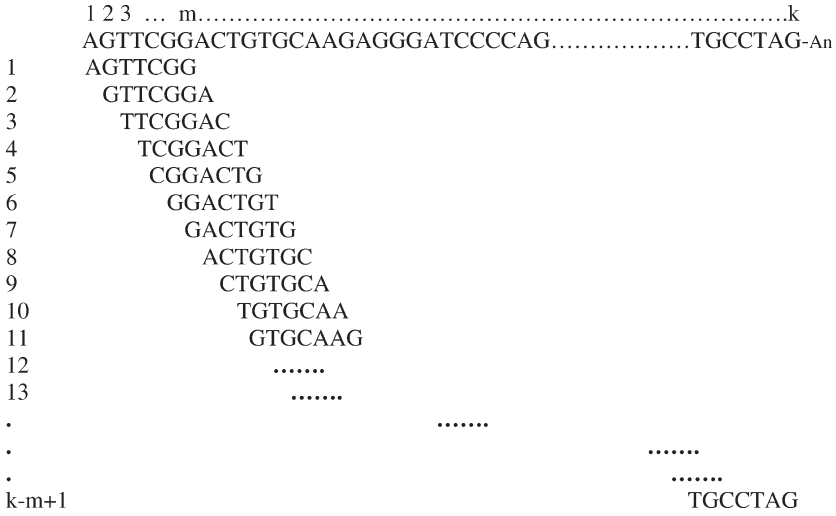


Fig. 1. Schematic illustration of the number of possible 7-base binding sites contained in any given mRNA 3' terminus of k bases in length, excluding bases in the poly A tail.

Although the event of not hitting the recognition site j and that of not hitting another recognition site j' ($j \neq j'$) may not be always independent, when $k \gg m$, we assume that they are. Under this assumption, the probability of the first arbitrary primer not hitting any of the mRNA recognition sites is:

$$(3) \quad P(H_1^c) = P(H_{11}^c \cap H_{12}^c \cap \dots \cap H_{1k-m+1}^c) = \prod_{j=1}^{k-m+1} P(H_{1j}^c) = \left(1 - \frac{1}{4^m}\right)^{k-m+1}$$

Now, we consider the second arbitrary primer randomly selected without replacement. The conditional probability that the second primer will not hit any of the mRNA recognition sites given the first primer did not hit the mRNA is:

$$(4) \quad P(H_2^c | H_1^c) = \left(1 - \frac{1}{4^m - 1}\right)^{k-m+1}$$

The probability that neither of these two primers hits the given mRNA is:

$$(5) \quad P(H_1^c \cap H_2^c) = P(H_1^c) P(H_2^c | H_1^c) = \left(1 - \frac{1}{4^m}\right)^{k-m+1} \left(1 - \frac{1}{4^m - 1}\right)^{k-m+1}$$

In general, the probability the i^{th} primer will hit any of the mRNA recognition sites j is:

$$(6) \quad P(H_{ij}) = \left(1 - \frac{1}{4^m - 1}\right), \text{ where } i = 1, 2, \dots, n$$

Under the assumption (3), the conditional probability that the i^{th} primer will not hit any of the mRNA recognition sites given the first $i-1$ primers did not hit the mRNA is

$$(7) \quad P(H_i^c | H_1^c \cap H_2^c \cap \dots \cap H_{i-1}^c) = [1 - P(H_{ij})]^{k-m+1} \left[1 - \frac{1}{4^m - (i-1)}\right]^{k-m+1}$$

The probability that none of the n randomly selected arbitrary primers will hit a given mRNA is:

(8)

$$\begin{aligned} & P(H_1^c \cap H_2^c \cap \dots \cap H_n^c) \\ &= P(H_1^c) P(H_2^c | H_1^c) \dots P(H_i^c | H_1^c \cap H_2^c \cap \dots \cap H_{i-1}^c) \dots P(H_n^c | H_1^c \cap H_2^c \cap \dots \cap H_{n-1}^c) \\ &= \left(1 - \frac{1}{4^m}\right)^{k-m+1} \left(1 - \frac{1}{4^{m-1}}\right)^{k-m+1} \dots \left[1 - \frac{1}{4^m - (i-1)}\right]^{k-m+1} \dots \left[1 - \frac{1}{4^m - (n-1)}\right]^{k-m+1} \\ &= \prod_{i=0}^{n-1} \left(1 - \frac{1}{4^m - i}\right)^{k-m+1} \end{aligned}$$

Thus, the probability that n arbitrary primers will hit the given mRNA at least once is:

$$(9) \quad P(k, m, n) = 1 - \prod_{i=0}^{n-1} \left(1 - \frac{1}{4^m - i}\right)^{k-m+1} = 1 - \left(1 - \frac{n}{4^m}\right)^{k-m+1}$$

Notice that if one uses all possible m -based primers ($n = 4^m$), then any given mRNA will be hit for sure as illustrated below:

$$(10) \quad P(k, m, 4^m) = 1 - \left(1 - \frac{4^m}{4^m}\right)^{k-m+1} = 1 - 0 = 1$$

3.4.2. Gene Coverage With the Consideration of Primer Mismatches at the 5'Ends

Now, we extend the mathematical model as described by **Eq. 9** to cover a more realistic DD process with the consideration of the additional base parings contributed by the 5' portion of an arbitrary primer. In this case, the primer “hits” the mRNA if m bases at the 3' end of the primer and at least x bases among the h bases at the 5' end of the primer match the mRNA target sequences.

Before proceeding, we define several addition notations. Let:

h = the number of bases at the 5' end of an arbitrary primer

x = the minimum number of bases at the 5' end of the primer that are required to match the mRNA target sequences.

$H^{5'}$ = the event that at least x bases at the 5' end of an arbitrary primer matches the corresponding mRNA target sequences.

$P(k, x, h, m, n)$ = the probability that n arbitrary primer consisting of h bases at the 5' end and m bases at the 3' end “hit” the mRNA tail with k bases for recognition at least once.

Clearly, given h bases at the 5' end of an arbitrary primer, the probability of the event that an exact x bases at the 5' end of the arbitrary primer match the mRNA corresponding bases follows the Binomial distribution:

$$(11) \quad b(x; h, p) = \binom{h}{x} p^x (1-p)^{h-x}, \text{ where } p = \frac{1}{4}$$

The probability of a match of at least x bases among h bases at the primer's 5' end with the corresponding bases of the mRNA is:

$$(12) \quad P(H^{5'}) = \sum_{l=x}^h b(l; h, p) = \sum_{l=x}^h \binom{h}{l} \left(\frac{1}{4}\right)^l \left(\frac{3}{4}\right)^{h-l}$$

Note that when $x = 0$, that is, if no additional match at the 5' end of the primer is required, then:

$$(13) \quad P(H^{5'}) = \sum_{l=0}^h b(l; h, p) = \sum_{l=0}^h \binom{h}{l} \left(\frac{1}{4}\right)^l \left(\frac{3}{4}\right)^{h-l} = 1$$

Given an mRNA tail with k bases for recognition and an arbitrary primer consisting of h bases at the 5' end and m bases at the 3' end, the event that m -bases at the 3' end of the primer match the mRNA corresponding bases and the event that a at least x bases at the 5' end of the primer match with the mRNA

corresponding bases are independent. Therefore, the event that the primer “hits” the mRNA is the intersection of the above two events. Because they are independent, the probability of the i^{th} primer “hits” the mRNA recognition site j is equal to the product of the probabilities of the two events. The probability of the event that m -bases at the 3' end of the primer match the mRNA corresponding bases is given by **Eq. 6**. Thus, with the consideration of gene degeneracy, the i^{th} primer hit any of the mRNA recognition sites j is:

$$(14) \quad P(H_{ij}) = P(H^{5'}) \frac{1}{4^m - (i-1)} = \frac{\sum_{l=x}^h \binom{h}{l} \left(\frac{1}{4}\right)^l \left(\frac{3}{4}\right)^{h-l}}{4^m - (i-1)}$$

It can be easily shown that the probability that n arbitrary primers consisting of h bases at the 5' end and m bases at the 3' end will “hit” the given mRNA at least once is:

$$(15) \quad P(k, x, h, m, n) = 1 - \prod_{i=0}^{n-1} \left(1 - \frac{\sum_{l=x}^h \binom{h}{l} \left(\frac{1}{4}\right)^l \left(\frac{3}{4}\right)^{h-l}}{4^m - i} \right)^{k-m-h+1}$$

When $x = 0$ and $h = 0$, then

$$(16) \quad P(k, 0, 0, m, n) = 1 - \prod_{i=0}^{n-1} \left(1 - \frac{1}{4^m - i} \right)^{k-m+1} = P(k, m, n)$$

Thus, **Eq. 9** is a special case of **Eq. 15**.

3.5. Results

3.5.1. Mathematical Models for Gene Coverage by DD

The strategy of DD is to use a series of short primers (10–13 bases) with arbitrary sequences to scan the 3' terminal sequences up to 1000 bases of different mRNAs in a cell (2). When these primers exhibit matches in sequences to the mRNA targets, along with one of the anchored oligo-dT primers that bind to the poly-A tails present in all eukaryotic mRNAs, the corresponding mRNA species are amplified (or “hit”) and visualized by DD. The experimental data on the priming characteristics of arbitrary primers agreed well with our original prediction that a minimal of six to seven bases at the very 3' ends of these primers are required to match (recognize) the target mRNAs, whereas the 5' bases

often exhibit mismatches to the mRNA target sequences (**16,20**) (**Table 1**). Our previous mathematical model of DD in gene coverage have, however, has discounted the contribution of these 5' bases of arbitrary primers, where additional base matches to mRNA occur (**16,17**). For a better illustration of our new mathematical model for DD, we shall consider two scenarios, one without and the other with taking into account the primer mismatches in hybridization, the latter of which will likely more accurately reflect the true gene coverage by DD.

For a better illustration of our new mathematical model for DD, we shall consider two scenarios, one without and the other with taking into account the primer mismatches in hybridization, the latter of which will likely more accurately reflect the true gene coverage by DD.

Let us consider the first scenario first. Given an mRNA tail with k bases for recognition, we randomly choose a short arbitrary primer with m bases ($m < k$) to scan the mRNA tail starting from the first recognition site (i.e., 1 to m) to the last recognition site (i.e., $k - m + 1$ to k) (**Fig. 1**). If there is a complete match in sequence between the primer and any of the mRNA recognition site(s), then the primer “hits” the mRNA. Thus, the probability that n arbitrary primers will “hit” any given mRNA by DD at least once, denoted as, can be represented with the following algorithms (for detailed mathematical modeling, see **Subheading 3.4**):

$$(9) \quad P(k, m, n) = 1 - \prod_{i=0}^{n-1} \left(1 - \frac{1}{4^m - 1} \right)^{k-m+1} 1 - \left(1 - \frac{n}{4^m} \right)^{k-m+1}$$

The mathematical model as described by **Eq. 9** for gene coverage by DD takes into account all three important variables, namely, k for the upper limit of the number of bases from an mRNA 3' tail to which the arbitrary primers hybridize, m for the number of 3' bases of an arbitrary primer, which are required to match the mRNA target sequences, n for the number of arbitrary primers randomly selected without replacement.

The importance of this mathematical model becomes apparent, for it allows us to predict the effects of m , k , and n on the gene coverage by DD. For example, if the length of mRNA 3' tails (k) being analyzed by DD is set at 600 bases (limited by separating power of a DNA sequencing gel) and the minimum number of required base matches (m) is set at 7, the probability of hitting any given mRNA using 80 and 160 randomly chosen arbitrary primers would be 94.5 and 99.7%, respectively (**Fig. 2A**). However, if assuming m still equals 7, but k is either decreased to 300 or increased to 1000 from 600 bases, the gene coverage can be decreased to 76.3% or increased to 99.2%, respectively, from 94.5%, using only 80 arbitrary primers (**Fig. 2B**). Similarly, if the number of bases (m) of an arbitrary primer required to match a target mRNA with 600 bases for recognition is either decreased to 6, or increased to 8 from 7 bases, the prob-

ability of hitting any given mRNA using 80 arbitrary primers increases to 99.9% or decreases to 51.5%, respectively, from 94.5% (**Fig. 2C**).

Clearly, **Eq. 9** only considers the gene coverage by DD as defined by the complete match between the bases at the 3' end (m) of an arbitrary primer with the target mRNA. In practice, as described earlier, in addition to the m -base match at the 3' end of the primers, additional base(s) at the 5' end of an arbitrary primer also exhibit matches to the target mRNA sequences (**Table 1**), and thus could contribute to mRNA template recognition. To take into account the 5' mismatch of the arbitrary primers to mRNA sequences, a more realistic DD model can be derived based on **Eq. 1**. Assuming that an arbitrary primer consists of two parts, with h bases at the 5' end and m bases at the 3' end, an arbitrary primer "hitting" a target mRNA can be redefined as, in addition to perfect matches between m bases at the 3' end of the primer and an mRNA target sequence, at least x out of h bases ($x \leq h$) from the 5' end of the primer are also required to match the mRNA target sequences. In this scenario, the probability that n arbitrary primers will "hit" the given mRNA at least once, denoted as, $P(k, x, h, m, n)$ can be represented as follows (for detailed mathematical modeling, see **Subheading 3.4.**):

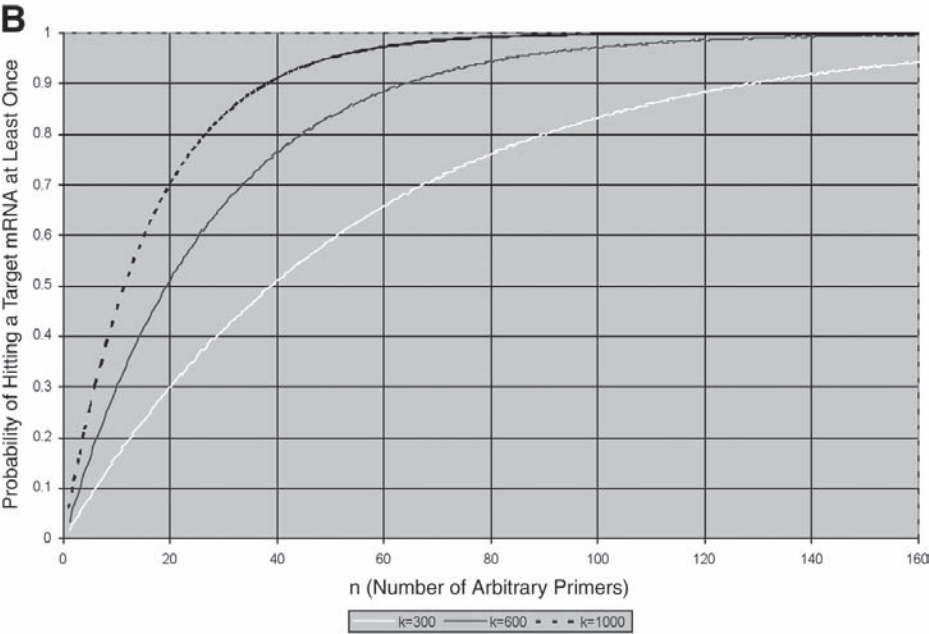
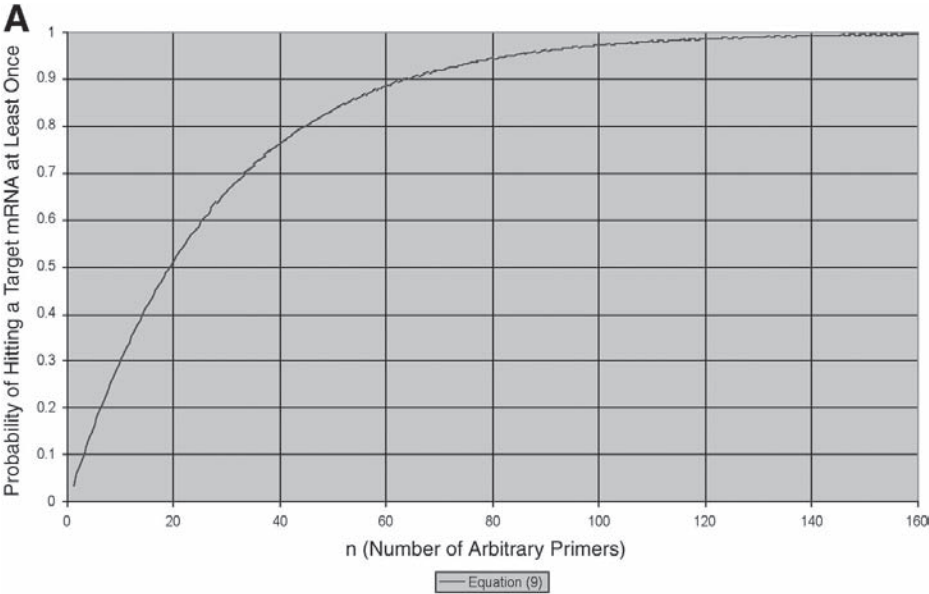
$$(15) \quad P(k, x, h, m, n) = 1 - \prod_{i=0}^{n-1} \left(1 - \frac{\sum_{l=x}^h \binom{h}{l} \left(\frac{1}{4}\right)^l \left(\frac{3}{4}\right)^{h-l}}{4^m - i} \right)^{k-m-h+1}$$

Clearly, based on **Eq. 15**, an additional requirement of the 5' base(s) of an arbitrary primer matching to an mRNA template would reduce the probability of the gene coverage by DD. As shown in **Fig. 3**, assuming $k = 600$, $m = 7$ and $h = 6$, the additional requirement, for example, of two bases ($x = 2$) between an arbitrary 13mer and any given mRNA template would reduce the probability of the gene coverage from 94.5 to 73.9% using 80 such arbitrary primers. In order to maintain the same 94.5% gene coverage, the number of arbitrary 13mers has to be increased from 80 to 172.

The resulting new DD mathematical model, **Eq. 15**, predicts that 80–160 arbitrary 13mers, when used in combinations with three one-base anchored oligo-dT primers, would allow any given 600-base mRNA within a eukaryotic cell to be detected with a 73.9–93.2% probability, respectively.

3.5.2. Computer Simulation of Gene Coverage by DD

To test the validity of **Eq. 1** and **Eq. 2**, computer simulations of DD process were conducted. The simulation programs were written in Java. Specifically,



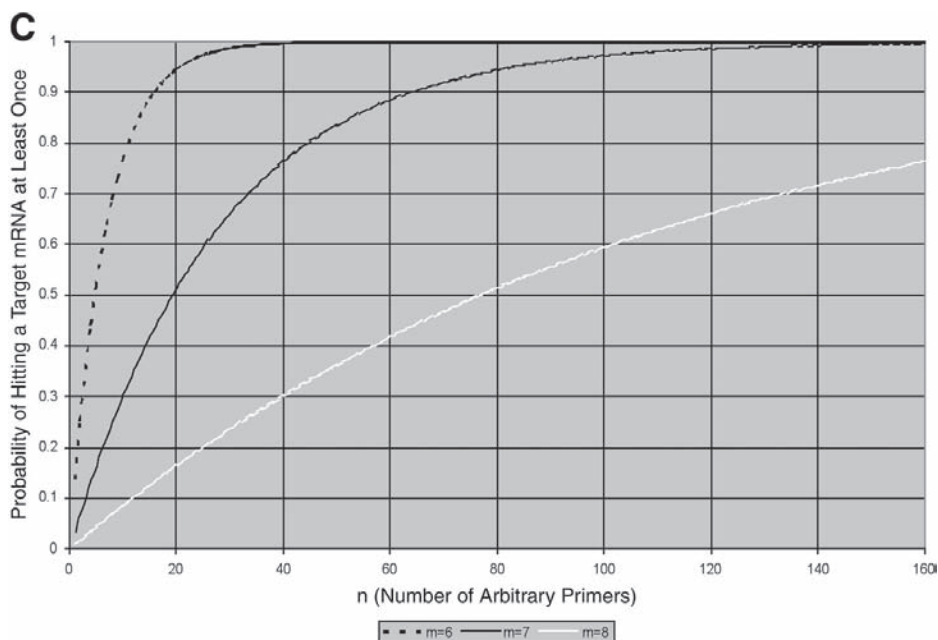


Fig. 2. Computer modeling of gene coverage by differential display with perfectly matching arbitrary primers. The probabilities of hitting any given mRNA at least once are presented as either a function of the number of arbitrary primers when $m = 7$ and $k = 600$ bp (A), the length of mRNA tails ($k = 300, 600$, or 900 bases) when $m = 7$ (B), or the number of base matches ($m = 6, 7$, or 8 bases) between an arbitrary primer and mRNAs when $k = 600$ bp (C).

Java method “IndexOf” was used to detect and record the match between an arbitrary primer and the target mRNA (see **Note 4**).

To test the validity of **Eq. 9**, we set $m = 7$, and $k = 600$. For each simulation experiment, we first randomly generated an mRNA tail with 600 bases and then randomly selected n ($n = 1, 2, \dots, 160$) m -base arbitrary primer(s) from 16,384 possibilities (4^7) without replacement to scan the target mRNA. If any of the n primers “hit” the mRNA, we recorded the experimental result as “success.” The experiment was repeated 5000 times for any given number of arbitrary primers n ($n = 1, 2, \dots, 160$). The probability for each primer “hitting” any given mRNA was approximated by the frequency of “success” (total number of “successes” recorded in 5000 independent trials/5000). The simulation results on the gene coverage by DD without the consideration of the primer degeneracy at the 5' end are in excellent agreement with model predictions (**Fig. 3**).

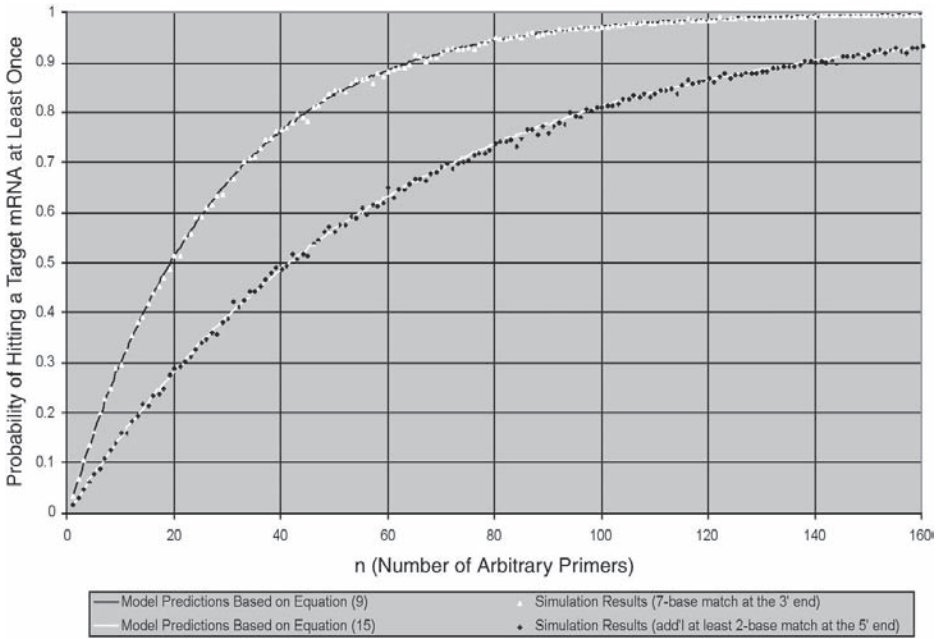


Fig. 3. Computer modeling and simulation of differential display (DD) with and without the consideration of primer mismatches at the 5' ends. Model predictions in gene coverage by DD without considering primer mismatches (**Eq. 9** in black solid line) and with considering two additional base matches from the 5' end of arbitrary 13mers (**Eq. 15** in white solid line) are compared with computer simulation results (white dots and black dots are simulations without and with consideration of primer mismatches at the 5' ends), assuming $k = 600$, $m = 7$, $h = 6$, and $x = 2$.

Very consistent results were also obtained for the DD simulation with consideration of the primer mismatches at the 5' end. Again, the simulation experiment started with a randomly generated mRNA tail with 600 bases. We then randomly selected n ($n = 1, 2, \dots, 160$) 13mers, each consisting of six randomly selected bases at the 5' end and seven bases at the 3' end (without replacement) to scan the mRNA tails. If one of the 13mers “hits” an mRNA, that is, in addition to a match of 7 bases at the 3' end of the arbitrary 13mer, at least two out of six bases at the 5' end of the 13mer also had to match the mRNA sequences, then we recorded the experiment as a “success.” The experiment was repeated 5000 times and the frequency of “success” in 5000 independent trials for a given number of arbitrary 13mers ($n = 1, 2, \dots, 160$) was recorded as the probability that any given mRNA is “hit.” As shown in **Fig. 3**, our math-

ematical model predictions based on **Eq. 15** were in excellent agreement with the simulation results.

3.5.3. Comprehensive Fluorescent Differential Display Analysis of p53 Tumor-Suppressor Gene Targets

To further test the gene coverage predicted by our mathematical model, we experimentally compared mRNA expression profiles of A2, a colon cancer cell line, with and without the induction of tumor-suppressor gene p53 (**21**). The A2 cell line contained a tetracycline-repressible wild-type p53. Automated FDD (**22**) with 164 combinations of primers (120 arbitrary primers with the G-anchored primer, 24 arbitrary primers with A- and C-anchored primers) was tested for the screening of genes induced by the tetracycline removal. Highly reproducible patterns of cDNAs were obtained that revealed over two dozen mRNAs that were clearly induced by the tetracycline removal (unpublished results). Subsequent cloning and sequencing of these cDNAs indicated that at least three of them corresponded to the p53- β -globin transgene, which was under tetracycline control (**Fig. 4**). In each case when p53 was detected as an inducible gene, different arbitrary primers (H-AP20, H-AP54, and H-AP63) hit different regions of the 3' tail of the p53- β -globin transgene in a fashion predicted by our model. The rest of the p53 targets genes identified will be described elsewhere. Because the mRNA transcript corresponding to p53-globin transgene ends with a "C" before its poly-A tail, the finding of it being hit by 3 out of 120 different randomly selected arbitrary primers in combination with the "G" anchor strongly supports the comprehensiveness of gene coverage by DD predicted by our mathematical model presented herein.

3.6. Discussion

Since its invention, differential display has become one the most popular methods for identifying and cloning differentially expressed genes (**7,20**). This work offers a mathematical principle behind the DD method and provides a theoretical foundation upon which a systematic and comprehensive analysis of gene expression can be carried out. The new mathematical model takes into account five major parameters: the length (number of bases) of the mRNA tails to be detected (k), the number of bases of an arbitrary primer critical for the recognition of target cDNAs (m), the degeneracy of arbitrary primers during DD (the additional x out of h 5' base(s) required to match an mRNA sequence), the number of arbitrary primers (n), and the probability of detecting any given mRNA at least once. Our model predicts that either increasing the number of arbitrary primers or the length of the cDNA target sequence to be analyzed can substantially increase the probability of detecting any given mRNA species in a cell by DD. However, the gene coverage as a function of the number of 3'

1	ACTGTGTCATG	GCGACTGTCC	AGCTTTGTGC	CAGGAGCCTC	GCAGGGGTTG	ATGGGATTGG
61	GGTTTTCCCC	TCCCATGTGC	TCAAGACTGG	CGCTAAAAGT	TTTGAGCTTC	TCAAAAGTCT
121	AGAGCCACCG	TCCAGGGAGC	AGGTAGCTGC	TGGGCTCCGG	GGACACTTTG	CGTTCGGGCT
181	GGGAGCGTGC	TTTCCACGAC	GGTGACACGC	TTCCCTGGAT	TGGCAGCCAG	ACTGCCTTCC
241	GGGTCACTGC	CATGGAGGAG	CCGCAGTCAG	ATCCTAGCGT	CGAGCCCCCT	CTGAGTCAGG
301	AAACATTTTC	AGACCTATGG	AAACTACTTC	CTGAAAACAA	CGTTCTGTCC	CCCTTGCCGT
361	CCCAAGCAAT	GGATGATTTG	ATGCTGTCCC	CGGACGATAT	TGAACAATGG	TTCACTGAAG
421	ACCCAGGTCC	AGATGAAGCT	CCCAGAATGC	CAGAGGCTGC	TCCCCGCGTG	GCCCCGTCAC
481	CAGCAGCTCC	TACACCGGCG	GCCCCGTCAC	CAGCCCCCTC	CTGGCCCCTG	TCATCTTCTG
541	TCCCTTCCCA	GAAAACCTAC	CAGGGCAGCT	ACGGTTTCCG	TCTGGGCTTC	TTGCATTTCTG
601	GGACAGCCAA	GTCCTGTGACT	TGCACGTACT	CCCCTGCCCT	CAACAAGATG	TTTTGCCAAC
661	TGGCCAAGAC	CTGCCCTGTG	CAGCTGTGGG	TTGATTCCAC	ACCCCCGCCC	GGCACCCGCG
721	TCCGCGCCAT	GGCATCTAC	AAGCAGTCAC	AGCACATGAC	GGAGGTTGTG	AGGCGCTGCC
781	CCCACCATGA	GCGCTGCTCA	GATAGCGATG	GTCTGGCCCC	TCCTCagcaT	cTTATCCGAG
HAP-63						
841	TGGAAGGAAA	TTTGCGTGTG	GAGTATTTGG	ATGACAGAAA	CACCTTTTCGA	CATAGTGTGG
901	TGGTGCCCTA	TGAGCCGCCT	GAGGTTGGCT	CTGACTGTAC	CACCATCCAC	TACAACTACA
961	TGTGTAACAG	TTCTTGCATG	GGCGGCATGA	ACCGGAGGCC	CATCCTCACC	ATCATCACAC
1021	TGGAAGACTC	CAGTGGTAAT	CTACTGGGAC	GGAacagcTT	TGAGGTGCGT	GTTTGTGCCT
HAP-54						
1081	GTCCTGGGAG	AGACCGGCGC	ACAGAGGAAG	AGAATCTCCG	CAAGAAAGGG	GAGCCTCACC
1141	ACGAGCTGCC	CCCAGGGAGC	ACTAAGCGAG	CACTGCCCAA	CAACACCAGC	TCCTCTCCCC
1201	AGCCAAAGAA	GAAACCACCTG	GATGGAGAAT	ATTTCACCCT	TCAGATCCGT	GGGCGTGAGC
1261	GCTTCGAGAT	GTTCCGAGAG	CTGAATGAGG	CCTTGAAGCT	CAAGGATGCC	CAGGCTGGGA
1321	AGGAGCCAGG	GGGGAGCAGG	GCTCACTCCA	GCCACCTGAA	GTCCAAAAGG	CTCCTGGGCA
1381	ACGTGctGgT	TGTTGTGCTG	TCTCTTCATT	TTGGCACAGA	ATTCACCTCT	CAGGTGCAGG
HAP-20						
1441	CTGCCTATCA	GAAGGTGGTG	GCTGGTGTGG	CCAATACCCCT	GGCTCACAAA	TACCACTGAG
1501	ATCGATCTTT	TTCCCTCTGC	CAAAAATTAT	GGGGACATCA	TGAAGCCCCT	TGAGCATCTG
1561	ACTTCTGGCT	AATAAAGGAA	ATTTATTTTC	ATTGCAAAAA	AAAAAAGCTT	
H-T₁₁G						

Fig. 4. Arbitrary 13mers hitting the inducible p53 tumor-suppressor transgene. The cDNA sequence of the wild-type p53- β -globulin (3' untranslated region) transgene under tetracycline control is shown with the locations where three different arbitrary 13mers (HAP-20, HAP-54, and HAP-63) and the G-anchored oligo-dT primer were found to recognize the p53- β -globulin cDNA. Mismatches are depicted in lower-case letters.

bases (m) required to completely match an mRNA sequence by an arbitrary primer is probably the most critical as the relationship is exponential (**Fig. 2C**). Our experimental data support that m is close to 7 (**Table 1**).

It is interesting to note that our new mathematical model for gene coverage by DD (**Eq. 9**) appears to be very similar to our previous mathematical model, $P = 1 - (1 - k/4^m)^n$ (**16,17**), except that n and k are switched in place. The error in our earlier model came from an incorrect assumption that the estimated numbers of primer binding sites (close to k) within an mRNA tail were all nonredundant. As a result, the prediction of the percentage of mRNAs recog-

nized by each arbitrary primer of m bases being $k/4^m$ does not hold in the old model. Furthermore, the earlier DD model did not take into account the additional 5' base matches of arbitrary primers to the mRNA sequences, which would lower the probability in gene coverage. Thus the new model presented here should provide a more accurate guidance for global analysis of gene expression by DD technology.

In deriving the mathematical model for DD, we had assumed that the event of an arbitrary primer not hitting one mRNA recognition site and that of not hitting any other mRNA recognition sites were independent. Although this assumption may not always hold (e.g., when k is close to m in value, which is unlikely to happen for an mRNA), our computer simulation results and empirical experimental findings both confirmed that our model is a very good approximation for gene coverage by DD. Furthermore, the new DD model should also provide us with valuable insight into future primer designs with optimal primer specificity and gene coverage by DD technology.

4. Notes

1. To avoid detecting differences that were irrelevant to p53 induction, two sets of cells, one washed with media containing tetracycline and the other without tetracycline were prepared. After incubating the cells with fresh media either with (no induction) or without (p53 induction) tetracycline, RNA samples were extracted at 4 and 8 h thereafter (21).
2. To avoid pipetting errors as much as possible, core FDD mixes containing everything except, cDNA and arbitrary primers were made, before putting on the robot, for each anchored primer labeled with fluorescence. Each core mix and the a set of arbitrary primers are then programmed with BioMek 2000 to be automatically mixed with different cDNAs on a 96-well PCR plate.
3. After PCR, FDD samples were manually loaded and separated on a 130-lane Jumbo gel. Digital images of highly reproducible FDD cDNA patterns were obtained with Hitachi FMBIO II fluorescent laser scanner. cDNA bands corresponding candidate p53 target genes were retrieved and reamplified after overlaying the gel on top of the true gel-size FDD image printout.
4. The Java code for DD simulation can be accessed at <http://pirate.shu.edu/~yangst/dd.htm>.

Acknowledgments

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Automation of Fluorescent Differential Display With Digital Readout

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Summary

Since its invention in 1992, differential display (DD) has become the most commonly used technique for identifying differentially expressed genes because of its many advantages over competing technologies such as DNA microarray, serial analysis of gene expression (SAGE), and subtractive hybridization. Despite the great impact of the method on biomedical research, there has been a lack of automation of DD technology to increase its throughput and accuracy for systematic gene expression analysis. Most of previous DD work has taken a “shot-gun” approach of identifying one gene at a time, with a limited number of polymerase chain reaction (PCR) reactions set up manually, giving DD a low-tech and low-throughput image. We have optimized the DD process with a new platform that incorporates fluorescent digital readout, automated liquid handling, and large-format gels capable of running entire 96-well plates. The resulting streamlined fluorescent DD (FDD) technology offers an unprecedented accuracy, sensitivity, and throughput in comprehensive and quantitative analysis of gene expression. These major improvements will allow researchers to find differentially expressed genes of interest, both known and novel, quickly and easily.

Key Words: Fluorescent differential display; DD; FDD; differential gene expression; automation; differential display on automated sequencer.

1. Introduction

How can a single fertilized egg containing a complete set of genes unique to a species give rise to so many different cell types that will ultimately organize into the different tissues and organs that define each specific organism? This has been one of the most elusive questions in biology, because even complete sequencing of many genomes, from a few thousand basepairs for bacteria to

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over 3 billion basepairs for human, has yet to provide enough clues to this mystery of life. Of the estimated 20,000–25,000 genes embedded in our genome, only a fraction of them, perhaps 10–15%, are “turned on” (expressed as mRNAs for protein synthesis) at any given time in each of our cells. Thus, interpretation of the genomic instructions in the post-genome era will have to rely, at least in large part, on tools that can allow us to determine when and where a gene is to be turned on or off in a cell as it divides, differentiates, and ages. Such tools are also important for the detection of when and where a seemingly precise interpretation of genomic instruction goes awry, which underlies many disease states such as cancer. Differential display (DD) technology (**1**) is one of the major tools that has already helped thousands of researchers all over the world interpret gene expression in their specific projects.

DD technology continues to be one of the most reliable methods for gene expression analysis available to biomedical researchers. Since its invention in 1992, the number of publications using DD has exploded to over 3600, easily outnumbering the publications using other competitive methodologies such as DNA microarrays (**2,3**), serial analysis of gene expression (SAGE) (**4**), and subtractive hybridization (**5**) (*see Table 1*). It is clear that the rapid and successful adoption of differential display has been largely attributed to the simplicity of the method. Simplicity ensures a higher probability of success and few artifactual differences caused by experimental errors. Essentially, starting from the RNA samples being compared, only two steps, reverse transcription and polymerase chain reaction (PCR), are needed before signals generated are analyzed on a gel matrix. No additional steps such as second-strand DNA synthesis, purification of cDNA, restriction enzyme digestion, adapter primer ligation, probe labeling/normalization, hybridization, or washing steps are required, because each of these steps could introduce and amplify errors or lead to the loss of mRNAs being detected.

DD takes advantage of three of the most simple, powerful, and commonly used molecular biological methods: reverse transcriptase (RT)-PCR, DNA sequencing gel electrophoresis, and cDNA cloning (**1,6**). The DD methodology, also referred to as DDRT-PCR or DD-PCR in PCR nomenclature (**7,8**), begins with total RNA being harvested from the cells/tissues of interest. A researcher will study at least two samples, but many more can be studied if the experiment suggests so. These samples will have morphological, genetic, or other experimental differences for which the researcher wishes to study the gene expression patterns, hoping to elucidate the root cause of the particular difference or specific genes that are affected by the experiment. Samples can be from any eukaryotic organism, including plants, fish, amphibians, reptiles, insects, yeast, fungi, and mammals. DD can be adapted for prokaryotic systems, but is more often used with eukaryotes.

Table 1
Impact of Major Technologies in Differential Gene Expression Analysis

Method	No. of citations ^a	Original publication ^b
Differential display	3606	Science (1992) 257 , 967–971
DNA microarrays	2296	Science (1995) 270 , 467–470
SAGE	1448	Science (1995) 270 , 484–487
Oligo arrays	742	Science (1996) 274 , 610–614

^aNumber of citations is the number of times the original publication has been cited by other papers, which reflects the number of times each technique has been used for publications.

^bSearch done with ISI Web of Knowledge Citation Search. Search conducted on January 19, 2005 at <http://isi15.isiknowledge.com/portal.cgi?DestApp=WOS&Func=Frame>.

The messenger RNAs (mRNAs) within the total RNA population are used as the templates for DD-PCR after first-strand cDNA synthesis by reverse transcription. The current methodology makes use of three “anchored” oligo-dT primers that target the poly-adenylation site of eukaryotic mRNA and have the form H-T₁₁M, where H is a *Hind*III restriction site (AAGCTT), T₁₁ is a string of 11 Ts (though the first two Ts come from the *Hind*III site), and M is G, C, or A (**9**). They are referred to as “anchor” primers because the non-T base after the string of 11 Ts enables the primer to be anchored to the same spot for each round of amplification, in contrast to standard oligo-dT primers that only contain a string of Ts and will anneal in multiple spots, creating a smear (*see Note 1*). The *Hind*III restriction site was added to the anchor primer design to make the primers longer and more efficient in annealing to the targeted poly-A site, as well as improving downstream applications such as cDNA cloning. Using the current anchor primer design, the cDNA populations are subsequently divided into three subpopulations that represent one-third of the potential mRNA expressed in the cell at any given time. Previous work indicated using anchor primers of the type T₁₁VN, where V can be A, G, or C and N can be any of the four nucleotides, as well as anchors of the type T₁₂MN, where M is a degenerate mixture of A, G or C, and N is any of the four nucleotides (**1**). Both of those primer designs result in larger subfractions of the mRNA population (12 for type T₁₁VN and 4 for T₁₂MN), which unnecessarily increases the amount of FDD-PCR reactions for the same level of gene coverage vs the H-T₁₁M primer design.

The next step in DD is the PCR-amplification of the cDNA subpopulations utilizing a combination of anchor primers (called H-T₁₁M) with a set of “arbitrary” primers that are random and short in length. The design of these arbitrary 13mers (H-AP primers) utilized in DD technology also includes a *Hind*III restriction site (AAGCTT) and a 7-basepair backbone of random base combinations. The *Hind*III restriction site is included in both the anchor and arbitrary

primers for more efficient primer annealing and easier downstream manipulation of the cDNA (9). The primers used in DD represent a random selection from over 16,000 (4^7) basepair combinations. Additionally, the length of an arbitrary primer is so designed that by probability each will recognize 50–100 mRNAs under a given PCR condition (10). As a result, mRNA 3' termini defined by any given pair of anchored-primer and arbitrary primer are amplified and displayed by denaturing polyacrylamide gel electrophoresis (PAGE). A mathematical model of estimated gene coverage utilizing various combinations of anchor and arbitrary primers was developed shortly after the advent of DD technology (10). This mathematical model indicated that approx 240 primer combinations (three anchor primers with 80 arbitrary primers) were needed to approach the level of estimated genome-wide screening for eukaryotes (approx 95%). However, a new mathematical model presented in the previous chapter of this book, predicts that more primer combinations are required to give that level of coverage; using 480 primer combinations (3 anchor primers with 160 arbitrary primers) would provide approx 93% coverage based on the new model.

DD was originally optimized with radioactivity using ^{35}S (1). ^{33}P labeling was then developed (9) for better sensitivity and resolution and has been the most commonly used for publications. However, fluorescent differential display (FDD) (*see Fig. 1*) was the next logical progression. In the development of FDD, it was crucial that the new platform have similar sensitivity to traditional DD with isotopic labeling, as well as other advantages that would make the platform a viable and improved alternative to the established DD methodology. FDD, optimized using fluorochrome-labeled anchor primers (generically called FH-T₁₁M) and higher dNTP concentrations in PCR, was shown to be essentially identical in both sensitivity and reproducibility to that of conventional DD (6) (*see Fig. 2*). Improvements such as elimination of radioactivity, digital data acquisition, and increased assay speed were goals that were successfully reached by the establishment of the FDD platform, representing a marked improvement over conventional DD.

After PCR amplification, gel electrophoresis is performed to separate the resulting PCR products by size. Reactions are run side by side so that the samples being compared are next to one another for each primer combination. Comparison of the cDNA patterns between or among relevant RNA samples reveals differences in the gene expression profile for each sample (*see Fig. 3*). Electrophoresis can be performed with denaturing polyacrylamide sequencing gels (1,11), nondenaturing polyacrylamide gels (7), or with agarose gels (12,13). Sequencing gels are the most commonly used method and are recommended here because they offer the best band resolution and allow for easy and efficient recovery of genes. In addition, their ability to accommodate a large number of reactions reduces the number of gels that must be run for FDD analysis.

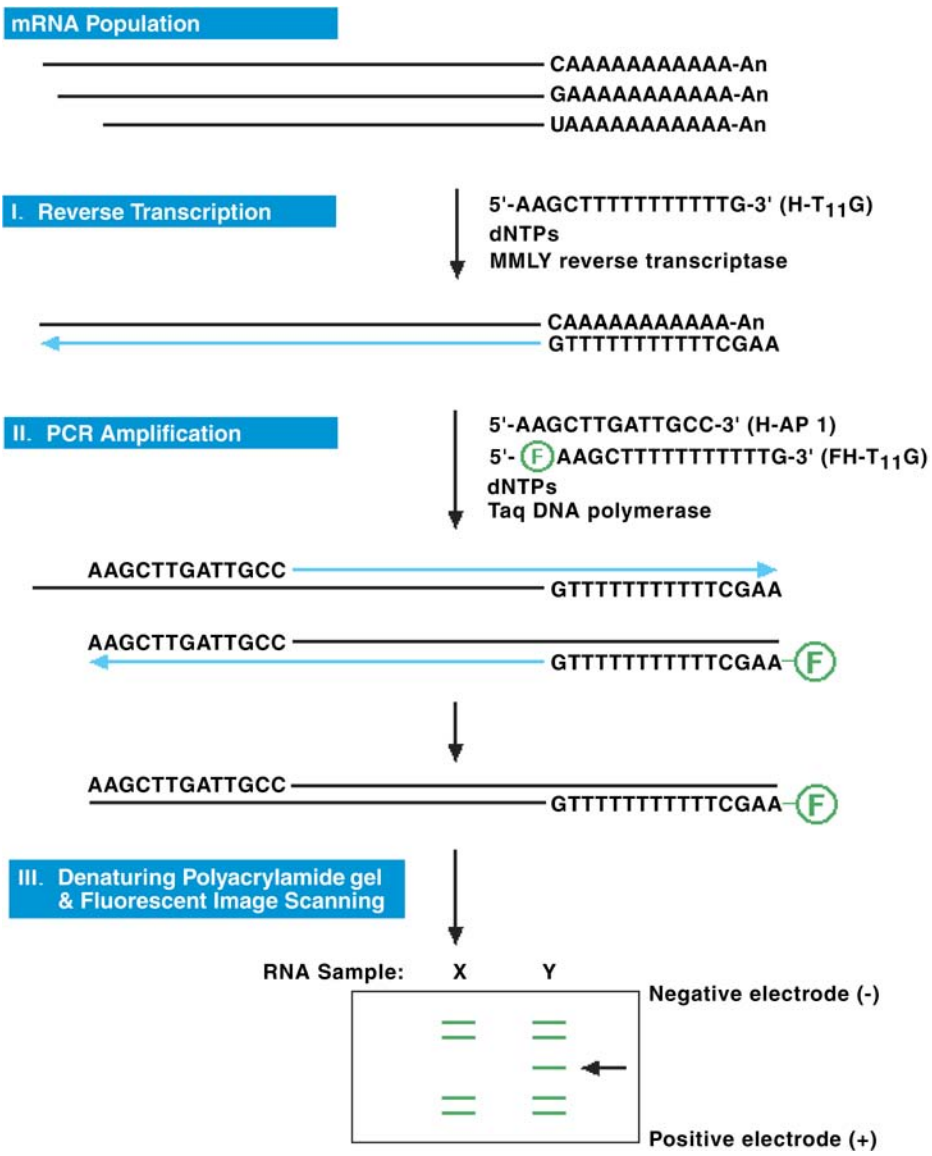
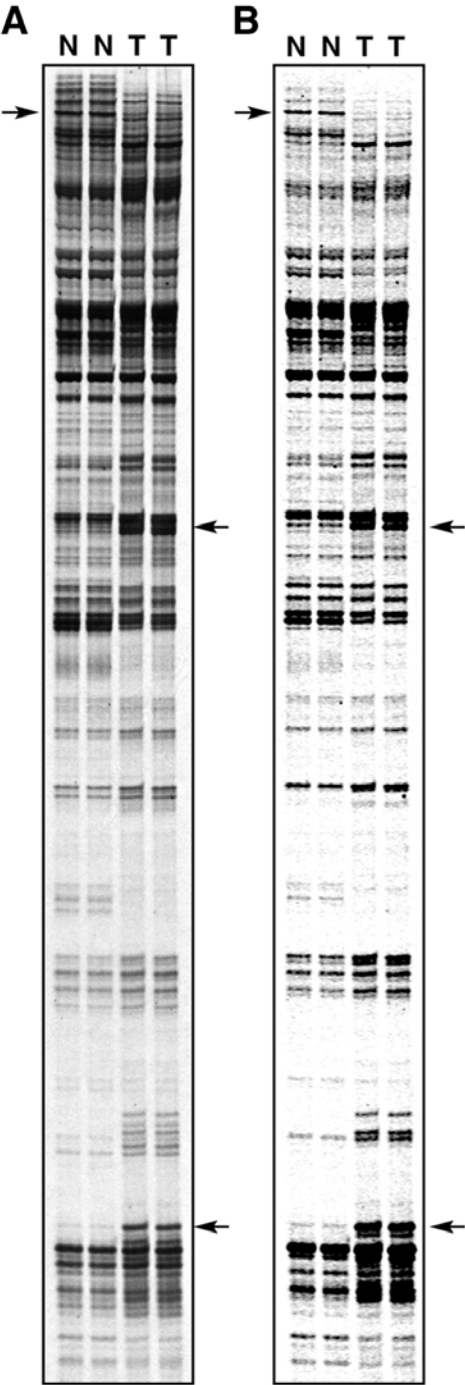


Fig. 1. Schematic representation of fluorescent mRNA differential display. Three fluorescently labeled one-base anchored oligo-dT primers with 5' *Hind*III sites are used in combination with a series of arbitrary 13mers (also containing 5' *Hind*III sites) to reverse transcribe and amplify the mRNAs from a cell.



Because the resulting cDNAs are fluorescently labeled, the use of a fluorescent imager scanner is required for this technology. Here the FMBIO[®] laser imager series (MiraiBio, Alameda, CA) is recommended for digital acquisition of the cDNA profiles. Although this is the recommended imager, other fluorescent scanners, such as the Typhoon[®] (Amersham Biosciences, Piscataway, NJ) and FLA-5000 (FUJIFILM Medical Systems, Stamford, CT) can also be used for FDD with similar sensitivity.

Another option for visualization of PCR reactions is to run samples on an automated sequencer. Our group has successfully used the Applied Biosystems ABI3100, a capillary array-based automated sequencer, for FDD band detection with several different fluorophores. These capillary electrophoresis (CE) machines have a laser at a fixed point and when a fluorescently labeled product passes the laser, a signal is detected. The results of FDD are seen as a series of spectral peaks for each lane, which can be compared to show differences in a very sensitive and reproducible way (*see Fig. 4*). The use of CE can dramatically cut down on the time and labor required for large-scale FDD screenings. However, the major drawback and bottleneck for using this technology with FDD is that, at this point, there is no way to retrieve bands from the CE results. One would still have to run a gel and detect bands using an alternate method. The most sophisticated attempt to solve this bottleneck was the development of a prototype computer-controlled CE system for positive band identification and retrieval by fraction collection by the Hitachi Japan group (*14*). But, to our knowledge, no further progress or commercialization has been made.

Upon completion of the gene expression profiles by gel electrophoresis, the next step is to begin characterization of the potential differentially expressed genes of interest. Bands are excised from the gel matrix and reamplified with the same primer combination as the original FDD-PCR and under the same reaction conditions. Generally, a PCR-product cloning step is recommended before differential gene confirmation and sequencing, but this is up to the preferences of the researcher. The PCR-TRAP[®] Cloning System (GenHunter Corporation, Nashville, TN) is recommended because it is designed specifically

Fig. 2. (*opposite page*) Comparison of radioactive and fluorescent differential display. DNA-free RNA from normal (N) and *ras* oncogene transformed (T) rat embryo fibroblasts were compared in duplicate by either conventional differential display with ³³P-labeled- α -dATP or FDD with fluorescein-labeled anchor primer under identical PCR conditions. The autoradiogram (A) and fluorescent images in grayscale (B) were compared in sensitivity and reproducibility as indicated. Reproducible differences are marked by arrows. The anchored primer, H-T₁₁G, was used in combination with arbitrary 13mer, H-AP29.

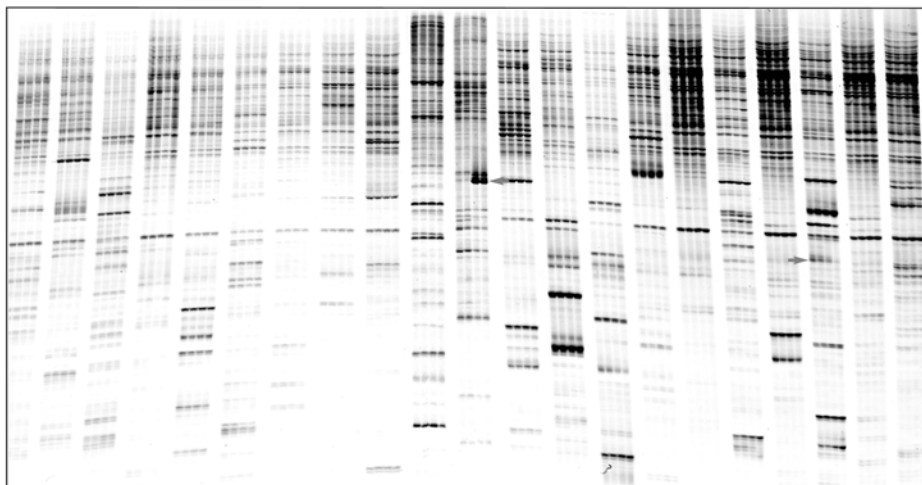


Fig. 3. Automated FDD result. Four RNA samples (before, and 6, 9, and 12 h after a drug treatment) were compared with 1 anchor primer in combination with 24 arbitrary primers (only 21 shown) using automation in liquid-handling, 132-lane electrophoresis unit, and digital acquisition of gel image. Grey arrows indicate reproducible differences worthy of pursuit.

for cloning DD bands and employs highly efficient positive-selection cloning. Because of the potential that more than one distinct cDNA is contained within an excised band, more than one colony should be screened for the correct size before it is characterized. Furthermore, if the screening results indicate that more than one cDNA is present in the colony population, each of the different cDNAs should then be further characterized.

Characterization of each potential gene includes sequencing of the cloned cDNAs of interest, with the results giving an indication of whether the cDNA is a known or unknown sequence. As with any differential gene expression technology, one has to be sure that the characterized sequences are actually differentially regulated, i.e., a “real difference,” and not a false positive. A variety of confirmation techniques, including Northern blot analysis, reverse Northern blot analysis, quantitative RT-PCR (qRT-PCR), or real-time PCR can be used. Although each has its own distinct advantages and disadvantages, Northern blot analysis is considered the gold standard for gene expression confirmation and is therefore recommended. Despite being labor-intensive, time-consuming, and requiring a significant amount of RNA, the Northern blot is by far the most accepted tool for confirmation. Northern blots have a distinct advantage over other confirmation methodologies in sensitivity, because both high- and low-level mRNA expression can be validated with this standard assay.

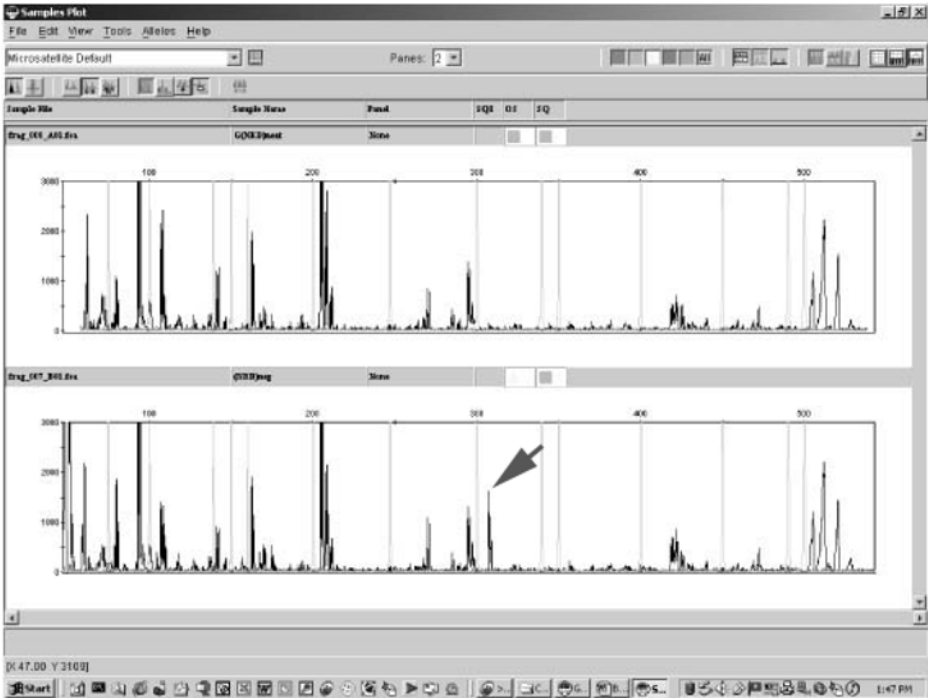


Fig. 4. Capillary electrophoresis of FDD reactions. RNA samples without (–) and with (+) p53 activation are compared by FDD and samples are run on ABI3100 Capillary Electrophoresis instrument. A candidate p53 target gene shows up regulation in the +p53 sample at approx 305 bp.

The optimized FDD technology is now able to compete with other gene expression tools such as DNA microarray technology because of improved high-throughput capabilities, while maintaining its inherent advantages over microarrays. Because the DD approach to differential gene expression analysis relies on randomly generated primers, no prior knowledge of the mRNA sequences is required, making the gene screening systematic, nonbiased, with the ability to find unknown genes. In addition, DD allows researchers to study more than two samples simultaneously, with only 10–20 μg total RNA required for a “complete coverage.” Disadvantages of microarray technology as compared to FDD are reproducibility, probe sensitivity, nonlinearity in signal detection (15), probe cross-hybridization owing to homologous cDNA sequences (16), and data management (17). Depending on the amount of desired gene coverage, FDD methodology enables quicker results when compared to traditional isotopic DD or other DD-related technologies, yet ensures

more reliable results when compared to microarray or other competing, non-DD technologies. Combined with robotics and digital data analysis, FDD has been shown to be even more accurate and high throughput (6,18). Elimination of manual reaction set-up, through the use of a robotic liquid dispenser, not only ensures reproducibility by reduction of pipetting errors, but, in combination with the elimination of conventional DD autoradiography, also decreases the amount of time required for a differential gene expression screening. This technology allows researchers to quickly and easily find the truly differentially expressed genes in their project so they can spend their time and effort on the downstream functional characterizations, where some of those mysteries of life can be pieced together.

2. Materials

2.1. Total RNA Isolation

1. Phosphate-buffered saline (PBS).
2. RNA isolation reagent: a phenol-guanidinium monophasic solution such as RNAPure® (GenHunter, cat. nos. P501 to P503) is recommended.
3. Chloroform.
4. Polytron™ Homogenizer for RNA extraction from tissue (Biospec Products Inc., Bartlesville, OK).
5. Diethyl pyrocarbonate-(DEPC)-treated water (GenHunter, cat. no. R105).
6. Isopropanol.
7. 100% ethanol.
8. 70% ethanol in DEPC-treated dH₂O.
9. 1.7 mL microfuge (Denville Scientific, Metuchen, NJ).

2.2. Removal of Genomic DNA From Total RNA

1. MessageClean® DNA Removal Kit (GenHunter, cat. no. M601) including RNase-free DNase I (10 U/μL), 10X reaction buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin), 3 M sodium acetate, pH 5.5, DEPC-treated water, and RNA loading mix.
2. Agarose, ultraPure (Invitrogen, Carlsbad, CA).
3. Distilled water (double distilled and autoclaved).
4. Phenol/chloroform (3:1) solution, Tris saturated: 30 mL melted crystalline phenol, 10 mL chloroform, 10 mL Tris-HCl, pH 7.0.
5. 10X MOPS Buffer: 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M ethylenediamine tetraacetic acid (EDTA), pH 6.5.
6. 12.3 M (37%) formaldehyde, pH >4.0.

2.3. Single-Strand cDNA Synthesis by Reverse Transcription

1. RNAspectra™ Fluorescent Differential Display Kit (GenHunter, cat. nos. R501-R510 and F501-F510) including distilled water, 5X RT buffer (125 mM Tris-Cl,

- pH 8.3, 188 mM KCl, 7.5 mM MgCl₂, and 25 mM dithiol threonine [DTT]), deoxyribonucleoside triphosphate (dNTP) mix (FDD), oligo-dT anchor primers (H-T₁₁M, 2 μ M), and MMLV reverse transcriptase (100 U/ μ L).
2. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter, catalog no. T101).
 3. Thermal cycler, GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA).

2.4. FDD-PCR

1. RNAspectraTM Fluorescent Differential Display Kit (GenHunter, cat. nos. R501-R510 and F501-F510) including distilled water, 10X PCR buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin), FDD dNTP mix, fluorescent anchor primers (R-H-T₁₁M or F-H-T₁₁M), and arbitrary primers (H-AP, 2 μ M).
2. *Taq* DNA polymerase (Qiagen, Valencia, CA, cat. no. 201207).
3. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter) or 96-well PCR plates (Thermo-Fast[®] 96 Detection Plate, ABgene Inc., Rochester, NY, cat. no. AB-1100).
4. Liquid-handling robot. GenHunter uses the Biomek 2000 (Beckman Coulter Inc., Fullerton, CA).

2.5. Gel Electrophoresis

1. Gel apparatus with low-fluorescent (borosilicate) glass plates such as Horizontal or Vertical FDD Electrophoresis Systems (GenHunter, cat. no. SA101 or SA201).
2. 5 M KOH.
3. 50% ethanol (EtOH).
4. Sigmacote[®] (Sigma, St. Louis, MO) or similar product.
5. 6% denaturing gel solution such as Sequagel 6 Ready-To-Use 6% Sequencing Gel[®] (National Diagnostics, Atlanta, GA, cat. no. EC-836).
6. 10X TBE: 0.89 M Tris-borate, pH 8.3, 20 mM disodium EDTA (Na₂EDTA).
7. 10% ammonium persulfate (APS).
8. FDD Loading Dye from RNAspectra Kit (GenHunter, cat. no. F201): 99% formamide, 1 mM EDTA, pH 8.0, 0.009% xylene cyanole FF, and 0.009%.
9. Fluorescent Laser Scanner. The FMBIO[®] II or III Series (MiraiBio, Alameda, CA) is recommended.
10. UV-transparent plastic wrap. Standard Glad[®] Cling Wrap (Glad Products Company, Oakland, CA) or Saran Wrap work well.
11. FDD locator dye (GenHunter, cat. no. F202 and R202).

2.6. Reamplification of Selected Differentially Expressed Bands

1. Distilled water.
2. 3 M sodium acetate, pH 5.5, from GenHunter MessageClean Kit.
3. Glycogen, 10 mg/mL (GenHunter, cat. no. S301).

4. 100% ethanol.
5. 85% ethanol.
6. Unlabeled anchor primers (H-T₁₁G, H-T₁₁A, H-T₁₁C; 2 μ M, from GenHunter RNAspectra Kit or cat. no H101-S).
7. Arbitrary primers (H-AP1 to H-AP80, 2 μ M, from GenHunter RNAspectra Kit or cat. nos. H-AP1 to H-AP80).
8. *Taq* DNA polymerase (Qiagen, cat. no. 201207).
9. dNTP Mix (FDD) from RNAspectra Kit.
10. 10X PCR buffer (GenHunter, cat. no. S201): 100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin.
11. Agarose.
12. 10X Agarose DNA loading dye: 40% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanole FF, 2.5 mM in distilled water.
13. 0.2 mL thin-walled PCR tube, RNase (GenHunter).

2.7. Cloning of Reamplified PCR Products

1. PCR-TRAP Cloning System (GenHunter, cat. no. P404) including insert-ready PCR-TRAP cloning vector, T4 DNA ligase (200 U/ μ L), distilled water, 10X ligase buffer (500 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP, 500 μ g/mL bovine serum albumin [BSA]), Lgh/Rgh primers (2 μ M), Colony Lysis Buffer (1X TE with 0.1% Tween-20), 10X PCR buffer, dNTP 250 μ M, tetracycline (20 mg/mL), and GH competent cells.
2. Luria Bertani (LB) media. Make 1 L LB with 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, and up to 1 L with dH₂O.
3. LB-Agar-TET plates. Make 1 L LB-Agar-TET plates with 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 9 g NaCl, 15 g Bacto-agar, and up to 1 L with dH₂O. Autoclave 60 min then and add 1 mL tetracycline (20 mg/mL) when liquid cools to approx 50°C. Pour plates.
4. Bacterial polystyrene Petri dish.
5. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter).
6. 1.7 mL microfuge tubes (Denville Scientific).
7. QIAEX™ II Gel Extraction Kit (Qiagen, cat. no. 20021).

2.8. Sequencing of Cloned PCR Products

1. AidSeq Primer Set C (GenHunter, cat. no. P203): includes Lseq and Rseq primers.
2. QIAquick PCR Purification Kit (Qiagen, cat. no. 28106).

2.9. Confirmation of Differential Gene Expression by Northern Blot

1. Lgh/Rgh Primers (2 μ M) from PCR-TRAP Cloning System or alone (GenHunter, cat. nos. L201 and L202).
2. *Taq* DNA polymerase.
3. dNTP 250 μ M from PCR-TRAP Cloning System or alone (GenHunter, cat. no. S501).

4. 10X PCR buffer from PCR-TRAP Cloning System or alone (GenHunter, cat. no. S201).
5. Colony lysis buffer from PCR-TRAP Cloning System or alone (GenHunter, cat. no. L102).
6. HotPrime[®] DNA labeling kit (GenHunter, cat. no. H501) including Klenow DNA polymerase (1 U/ μ L), 10X labeling buffer, dNTP (-dATP) or dNTP (-dCTP) (500 μ M), stop buffer, and distilled water.
7. QIAEX[™] II Gel Extraction Kit.
8. Agarose.
9. 10X MOPS buffer.
10. 12.3 M (37%) formaldehyde, pH >4.0.
11. Distilled water.
12. Lock-top microfuge (USA Scientific, Ocala, FL, cat. no. 1415-5100).
13. α -[³²P] dATP (3000 curies/mmol) (PerkinElmer Life Sciences, Boston, MA, cat. no. BLU512H).
14. Sephadex G50 column (Roche Applied Science, Indianapolis, IN, cat. no. 1814419).
15. Salmon sperm DNA (10 mg/mL) (GenHunter, cat. no. ML2).
16. Nylon membrane: Nytran SuperCharge Nylon Transfer Membrane (Schleicher and Schuell, Keene, NH, cat. no. 10 416 216).
17. Paper towels.
18. UV-transparent plastic wrap.
19. Single-emulsion scientific imaging film. Kodak Biomax MS (Kodak-Eastman, Rochester, NY, cat. no. 8715187) is recommended.
20. 20X saline-sodium citrate (SSC): 3 M NaCl, 0.3 M trisodium citrate. Adjust pH to 7.0 with 1 M HCl.
21. Formamide prehybridization/hybridization solution (GenHunter, cat. no. ML1).
If preparing in the lab, use the following protocol for 500 mL:

20X Saline-sodium phosphate-EDTA (SSPE)	125 mL
50X Denhardt's Solution	50 mL
20% Sodium dodecyl sulfate (SDS)	2.5 mL
Formamide	250 mL
Distilled water	up to 500 mL

Mix well, aliquot into smaller volumes, and store at -20°C until use.

To make 20X SSPE: 3 M NaCl, 0.1 M NaH₂PO₄ (dibasic), 0.01 M EDTA.

To make Denhardt's Solution, 50 mL:

Ficoll	0.5 g
Polyvinylpyrrolidone	0.5 g
BSA (Pentax Fraction V)	0.5 g
Distilled water	Up to 50 mL

22. 1X SSC, 0.1% SDS (w/v).
23. 0.25X SSC, 0.1% SDS (w/v).

3. Methods

3.1. Total RNA Isolation

Although FDD takes advantage of the poly-adenylation (poly-A+) site of eukaryotic mRNA, total RNA is preferred over poly-A+ RNA (mRNA) for several reasons. These reasons include the overall ease of purification, the ability to verify RNA integrity, and the cleaner background signal (*see Note 2*). To this end, total RNA is suggested for FDD analysis.

If one is planning to do a 240-primer combination screening with FDD, approx 12 µg of “cleaned” total RNA is required. The term “cleaned” refers to being clean of DNA achieved by DNase I treatment described in **Subheading 3.2**. Generally, 50–80% of the beginning amount of total RNA can be retrieved after cleaning. In addition, it is important to make sure there is plenty of RNA left over for whatever confirmation step is chosen. To ensure there is enough RNA for all steps, it is suggested to isolate approx 50 µg of total RNA. The amount of total RNA that can be isolated from a sample can vary widely depending on the tissue/cell type, procedure used, organism, and proficiency at that particular procedure. However, using a reagent based on the standard phenol/guanidine thiocyanate technique such as RNeasy[®], one can achieve an average yield of 50 µg of total RNA from 25 mg of tissue or 2.5×10^6 cells (*see Note 3*).

3.1.1. RNA Extraction From Various Sources

3.1.1.1. EXTRACTION OF RNA FROM TISSUE CULTURES

1. If using regular “attached” cells, pour off medium. Set the plate on ice. If cells are in suspension, spin down cells, remove the medium, then move on to **step 4**.
2. Rinse cells with 10–20 mL of cold PBS.
3. Pour off rinsed PBS and remove the residual PBS with a 1000 µL pipet (*see Note 4*).
4. Add 2 mL of RNeasy RNA isolation reagent per 100- to 150-mm plate to lyse the cells. Spread the solution by shaking the plate. This volume is sufficient for 1–10 million cells.
5. Let sit on ice for 10 min.
6. Pipet the lysate into two labeled 1.5-mL microfuge tubes.

3.1.1.2. EXTRACTION OF RNA FROM TISSUES

1. Add at least 2 mL of RNeasy RNA isolation reagent to the tissue in a 50 mL conical tube on ice. Ideally, the volume ratio of RNA isolation reagent to tissue should be at least 10:1.
2. Homogenize the tissue with a Polytron[™] Homogenizer until the tissue is dispersed.
3. Let sit on ice for 10 min.
4. Transfer 1 mL aliquots of the lysate into labeled 1.5 mL centrifuge tubes.

3.1.1.3. EXTRACTION OF RNA FROM BLOOD

1. Spin down blood products and remove the plasma.
2. Follow the instructions in **Subheading 3.1.1.2.**

3.1.2. RNA Purification

1. Add 150 μL of chloroform/mL of lysate. Vortex for 10 s. Protocol can be stopped here by placing the lysates at -80°C .
2. Centrifuge the tubes at 4°C with maximum speed for 10 min (*see Note 5*).
3. Carefully remove the upper phase (*see Note 6*) into a clean, labeled 1.5-mL centrifuge tube. If RNA is being isolated from tissues, a second extraction is generally recommended to remove any RNases (*see Note 7*).
4. Add an equal volume of isopropanol. Mix vigorously or vortex for 30 s. Let sit on ice for 10 min.
5. Centrifuge for 10 min at 4°C with maximum speed.
6. Rinse the RNA pellet with 1 mL of cold 70% ethanol (in DEPC-treated water). Centrifuge 2 min at 4°C with maximum speed.
7. Remove the ethanol. Spin briefly and remove the residual wash solution with a pipet.
8. Resuspend the RNA in DEPC-treated water. The amount used for resuspension will depend on the amount of RNA isolated, but the RNA should be at a concentration greater than 1 $\mu\text{g}/\mu\text{L}$, so adjust accordingly. Do not use SDS in resuspension if using RNA for any PCR application.
9. Measure the concentration by taking 1 μL of the RNA (using P10 pipet) and diluting to 1 mL of water (a 1:1000 dilution). Read at 260 nm. 1 OD_{260} = 40 μg
10. Move on to next steps and store RNA that will not be “cleaned” in aliquots at -80°C until next use.

3.2. Removal of Genomic DNA From Total RNA

For the purposes of FDD gene expression analysis, as well as any other RNA-based gene expression technologies, contaminating genomic DNA must be removed before single-strand cDNA synthesis by reverse transcription and subsequent PCR reactions. If left unchecked, any primers with matching sequence to the contaminating DNA will anneal during the FDD-PCR reactions, causing amplification of DNA sequences and leading to a higher false-positive rate. Therefore, the following protocol is one of the most important procedures in preventing irregularities or artifacts during the FDD-PCR reactions by removal of the contaminating genomic DNA. It is important to note that one will typically retrieve 50–80% of the total RNA put into the reaction, so the amount to be cleaned must be adjusted to the amount needed for FDD.

3.2.1. DNase I Digestion of Total RNA

1. If necessary, dilute desired amount of RNA to be digested (maximum of 50 μg) with DEPC-treated water to a volume of 50 μL .

2. In a 1.5-mL centrifuge tube, add the following *in order* (total reaction volume is 56.7 μ L):

Total RNA (10–50 μ g)	50 μ L
10X Reaction buffer	5.7 μ L
DNase I (10 U/ μ L)	1.0 μ L

3. Mix gently and incubate at 37°C for 30 min (*see Note 8*).

3.2.2. Extraction and Ethanol Precipitation of DNA-Free RNA

1. Prepare phenol/chloroform solution (*see Note 9*) by melting crystalline phenol at 65°C.
2. Add 30 mL melted phenol to 10 mL chloroform and mix well.
3. Add 10 mL Tris-HCl, pH 7.0 and mix well. Allow saturation phase to form before using.
4. Add 40 μ L of phenol/chloroform solution to each DNase I reaction (*see Note 10*). Vortex for 30 s.
5. Let sit on ice for 10 min.
6. Centrifuge with maximum speed for 5 min at 4°C.
7. Collect upper phase (*see Note 6*) and place in a clean, labeled 1.5-mL microfuge tube.
8. Add 5 μ L 3 M sodium acetate and 200 μ L 100% ethanol. Mix well.
9. Let sit at least 1 h at –80°C. Overnight to a few days at –80°C is fine.
10. Centrifuge at 4°C for 10 min with maximum speed to pellet RNA.
11. Carefully remove the supernatant and rinse the RNA pellet with 0.5 mL of 70% ethanol (in DEPC-treated water). Do not disturb the pellet.
12. Centrifuge for 5 min at 4°C with maximum speed and remove supernatant. Centrifuge again briefly, removing the residual liquid without disturbing the RNA pellet.
13. Resuspend the RNA in 10–20 μ L of DEPC-treated water.

3.2.3. RNA Quantification and Integrity Verification

After cleaning, it is crucial to be able to determine both the quantity and quality of the RNA retrieved. The amount can easily be quantified by OD₂₆₀. The quality/integrity of the RNA is determined most accurately by running the RNA on an “RNA gel” and looking for the appearance of sharp ribosomal RNA bands.

1. Quantitate the RNA amount by OD₂₆₀ after 1:1000 dilution of the DNA-free RNA sample with distilled water.
2. Prepare an “RNA gel” (denaturing formaldehyde agarose gel with MOPS and formaldehyde) by the following protocol:
 - a. Add the following to a microwave-safe container:

10X MOPS	10 mL
Agarose	1–1.5 g
Distilled water	83 mL

- b. Microwave for approx 3 min or until agarose is melted.
 - c. Let agarose cool to at least 50°C (barely touchable by hand).
 - d. Add 7 mL of a 12.3 M (37%) formaldehyde solution. Gently mix.
 - e. Pour into prepared gel casting plate and add gel comb.
 - f. Running buffer (1 L) is made by diluting 100 mL of 10X MOPS with 900 mL of distilled water to a 1X concentration. Cover agarose gel with running buffer.
3. Check the integrity of the RNA (*see Note 11*) by resolving 2–3 µg of both pre-DNase and post-DNase RNA samples on a 7% formaldehyde agarose gel with RNA Loading Mix by the following protocol:
 - a. Add 1–10 µL (2–3 µg) of RNA to 20 µL RNA Loading Mix in a labeled 1.5-mL microfuge tube. Mix well.
 - b. Incubate at 65°C for 10 min.
 - c. Centrifuge sample briefly to collect condensation.
 - d. Put samples on ice for 5 min.
 - e. Load entire amount onto RNA gel.
 - f. Run at 50–60 V for approx 45 min or until resolution of the ribosomal subunits is achieved.

3.3. Single-Strand cDNA Synthesis by Reverse Transcription

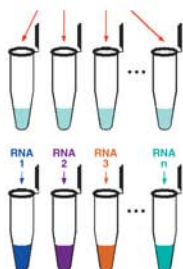
Generally, two RT reactions are done per sample (called “in-duplicate”) to ensure reproducibility and as a way of reducing any false positives. It is recommended to set up separate RT core mixes for each individual H-T₁₁M in 200 µL volume RT reactions if 240 primer combinations will be performed. Therefore, if two samples are being studied, set up four 200 µL RT reactions for H-T₁₁G, four 200 µL RT reactions for H-T₁₁A, and four 200 µL RT reactions for H-T₁₁C. If smaller or larger numbers of primer combinations are chosen, adjust accordingly.

1. Dilute 40 µL of each RNA sample to a final concentration of 0.1 µg/µL with DEPC-treated water and mix thoroughly. Place on ice.
2. For an RT core mix with two samples in-duplicate for one H-T₁₁M primer (H-T₁₁G will be shown here), add the following:

376 µL distilled water
160 µL 5X RT buffer
64 µL FDD dNTP mix
<u>80 µL H-T₁₁G primer</u>
680 µL total volume
Mix well.

3. Divide the above 680 µL evenly into four tubes labeled with sample name (for example: RTG-1a, RTG-1b, RTG-2a, RTG-2b), aliquoting 170 µL per tube (*see Fig. 5* for step-by-step schematic of RT and FDD-PCR setup).

I. RT Reaction

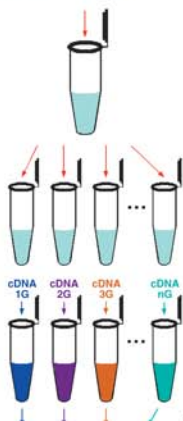


a) Add 170 μL RT Core Mix G to all

b) Add 20 μL of different total RNAs

c) Begin RT Reaction. After 10 min. at 37°C,
add 10 μL MLV RT to each reaction.

II. DD-PCR

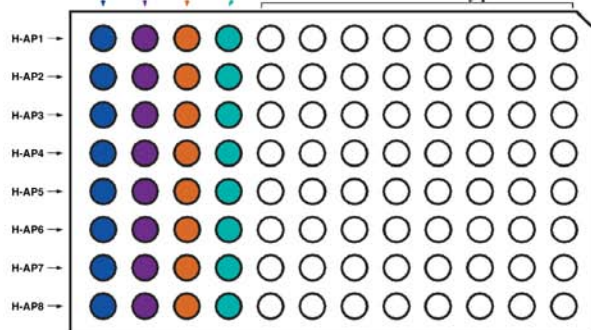


a) Add 1896 μL FDD Core Mix G & 24 μL Taq to a tube

b) Add 480 μL of FDD Core Mix G/Taq Mixture to all

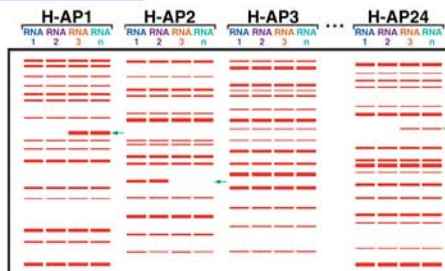
c) Add 60 μL of corresponding cDNA

Additional wells are used for additional
reactions with H-AP arbitrary primers 9-24



d) Add 18 μL of
cDNA/FDD Core
Mix/Taq Mixture to
each of the 8 wells
down & 2 μL of each
H-AP Arbitrary
primer to each of the
4 wells across.
Repeat for H-AP
primers 9 to 24

III. Electrophoresis



a) Run out all samples on a 6%
denaturing polyacrylamide gel

b) Cut out any bands of interest
for reamplification, cloning,
and confirmation.

4. Add 20 μL of corresponding total RNA (0.1 $\mu\text{g}/\mu\text{L}$, freshly diluted, *see* **Note 12**) to each tube. For example, add 20 μL of RNA 1 to each of tubes RTG-1a and RTG-1b followed by 20 μL of RNA 2 to each of tubes RTG-2a and RTG-2b. Mix each tube well.
5. Program the thermal cycler to: 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, 4°C soak (*see* **Note 13**).
6. Place tubes on thermal cycler and begin program.
7. After the tubes have been at 37°C for 10 min, pause the thermal cycler and add 10 μL of MMLV reverse transcriptase to each tube. Quickly mix well by finger-tipping or pipetting up and down before continuing incubation program.
8. At the end of the reverse transcription, spin the tube briefly at maximum speed to collect condensation. Set the tubes on ice or store at -20°C for later use.
9. Repeat **steps 1–8** for H-T₁₁A and H-T₁₁C primers.

3.4. FDD-PCR

This protocol is designed for 240 primer combinations in duplicate per sample using three fluorescent dye-labeled anchor primers (FH-T₁₁M) and 80 upstream arbitrary primers (H-AP). This would yield approx 74% coverage of all possible genes. For a complete, genome-wide screening, 480 primer combinations or more must be completed per sample. It is ideal to set up PCR reactions in 96- or 384-well PCR plates using a robot to ensure reproducibility and increase throughput. Depending on the number of samples and the plate being used, one may be able to combine more or less than 24 primer combinations into one experiment. However, for simplicity, a 24-primer combination experiment with one anchor primer and two RNA samples in duplicate using a 96-well plate will be discussed. Therefore, this protocol will need to be repeated 10 times using varying anchor-arbitrary primer combinations.

1. A separate FDD-PCR core mix for each individual FH-T₁₁M primer is made. Here, a core mix for all 80 H-AP primers for FH-T₁₁G primer is shown. This will be called “FDD Core Mix G.”

4080 μL distilled water
800 μL 10X PCR buffer

Fig. 5. (*opposite page*) Schematic for reverse transcription (RT) and fluorescent differential display (FDD) reaction setup. This schematic shows individual steps involved and quantities required for standard RT and fluorescent differential display FDD reaction setups. These numbers are based on comparing two samples in duplicate (or four samples not in duplicate) with FH-T₁₁M anchor primer in combination with 24 H-AP arbitrary primers. These steps would be repeated 10 times until all 240-primer combinations (three anchor primers and 80 arbitrary primers) have been completed.

640 μ L dNTP Mix (FDD)

800 μ L FH-T₁₁G primer

6320 μ L total volume

Mix well.

2. Aliquot 1896 μ L of FDD Core Mix G into three separate tubes labeled “FDD Core Mix G” (*see Fig. 5* for step-by-step schematic of RT and FDD-PCR setup). Aliquot the remaining amount into a fourth tube labeled “FDD Core Mix G-remainder” (approx 632 μ L).
3. To one of the tubes labeled “FDD Core Mix G,” add 24 μ L *Taq* DNA polymerase. Mix well. Freeze the other three tubes aliquoted in **step 2** at -80°C for later PCR reactions.
4. Aliquot 480 μ L of “FDD Core Mix G/*Taq*” mixture to four separate tubes labeled corresponding to the RT reactions. For example, use FDDG-1a, FDDG-1b, FDDG-2a, FDDG-2b.
5. Add 60 μ L of corresponding cDNA from RT to each of the four tubes. For example, 60 μ L of RTG-1a tube would go into the tube labeled FDDG-1a. Mix well.
6. Using either a robot or by hand, add 2 μ L of H-AP primers 1–24 to corresponding wells of 96-well plate (*see Fig. 5*).
7. Using either a robot or by hand, add 18 μ L of corresponding FDD Core Mixes to corresponding wells of 96-well plate (*see Fig. 5*).
8. Total reaction volume will be 20 μ L. Add 25 μ L of mineral oil if needed.
9. Program the thermal cycler to:
 - 94 $^{\circ}\text{C}$ for 15 s (*see Note 14*)
 - 40 $^{\circ}\text{C}$ for 2 min
 - 72 $^{\circ}\text{C}$ for 60 s
 - for 40 cycles
 - 72 $^{\circ}\text{C}$ for 5 min
 - 4 $^{\circ}\text{C}$ soak.
10. Put 96-well plate on thermal cycler and begin program. Once completed, store reactions at -20°C in the dark.
11. **Steps 3–10** will then be repeated for H-AP primers 25–48 and 49–72.
12. The same process will then be done for H-AP primers 73–80 as follows (*see Note 15*):
 - a. Add 8 μ L *Taq* DNA polymerase to the 632 μ L of “FDD Core Mix G-remainder.” Mix well.
 - b. Aliquot 160 μ L of that mixture to four separate tubes labeled the same as **step 4**.
 - c. Add 20 μ L of cDNA from RT to each of the four tubes corresponding cDNA as in **step 5**. Mix well.
 - d. Using either a robot or by hand, add 2 μ L of H-AP primers 73–80 to corresponding wells of 96-well plate.

- e. Using either a robot or by hand, add 18 μL of corresponding FDD Core Mixes to corresponding wells of 96-well plate.
- f. Follow **steps 8–10**.

13. Repeat **steps 1–12** for FH-T₁₁A and FH-T₁₁C primers.

3.5. Gel Electrophoresis

Because performing a large-scale FDD experiment requires many hundreds of PCR reactions (960 in the previously mentioned experiments), one of the areas for improvement in making it more high-throughput is in the gels themselves. Using a gel apparatus with many lanes can speed up this process tremendously. One system that has been successfully used is the Horizontal FDD Electrophoresis System with 132 lanes and the “Microtrough System” containing grooved glass plates. This allows one to load at least one entire 96-well plate on one gel. In addition, the Microtrough System allows the researcher to use standard 10 μL pipet tips for sample loading instead of the difficult-to-use flat gel-loading tips required by standard sequencing apparatuses. Hand position during loading is more stable and relaxed with this system.

A multichannel pipettor for gel loading has also been tried. Matrix Technologies (Hudson, NH) manufactures several pipettors with width-expandable channels called “Matrix Equalizers.” The 8-channel Matrix Equalizer 384 with 0.5–12.5 μL volume range works fairly well. These pipettors have tips that move independently and can be spaced anywhere from 4.5 to 14.15 mm apart. For the gel loading, the tips were spaced at 9 mm for liquid uptake from a 96-well plate and then collapsed together to 4.5 mm for gel loading. However, this 4.5 mm distance only allows 87 lanes per gel, not enough to load an entire 96-well plate. A pipettor that could contract to 3 mm for gel loading would be ideal, but so far Matrix has not manufactured this. Therefore, using one of these pipettors has trade-offs: although it decreases the time required for gel loading and the chance of incorrect loading, fewer reactions can be run on the same gel. The other option is to load the PCR reactions using the Matrix pipettor at the 6 mm distance, loading every other well, but this requires re-configuration of the reaction setup.

For the aforementioned experiments that have 960 PCR reactions on 10 96-well plates, it is recommended to run 10 separate gels, each with one 96-well plate. One to two gels can generally be run per day, requiring 5–10 d to run all 10 gels.

For ease of use, the Sequagel 6 Ready-To-Use 6% Sequencing Gel is recommended for denaturing gel electrophoresis. However, a general protocol is given here for the 6% denaturing polyacrylamide gel that is recommended for resolution of cDNA profiles.

1. Thoroughly clean both sides of glass plates to be used with warm water and soap, ensuring that there is no previous gel debris or streaks (*see Note 16*). Be sure to rinse thoroughly afterward because soap residue may cause problems. KOH can be used occasionally for this purpose to strip off hard-to-clean residue.
2. Further clean the glass plates by wiping with a 50% ethanol (EtOH) solution. Make sure plates are completely dry.
3. Coat the interior surface of one of the plates (usually the notched one) with 500 μ L Sigmacote or similar product using a Kim-Wipe to smoothly spread over the surface. Let dry for 1 min. This coating step allows the gel to preferentially stick to the noncoated plate upon separation of plates for band-cutting after running the gel.
4. Use 60 mL of the gel mixture for a $45 \times 28 \times 0.04$ cm gel.
5. Add 0.5 mL of 10% APS solution and mix thoroughly.
6. Pour gel into sequencing gel cast and let polymerize 1–2 h or overnight (*see Note 17*).
7. After polymerization, load the glass plates into the sequencing apparatus and add 1X TBE buffer to upper and lower buffer chambers.
8. Flush the urea from the gel wells and pre-run the sequencing gel in 1X TBE buffer for 30 min.
9. Add 3.5 μ L of each FDD-PCR reaction with 2 μ L of FDD loading dye. Alternatively, an appropriate ratio of loading dye (8 μ L for 20 μ L PCR reactions) can be added directly to the PCR reaction if the reactions will only be used for running gels. Incubate at 80°C for 2 min immediately before loading onto the gel. This step is to denature the DNA samples before gel loading.
10. After heat-denaturation, put samples on ice for 1–2 min.
11. Load maximum amount of sample (usually 3–4 μ L) into wells. It is crucial that all urea be removed from the wells before loading samples (*see Note 18*). For best results, load 4–6 lanes and then stop briefly to reflush wells to remove urea. Load in appropriate groups, usually by primer combination.
12. Electrophoresis for 1.5–3 h at 60 W constant power (voltage not to exceed 2000 V) until the xylene cyanole dye (the slower moving dye) reaches the bottom of the gel. In a 6% gel, the xylene cyanole will co-migrate with DNA of approx 106 bp as a reference point. If voltage exceeds 2000, lower the wattage. Gel should be kept in the dark while running to prevent photo-bleaching of samples (*see Note 19*) either by using a dark room or covering the gel apparatus with a cardboard box.
13. Turn off power supply and remove the plates from the gel apparatus. Take off gel tape and remove spacers and comb (*see Note 20*). Clean the outside of the glass plates very well with warm water and 50% ethanol to remove any residue from gel or tape. Thorough cleaning is required to reduce background signal (*see Note 16*).
14. Scan the gel on a fluorescence imager with an appropriate filter, following the manufacturer's instructions based on the particular fluorophore being used.

3.6. Reamplification of Selected Differentially Expressed Bands

Assuming differentially expressed bands of interest are seen, those bands should be excised from the gel. Following excision, the cDNAs will be reamplified using the same anchor-arbitrary primer combinations and reaction conditions as the initial FDD-PCR reactions. The reamplification products can then be cloned and sequenced for further characterization.

1. Separate the glass plates by taking off the notched/smaller glass plate (*see Note 21*) leaving the gel attached to the unnotched/larger plate.
2. Place a layer of UV-transparent plastic wrap on top of the gel. This prevents contamination of the gel as well as making gel cutting easier.
3. Spot 0.5 μL of FDD locator dye at the upper and lower corners of the gel to allow orientation of the picture with the gel. The FDD locator dye, with its combination of fluorescent and visible dyes, can be used to easily align the gel with the printed template for band excision.
4. Rescan the gel with the gel facing up.
5. Print a real-size image on appropriately sized paper (*see Note 22*) using a quality ink jet or laser printer. This printed image will be used as the template to excise differentially expressed cDNAs.
6. Choose and label any bands to be cut (*see Note 23*). A logical band-naming nomenclature should be used such as RN-G-1A (RN = researcher name; G = FH-T₁₁G anchor primer; 1 = H-AP1 arbitrary primer; A = top differentially expressed band in lane).
7. Place the printout on the table top and lay the glass plate on top of it. Orient the plate so that the spots on the printout match up with those on the gel.
8. Excise each band with razor or other sharp utensil and place into a 1.5-mL microfuge tube labeled with the corresponding band name.
9. For each band being reamplified, add 100 μL of distilled water to the tube containing the corresponding gel slice.
10. Let soak for 10 min at room temperature.
11. Boil the tightly closed tube (with parafilm or lock-top tube) for 15 min to elute the cDNA from the gel slice.
12. Spin for 2 min at maximum speed to collect condensation and pellet the gel.
13. Transfer supernatant to a fresh 1.5-mL microfuge tube labeled tube. Discard tube with gel slice. Add 10 μL of 3 M sodium acetate, 5 μL of glycogen, and 450 μL of 100% ethanol per tube. Let sit for at least 30 min on dry ice or in a -80°C freezer.
14. Spin for 10 min 4°C at maximum speed to pellet the DNA. Remove the supernatant and rinse the pellet with 200 μL of ice-cold 85% ethanol. Spin briefly and remove the residual ethanol.
15. Dissolve the pellet in 10 μL of dH₂O.
16. Make a reamplification core mix for each of the anchor primers that is large enough to reamplify all FDD bands with that particular anchor primer:

a. A standard reamplification reaction will contain:

Distilled water	23.3 μ L
10X PCR buffer	4.0 μ L
dNTP mix (FDD)	0.3 μ L
H-AP primer (2 μ M)	4.0 μ L
H-T ₁₁ M (2 μ M) (see Note 24)	4.0 μ L
cDNA template	4.0 μ L
Taq DNA polymerase	0.4 μ L
40 μ L total volume	

- b. Determine how many bands of each anchor primer will be reamplified. Multiply each of these by 10% to give a cushion for any pipetting inaccuracies. The number of bands for H-T₁₁G $\times$ 10% = g; the number of bands for H-T₁₁A $\times$ 10% = a; and the number of bands for H-T₁₁C $\times$ 10% = c.
- c. Make a Reamplification Core Mix for each H-T₁₁M by multiplying the numbers for a “Standard Reamplification Reaction” by g, a, and c accordingly. However, for the core mixes, the H-AP primers and cDNA templates will not be added, because these will vary with each band. Make the core mix as follows:

Distilled water	23.3 μ L $\times$ g, a, or c
10X PCR buffer	4.0 μ L $\times$ g, a, or c
dNTP mix (FDD)	0.3 μ L $\times$ g, a, or c
H-T ₁₁ M (2 μ M)	4.0 μ L $\times$ g, a, or c
Taq DNA polymerase	0.4 μ L $\times$ g, a, or c
32 μ L $\times$ g, a, or c (total volume)	

- d. As an example, if there were 20 bands chosen for reamplification from FH-T₁₁G, g would be 22 (20 $\times$ 10% = 22). A core mix should be made for 22 bands by multiplying the numbers from **step c** by 22:

Distilled water	23.3 μ L $\times$ 22 = 512.6 μ L
10X PCR buffer	4.0 μ L $\times$ 22 = 88 μ L
dNTP mix (FDD)	0.3 μ L $\times$ 22 = 6.6 μ L
H-T ₁₁ M (2 μ M)	4.0 μ L $\times$ 22 = 88 μ L
Taq DNA polymerase	0.4 μ L $\times$ 22 = 8.8 μ L
32 μ L $\times$ 22 = 704 total volume	

- e. Make appropriate amounts of core mixes for both FH-T₁₁A and FH-T₁₁C.
14. After core mixes are made, aliquot 32 μ L into 0.2-mL tubes (individually, as strip tubes, or in a 96-well plate) labeled with the proper band name.
15. Add 4 μ L of the corresponding cDNA template from **step 11** as well as 4 μ L of the corresponding H-AP primer.
16. Place the reamplification reactions on the thermal cycler and perform using same conditions as FDD-PCR.
17. Make a 1.5% agarose gel with ethidium bromide by adding 1.5 g of agarose to 100 mL of 1X TAE. When the agarose/1X TAE mix cools to approx 50°C (barely

touchable by hand), add 3 μL of ethidium bromide (EtBr), swirl to mix, and pour solution into plastic agarose-casting tray.

18. Add 30 μL of the reamplification reaction to 5 μL of agarose DNA loading dye in a 0.5 mL microfuge tube. Load the 35 μL volume onto the 1.5% agarose gel. Save the remaining 10 μL of the PCR samples at -20°C for future cloning.
19. Electrophorese at 70 V for approx 45–60 min.
20. Confirm correct cDNA reamplification by visualizing gel using a UV transilluminator. Reamplified band should be approx the same size as the band cut from the original FDD gel.

After successful reamplification, each band must be confirmed to be a “real” difference by Northern blot or another technique. In addition, the band will need to be sequenced to determine if it is a known or a novel sequence. The order in which these are done can vary and is generally up to the preference of the researcher. Direct sequencing of the reamplified PCR products can sometimes be done here (*see Note 25*), but a cloning step is recommended first. The following steps are presented in the recommended order, but this can be modified based on the situation.

3.7. Cloning of Reamplified PCR Products

Clone differentially expressed cDNAs into recommended PCR-TRAP cloning vector, or other suitable cloning vector, following manufacturer’s protocol. The PCR-TRAP cloning vector (*see Note 26*) protocol is listed next for simplicity.

3.7.1. Ligation

The reamplified PCR products from differential display should be used directly for cloning without any post-PCR purification, modification, or dilution. Gel-purified PCR products contain inhibitors of DNA ligase and will lead to significantly reduced cloning efficiency (*see Note 27*).

1. For a 20 μL ligation reaction, add in order:

dH ₂ O	10 μL
10X ligase buffer	2 μL
PCR-TRAP Vector	2 μL
PCR product	5 μL
T4 DNA ligase (add last)	1 μL
	20 μL

2. Mix well by finger tipping. Spin briefly.
3. Ligate overnight at 16°C .
4. Use directly for transformation or store at -20°C .

3.7.2. Transformation

1. Thaw the GH-competent cells in ice-water slush for 10–15 min. While the cells are melting, label the appropriate number of 1.5-mL microfuge tubes and set on ice. Quickly mix the cells by finger-tipping and aliquot 100 μ L into each 1.5 mL microfuge tube. Immediately refreeze (at -70°C) the remaining competent cells for future use.
2. Spin ligation tubes briefly to collect condensation. Add 10 μ L of each ligation mix to a tube containing the competent cells.
3. Mix well by finger-tipping and incubate on ice for 45 min.
4. Heat-shock the cells for 2 min at 42°C and then set the tubes back on ice for 2 min.
5. Add 0.4 mL of LB medium without tetracycline and incubate the cells at 37°C for 1 h. It is important that no tetracycline be in the LB during this step because the bacteria with recombinant plasmids need time to express the tetracycline-resistance gene (see **Note 28**). It is recommended that the LB-Tet plates be warmed up at 37°C for 1 h before plating, so this is a good time to do so.
6. After vortexing briefly, plate 200 μ L of cells on an LB-Tet plate (containing 20 $\mu\text{g/mL}$ of tetracycline). Store un plated cells at 4°C if re-plating is needed within 1 wk.
7. Once the plate surface is dry, incubate the plate upside down overnight at 37°C .
8. Score the Tet^R colonies and save the plate upside down at 4°C for further analysis.

3.7.3. Checking for the Insert

Checking for plasmids containing a DNA insert is easily done by the colony-PCR method using primers that flank the cloning site of the PCR-TRAP Vector. Therefore, it is unnecessary to wait another day before plasmid miniprep and restriction enzyme digestion can be performed.

3.7.3.1. COLONY LYSIS

1. On the bottom of the plate, number each Tet^R colony to be analyzed and then aliquot 50 μ L of colony lysis buffer into a corresponding microfuge tube.
2. Pick each colony with a clean pipet tip (try not to pick too much of the colony; a tiny amount that can be seen by the eye is usually more than enough) and transfer the cells into the colony lysis buffer of the corresponding numbered tube.
3. Incubate the tubes in boiling H_2O for 10 min.
4. Spin at room temperature for 2 min to pellet the cell debris, then transfer the supernatant into a clean tube with corresponding number.
5. Use the lysate immediately for PCR analysis or store at -20°C for future amplification.

3.7.3.2. PCR REACTION

1. For each colony lysate add (*see Note 29*):

dH ₂ O	20.4 μ L
10X PCR buffer	4.0 μ L
dNTPs (250 μ M)	3.2 μ L
Lgh primer	4.0 μ L
Rgh primer	4.0 μ L
Colony lysate	4.0 μ L
<i>Taq</i> DNA polymerase	0.4 μ L
	40 μ L

Mix well and add 30 μ L mineral oil if required by the thermal cycler.

2. PCR parameters are as follows: 94°C for 30 s, 52°C for 40 s, 72°C for 1 min for 30 cycles, followed by a 5 min extension at 72°C, and a final incubation at 4°C. For checking cloned PCR products longer than 700 bp, increase the elongation time at 72°C from 1 to 2 min.
3. Analyze 20 μ L of the PCR product on a 1.5% agarose gel with EtBr staining, while saving the rest for sequencing. Plasmids containing an insert should result in an easily visible band. Verify the insert size by comparing the molecular weight of the PCR product before and after cloning. The PCR product after colony PCR should be 120 bp larger than the original PCR insert before cloning owing to the flanking vector sequence being amplified.
4. The bands should then be purified from the agarose gel using a QIAEX II kit and saved for Northern blot probe generation using GenHunter's HotPrime® DNA Labeling Kit.

3.7.4. Storing the Cloned PCR Products

1. After a plasmid has been determined to contain an insert of interest, the corresponding Tet^R colony should be re-streaked to single colonies on a new LB-Tet plate:
 - a. Locate the colony marked with the number on the original plate, pick it with a clean pipet tip, and streak the cells on a new LB-Tet plate.
 - b. Change to another clean tip, rotate the plate 90°, and streak a second time in order to obtain single colonies.
 - c. Incubate the plate overnight at 37°C.
2. Inoculate a single Tet^R colony into a 5 mL LB culture (no Tetracycline, *see Note 29*) and use 3 mL for plasmid miniprep. Save the remainder for glycerol (50%) cell stock at -70°C.

3.8. Sequencing of Cloned PCR Products

If using the PCR-TRAP Cloning System, sequencing can be performed utilizing vector-specific primers such as Lseq/Rseq or Lgh/Rgh. If using a clon-

ing vector other than the one recommended, consult manufacturer's guidelines for sequencing instructions.

1. For bands that are the correct size, purify the remaining 20 μ L of the saved colony PCR reaction using the QIAquick PCR Purification Kit.
2. This purified product can then be used directly for sequencing.
3. Determine the sequence of your band by finding the H-AP and H-T₁₁M sequences. The band can be cloned in either direction into the PCR-TRAP vector, so ensure that the sequence is in the correct orientation.

3.9. Confirmation of Differential Gene Expression by Northern Blot

To confirm differential expression of the selected cDNAs, Northern blot analysis is suggested rather than other confirmation techniques such as reverse northern hybridization (19), quantitative RT-PCR, or real-time PCR. The Northern blot technique is technically simple and straightforward in approach, requiring no manipulation of the RNA sequences from which differential gene expression has been detected. Additionally, Northern blot analysis is the most accepted confirmation technique for differential gene expression, often being referred to as the "gold standard" of gene expression confirmation assays. If using the recommended PCR-TRAP cloning vector, the probe template is produced by a PCR reaction of the cDNA construct within the cloning vector. The required primers are supplied with the cloning system. Additionally, the HotPrime DNA Labeling Kit, a random prime labeling kit with major improvements over the traditional random priming kit, is suggested. It is specifically designed to efficiently label DNA probes isolated from DD for Northern blot analysis. This method makes use of random decamers, rather than the traditional hexamers used in random priming, incorporates the anchored oligo-dT primers (H-T₁₁M) into the labeling buffer to ensure full-length anti-sense cDNA probe labeling, and uses radioactive dATP to take advantage of the AT-rich nature of DD bands. These improvements greatly increase the chance for signal detection on the Northern blot analysis.

3.9.1. Generation of cDNA Probes

The product that was gel-purified with the QIAEX II kit in **Subheading 3.7.3.2., step 4** will be used as a template to generate a probe using the HotPrime DNA Labeling Kit. If a different vector was used for cloning, consult manufacturer's suggestions for generating cDNA probes for Northern blot hybridization.

3.9.2. Labeling of cDNA Probes

1. If using the recommended HotPrime DNA Labeling Kit, thaw all components completely and immediately set them on ice.

2. Set up the following reaction in a 1.5-mL microfuge tube with a locking cap (so the cap will not loosen during boiling):

Distilled water	11 μ L
10X Labeling buffer	3 μ L
DNA template to be labeled (10–50 ng)	7 μ L

3. Incubate the mixture in a boiling water bath for 10 min.
4. Quickly chill the tubes on ice. Spin the tube briefly to collect the condensation.
5. To the reaction, add the following *in order*:

dNTP (-dATP) (500 μ M)	3 μ L
α -[^{32} P] dATP (3000 Ci/mmol)	5 μ L
Klenow DNA polymerase	1 μ L

If using α -[^{32} P] dCTP instead of α -[^{32} P] dATP, substitute dNTP (-dCTP) for dNTP (-dATP).

6. Incubate for 20 min at room temperature, followed by incubation at 37°C for an additional 10 min.
7. Add 6 μ L of the Stop buffer and mix well.
8. Purify the labeled probe with a Sephadex G50 column. Collect the purified probe in a 1.5-mL microfuge tube with a lock-on cap. Count 1 μ L of labeled probe in a scintillation counter. A total of 10 million or more CPM can be obtained for most of the labeled DNA probes.

3.9.3. Probe Hybridization

1. Protocols for the preparation of a denaturing agarose (RNA) gel, including sample loading and electrophoresis conditions, and RNA transfer to nitrocellulose or nylon membrane has been previously described (20).
2. If the prehybridization buffer has been stored at -20°C, thaw at 37°C for 20 min.
3. Denature the salmon sperm DNA by incubating for 10 min in a boiling water bath.
4. Add salmon sperm DNA (to a final concentration of 100–200 μ g/mL) in the prehybridization solution. Mix well.
5. Use 5 mL of prehybridization solution or enough to cover the membrane.
6. Prehybridize at 42°C for at least 4 h.
7. Denature the purified probe in a 1.5-mL microfuge tube with a lock-on cap (otherwise the cap may loosen) by boiling for 10 min in a water bath.
8. Chill on ice for 2 min.
9. Spin down the condensation and add the probe directly to the prehybridization solution.
10. Hybridize overnight.
11. Carefully decant the radioactive hybridization solution and dispose of in an appropriate container for radioactive waste.
12. Wash with 1X SSC containing 0.1% SDS *twice* at room temperature, each time disposing of wash solution in an appropriate container.

13. Wash for 15–20 min with 0.25X SSC containing 0.1% SDS *prewarmed* to the final washing temperature of 50–55°C.

3.9.4. Blot Exposure

1. Blot the membrane dry with paper towels and cover using UV-transparent plastic wrap.
2. Expose blot to single emulsion film with intensifying screen at –70°C for best signal detection.

It is hoped that most of the bands will be confirmed to show differential gene expression. Those that are confirmed are considered “real” differences as opposed to any “false positives” (*see Note 30*). The real differences, of course, will warrant further study and require downstream functional characterizations, where some of those mysteries of life can be pieced together.

4. Notes

1. Nonanchored oligo-dT primers have been used for DD, but their disadvantages far outweigh the advantage of needing only one primer for RT and PCR. Without the non-T base at the 3' end of the primer to “anchor” their position, they can anneal anywhere on the poly-A tail for PCR and will thus create many different sized DNA fragments for the identical cDNA species. This leads to a background smear, which is aesthetically unappealing, but more importantly will create problems for downstream reamplification of the wrong cDNA.
2. Although, poly-A+ RNA (mRNA) is what is actually being reverse transcribed in DD, it is rarely used as the RNA input. It can be purified and used for DD, but it provides no significant advantages and therefore total RNA is the preferred RNA source for DD for a number of reasons. First, it is much easier to purify than poly-A+ (mRNA), because simple RNA isolation reagents exist from many commercial sources, including RNeasy. Most of the protocols for purifying mRNA require purification of total RNA first, so it requires additional steps. Second, total RNA allows for easy evaluation of overall RNA integrity by running an “RNA gel” and visualizing the ribosomal RNA bands. If these bands are sharp and without a background smear, it can be assumed that the mRNA is also intact. There are ways to evaluate mRNA integrity, but they require expensive and sophisticated instruments such as the Agilent Bioanalyser. Finally, the methods used for mRNA purification generally require an oligo-dT binding step so that only the mRNA will be captured. This always leads to some oligo-dT contamination in the RNA sample, which will cause problems for the same reasons listed in **Note 1**. For all these reasons, total RNA is the RNA type of choice for DD.
3. The RNeasy reagent is a simple mono-phasic solution for rapid isolation of intact total RNA that is similar to other phenol/guanidine thiocyanate-based RNA isolation products, but has several major advantages. These include special cell lysis chemicals giving better yield, a yellow color allowing easier visualization during phase separation, and better stability with less corrosiveness. The high-quality RNA isolated can be used for DD, Northern blot and reverse Northern blot analysis, and other applications.

4. During RNA isolation from cells, it is crucial to completely remove any residual PBS after rinsing. Otherwise, the ratio of RNAPure to cells will be altered. Let the plate sit on an angle for 1 min and remove the residual PBS with a 1000- μ L pipet.
5. During RNA purification steps, many of the centrifugation steps are done at 4°C. We put our centrifuge in the refrigerator a few hours before these steps will be done. However, we have noticed that if you leave the centrifuge in the refrigerator continuously, it will not spin as fast. We assume this would be caused by either temperature or moisture. Therefore, if you are using a standard lab centrifuge designed for room temperature use, do not keep the centrifuge in the refrigerator long-term.
6. When removing the upper phase, it is crucial that you do not touch the interphase, which may contain proteins including RNases/DNases. It is much better to lose some RNA, but ensure that what RNA you do retrieve will be free of RNase/DNase, than to try to get as much of the upper phase as possible and risk RNase/DNase contamination.
7. Because tissues generally contain higher amounts of RNases than cells, we have noticed that a second-extraction phenol extraction step will significantly improve the DD results in terms of reproducibility and overall quality. This second extraction can be done directly after taking the upper phase and using more RNAPure reagent. Just add 1 mL of RNAPure reagent per 100 μ L of upper phase and follow the protocol starting at **Subheading 3.1.2.** again.
8. For the DNase I digestion step at 37°C, we recommend sticking to this 30-min time as closely as possible in case there is any RNase contamination. However, it is also crucial to do the full 30-min incubation to completely digest all DNA.
9. We have found that Phenol/ CHCl_3 (3:1) is superior to Phenol/ CHCl_3 (1:1) or Phenol/ CHCl_3 /isoamyl alcohol (25:24:1). However, these other options can be used, but the extraction should be repeated twice to insure complete removal of proteins. Phenol/ CHCl_3 /isoamyl alcohol is normally used for DNA or plasmid purification. It is recommended that all reagents for RNA work be separated from DNA work to avoid RNase contamination.
10. There are nonphenol/chloroform-based based protocols to inactivate or remove DNase including heat-inactivation, chemical-inactivation, or column-based purification. However, phenol/chloroform-based purification is the gold standard for protein removal and the only way to ensure that all DNase is removed. The other protocols may inactivate or remove most of the DNase, but for RT-PCR applications, even minute amounts of DNase will cause major problems with results. Therefore, we only recommend phenol/chloroform-based purification.
11. To check for RNA integrity, look for the clear appearance of the ribosomal RNA bands, with little to no smearing. RNA from different species can look significantly different, but mammalian RNA should have 28S and 18S rRNA bands in close proximity at the top of the gel and a 5S rRNA band lower. If the RNA appears degraded, this can be caused by many things:

- a. RNA was degraded before treatment with DNase I. Check integrity at all stages (before digestion, after digestion, after Phenol/CHCl₃ extraction, and so on). Make sure RNA is stored at -80°C at concentrations of at least $1\text{ }\mu\text{g}/\mu\text{L}$.
 - b. DNase I was contaminated. DNase I from many vendors contain detectable RNase contamination. The DNase I from the MessageClean Kit is guaranteed to be RNase-free.
 - c. RNA was degraded by reagents or equipment. Make sure all solutions and buffers are made with DEPC-treated dH₂O and all vessels, including tubes, tips, and gel boxes, are free of RNase.
 - d. The RNA sample itself is contaminated with RNase. This is a common problem with RNA extracted from large amounts of tissue, which is why at least two extraction steps are recommended for tissues. To confirm RNase contamination, incubate RNA with $1\text{--}2\text{ mM MgCl}_2$ in Tris-Cl, pH 8.0, at 37°C for 30 min. This will activate any RNase in the RNA. If this is confirmed, if enough “uncleaned” RNA remains, do an additional phenol/CHCl₃ extraction with RNA sample following the same procedures in **Subheading 3.2.2**. If not, start a new RNA extraction and increase the RNA extraction solution (RNApure) to tissue ratio and do an additional phenol/CHCl₃ extraction step.
 - e. The RNA sample sometimes appears to be degraded after agarose gel analysis, when the actual problem is the pH of the buffer, too much salt in the RNA, or bad loading dye, which has caused the ribosomal RNAs (28S and 18S) to migrate strangely. We recommend using the RNA Loading Mix. Confirm the pH of the MOPS buffer, which should be between pH 6.5 and 7.0. Also, make sure formaldehyde is added to the gel and the RNA sample is denatured by incubating in RNA Loading Mix at 65°C for 10 min before loading.
12. RNA samples should be freshly diluted with dH₂O or DEPC-treated H₂O to $0.1\text{ }\mu\text{g}/\mu\text{L}$ directly before RT reaction setup. Do not reuse the diluted RNA after freezing and thawing because the RNA will be degraded and yield poor results.
 13. For the RT reaction, the initial 65°C incubation is intended to denature the RNA secondary structure. The final incubation at 75°C is to inactivate the reverse transcriptase without denaturing the cDNA/mRNA duplexes. Therefore “hot start” PCR is neither necessary, nor helpful for the subsequent PCR reactions using cDNAs as templates.
 14. If not using the recommended thermal cycler, you may need to adjust the denaturation (94°C) time to 30 s.
 15. The PCR setup for H-AP primers 73–80 can be done at the same time for all 3 FH-T₁₁M primers so they can all be put on one 96-well plate.
 16. Gel debris and streaks on the glass plates will usually fluoresce and can cause major background problems. Therefore, thorough cleaning is required.
 17. If overnight gel polymerize is done, plastic wrap, such as Saran Wrap, should be used to prevent the gel from drying out.
 18. During sample gel-loading, it is crucial that the urea in the wells be completely flushed immediately before loading your samples. Because urea is heavier than water, it will fall to the bottom of the well fairly quickly. If a sample is loaded

without flushing a well, it will sit on top of the urea, which in turns causes strange migration and poor resolution. For best resolution, flush every 4–6 wells loaded using a syringe or pipet while trying not to disturb samples that have already been loaded.

19. Fluorescent dyes are light-sensitive. We recommend keeping primers and samples in the dark or covered with aluminum foil. While running the gel, the apparatus should also be kept in the dark as much as possible. This can be done by running gels in a dark room or using a cardboard box to cover the entire apparatus.
20. When scanning the gel, it is best to remove the gel tape, spacers, and comb, which will fluoresce and can cause background problems. However, if you think you might run the gel longer for better separation, you should do a quick scan before removing gel tape, spacers, and comb to determine if it has been run long enough.
21. To separate the glass plates, we have found that small plastic wedges, which can be purchased from several gel companies, work well. It is important to do this slowly to make sure the gel is sticking only to one side.
22. When printing out the real-size image, you will need a paper large enough to fit the whole gel. We use 11 × 17 paper on an ink-jet printer, which allows plenty of space for the entire gel. If necessary, you could also print the gel on two to three pieces of paper and tape them together.
23. When selecting bands to cut, if there is a chance that pursuit of the band is worthwhile, it is recommended to cut it. Later, a decision can be made whether or not to reamplify that band. However, if one later decides to pursue a band that was not cut, the gel will have to be run again because gels can only be stored for a few days before drying out. When a large quantity of gels is being run, it usually makes sense to run all the gels first, cutting any interesting bands along the way, and storing those bands in the refrigerator. When all gels have been completed, a decision can be made on which bands are worthwhile reamplifying and then they can be done together.
24. For the reamplification reaction, note that the unlabeled (without 5' fluorophore) H-T₁₁M primers are used. Otherwise, the fluorophore can interfere with future cloning.
25. Direct sequencing can sometimes be done following successful reamplification. If the reamplified product is a single, clean band, direct sequencing with the H-AP primer can work, generally about 50% of the time. However, if the reamplified product has multiple bands, a cloning step will have to be done first.
26. The PCR-TRAP Cloning System is by far the most efficient cloning method for PCR products that we have tested. It utilizes a third-generation cloning vector that features positive selection for DNA inserts. Only recombinant plasmids confer antibiotic resistance. The principle of this unique cloning system is based on the phage Lambda repressor gene, cI, which is cloned on the PCR-TRAP vector and codes for a repressor protein. The repressor protein binds to the Lambda right operators Or1 to Or3 of the *cro* gene, thereby turning off the promoter that drives the Tet^R gene on the plasmid. Therefore, cloning of the PCR products

directly, without any post-PCR purification, into the cI gene leads to inactivation of the repressor gene, thus turning on the Tet^R gene. This allows the *Escherichia coli* containing recombinant plasmids to grow on Tet plates.

27. For PCR product cloning of DD fragments, we do not recommend any post-PCR purification, modification, or dilution. However, if you decide to gel-purify, cloning can still be done, but with much-reduced efficiency. GenHunter always recommends using the PCR product directly in the ligation step. Some reagents used in gel purification, such as NaI, are potent inhibitors of DNA ligase. If purified PCR products must be used (whether by gel or column purification), adding more ligase (two to five times as much) may help.
28. Although PCR-TRAP is a high copy-number cloning vector, cells harboring the plasmid are more sensitive to tetracycline in liquid culture than on plates. Do not add any tetracycline in liquid cell culture, because it may significantly inhibit cell growth.
29. For the colony-PCR reactions, it is recommended that a core mix containing everything except the colony lysate be made in order to minimize pipetting errors and to be able to analyze many colony lysates at one time.
30. If a band chosen from DD does not show differential expression on a Northern blot, it does not mean that it is necessarily a “false positive.” We have seen several examples where bands show no noticeable differential expression on Northern blots, but upon review, something else is involved such as polymorphism at primer binding site, short sequence deletion/insertion, splicing difference, and so on. The message is if a band looks convincing on the DD gel, but does not show differential expression by Northern blot, it could be a false positive, but could also be something very interesting, and worth pursuing.

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Ordered Differential Display

Mikhail V. Matz and Ella A. Meleshkevitch

Summary

Ordered differential display (ODD) is one of the approaches that uses systematic, rather than random, sampling of transcripts for display and thereby provides means to browse through essentially all the transcripts in the compared mRNA pools. It is specifically adapted for small amounts of starting material. The protocol outlined here, in addition to ODD procedure itself, also describes isolation of RNA and synthesis of double-stranded cDNA from small biological samples.

Key Words: Ordered differential display; differential gene expression; microscopic samples; cDNA amplification; reproducibility; invertebrates.

1. Introduction

Ordered differential display (ODD) (*1*) belongs to a diverse group of methods that can be generally called “systematic differential display approaches” (*2*). All these techniques, similar to very popular “classical” differential display (DD), or DDRT-PCR, rely on comparison of band patterns on gels to pinpoint cDNA fragments representing differentially expressed genes, but utilize radically different principles of the pattern production. Rather than randomly picking cDNAs by means of polymerase chain reaction (PCR) with short arbitrarily matching primers, systematic techniques attempt to accurately subdivide the whole cDNA population into known number of nonoverlapping subsets, which would be of sufficiently low complexity to be successfully separated on polyacrylamide gel. The major advantage of doing this is that one can be completely sure that, upon looking at all possible subsets, essentially all cDNA species have been screened (as far as the method’s sensitivity allows). There are several more advantages to the systematic techniques, and to ODD

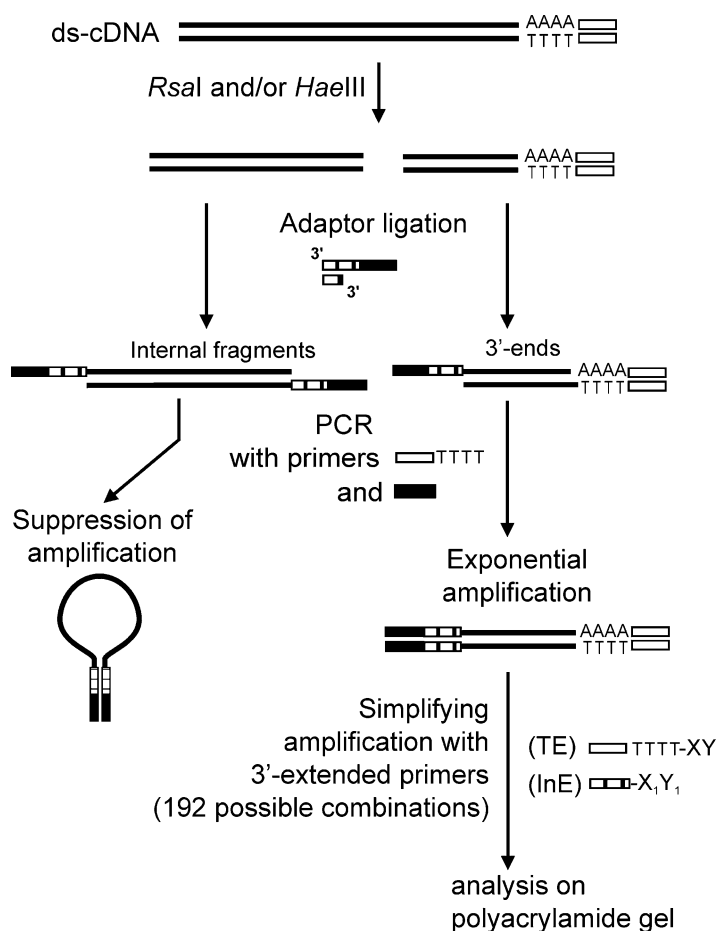


Fig. 1. Schematic representation of ordered differential display (ODD) protocol. (Adapted with permission from ref. 3.)

in particular, which for certain experimental setups compensate for the higher labor requirements in comparison to DDRT-PCR. ODD has been successfully applied to study various biological models (3–14).

The principle of ODD is depicted in **Fig. 1**. Briefly, after synthesizing double-stranded cDNA by any conventional technique (only using ODD-cDNA synthesis primer for initiating first-strand synthesis). The DNA is digested by four-base-recognizing restriction endonuclease. Then, a pseudo-double-stranded adaptor (15,16) is ligated to the cDNA fragments, and PCR is performed using the cDNA-synthesis primer and a primer corresponding to the distal half of the adaptor. This setup invokes PCR-suppression effect (17),

which prohibits amplification of the molecules that do not contain annealing site for cDNA synthesis primer. As a result, an amplified sample containing only 3'-restriction fragments of cDNAs is obtained. This amplification technique replaces the laborious and relatively inefficient procedures of physical separation of cDNA fragments required by some other systematic display methods, and obviates the problem of genomic DNA contamination (15). Most importantly, its use effectively lowers the requirement for initial amount of total RNA to 20–50 nanograms: this amount is sufficient for contemporary cDNA synthesis methods to produce a well-representative pool of cDNAs (10^6 or more individual cDNA molecules) to be used as starting material for the amplification (15,18). The straightforward indication of the initial cDNA pool representation is the number of PCR cycles required for amplifying it to the DNA concentration of about 10–15 ng/ μ L: if this number is less than 20, there were more than 10^6 molecules at the start (2,15,18). Having a well-representative sample is very important, because the DD patterns tend to exhibit fluctuations of bands' intensity (up to total disappearance of some bands) when working with under-represented samples (Fig. 2A). As long as the sufficient representation of amplified sample of 3'-ends is ensured, the ODD analysis becomes very reproducible (Fig. 2B).

2. Materials

1. Dispersion buffer ("buffer D"): 4 M guanidine thiocyanate, 30 mM disodium citrate, 30 mM β -mercaptoethanol, pH 7.0–7.5 (see Note 1).
2. Buffer-saturated phenol, pH 7.0–8.0 (GIBCO/Life Technologies).
3. Chloroform-isoamyl alcohol mix (24:1).
4. 96% ethanol.
5. 80% ethanol.
6. 12 M lithium chloride.
7. Co-precipitant: See DNA reagent (Amersham) or glycogen.
8. Fresh milliQ water.
9. Agarose gel (1%) containing ethidium bromide (EtBr).
10. SuperScript II reverse transcriptase, 200 U/ μ L (Life Technologies) or 20X PowerScript reverse transcriptase (Clontech) with provided buffer.
11. 0.1 M dithiothreitol (DTT).
12. Deoxyribonucleoside triphosphate (dNTP) mix, 10 mM each.
13. 5X Second-strand buffer: 500 mM KCl, 50 mM ammonium sulfate, 25 mM MgCl_2 , 0.75 mM β -NAD, 100 mM Tris-HCl, pH 7.5, 0.25 mg/mL bovine serum albumin (BSA).
14. 20X Second-strand enzyme cocktail: 6 U/ μ L DNA polymerase I, 0.2 U/ μ L RNase H, 1.2 U/ μ L *Escherichia coli* DNA ligase.
15. T4 DNA polymerase (1–3 U/ μ L).
16. 3 M sodium acetate, pH 5.0.

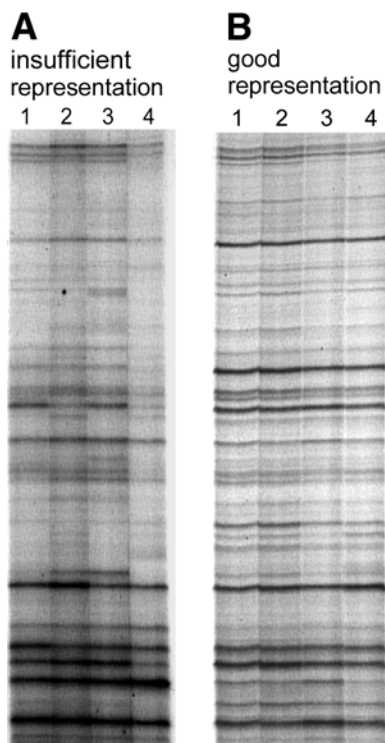


Fig. 2. Effect of initial cDNA sample representation on ordered differential display (ODD) reproducibility. In both cases, the pattern of the same subset is shown for two sets of analogous cDNA samples. (A) Complete quadruplicate (starting from RNA isolation) of growing blastema (regenerating tissue) of freshwater planarian *Dugesia (Girardia) tigrina*. A single blastema was used for each sample, which yielded cDNA amplified in more than 20 polymerase chain reaction (PCR) cycles (under-represented). (B) Same as (A), but 20 blastemata were used to prepare each sample, yielding a representative cDNA sample amplified in 18 PCR cycles. Note that the pattern in panel A is significantly more “noisy” and therefore prone to produce false positives. The increased sharpness of the bands in (B) is actually because ^{33}P isotope was used instead of ^{32}P as in (A) (Adapted with permission from ref. 3.)

17. Oligonucleotides: see **Table 1** and **Note 2**.
18. Restriction endonucleases *RsaI*, *HaeIII*, *MboI*, and *AluI* (New England Biolabs).
19. T4 DNA Ligase (New England Biolabs) with provided buffer.
20. QiaQuick-spin PCR purification kit.
21. T&M solution: 10 mM Tris-HCl, pH 8.0, 1 mM MgCl_2 .
22. KlenTaq Polymerase (Ab Peptides Inc.) or its analog.
23. Agarose and DNA sequencing gel equipment.

Table 1
ODD Oligos List

Adpater (10 μ M mixture of two oligos)

5'-tgt agc gtg aag acg aca gaa agg gcg tgg tgc gga ggg cggt
5'-acc gcc ctc cg

Distal adapter-specific primer (DAd)

5'-tgt agc gtg aag acg aca gaa

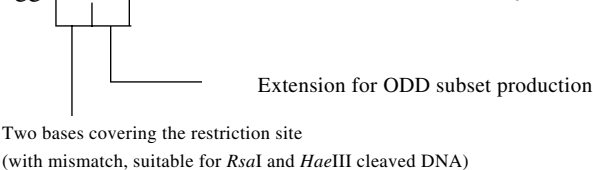
TRsa (nonextended T-primer for cDNA synthesis and amplification)

5'-cgc agt cgg tac (t)₁₃

Inner adapter-specific extended primers (InE-primers)

5'- gcg tgg tgc gga ggg cggt gc GG
5'- gcg tgg tgc gga ggg cggt gc GA
5'- gcg tgg tgc gga ggg cggt gc GT
5'- gcg tgg tgc gga ggg cggt gc GC (Note: G→ T substitutions in the 3rd base from 3'end)
5'- gcg tgg tgc gga ggg cggt gc AG
5'- gcg tgg tgc gga ggg cggt gc AA
5'- gcg tgg tgc gga ggg cggt gc AT
5'- gcg tgg tgc gga ggg cggt gc AC (Note: G→ T substitutions in the 3rd base from 3'end)
5'- gcg tgg tgc gga ggg cggt gc TG
5'- gcg tgg tgc gga ggg cggt gc TA
5'- gcg tgg tgc gga ggg cggt gc TT
5'- gcg tgg tgc gga ggg cggt gc TC

5'- gcg tgg tgc gga ggg cggt gc CG
5'- gcg tgg tgc gga ggg cggt gc CA
5'- gcg tgg tgc gga ggg cggt gc CT
5'- gcg tgg tgc gga ggg cggt Tc CC (Note: G→ T substitutions in the 3rd base from 3'end)



Extended T-primers (TE-primers):

5'-cgc agt cgg tac (t)₁₃ AG
... AA
... AT
... AC

And so on, all extension variants except those with T in the distal position.

24. Thermocycler.
25. 10X polynucleotide kinase buffer (500 mM Tris-HCL, pH 7.6, 100 mM MgCl₂, 10 mM spermidine, 10 mM ethylenediaminetetraacetic acid (EDTA)).
26. $\gamma^{33}\text{P}$ -ATP or $\alpha^{33}\text{P}$ -dATP, 10 uCi/ μL (3000–5000 Ci/mmol).
27. T4 polynucleotide kinase (5 U/ μL).
28. 10X PC2 buffer: 200 mM Tricine-KOH, pH 9.1, 160 mM ammonium sulfate, 30 mM MgCl₂, 0.5 mg/mL bovine serum albumin (BSA).
29. TaqStart antibodies (Clontech).
30. 1X TBE buffer: 0.089 M Tris-borate, 2 mM EDTA, pH 8.5.
31. 0.5X TBE buffer: 0.0445 M Tris-borate, 1 mM EDTA, pH 8.5.
32. Standard sequencing stop-solution.
33. X-ray film with appropriate exposure and development equipment and reagents.
34. QT: 10 mM Tris-HCl, pH 8.0.
35. Purified yeast tRNA.

3. Methods

3.1. Total RNA Isolation

The protocol provided has been extensively tested on a variety of animal-derived material and is more likely to work on a nonstandard object than other methods and commercially available kits (*see Note 3*). Still, RNAqueous kit from Ambion seems to approach this protocol in terms of versatility, so it can be used instead in most cases, even for microscopic samples.

1. Dissolve the tissue sample in buffer D (*see Note 4*).
2. Spin the sample at maximum speed on table microcentrifuge for 5 min at room temperature to remove debris. Transfer the supernatant to a new tube.
3. Put the tube on ice, add equal volume of buffer-saturated phenol, and mix. There will be no phase separation at this moment. Add 1/5 volume of chloroform-isoamyl alcohol (24:1) and vortex the sample. Two distinct phases will separate. Vortex three to four more times with about 1-min intervals between steps. Incubate the tube on ice between steps. Spin at maximum speed on table microcentrifuge for 30 min at +4°C. Remove and save the upper, aqueous phase. Take care to avoid warming the tube with your fingers, or the interphase may become invisible.
4. Repeat **step 3**.
5. Add 1 μL of co-precipitant, and then add an equal volume of 96% ethanol and mix. Spin immediately at maximum speed on table microcentrifuge at room temperature for 10 min. The precipitate may not form a pellet, instead being spread over the back wall of the tube and thus being almost invisible even with co-precipitant added. Wash the pellet once with 0.5 mL 80% ethanol. Dry the pellet briefly until no liquid is seen in the tube (do not over-dry).
6. Dissolve the pellet in 100 μL of fresh milliQ water. If the pellet cannot be dissolved completely, remove the debris by spinning the sample at maximum speed on a table microcentrifuge for 3 min at room temperature. Transfer the supernatant to a new tube, then add equal volume of 12 M LiCl and chill the solution at –20°C

for 30 min. Spin at maximum speed on table microcentrifuge for 15 min at room temperature. Wash the pellet once with 0.5 mL 80% ethanol, and dry as previously done. The precipitated RNA is usually invisible, because co-precipitant does not precipitate in LiCl.

7. Dissolve the pellet in 40 μ L of fresh milliQ water.
8. Load 2 μ L of the solution onto a standard (nondenaturing) 1% agarose gel to check the amount and integrity of the RNA. Add EtBr to the gel to avoid the additional (potentially RNase-prone) step of gel staining. Load a known amount of some DNA on a neighboring lane to use as standard for determining the RNA concentration. Intact RNA should exhibit sharp band(s) of ribosomal RNA (*see Fig. 3 and Notes 5–9*).

3.2. Double-Stranded cDNA Synthesis

This protocol describes a standard procedure of double-stranded cDNA synthesis that uses RNaseH/DNA polymerase I/DNA ligase cocktail for synthesis of the second cDNA strand. Any commercially available kit that utilizes the same principle (such as Marathon kit by BD Biosciences Clontech) can be used at this step, except that the primer for first-strand synthesis provided in the kit should be substituted for TRsa.

3.2.1. First-Strand cDNA Synthesis

1. To 5 μ L RNA solution in water (0.03–3 μ g of total RNA), add 1 μ L of 10 μ M primer TRsa and cover with mineral oil. Incubate at 65°C for 3 min, and then put the tube on ice.
2. Add 2 μ L 5X first-strand buffer (provided with reverse transcriptase), 1 μ L of 0.1 M DTT, 1 μ L of reverse transcriptase, 0.5 μ L of dNTP mix (10 mM each) and incubate at 42°C for 1 h, then put the tube on ice.

3.2.2. Second-Strand cDNA Synthesis

1. To the first-strand cDNA solution, add 49 μ L of milliQ H₂O, 1.6 μ L of dNTP mix (10 mM each), 16 μ L of 5X second-strand reaction buffer, and 4 μ L of 20X second-strand enzyme cocktail. (The total volume of the reaction mix is about 80 μ L.) Incubate at 16°C for 1.5 h, and then put the tube on ice.
2. Add 1 μ L T4 DNA polymerase, incubate 0.5 h at 16°C to polish ends.
3. Stop the reaction by heating at 65°C for 5 min.
4. Take the reaction mix from under the oil, put in new tube, and add 0.5 vol phenol then 0.5 vol chloroform-isoamyl alcohol (24:1). Vortex the solution and spin at maximum speed on a table microcentrifuge for 10 min. Transfer the upper, aqueous phase into new tube.
5. Add carrier (SeeDNA, Amersham, or glycogen) and precipitate DNA by adding 0.1 vol (8 μ L) 3 M sodium acetate, pH 5.0, and 2.5 vol (200 μ L) 95% ethanol at room temperature. Spin immediately for 15 min at maximum speed on a table microcentrifuge at room temperature.
6. Wash the pellet with 80% ethanol; air-dry the pellet for about 5 min at room temperature. Dissolve pellet in 6 μ L H₂O.

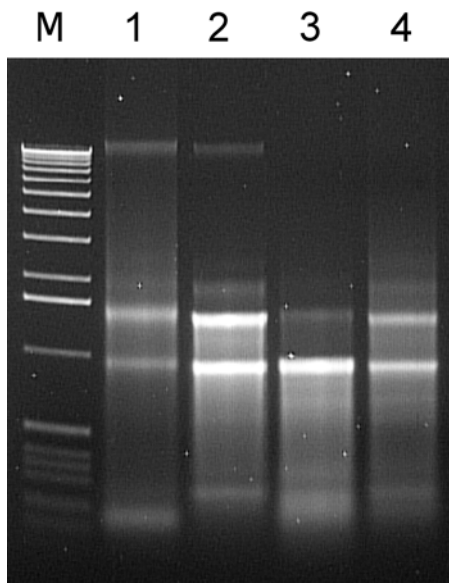


Fig. 3. Nondenaturing agarose electrophoresis of total RNA from various invertebrate sources. Lane 1, unidentified sponge; lane 2, comb jelly *Bolinopsis infundibulum* (phylum Ctenophora); lane 3, planarian *Girardia tigrina* (phylum Platyhelminthes); lane 4, stony coral *Montastraea cavernosa* (phylum Cnidaria). M, 50 ng of 1 kb DNA ladder (GIBCO/Life Technologies).

3.3. cDNA Digestion and Adapter Ligation

In ODD, cDNA species are discriminated by the length of fragment between poly-A attachment site and the first occurrence of site for some four-base specific restriction endonuclease. Two such enzymes fit the designed oligos set: *RsaI* (GT/AC) and *HaeIII* (GG/CC) (see **Note 10**).

1. To cDNA dissolved in 20 μL QT, add 2.2 μL of reaction buffer provided with the restriction endonuclease and 1.5 μL of the restriction endonuclease. Incubate at 37°C for 1.5 h.
2. Inactivate the restriction endonuclease by heating (for *RsaI*, 2 min at 60°C) and phenol-chlorophorm extraction, precipitate the cDNA by ethanol-NaAc, wash once with 200 μL of 80% ethanol, dry, and dissolve in 5 μL QT.
3. Add 2 μL of 10 μM ODD adapter (mixture of two adapter oligos 10 μM each, see oligos list), 1 μL of 10X ligation buffer (provided with the ligase) and 1 μL (1–5 U) of ligase. Leave the reaction at 16°C overnight.
4. Add 90 μL of water to the ligation mixture and purify it by QiaQuick-spin PCR purification column, according to the provided protocol for PCR products. Elute

with 30 μL of T&M solution (*see Note 11*). Alternatively, the ligation mixture may be diluted fivefold with water and 1 μL of this dilution may be taken for subsequent PCR.

3.4. Amplification of Basic Samples

This is the step in which the success of all the previous manipulations can be evaluated for the first time. When substantial amount of product representing the basic sample of 3'-cDNA ends can be amplified in less than 20 cycles, it means that the samples are of sufficient representation for robust ODD analysis; if not, the analysis will be irreproducible, if possible at all (*see Note 12*). Because it is the same for all samples under comparison except for the DNA template, the PCR reaction mixture can be prepared as a master mix.

1. Each amplification reaction contains: 2.5 μL of 10X PC2 buffer; 0.5 μL of 5 mM dNTPs; 0.25 μL of 10 μM primer TRsa; 0.25 μL of 10 μM DAd-primer; 12 μL of deionized water; 0.25 μL of KlenTaq 25 U/ μL (Ab Peptides); 10 μL of QIA-purified cDNA sample (*see Note 13*).
2. Perform PCR with the following program: 94°C 40 s $\rightarrow$ 65°C 1 min $\rightarrow$ 72°C 1.5 min (15 cycles).
3. Load 3 μL of each reaction on agarose gel and run for a short while: about 1 cm from wells. (Meanwhile, the PCR reactions may be kept on table.) If some or all products are poorly visible, put the corresponding samples back into thermocycler and perform additional cycles (*see Notes 14 and 15*).
4. Dilute the obtained PCR products 30–100-fold in QT with 20 ng/ μL purified yeast tRNA added (*see Note 16*).

3.5. ODD Reactions

Two alternative methods of incorporating a radioactive label into the cDNA fragments are possible, which are usually called terminal and in-strand labeling (*see Notes 17 and 18*).

3.5.1. Terminal Labeling

1. For 10 ODD reactions mix: 2 μL of 10 μM extended primer; 1 μL of 10X polynucleotide kinase buffer, 5 μL of $\gamma^{33}\text{P}$ -ATP 10 uCi/ μL (3000–5000 Ci/mmol); 1 μL of water; 1 μL of T4 polynucleotide kinase (5 U/ μL).
2. Incubate 30 min at 37°C, and then stop the reaction by heating for 2 min at 70°C.
3. For ODD subset amplification (10 reactions) mix: 4 μL of 5 mM dNTPs; 10 μL of 10X PC2 buffer; 10 μL of kinase reaction; 2 μL of 10 μM second (not labeled) extended primer; 74 μL of water; 3 μg of TaqStart antibodies (Clontech); 1 μL of KlenTaq 25 U/ μL (Ab Peptides).
4. Dispense the mix into 9- μL aliquots and add 1 μL of diluted basic samples to each.
5. Perform PCR: 94°C 40 s $\rightarrow$ 65°C 1 min $\rightarrow$ 72°C 1.5 min (25 cycles).

3.5.2. In-Strand Labeling

1. For 10 reactions mix: 10 μL of 10X PC2; 2 μL of 5 mM dNTPs; 2 μL of 10 μM TE-primer; 2 μL of 10 μM InE-primer; 79 μL of water; 5 μL of $\alpha^{33}\text{P}$ -dATP 10 uCi/ μL (3000–5000 Ci/mmol); 3 μg of TaqStart antibodies (Clontech); and 1 μL of KlenTaq 25 U/ μL (Ab Peptides).
2. Dispense the mix into 9- μL aliquots and add 1 μL of diluted basic samples to each.
3. Perform PCR: 94°C 40 s $\rightarrow$ 67°C 1 min $\rightarrow$ 72°C 1.5 min (25 cycles).

3.6. Electrophoresis

1. Prepare 0.4 mm-thick 5% sequencing gel. The buffer should be 1X TBE in gel and lower chamber, 0.5X TBE in upper chamber.
2. Mix 5 μL of labeled ODD reaction with 5 μL of standard sequencing stop-solution. Heat these samples at 90°C for 5 min before loading (do not let the samples evaporate while heating).
3. Pre-run the gel (*see Note 19*). Upon removing the comb from the gel, wash wells with 0.25X TBE (twice-diluted upper chamber buffer), load reference (2 μL of stop-solution) into one well, and run the gel at maximum voltage until bromophenol migrates about 0.5 cm. Then wash wells again with 0.25X TBE and load the samples (3–5 μL /lane).
4. Run-in (until both dyes migrate into the gel) at one-third of running voltage, then switch to maximum. Run the gel until xylene-cyanol migrates about two-thirds of the gel length.
5. After running, dry the gel on a Whatman paper at 60°C and expose to X-ray film overnight. Attach fluorescent or radioactive position markers to the gel so that the autograph can be aligned with the gel for band excision.

3.7. Band Excision and Amplification

1. Align the autograph with the gel. Using hot needle, pierce the film twice to mark the middle three-fourths of the band of interest (avoid taking the edges of the band). Remove film; excise the piece of the gel-paper sandwich surface between the two marks. As a negative control, cut out the same region of the neighboring lane, which does not contain the band (this is especially important for weak differential bands).
2. Put the excised piece into 30 μL of QT; incubate the tube at 55°C for 2 h or at 4°C overnight.
3. Take 1 μL of the eluate for PCR with corresponding extended primers (in 25 μL). PCR conditions are the same as described for basic samples' production (**Subheading 3.4.**). Intense and low (meaning the fragment length is small) bands are usually amplified in 15–17 cycles, weak and high ones in 19–22 cycles (*see Notes 20 and 21*).

4. Notes

1. Normally the dispersion buffer does not require titration. If pH comes out significantly lower than 7.0, try another batch of guanidine or disodium citrate. The buffer may be stored for years at 4°C in the dark.
2. The set of oligonucleotides presented in **Table 1** has been extensively tested on a number of various invertebrates and consistently produced good results. It is very important to have them purified using high-performance liquid chromatography (HPLC) or polyacrylamide gel to ensure best possible quality.
3. There is widespread belief that RNA is very unstable and therefore all the reagents and materials for its handling should be specially treated to remove possible RNase activity. We have found that purified RNA is rather stable and, ironically, too much anti-RNase treatment can become a source of problems. This especially applies to DEPC-treating of aqueous solutions, which often leads to RNA preparations that are very stable but completely unsuitable for cDNA synthesis. Simple precautions such as wearing gloves, avoiding speech over open tubes, using aerosol-barrier tips, and using fresh milliQ water for all solutions are sufficient to obtain stable RNA preparations. All organic liquids (phenol, chloroform, and ethanol) can be considered essentially RNase free by definition, as well as the dispersion buffer containing 4 M guanidine thiocyanate.
4. The volume of tissue should be not more than one-fifth of the dispersion buffer D volume. To avoid RNA degradation, tissue dispersion should be done as quickly and completely as possible, ensuring that cells do not die slowly on their own. To adequately disperse a piece of tissue usually takes 2–3 min of triturating using a pipet, taking all or nearly all volume of buffer into the tip each time. The piece being dissolved must go up and down the tip, so it is sometimes helpful to cut the tip to increase the diameter of the opening for larger tissue pieces. Tissue dispersion can be done at room temperature. The dispersed samples can be stored at 4°C for 1–2 d.
5. The tissue dispersed in buffer D produces a highly viscous solution. The viscosity is usually owing to genomic DNA. This normally has no effect on the RNA isolation (except for dictating longer periods of spinning at the phenol-chloroform extraction steps), unless the amount of dissolved tissue was indeed too great. However, in some cases (for example, freshwater planarians or mushroom anemones) mucus produced by the animal contributes to viscosity. This substance tends to co-purify with RNA, making it very difficult to collect the aqueous phase at the phenol-chloroform extraction step. It likewise lowers the efficiency of cDNA synthesis. The RNA sample contaminated with such mucus, although completely dissolved in water, does not enter agarose gel during electrophoresis. The EtBr-stained material stays in the well, probably because the mucus adsorbs RNA. Including cysteine in buffer D can diminish the mucus problem. To buffer D, add 0.1 vol of solution containing 20% L-Cysteine hydrochloride hydrate and 50 mM

tricine-KOH, pH 7.0 (takes a lot of titration). The cysteine solution should be freshly prepared. After dissolving the tissue, incubate the sample for 2 h at 4°C, and then proceed with the previously mentioned protocol.

6. RNA degradation can be assessed using nondenaturing electrophoresis. The first sign of RNA degradation on the nondenaturing gel is a slight smear starting from the rRNA bands and extending to the area of shorter fragments, such as seen on **Fig. 3**, lanes 3 and 4. The RNA showing this extent of degradation is still good for further procedures. However, if the downward smearing is so pronounced that the rRNA bands do not have a discernible lower edge, the RNA preparation should be discarded. The amount of RNA can be roughly estimated from the intensity of the rRNA staining by EtBr in the gel, assuming that the dye incorporation efficiency is the same as for DNA (the ribosomal RNA may be considered a double-stranded molecule owing to its extensive secondary structure).
7. The rule for vertebrate rRNA is that in intact total RNA the upper (28s) rRNA band should be twice as intense as the lower (18s) band, but this rule does not apply to invertebrates. The overwhelming majority of them have 28s rRNA with a so-called "hidden break." It is actually a true break right in the middle of the 28s rRNA molecule, which is called hidden because under nondenaturing conditions the rRNA molecule is being held in one piece by the hydrogen bonding between its secondary structure elements. The two halves, should they separate, are each equivalent in electrophoretic mobility to 18s rRNA. In some organisms, the interaction between the halves is rather weak, so the total RNA preparation exhibits a single 18s-like rRNA band even on nondenaturing gel (**Fig. 3**, lane 3). In others the 28s rRNA is more robust, so it is still visible as a second band, but it rarely has twice the intensity of the lower one (**Fig. 3**, lanes 1, 2, and 4).
8. Curiously, genomic DNA contamination is reproducible for a particular species, but varies between species. However, it never exceeds the amount seen in **Fig. 2A**, lanes 1 and 2: a weak band of high molecular weight. Such extent of contamination does not affect further procedures. In fact, the methods of cDNA amplification described here tolerate genomic DNA up to 50% of the total sample mass, without losing specificity or efficiency.
9. To store the isolated RNA, add 0.1 vol of 3 M sodium acetate and 2.5 vol of 96% ethanol to the RNA in water, and mix thoroughly. The sample may be stored for several years at -20°C.
10. In ODD, cDNA species are discriminated by the length of fragment between poly-A attachment site and the first occurrence of site for some four-base-specific restriction endonuclease. Two such enzymes fit the designed oligos set: *RsaI* (GT/AC) and *HaeIII* (GG/CC). The choice of the restriction enzyme should depend on the average GC-content of cDNA, so that most of cDNA species should produce 3' fragments shorter than 1 kb. For most animal species *RsaI* suits best, though for some special cases with very high GC content *HaeIII* may be the enzyme of choice. When more than 300 ng of RNA was put into cDNA synthesis, the volume of cDNA solution may be adjusted to 40 µL by QT and the following two steps (digestion-ligation and PCR) may be made in parallel with two differ-

ent restriction endonucleases, to see which would produce the amplified cDNA sample consisting of shorter fragments.

11. This stage is required to remove the residual adapter oligos, so that larger volume of ligated cDNA could be put into the subsequent PCR. It is essential when the initial amount of RNA was very low (100–200 ng).
12. The product of amplification (smear with some bands) should become well-visible on agarose gel (i.e., reach the DNA concentration of about 25 ng/ μ L) after not more than 15–20 PCR cycles, to be sure that the cDNA samples are well-representative. Poor representativity of the samples obtained in more than 20 PCR cycles leads to the great increase of “statistical noise” during display, which may lead to false positives. It is very important to use KlenTaq (Ab Peptides Inc.) or its analog in all the ODD PCRs. No other polymerase makes it possible to obtain such clear and reproducible pictures as KlenTaq.
13. If no QIA purification was made, 1 μ L of fivefold dilution of the ligated cDNA sample is added to 24 μ L of the mixture (adjust the volume with deionized water).
14. To estimate the required number of additional cycles, it can be roughly supposed that each PCR cycle raises the product amount twofold. If a product is just barely visible even on such a short run, the sample should be given four more cycles. If there is no product visible at all, add six more cycles. Then run the products on a gel again and evaluate their relative amounts once more. If there was no or very little product visible after 20 cycles, there was too little cDNA in the sample to be used in ODD.
15. The PCR products should look like smears shorter than 1 kb, with maximum of their intensity at 300–700 bp. If the maximum intensity is at about 1 kb or higher, alternative restriction enzyme should be considered to produce shorter 3'-terminal fragments.
16. The exact extent of dilution is selected in such a way that all the diluted samples would contain DNA in the same concentration. The diluted samples serve as a basic cDNA source for ODD patterns production. They can be stored at -20°C for years.
17. Terminal labeling assumes that one of the ODD primers is phosphorylated using T4 polynucleotide kinase and $\gamma^{33}\text{P}$ -ATP in advance and then used in ODD reactions. In-strand labeling is simply adding $\alpha^{33}\text{P}$ -dATP into the ODD-PCR reaction so it becomes incorporated into the newly synthesized fragments. We recommend terminal labeling, because of two reasons: first, in this way only one strand of the PCR product become labeled and visible on gel, which to some extent lowers the complexity of the pattern; and second, PCR products appear on gel with the intensity depending solely on the cDNA species' concentration in the sample. With in-strand labeling, the intensity depends also upon the length of the fragment, so that the shorter products become much less pronounced than the longer ones. As for the isotope, ^{33}P is highly recommended.
18. It is recommended, before preparing labeled reactions, to test several extended primers combinations without labeling (PCR as just described but without radioactivity) and look at the products on agarose gel. The products should be fully visible after 23–25 PCR cycles, have the same intensity within a particular prim-

ers combination, and substantial pattern differences should be observed between primer combinations.

19. The pre-run is needed to create a buffer gradient under the wells, so the samples become concentrated as they migrate into gel. This greatly improves the resolution.
20. If the product of re-amplification is fully seen at agarose after 20 cycles or less and looks like a single band, while the negative control is empty, such product is likely to be successfully sequenced directly by any appropriate method using extended primers. If the re-amplification takes more than 21 cycles and/or when additional weak bands are visible along with the major product, secondary excision from polyacrylamide gel is recommended before direct sequencing. Alternatively, the product may be cloned, and the clones containing the most frequent insert (which is the target one) may be either identified after sequencing 10 randomly picked clones, or in the way that we call "chop check," consisting in the following. After cloning, screen 10–12 colonies by means of PCR with vector-specific primers to identify clones containing inserts of correct length. Put 2 μ L of such PCR product into 10 μ L of restriction mixture, containing cocktail of four-cutters: *Mbo*I, *Alu*I, and *Hae*III (or *Rsa*I, if *Hae*III was used in ODD), in NEBuffer 2 (New England Biolabs). The products of restriction (all 10 μ L) can be resolved on a gel containing 3% NuSieve GTG/1% standard agarose or 2.5% Metaphor agarose. Identical clones will show identical fragment pattern.
21. Primary proof of band "differentiality" is provided by the reproducible pattern seen in parallel duplicates already on ODD gel. Only "reproducibly differential" bands should be excised and analyzed. The easiest way to check the sequence for being differential is quantitative RT-PCR with the primers synthesized for the sequence that was found in the band. First, quantitative PCR with band-specific primers should be performed using the original basic samples as template DNA. After getting positive result with ODD samples, quantitative RT-PCR should be performed on independently isolated RNA samples that were not used in ODD. Alternatively, Northern blot hybridization may be used. It should be noted that the product of band re-amplification could not be used to prepare a probe for Northern hybridization, because it will generate too much background; cloned sequence is required.

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GeneCalling[®]

Transcript Profiling Coupled to a Gene Database Query

Richard A. Shimkets

Summary

We describe here the GeneCalling[®] method for the discovery of differentially expressed genes, both known and novel, from any species and with useful sequence information to determine the potential function of novel genes captured. The method relies on transcript visualization coupled to a database query to rapidly and quantitatively identify differentially expressed transcripts. The method has been applied to a wide variety of disease models in a wide variety of species, addressing problems as diverse as identifying novel human cancer gene targets, understanding how drugs and diet affect animal models of disease, and understanding the basis of trait differences in related strains of corn.

Key Words: GeneCalling; cDNA; mRNA; bioinformatics; disease.

1. Introduction

The comprehensive discovery of differences in gene expression among samples is a powerful method of identifying genes associated with diseases, traits, and biological responses to chemicals. Existing methods for expression analysis fall into three general classes: transcript sampling by sequencing (1–3), transcript amplification and imaging (4–8), and hybridization-based approaches (9–13). Serial analysis of gene expression (SAGE) (2), a cost-effective transcript-counting technique, is limited by the small amount of sequence information obtained for each gene. Transcript sequencing following subtractive hybridization also identifies differentially expressed genes, but is limited to binary comparisons (3). Transcript-imaging approaches such as dif-

ferential display (DD) (4), partitioning by type IIS restriction enzymes (6), representational difference analysis (RDA) (7), and amplified fragment length polymorphism (AFLP) (8) are rapid and, in theory, are comprehensive because they utilize banding patterns that are dependent on gene expression. However, each of these requires a time-consuming cloning and confirmation process for determination of the identity of differentially expressed gene fragments.

The development of microarrays has revolutionized the capacity of hybridization techniques (9–13) to identify differences in gene expression. Hybridization approaches are rapid and provide the identity of differentially expressed genes of known sequence immediately. In practice, however, hybridization methods are limited by an inability to detect or discover completely novel genes with no EST representation, thus making work in most organisms impossible.

We describe here the GeneCalling[®], method for the discovery of differentially expressed genes, both known and novel, from any species and with useful sequence information to determine the potential function of novel genes captured (Fig. 1) (14). The method has been applied to a wide variety of disease models in a wide variety of species, addressing problems as diverse as identifying novel human cancer gene targets (15,16), understanding how drugs and diet affect animal models of disease (17,18), and understanding the basis of trait differences in related strains of corn (19,20).

2. Materials

1. Trizol (Invitrogen, Carlsbad, CA).
2. Bromochloropropane (Molecular Research Center Inc., Cincinnati, OH).
3. DNase I (Promega).
4. 0.01 M dithiothreitol (DTT) (Invitrogen).
5. RNasin (Promega).
6. OliGreen (Molecular Probes).
7. Oligo(dT) magnetic beads (PerSeptive, Cambridge, MA).
8. Oligo(dT)₂₅V (V = A, C, or G) (Keck Center, New Haven, CT).
9. Superscript II reverse transcriptase (Invitrogen).
10. Phenol/chloroform (Sigma-Aldrich, St. Louis, MO).
11. Urea (Sigma-Aldrich).
12. Restriction endonucleases (Invitrogen).
13. 10X restriction endonuclease buffer (Invitrogen).
14. *Escherichia coli* DNA ligase (Invitrogen).
15. 10X ligase buffer (Invitrogen).
16. *E. coli* DNA polymerase (Invitrogen).
17. T4 DNA polymerase (Invitrogen).
18. *E. coli* RNase H (Invitrogen).
19. Arctic shrimp alkaline phosphatase (USB, Cleveland, OH).
20. PicoGreen (Molecular Probes).

21. KlenTaq (Clontech Advantage).
22. PFU (Stratagene, La Jolla, CA).
23. MPG streptavidin beads (CPG).
24. FAM (PE-Applied Biosystems, Foster City, CA).
25. TAMRA- and ROX-tagged molecular size standard (PE-Applied Biosystems).
26. 10X TB Buffer: 500 mM Tris, 160 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgCl_2 , pH 9.15 (Sigma-Aldrich).
27. Buffer 1: 3 M NaCl, 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (Sigma-Aldrich).
28. Buffer 2: 10 mM Tris, 1 mM EDTA, pH 8.0 (Sigma-Aldrich).
29. Buffer 3: 80% (v/v) formamide, 4 mM EDTA, 5% TAMRA- or ROX-tagged molecular size standard (Sigma-Aldrich).
30. 10 mM ATP (Sigma-Aldrich).
31. 2.5% polyethylene glycol (PEG) (Sigma-Aldrich).

3. Methods

3.1. GeneCalling Chemistry

1. Total cellular RNA is isolated with Trizol using one-tenth volume of bromochloropropane for phase separation.
2. Contaminating DNA is removed by treatment with DNase I in the presence of 0.01 M DTT and 1 U/ μL Rnasin. Following phenol/chloroform extraction, RNA quality was evaluated by spectrophotometry and formaldehyde agarose gel electrophoresis, and RNA yield was estimated by fluorometry with OliGreen. Poly-A⁺ RNA was prepared from 100 μg total RNA using oligo(dT) magnetic beads, and quantitated with fluorometry.
3. First-strand cDNA is prepared from 1.0 μg of poly(A)⁺ RNA with 200 pmols oligo(dT)₂₅V (V = A, C, or G) using 400 U of Superscript II reverse transcriptase.
4. Second-strand synthesis is performed at 16°C for 2 h following the addition of 10 U of *E. coli* DNA ligase, 40 U of *E. coli* DNA polymerase, and 3.5 U of *E. coli* RNase H. Five units of T4 DNA polymerase is then added, and incubation at 16°C is continued for 5 min. The reaction is then treated with 5 U of arctic shrimp alkaline phosphatase at 37°C for 30 min, and cDNA-purified by phenol/chloroform extraction.
5. The yield of cDNA is estimated using fluorometry with PicoGreen.
6. cDNA fragmentation, tagging, and amplification are performed in a three-step process. Fragmentation is achieved by restriction enzyme digestions in a 50 μL reaction mix containing 5 U of each restriction enzyme, 1 ng of double-stranded cDNA, and 5 μL of the appropriate 10X restriction endonuclease buffer. Coverage of most mRNAs is achieved by performing 80 separate sets of cDNA fragmentation reactions, each with a different pair of restriction enzymes (*see Note 1*).
7. Tagging is achieved by ligation of amplification cassettes with ends compatible to the 5' and 3' ends of the cDNA fragments. Incubation of the ligation occurs at 16°C for 1 h in 10 mM ATP, 2.5% PEG, 10 U T4 DNA ligase, and 1X ligase buffer.

8. Amplification is achieved by the addition of the following reagents: 2 μ L 10 mM dNTP, 5 μ L 10X TB buffer, 0.25 μ L KlenTaq: PFU (16:1), 32.75 μ L H₂O, primer 1 labeled with FAM, primer 2 labeled with biotin. Twenty cycles of amplification (30 s at 96°C, 1 min at 57°C, 2 min at 72°C) are followed by 10 min at 72°C.
9. PCR product purification is performed using MPG streptavidin beads. After washing the beads twice with buffer 1, 20 μ L of buffer 1 is mixed with the PCR product for 10 min at room temperature, separated with a magnet, and washed once with buffer 2. The beads are then dried and resuspended in 3 μ L of buffer 3.
10. Following denaturation (96°C for 3 min), samples are loaded onto 5% polyacrylamide, 6 M urea, 0.5X TBE ultrathin gels and electrophoresed on a Niagara instrument. The primary components of the Niagara gel electrophoresis system are an interchangeable horizontal ultrathin gel cassette mounted in a platform employing stationary laser excitation and a multi-color CCD imaging system. Each gel cassette is loaded in four cycles of 12 wide (48 lanes total) directly from a 96-well plate using a robotic arm. The Niagara system has the advantage of high throughput, with separation of fragments between 30 and 450 bases in 45 min (*see Note 2*).
11. PCR products are visualized by virtue of the fluorescent FAM label on primer1, which ensures that all detected fragments have been digested by both enzymes (*see Note 1*).

3.2. Gel Interpretation

The output of the electrophoresis instruments is processed using the Java-based internet-ready Open Genome Initiative (OGI) software suite. Gel images are first visually checked and tracked. Each lane contains the FAM-labeled products of a single GeneCalling reaction plus a sizing ladder spanning the range from 50–500 bp. The ladder peaks provide a correlation between camera frames (collected at 1 Hz) and DNA fragment size in basepairs. After tracking, lanes are extracted and the peaks in the sizing ladder are found. Linear interpolation between the ladder peaks is used to convert the fluorescence traces from frames to base pairs. A final quality control step checks for low signal-to-noise, poor peak resolution, missing ladder peaks, and lane-to-lane bleed. Data that pass all of these criteria are submitted as point-by-point length vs amplitude addresses to an Oracle 8 database.

3.3. Difference Identification

For each restriction enzyme pair in each sample set, a composite trace is calculated compiling all of the individual sample replicates followed by application of a scaling algorithm for best fit to normalize the traces of the experimental set vs that of the control. The scaled traces are then compared on a point-by-point basis to define areas of amplitude difference that meet the minimum prespecified threshold for a significant difference. Once a region of dif-

ference is identified, the local maximum for the corresponding traces of each set is then identified. The variance of the difference is determined by:

$$(1) \quad \sigma_{\Delta}^2(j) = \lambda_1(j)^2 \sigma_{Total}^2(j:S_1) + \lambda_2(j)^2 \sigma_{Total}^2(j:S_2)$$

where $\lambda_1(j)$ and $\lambda_2(j)$ represent scaling factors and $(j:S)$ represents the trace composite values over multiple samples. The probability that the difference is statistically significant is calculated by:

$$(2) \quad p(j) = 1 - \int_{-\Delta}^{\Delta} dy \frac{1}{\sqrt{2\pi\sigma_{\Delta}^2}} \exp\left(\frac{-y^2}{2\sigma_{\Delta}^2}\right)$$

where y is the relative intensity. All difference peaks are stored as unique database addresses in the specified expression difference analysis.

3.4. Gene Identification

cDNA fragments representing differentially expressed genes can be identified by database searching with the six basepair restriction enzyme recognition sequences at the fragment ends and the exact length of each fragment (determined electrophoretically, subtracting linker length) (*see Note 1*). Database searching for genes predicted to have restriction fragments of matching lengths enables the immediate identification of all of the genes whose sequences reside in that database, and flags fragments derived from novel genes by virtue of their absence from the database. Given a three-nucleotide size window, database lookup can provide a unique assignment of gene identity. The detection of multiple fragments derived from the same gene that show differential expression of the same directional modulation increases the likelihood that the prediction of the gene identity is correct (*see Fig. 2*).

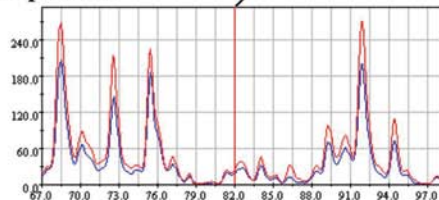
Database lookup can provide a unique assignment of gene identity, and the detection of multiple fragments derived from the same gene that show differential expression of the same directional modulation increases the likelihood that the prediction of the gene identity is correct.

3.5. Gene Confirmation by Oligonucleotide Poisoning

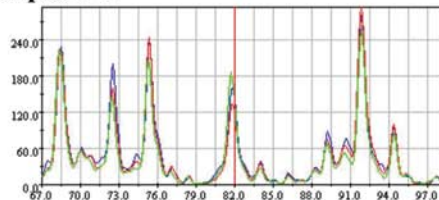
Restriction fragments that map in end sequence and length to known genes in the species of interest are used as templates for the design of unlabeled oligonucleotide primers. An unlabeled oligonucleotide designed against one end of the restriction fragment is added in excess to the original reaction, and is reamplified for an additional 15 cycles. This reaction is then electrophoresed and compared to a control reaction reamplified without the unlabeled oligonucleotide to evaluate the selective diminution of the peak of interest.

Transcript

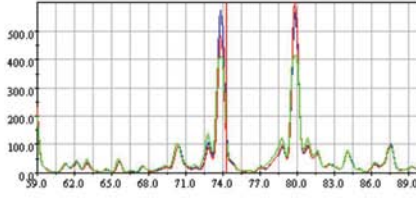
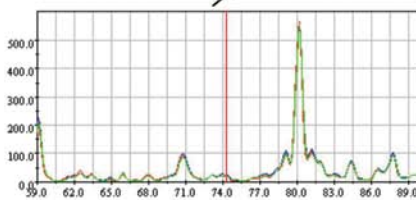
Samples 1-3



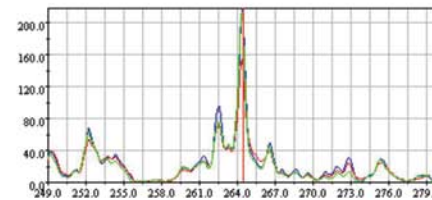
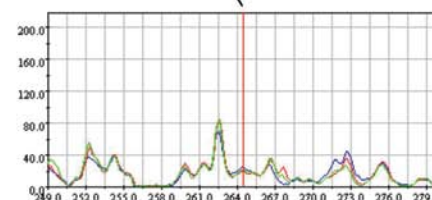
Samples 4-6



Enzyme 1 + Enzyme 2
82 nucleotide fragment



Enzyme 3 + Enzyme 4
74 nucleotide fragment



Enzyme 5 + Enzyme 6
264 nucleotide fragment

Fig. 2. GeneCalling data example. Multiple electrophoretic peaks that represent differences in mRNA abundance map informatically to a single gene in the gene transcript database by virtue of end sequences and fragment length.

4. Notes

1. Because the biotin label is necessary for purification and the FAM label is necessary for detection, all detected fragments result from restriction digestion with both enzymes. Typically 96 GeneCalling reactions are performed, each with a separate pair of endonucleases, on triplicate samples.
2. Other forms of electrophoresis, such as vertical gel electrophoresis and capillary electrophoresis, are compatible with this protocol.

The principle advantages of GeneCalling include the flexibility to discover known and novel dysregulated genes, the ability to apply this technology to any organism containing tangible RNA; the capturing of the transcript's center, which provides protein-coding information; the ability to sensitively distinguish rare and abundant transcripts, the ability to independently measure transcript abundance multiple independent times in a single experiment, and the ability to comprehensively measure the majority of transcripts in a cell. These characteristics make GeneCalling an attractive system for the drug discovery industry as well as a variety of other molecular biology applications.

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High-Density Sampling Differential Display of Prokaryotic mRNAs With RAP-PCR

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Summary

A high-throughput approach to prokaryotic differential display has been developed. A large number of reverse transcription polymerase chain reactions (RT-PCR) are performed on total RNA isolated from induced and control bacterial cultures. Each RT-PCR reaction uses a single oligonucleotide primer and constitutes an independent sampling of the mRNA population. The large number of reactions performed allows the repeated sampling of the targeted polycistronic mRNA, which is clearly identified among possible false positives.

Key Words: RAP-PCR; prokaryotic differential display; high-throughput differential display.

1. Introduction

The absence of stable poly-A tails at the end of mRNA of prokaryotes prevents the practice of the original differential display (DD) technique described by Liang and Pardee (1). RNA fingerprinting by arbitrarily primed polymerase chain reaction (RAP-PCR) (2), an alternative approach to DD, uses arbitrary primers for both the first reverse transcription (RT) step and the second extension step (2) and it can be used for prokaryotes as demonstrated (3). Although mRNA sampling techniques have been used relatively infrequently for gene discovery in prokaryotes, three factors are advantageous to the application of DD in bacteria and increase the probability of successful identification of the genes of a metabolic or functional pathway. First, bacterial genomes are smaller than those of eukaryotes and decrease the probability of generating false positives. Second, the multicistronic mRNAs that result from the organization of

bacterial genes in operons provide a larger target for the arbitrary amplification of DNA fragments. Third, the fact that the genes in operons usually encode functionally related proteins facilitates the recognition of candidate genes by sequence similarity to proteins of known functions.

A protocol for RAP-PCR has been developed that uses a large number (81–240) of primers to allow for a high density of sampling of the RNA population. Operons that are expressed under inducing conditions can be sampled multiple times and stand out in the DD analysis of gene expression. We have used this approach for the discovery of metabolic pathways in bacteria for which genetic techniques have not been developed (4) (Brzotowicz and Rouvière, Walters and Rouvière in preparation) as well as in enrichment cultures (5). Typically, 20–40% of the differentially amplified bands identified correspond to the metabolic pathway targeted.

This protocol has been designed to fit a 96-well plate format and use, to the greatest extent possible, commercial kits, pre-made solutions, “one-tube” enzymes formulations, pre-cast gels, and so on. Equivalent kits from alternate suppliers can be used. If using these supplies is not possible, individual steps can be performed with noncommercial “lab-made” tools as described (6). Once the optimal induction conditions for the pathway of interest are identified, the entire DD protocol, from the isolation of RNA to the sequencing of the clones, takes between 2 and 3 wk.

2. Materials

1. Bead Beater (Biospec Products, Bartlesville, OK).
2. 0.1 mm zirconia beads (Biospec Products).
3. Qiagen RNeasy Midi kit, (Qiagen, Valencia, CA).
4. Qiagen Buffer RLT (Qiagen).
5. Qiagen Buffer RW1 (Qiagen).
6. Qiagen Buffer RPE (Qiagen).
7. RNase-free water (Ambion, Austin, TX).
8. RNase-free DNase (Ambion).
9. SuperScript™ one-step RT-PCR kit (Invitrogen, Carlsbad, CA).
10. 2X reaction mix: a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO₄ (Gibco, Carlsbad, CA).
11. RT/Platinum taq mix (Gibco).
12. 6X TBE loading buffer.
13. Taq polymerase (Qiagen).
14. RNase-free MgCl₂ (Ambion).
15. RNase-free KCl (Ambion).
16. RNase-free Tris-HCl, pH 8.3 (Ambion).
17. PCR machine equipped for 96-well plates (Gene Amp 9700 PCR machine, Applied Biosystems, Foster City, CA).

18. Set of oligonucleotide primers pre-aliquoted in a 96-well PCR plate.
19. Pre-cast polyacrylamide gels (Excel gels, Amersham-Biosciences, Piscataway, NJ).
20. DNA silver staining kit (Plus-One staining kit, Amersham-Biosciences).
21. GeneAmp® 10X PCR buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 0.01 % (w/v) gelatin (Perkin Elmer).
22. AmpliTaq® DNA polymerase (Perkin Elmer).
23. dNTPs (Perkin Elmer).
24. PCR fragment cloning kit for sequencing (TOPO TA cloning kit, Invitrogen).
25. Sequence assembly software (Sequencher, Genecodes, Ann Arbor, MI).
26. Multichannel pipettor (Apogent, Hudson, NH).
27. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

3. Methods

The methods detailed in **Subheadings 3.1–3.9.** outline: (1) the growth of cells and characterization of the induction conditions of the pathway of interest; (2) isolation the total cellular RNA; (3) removal of contaminating DNA from the RNA preparation; (4) design of the RT-PCR oligonucleotide set; (5) arbitrarily primed reverse transcription and PCR amplification; (6) analysis of the differentially amplified bands; (7) elution of the DNA from the polyacrylamide gel slices and reamplification of the DNA fragments; (8) cloning and sequencing of the differentially expressed fragments; and (9) the sequence analysis of differentially amplified bands.

3.1. Growth of Cells and Characterization of Induction Conditions

Because DD exploits the differences in gene expression of cells exposed to different physiological conditions, initial accurate characterization and optimization of the induction conditions of the genes and pathway of interest are critical to the success of the DD analysis. Induction conditions should be chosen in order to minimize physiological changes other than those targeted. This specific induction can be accomplished by varying only a single physiological parameter, by using a single culture split at the time of induction, by minimizing the time of induction, and so on. Growth conditions should maximize the relative abundance of specific mRNA targeted, for example, by using a relatively rich medium in which most biosynthetic pathways will not be induced. Finally, the RNA should be extracted from the very culture that was used to characterize the induction.

The following protocol is derived from a published work on the identification of nitrophenol degradation genes in a Gram positive bacterium (4). It is presented as a generic example to emphasize critical experimental points.

1. Inoculate 50 mL of defined medium complemented with yeast extract in an Erlenmeyer flask with a single colony.
2. Grow the culture until the culture reaches exponential phase (OD_{600} approx 0.1).
3. Add 200 mL of fresh medium and split the culture into two identical flasks.
4. Perform induction to one of the cultures by adding inducing agent, leaving the other culture unchanged.
5. Grow cultures for two generations.
6. Remove two 15-mL samples from each culture at timed intervals and transfer the samples into conical centrifuge tubes pre-chilled on ice.
7. Immediately harvest the cells of the first sample by centrifugation at 12,000g for 3–5 min in a rotor cooled to -4°C . Keep the cells of the second sample on ice.
8. Discard the supernatant. Freeze the tubes in a dry ice/ethanol bath and store at -80°C . The total RNA will later be isolated from the cell samples taken at the timepoint corresponding to optimum induction.
9. Transfer the second sample to an Erlenmeyer flask pre-chilled on ice. This sample will be used to characterize the induction of the pathway of interest. Cells should be processed according to the method chosen to characterize the induction of the targeted genes. For example, when searching for specific catabolic genes, respirometry is a practical technique and the cells can be stored on ice until all RNA samples are stored. When an analytical technique like high-performance liquid chromatography (HPLC) is used, the samples can be quickly extracted by ice-cold methanol. When polyacrylamide gel electrophoresis (PAGE) is used to monitor protein biosynthesis by protein or activity stain, the cells can simply be centrifuged and frozen.
10. Characterize the induction by the chosen method for the samples taken at various time-points. Proceed to extract RNA from the samples corresponding to the timepoint of optimum induction of the genes targeted.

3.2. Isolation of Total Cellular RNA

Prokaryotic mRNAs are short-lived, with a half-life on the order of minutes in fast-growing species. The procedures for RNA extraction must, thus, be very rapid. Techniques of cell disruption based on a lysozyme treatment to digest bacterial cell walls are too slow (tens of minutes to hours) and lead to highly degraded mRNA. The protocol for the isolation of total cellular RNA uses a physical disruption of bacterial cells with zirconia beads in a bead beater in the presence of the denaturing buffer of an RNA extraction kit, here the guanidine isothiocyanate containing buffer RLT from the Qiagen RNeasy Midi extraction kit. Because RAP-PCR samples gene fragments and allows the reassembly of DNA fragments during the PCR amplification, the sheering of long mRNA during the bead-beating step is not a problem. This bead-beating approach works well even for bacteria of the High G+C group that have extremely resistant cell envelopes. Once the cells are disrupted in the denaturing buffer the remaining manipulations including the elution of the RNA in

RNase-free water can be done at room temperature. **Steps 4–9** in the protocol below are essentially identical to the steps outlined in the RNeasy Qiagen manual.

1. Add 1.5 mL of buffer RLT to each frozen cell pellet (add the buffer to the cells stored on dry ice). Resuspend the pellet on ice with a pipet and transfer the suspension to a 2.5-mL bead-beater tube containing about 500 μL of 0.1 mm diameter zirconia beads. Speed is essential at this step to avoid mRNA degradation.
2. Disrupt cells by bead beating for 2 min at maximum speed (2400 rpm).
3. Centrifuge the bead beater tubes for 1 min at 10,000g. Remove the supernatant and transfer to a 15-mL conical tube.
4. Bring the volume to 3.8 mL with buffer RLT when there are greater than 5×10^9 cells.
5. Add 2.8 mL of ethanol. Mix by vortexing or shaking. Do not centrifuge.
6. Apply sample, including any precipitate that may have formed, to the RNeasy midi spin column sitting in a 15-mL centrifuge tube. The maximum loading volume is 3.8 mL. Close the tube and centrifuge for 5 min at 3000–5000g. Discard the flow-through liquid. Repeat with the remainder of the sample.
7. Add 3.8 mL of buffer RW1 to the RNeasy midi spin column and centrifuge for 5 min at 3000–5000g to wash the column. Discard the flow-through.
8. Add 2.5 mL of buffer RPE to the RNeasy midi spin column and centrifuge for 2 min at 3000–5000g to wash the column.
9. Add an additional 2.5 mL of buffer RPE to the RNeasy midi spin column and centrifuge 5 min at 3000–5000g to dry the column. Make sure the column is completely dry before proceeding.
10. Elute the RNA by placing the RNeasy midi column on top of a new 15-mL collection tube. Add 500 μL of RNase-free water directly onto the spin column membrane. Close the tube gently and let it stand for 10 min before centrifuging it for 3 min at 3000–5000g.
11. Quantitation of the RNA is performed with a Nano-Drop on 1 μL of RNA from each RNA pool. The concentration of the RNA pools are adjusted to the same concentration by addition of RNase-free water. Because prokaryotic RNA is unstable, any subsequent manipulations should be performed on ice and kept to a minimum to avoid different differential degradation in the two RNA pools to be compared by DD. RNA preparations are kept at -80°C .

3.3. Removal of Contaminating DNA From RNA Preparation

The technique of differential display is sensitive to contaminating DNA in the total RNA samples, because the DNA will lead to the artifactual amplification of DNA fragments differing between the control and induced sample. Contaminating DNA is removed from the RNA samples by DNase treatment. The absence of DNA must be confirmed by several parallel PCR and RT-PCR reactions. The RT-PCR reactions catalyzed by a mixture of reverse transcriptase and DNA polymerase will yield DNA fragments. The PCR reactions

catalyzed by an enzyme mixture in which the reverse transcriptase has been heat-killed should not generate PCR fragments.

1. Digest DNA in the total 500 μL RNA preparation with 6 U of RNase-free DNase in a buffer containing 10 mM MgCl_2 and 50 mM KCl for 1 h at 37°C. It is our experience that the DNase does not need to be heat-killed.
2. Assemble 10 amplification reactions on ice as follows: 70 μL water, 5 μL of RT/Taq mix, and 125 μL of 2X reaction mix.
3. Divide the amplification reaction mix in 5×40 μL .
4. Choose five primers from the primer set described later with varied 3'-end sequences.
5. To each of the five tubes, add 8 μL of one of the five primers (0.8 μM final concentration).
6. Divide each reaction mix into two PCR tubes.
7. Heat one of the tubes to 95°C for 5 min to inactivate the reverse transcriptase. Keep the other tube on ice.
8. Add 1 μL (approx 20 ng) of template RNA to both of the tubes.
9. Place the reaction tubes in the PCR machine pre-cooled to 4°C and perform PCR amplification with the following parameters: 4°C for 2 min, 5 min ramp to 37°C, hold at 37°C for 1 h, 95°C for 3 min; 1 cycle 94°C for 1 min, 40°C for 5 min, 72°C for 5 min; 1 cycle 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; 40 cycles then hold at 4°C.
10. Analyze the DNA fragments on a low-resolution 1% agarose gel stained with ethidium bromide (EtBr). A high-resolution gel is not needed at this point.
11. Proceed only if there are no DNA fragments produced in the RT-PCR reactions containing the heat-treated enzymes. Several bands should be present when the products of the control reactions are run on an agarose gel.

3.4. Design of the RT-PCR Oligonucleotide Set

A large number of primers are used in many independent RT-PCR reactions, each initiated by a single primer to increase the probability of sampling a targeted gene or operon. The sequence of the primers can be completely arbitrary. In published work, a collection of 240 primers with the sequence 5'-CGGAGCAGATCGVVVVV-3' was used. VVVVVV represents all the combinations of the three bases A, G, and C at the last five positions of the 3' end. Three primers forming the strongest primer dimers were omitted, which allowed all of the RT-PCR reactions to fit on five 96-well PCR plates. The 5' end sequence common to all primers was originally designed to minimize homology to both orientations of the 16S rDNA sequences from bacteria from widespread phylogenetic position in order to avoid artifactual amplification of the overwhelmingly abundant, stable RNA species. In practice, differentially amplified bands corresponding to 16S and 23S rRNA are nevertheless generated and account for 10–20% of the DD fragments. The sequence common to

each primer can be used later to reamplify the differentially amplified bands. Other collections of primers can be designed, in particular, some of the primers can be designed to specific sequences such as transcription initiation sequences, start and stop codons, or sequences coding conserved amino acid motifs.

Array the primers (4 μL of 2.5 μM) on a 96-well format as represented in **Fig. 1**. Each primer is placed in two adjacent wells where the RT-PCR reactions using RNA from the control cells and from the induced cells will be performed side by side. If several DD experiments are to be performed, it is advantageous to assemble a “primer stock” plate containing each primer and from which multiple PCR plates can be prepared by transferring 4 μL of each oligonucleotide primer at 2.5 μM with a multichannel pipettor. These plates can be sealed with adhesive tape sheets and stored indefinitely at -20°C .

3.5. Arbitrarily Primed Reverse Transcription and PCR Amplification

The reverse transcription and PCR amplifications were performed with RT-PCR enzyme mixes that contained a reverse transcriptase and a thermostable DNA polymerase in a compatible buffer. The use of a single-tube enzyme formulation to perform both the RT and the PCR steps decreases the chances of pipetting imprecision that leads to differences in the band pattern generated for the RNA from control and induced cells. To set up RT-PCR reactions as reproducibly as possible, stock RT-PCR reaction mixtures are prepared and split in order to add only the specific component to each tube. The protocol below describes the approach for a set of 240 primers, but can be scaled down to use fewer primers.

Several steps were integrated into the technique that simplify the preparation of 480 RT-PCR reactions and decrease tube-to-tube variations. All reactions below are assembled on ice. Prepare an RT-PCR master mix. For 110 reactions (enough reaction mix for a 96-well plate), mix 1375 μL of Invitrogen Superscript One-step RT-PCR 2X reaction mix, 825 μL water, 55 μL RT/Taq mix.

1. Split the master mix into two tubes (1127 μL /tube).
2. Add 27.5 μL template RNA from control cells (at approx 20 ng/ μL) to one tube (“control” master mix) and 27.5 μL of RNA from the induced cells to the other tube (“induced” master mix).
3. Using a multipipettor, dispense 21 μL of the “control” master mix into all wells of lanes 1, 3, 5, and 7. Dispense 21 μL of the “induced” master mix into all wells of lanes 2, 4, 6, and 8 of the RT-PCR plate.
4. Place the 96-well PCR plate with the assembled reactions in the block of a PCR machine cooled to 4°C .
5. The RT-PCR protocol is the same as described in **Subheading 3.1.2**. (Note: the reverse transcriptase is not inactivated for any of the samples prior to the run.)

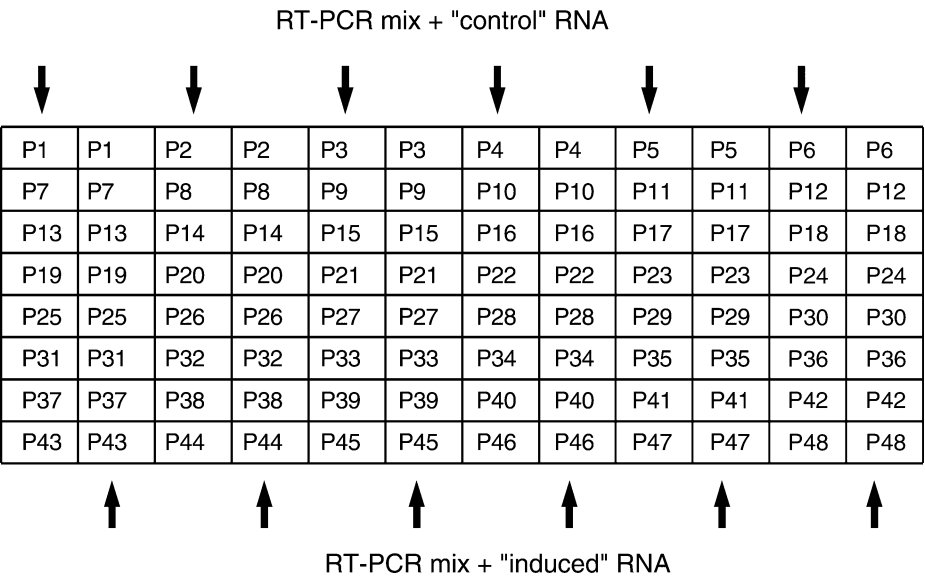


Fig. 1. Array of 48 pre-aliquoted primer on 96-well format. Five such plates cover the 240 primer set. Plate no. 1 contains primers number P1 to P48. Reverse transcriptase polymerase chain reaction mixes are added in the well columns as indicated.

3.6. Analysis of the Differentially Amplified Bands

At the completion of the RT-PCR reactions described in **Subheading 3.5.**, the products are analyzed by gel electrophoresis. Although vertical acrylamide sequencing gels or high-resolution agarose gels can be used, we have used pre-cast horizontal polyacrylamide gels (Excel gels, Amersham-Biosciences) because of their speed and ease of use. Each gel can accommodate 48 samples so that the analysis of the 240 pairs of RT-PCR reactions can take place on 10 gels. The DNA is visualized on the gel by silver stain. The advantage of silver stain is that the DNA bands can be excised with great accuracy from the gel. In a typical high-density mRNA sampling experiment, 50–100 bands are chosen for further analysis.

1. Transfer 6 μ L of each RT-PCR reaction into a new 96-well PCR plate where each tube contains 2 μ L of 6X TBE loading buffer containing bromophenol blue.
2. Load the content of each tube (RT-PCR sample + loading buffer) on the flat-bed gel.
3. Run electrophoresis at 600 V on a Multiphor II electrophoresis apparatus cooled to 4°C. During electrophoresis, the gel is cooled to 4°C to avoid distortion. The gel is run until 30 min after the bromophenol blue runs off of the gel.

4. Stain the gel with Plus One Silver Staining kit following the manufacturer's protocol.
5. Rinse the gels extensively for 1 h with four successive volumes of distilled water. The gels can be stored in water at room temperature for several days.

The silver stain can detect DNA concentrations as low as 20–50 pg DNA/band. A typical RT-PCR reaction yields between 10 and 30 discrete, resolved DNA bands with molecular weights ranging from 100 to 1200 bp. With the exception of a few bands present in one of the two reactions, the banding pattern generated from the RT-PCR reaction using the RNA from the control and the induced cells are very similar. DNA bands generated in reactions, including RNA from induced cells but not in reactions, including RNA from control cells are recorded as potential differentially amplified bands and excised from the gel. Similarly, bands absent in the “induced” sample, but present in the control sample, are candidates for repressed genes.

3.7. Elution of DNA From Polyacrylamide Gel Slices and Reamplification of the DNA Fragments

Silver staining of DNA allows the direct and accurate excision of the DNA band from the gel and limits the subsequent reamplification of contaminating DNA fragments and background. The primer used in the reamplification reaction is the primer initially used in the RT-PCR reaction, to generate the fragment. Alternatively, a primer with a sequence common to all RT-PCR primers (here 5'-CGGAGCAGATCG-3') can be used.

1. Excise a thin slice (0.5 mm) from the core of the stained bands using a new razor blade. Care should be taken to avoid excising large slices that will contain contaminating DNA.
2. Transfer each band to a microfuge tube containing 50 μ L of 10 mM KCl and 10 mM Tris-HCl, pH 8.3.
3. Heat the tubes to 95°C for 20 min to allow some of the DNA to diffuse out of the gel.
4. Set up a series of reamplification reactions using the eluate from each gel slice as a template with the following conditions: 5 μ L of the original 2.5 μ M primer that led to amplification of the band, 5 μ L of 10X PCR buffer, 5- μ L of 2.5 μ M dNTPs, 29 μ L water, 0.5 μ L taq DNA polymerase, and 5 μ L of the slice eluate or a 1:10 dilution of the eluate.
5. The PCR parameters are 94°C (1 min), 60°C (1 min), 72°C (5 min) for 40 cycles.
6. Before cloning the PCR products, confirm the reamplification of the DNA. Run 6 μ L of each PCR product on an agarose gel. Stain the gel with EtBr before visualizing.

Note: band identification and nomenclature is important for keeping track of the origin of the band in many subsequent reactions. Names can typically be based on the primer that generated the band (i.e., ACG18). If multiple bands

are excised from the same RT-PCR reaction, the addition of a b1 or b2 to the band name is easily added (i.e., ACG18b2).

3.8. Cloning and Sequencing of the Differentially Expressed Fragment

The products of the reamplification PCR reactions are cloned. The plasmid from several clones from each cloning reaction are purified and sequenced to compensate for the possible reamplification of contaminating background DNA.

1. Clone the product of the reamplification PCR using a cloning kit for PCR fragments such as the blue/white cloning vector kit TOPO TA Cloning kit for Sequencing, which contains the pCR4-Topo vector (Invitrogen, San Diego, CA).
2. Transform *Escherichia coli* Top10 cells with the cloning reactions. When more than 96 bands are cut out from the polyacrylamide gels, it is advantageous to use Invitrogen's MultiShot TOP10 chemically competent *E. coli*. The competent cells are arrayed in a 96-well block, allowing the easy cloning of a large numbers of plasmids.
3. The plasmids from eight transformants from each cloning reaction are isolated from the cells and submitted for sequencing analysis. Name each plasmid according to the cloned band.
4. Sequencing is carried out from the vector from one or both directions, for example using primers specific for the pCR4-Topo vector, T3 and T7.

Notes: cost savings can be achieved by using only 0.3–0.5 μL of vector per cloning reaction instead of the recommended 1 μL per reaction (only a few transformants are required) and by sequencing only three or four transformants instead of eight.

3.9. Sequence Analysis of the Differentially Amplified Bands

The sequences from all of the clones (trimmed of vector and primer sequences) are compared and assembled into contiguous sequences (contigs) using sequence assembly software like Sequencher (Genecodes, Ann Arbor, MI). Each contig is assembled from the overlapping sequences of the multiple clones of the same differentially amplified band. Some contigs are also assembled from the sequences of different bands generated in distinct RT-PCR reactions with different arbitrary primers, indicating that the corresponding mRNA has been sampled more than once. The translated nucleotide sequence of all the contigs is compared to sequences in the public database using the BlastX algorithm (<http://www.ncbi.nlm.nih.gov/blast/>).

Several criteria are used to rank the contigs in order of relevance for the physiological function targeted.

1. Select contigs assembled from the sequence of DNA bands differentially amplified in independent RT-PCR reactions. These contigs correspond to a single mRNA sampled multiple times at the same position. Hot-spots of amplification are often

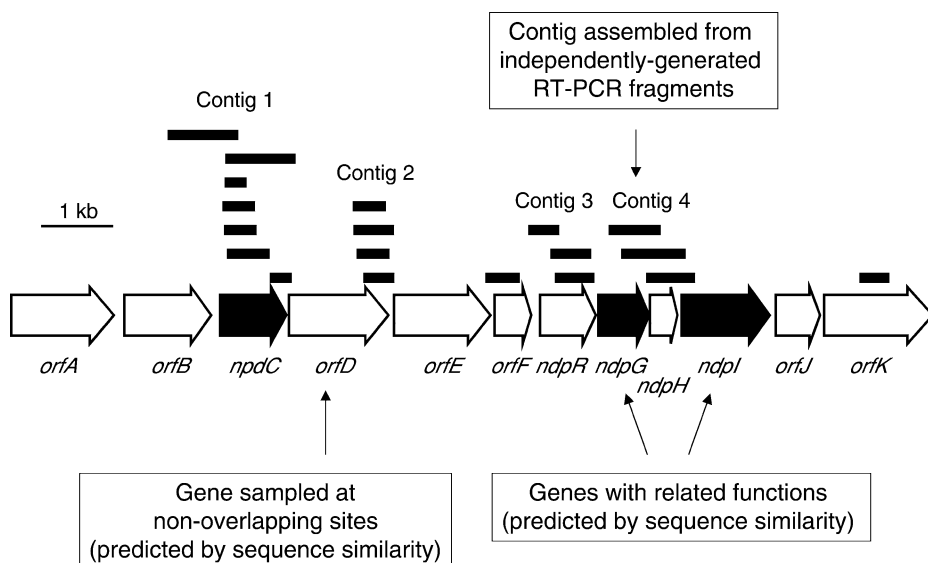


Fig. 2. High-density sampling of a metabolic operon redrawn from (4). The three criteria described in **Subheading 3.9.** to identify the targeted genes are encountered here. Black bars represent differentially amplified sequences. Contigs 1–4 represent mRNA sequences sampled multiple times in independent RT-PCR reaction. Genes in black encode functionally related F420-dehydrogenases.

- observed (4) and may correspond to particular mRNA secondary structures.
2. Select contigs with derived amino acid sequence having similarity to the same proteins. These contigs are likely to correspond to a single mRNA sampled multiple times at different nonoverlapping positions.
3. Select contigs with derived amino acid sequences that have similarity to proteins related functionally, i.e., expected to be found in the targeted metabolic pathway and likely to be present in a single operon.

All three situations are usually encountered in each experiment. Typically, 20–40% of the DD bands sample the same polycistronic mRNA. **Figure 2** presents the results of our search for the genes responsible for the degradation of di- and tri-nitrophenol using DD. Four genes were sampled multiple times in an overlapping region (contigs 1–4). Another gene, *orfD* was sampled both at its 5' and 3' ends as observed by the similarity of sequence from two contigs to the same aldehyde dehydrogenase. Furthermore, two contigs encoded sections of proteins that were homologous to F420-dependent dehydrogenases (*npdC*, *ndpG*, *ndpI*), suggesting that they corresponded to functionally related genes. Satisfying these three criteria was a very strong indication that the targeted operon had been identified. Indeed sequencing the DNA region around the

F420-dependent genes showed that 9 out of 12 genes had been sampled by DD, with the 3 genes shaded in black being F420-dependent genes (**Fig. 2**). The role of the operon in the degradation of nitrophenols was subsequently confirmed.

4. Notes

This high-density of mRNA sampling approach to RAP-PCR can be adapted according to the particular situation of each laboratory, in particular, with respect to the balance between cost of consumables and of manpower. In industrial settings, it is often more cost effective to use pre-made supplies and to sequence many clones. If the budget for outside supplies and services is limiting, several steps can be adapted to lower the cost of outside supplies and services in addition to those previously mentioned.

1. The most efficient way to lower the number of RT-PCR bands to be further investigated is by performing the RT-PCR reactions from RNA pools obtained from completely independent duplicate cultures. Only RT-PCR DNA fragments amplified in both RT-PCR are further considered. This approach greatly reduces the number of false-positives and allows to use a smaller set of primers.
2. The false-positives that result from the artifactual differential amplification of 16S and 23S RNA can be eliminated by dot blot analysis as described (7).
3. The number of clones sequenced can be limited by confirming that the differentially amplified bands are truly differentially expressed using a dot blot analysis.

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Vertical Arrays

Microarrays of Complex Mixtures of Nucleic Acids

Rosana Risques, Gaelle Rondeau, Martin Judex, Michael McClelland, and John Welsh

Summary

Vertical arrays are microarrays that have complex mixtures of nucleic acids as array elements, and that are hybridized with single sequence probes. Like dot blots, many different experiments can be spotted on a single vertical array, allowing single genes to be compared across many conditions. Vertical arrays have two additional advantages over dot blots. First, they are printed on glass slides, allowing the use of low-volume, high-concentration hybridization reactions. Second, they can be made using low-complexity representations of the original nucleic acid population. This increases signal-to-noise relative to the usual use of dot blots, wherein the entire complexity of the population is usually spotted. Whereas standard microarrays achieve horizontal coverage of many genes and are repeated to cover many experiments, vertical arrays achieve vertical coverage of many experiments and are repeated to cover many genes. In cases where the number of genes is limited, but the number of experiments is very large, vertical arrays may be advantageous.

Key Words: Microarray; RNA arbitrarily primed polymerase chain reaction; PCR; RAP-PCR; differential display; DD.

1. Introduction

RNA fingerprinting methods, including differential display (DD) (1), RNA fingerprinting by arbitrarily primed polymerase chain reaction (RAP-PCR) (2–4), and restriction-ligation PCR (5) were originally used to detect evidence for differential gene expression in “fingerprints” typically resolved by gel electro-

phoresis. Quantitative measurements of gene expression using these methods are limited by gel resolution and background, by the problem of associating a differentially regulated gel "band" with its sequence, and by the organizational challenges of running and analyzing many sequencing gels. Consequently, thorough analysis of differential gene expression using an electrophoresis-based approach and more than just a few biological variables is impractical for most labs. Expression microarrays are more convenient and provide better analytical power for most transcripts.

However, fingerprinting methods can be combined with arrays in a number of ways to provide powerful new tools. Fingerprinting methods sample only subsets of the total complexity of an RNA population; that is, they have lower sequence complexity. Thus, any single sequence present in the fingerprinting reaction product, referred to henceforth as "low complexity representations" or "LCRs," is often more highly represented than it is in the corresponding total RNA population. Consequently, when LCRs are used as solution-phase radioactive or fluorescent probes in cDNA array experiments, these over-represented sequences have favorable signal-to-noise behavior (6,7). Iteration using several LCRs generated using different subset selection criteria, such as different arbitrary primers in DD or RAP-PCR, or different restriction enzymes and different 3' primer extensions in restriction-ligation PCR, allows arrays to measure changes in gene expression in transcripts with abundances that are too low to be measured using standard methods of probe preparation, such as oligo dT- or random priming.

We took advantage of the favorable signal-to-noise properties of LCRs to devise the "vertical array." Vertical arrays were conceived as a high-throughput, high-sensitivity alternative to dot blots. High throughput is achieved by using a glass slide microarray format that can accommodate thousands of "dots." However, rather than using the full complexity of the mRNA, as is used in traditional dot blots, high sensitivity is achieved by placing LCRs of an mRNA population in each dot. This enhances sensitivity because each individual sequence in a dot is more highly represented in an LCR than in the full complexity of the mRNA. In principle, 20 nonoverlapping LCRs from a single mRNA sequence would lead to approx 20 times the sensitivity that could be achieved using the full mRNA complexity. In practice, the method to prepare LCRs that we will present here, RNA arbitrarily primed PCR (RAP-PCR), leads to overlapping representations. In LCRs produced by RAP-PCR, sequences from the high-complexity, low-abundance class tend to be over-represented in the products, at the expense of the more abundant transcripts, and this provides additional enhancement of the expected signals from rare transcripts. An example of vertical array hybridization is shown in **Fig. 1**.

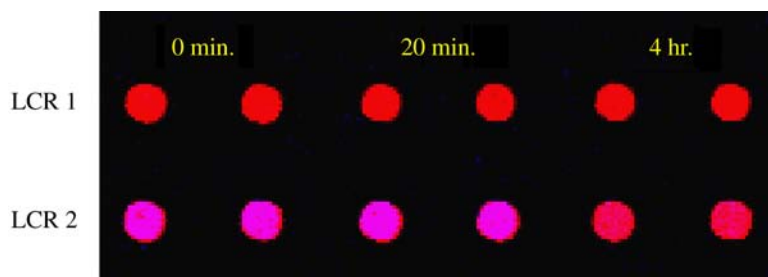


Fig. 1. Vertical array detection of differential regulation in response to the addition of serum to serum-starved fibroblasts. The top row shows an LCR in which a sequence homologous to AA42873 is absent: the (red) fluorescent signal is from the positive hybridization control. The bottom row shows hybridization at $t = 0$ and $t = 20$ min (violet), but not at 4 h using an LCR in which the transcript is sampled.

RAP-PCR has been described, and readers are encouraged to examine the original and later publications for more detailed discussions. In brief, RNA and DNA polymerases generally do not require perfect 20 nucleotide long matches to prime synthesis. Either class of enzyme will extend from oligonucleotide primers having rather poor matches, given enough time, and lowering hybridization stringency can enhance extension from mismatched oligonucleotides. Although the requirement for complementarity is not absolute, neither is it nonexistent, the result of which is that a given arbitrary primer always leads to synthesis from the same positions in a complex template. Note that an arbitrary primer is not the same thing as a random primer. A random primer is degenerate at each position, whereas, an arbitrary primer has a nondegenerate sequence that has been chosen arbitrarily (*see Note 1*). If arbitrary priming is used on oligo dT-primed first-strand cDNA, the products can be amplified by PCR using the oligo dT primer (or a 5' extension) and the arbitrary primer, giving rise to DD. If two arbitrary priming steps are used, either on oligo dT-primed first-strand cDNA or on RNA using reverse transcriptase for the first arbitrary priming step, the products can be amplified using only the arbitrary primers, giving rise to RAP-PCR. Restriction-ligation PCR on double-stranded cDNA can also be used to produce LCRs. Any of these methods leads to a reproducible sampling of the original mRNA population. LCRs representing different sequences can be made by varying the sequence of the arbitrary primer in DD and RAP-PCR, or by varying either the restriction enzyme or 3' extensions in primers used to amplify restriction-ligation products. In this essay, we concentrate on the RAP-PCR approach.

The sequence constraints on arbitrary primers are not well established beyond a few rules of thumb. Typically, 10- to 12mers are easier to use than

longer primers (e.g., 18- to 35mers), primers should be free of 3' self-complementarity to avoid PCR artifacts, and the primers can contain degeneracy at any position as long as 3' complementarity is avoided. Greater than 50% G + C content also appears to be beneficial. Finally, it is usually advisable to avoid primers that have significant homology with repetitive elements.

Vertical arrays are promising as an alternative to standard microarrays in a variety of circumstances. If the expression of a moderately large number of genes, 100, must be explored under a large number of conditions, 1000, standard microarrays will require 1000 hybridizations, without replicates. In vertical arrays, LCRs from the mRNAs from the 1000 experiments would be placed on an array and hybridized with 100 fluorescent probes. The trade-off is that multiple LCRs need to be prepared for each RNA, possibly as many as 20 for each experimental variable, to achieve adequate representation, and this can be a large undertaking. We prepare LCRs robotically, thereby reducing the impact of this variable. Another trade-off is that the vertical arrays only examine a predetermined set of sequences, and do not provide the same richness of discovery opportunities that 1000 hybridizations to comprehensive standard arrays may provide. The reader will recognize that quantitative RT-PCR is another alternative, but continuing with the same example, RT-PCR would require the preparation and fluorescence monitoring of 100,000 reactions, which is out of reach for most labs. Vertical arrays fit a natural niche as a drug screening tool, where even libraries of moderate size are difficult to address using standard microarrays.

2. Materials

2.1. Equipment

1. Microarray printer.
2. Microarray scanner.
3. StrataLinker (Stratagene, La Jolla, CA).
4. Coplin jars.
5. Corning Hybridization Chambers (Corning, Corning, NY).
6. Water baths at 37°C and 42°C.
7. Orbital shaker.
8. Microcentrifuge.
9. Low-speed centrifuge with microtiter plate adaptors.
10. Thermocycler for 96-well plates.
11. UV spectrophotometer.
12. Oven (at 80°C).
13. Agarose gel electrophoresis equipment.
14. Polyacrylamide gel electrophoresis equipment.
15. Bacterial and eukaryotic cell culture facilities.

2.2. Labware

1. 96-well and 384-well plates for PCR.
2. UltraGAPS coated slides (Corning).
3. Cover slips.
4. ddH₂O and ddH₂O adjusted to pH 9.5 with NaOH.
5. 50-mL Falcon tubes.
6. Aluminum foil.

2.3. Kits

1. PSI Ψ Clone PCR 96 purification kit (Princeton Separations, Adelphia, NJ).
2. RNeasy Mini Kit (Qiagen, Valencia, CA).
3. QIAquick PCR purification kit (Qiagen).
4. Megascript kit (Ambion, Austin, TX).

2.4. Enzymes and Reagents

1. T7 RNA Polymerase (Promega, Madison, WI).
2. Exonuclease-free DNA polymerase, Klenow fragment (New England Biolabs Inc., Beverly, MA).
3. *EcoRV* (Promega).
4. *ClaI* (Promega).
5. Random hexamers (1 $\mu\text{g}/\mu\text{L}$).
6. Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Buckinghamshire, UK).
7. M13F (GTTTTCCCAGTCACGACGTTG).
8. M13R (TGAGCGGATAACAATTCACACAG).
9. 100 μM arbitrary primer solutions (*see Note 2*). A few oligonucleotide sequences that work well are: 5'GCACCAGGGG, 5'TCACCAGCCA, 5'ACGGGCCAGT, 5'CAAGGGCAGT, 5'GGCAGGCTGT, 5'GGGCACCAGG, 5'GGGGCACCAC, 5'CTGACTGCCT.
10. Poly dT (Amersham Pharmacia Biotech).
11. Human Cot-1 DNA (Invitrogen, Carlsbad, CA).
12. Bacterial DNA (for use as a carrier in hybridization controls; *see Note 6*).
13. Dimethyl Sulfoxide (DMSO).

2.5. Solutions

1. DNase I solution: 8.5 U/ μL DNase I (Gibco BRL), 1.2 U/ μL RNase inhibitor (Roche), 0.1 M Tris, pH 8.3, and 0.05 M MgCl₂.
2. 1X M-MLV buffer (Promega, Madison, WI): 250 mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl, 15 mM MgCl₂, 50 mM dithiothreitol (DTT).
3. 1X RRAP mixture: 10 μL 5X MMLV buffer, 1 μL 10 mM dNTPs, 2.5 μL 100 μM arbitrary primer, 0.1 μL of α -[³²P]-dCTP, 5 μL 10 U/ μL AmpliTaq Stoffel Fragment (Applied Biosystems, Foster City, CA), 0.25 μL 200 U/ μL M-MLV-Reverse Transcriptase (Promega, Madison, WI), 27.15 μL H₂O.

4. 4X Random priming mix: 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 30 mM DTT, 0.1 mM each of dATP, dCTP, dGTP, and 0.036 mM dTTP, 0.16 mM Cy3- or Cy5-linked dUTP (Amersham, Arlington Heights, IL) and 1.6 U/μL of exonuclease-free Klenow (New England Biolabs Inc.).
5. 2X Insert PCR mix: 20 mM Tris-HCl (pH 9.0 at 25°C), 100 mM KCl, and 0.2% Triton X-100, 3 mM MgCl₂, 0.4 mM each dNTP, 0.4 μM M13F, 0.4 mM M13R (see **Note 3**), and 0.5 U/μL Taq DNA Polymerase (Promega).
6. Liquid LB with ampicillin (50 μg/mL).
7. Pre-hybridization solution (100 μL): 25 μL of formamide, 25 μL of 20X saline-sodium citrate (SSC), 1 μL of 10% sodium dodecyl sulfate (SDS), 1 μL of bovine serum albumin (BSA) (10 mg/mL), 48 μL of distilled water.
8. Hybridization solution (71 μL): 25 μL formamide, 25 μL 20X SSC, 1 μL 10% SDS, 10 μL of Poly dT (1 mg/mL), 10 μL human Cot-1 DNA (1 mg/mL).
9. 20X SSC: 3 M NaCl, 0.3 M sodium citrate.
10. 10% SDS.

3. Methods

3.1. Preparation of LCRs

LCRs are prepared using a variant of RAP-PCR wherein both reverse transcription and PCR are supported by a single reaction mixture. This has the advantage that the tubes do not need to be opened between steps, which facilitates robotic preparation of LCRs. Screening for effective primers can be facilitated by buying oligonucleotides already arrayed in 96-well plates.

1. Isolate total RNA from adherent cells growing in culture using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's directions. Elute RNA from the columns with 80 μL ddH₂O.
2. Treat the 80 μL RNA with 20 μL of DNase I solution. Incubate 1 h at 37°C. Repurify this DNase I-treated RNA using an RNeasy Mini kit and the clean-up protocol recommended by the manufacturer. Elute the RNA from the columns with 40 μL ddH₂O.
3. Check the concentration by absorption spectrophotometry at 260 nm assuming that an extinction coefficient of 1 = 40 μg/mL.
4. Adjust the total RNA concentration to 25 ng/μL by the addition of water.
5. Combine 4 μL of 25 ng/μL RNA with 46 μL 1X RRAP. Incubate at 37°C for 1 h, followed by 3 min at 94°C, and thermocycling at 94°C for 15 s, 35°C for 2 min, 72°C for 2 min, for 35 cycles, followed by 5 min at 72°C.
6. Purify the products using the PSI Ψ Clone PCR 96 purification kit following the manufacturer's recommendations. Elute products with 80 μL of distilled water, pH 9.5.
7. Measure the concentration of DNA for at least one replicate for each different primer (see **Note 4**). The concentration should be about 30 ng/μL.
8. Mix 2 μL of purified RAP with 9 μL of formamide dye solution, denature at 85°C for 4 min and chill on ice. Electrophoresis 1.5 μL through a 4% polyacryla-

midex, 8 M urea sequencing gel at 50–70 W until the xylene cyanol tracking dye reaches the bottom of the gel. Dry under vacuum at 85°C and expose to phosphorimage screen (approx 16 h) or X-ray film (1–2 d). Replicates should be almost identical by eye, except for occasional changes reflecting differential gene expression in abundant products (see **Note 5**).

3.2. Preparation of Hybridization Controls

Three types of controls should be prepared: (1) a control for the amount of hybridizable material in each spot. This is achieved by combining small amounts of each LCR, followed by fluorescent labeling of the mixture, resulting in a complex probe that will hybridize to any type of LCR spotted on the vertical array in proportion to the amount of DNA in a spot. This is used to normalize signal intensities according to the amount of hybridizable DNA in a spot. (2) Positive and (3) negative controls to allow the specificity of hybridization of a probe to the homologous sequence to be assessed.

1. Take an equal aliquot of all the RAP reactions to be printed in the vertical arrays and label the mixture with Cy3-dUTP using the following random primed synthesis protocol: mix 500 ng of RAP-PCR product with 8 µg of random hexamer, adjust the volume to 30 µL, and heat for 5 min at 95°C. Add 10 µL 4X Random Priming Mix, incubate at 37°C overnight. Purify using a QIAquick PCR Purification Kit. Check incorporation by spectrophotometry at 550 nm for incorporated Cy3. This will be used as a control for the amount of DNA in a spot on the array, which can vary owing to printing errors and array surface behavior. Make aliquots and store frozen, protected from light.
2. Negative hybridization controls can be prepared using bacterial DNA (see **Note 6**) digested with restriction enzymes to mimic the DNA fragments produced during LCR preparation. Digest 4.5 µg of bacterial DNA with 25 U of *EcoRV* (Promega, Madison, WI) and 25 U of *ClaI* in 1X Multicore buffer, with 0.1 mg/mL Acetylated BSA in a final volume of 100 µL, at 37°C for 3 h. Purify using a QIAquick PCR purification kit. Measure the DNA concentration spectrophotometrically and adjust to 50 ng/µL.
3. Positive controls consist of the same digested bacterial DNA used as negative controls “spiked” with specific gene sequences, in serial dilutions. Prepare a dilution series for genes of interest using purified PCR products. These are the genes whose expression will be monitored. Fragments from these sequences can be prepared by PCR from bacterial clones or plasmids according to **Subheading 3.4., step 1**. Adjust the concentrations of the purified PCR products to 15 ng/mL and prepare fivefold dilutions 3 ng/µL, 0.6 ng/µL, 0.12 ng/µL, 24 pg/µL, and 4.8 pg/µL. Combine 15 µL of each dilution DNA with 45 µL of digested bacterial DNA giving dilution factors of 1/10, 1/50, 1/250, 1/1250, 1/6250, and 1/31,250. Negative controls are simply the bacterial DNAs with the specific target gene sequences omitted.

3.3. Printing Vertical Arrays

1. Place the purified RAP-PCR products, and the positive and negative controls in a microtiter plate and dry at 70°C in a thermocycler with the lid opened. Redissolve each in 22 μL of distilled water. The final average DNA concentration per well should be approx 100 ng/ μL .
2. Combine 4 μL of DNA from each well in the microtiter plate with 4 μL of DMSO in a suitable microtiter plate for your particular microarray printer (see **Note 7**).
3. Print this onto Ultra GAPS coated slides (Corning).
4. After printing, cross-link the printed DNA to the plate by UV irradiation (we use a Stratalinker at 300 mJ) and bake for 3 h at 80°C under vacuum.
5. Rinse the slides in distilled water for 2 min and dry them by centrifugation. This removes the NaOH introduced during the PSI kit purification step and increases the useful lifetime of the slides.

3.4. Fluorescent Probe Preparation

The inserts from plasmid clones containing the sequences of the genes of interest must be converted into fluorescent probes. Plasmids differ with respect to the sequences of their cloning sites, therefore, the oligonucleotide primers used in this section must be chosen to contain the correct sequence. We have had better success with in vitro transcription for probe preparation than with random primed synthesis.

1. Grow bacteria harboring the plasmid in LB medium with ampicillin at 37°C overnight. Mix 10 μL of bacterial culture with 90 μL of distilled water, boil for 5 min, and centrifuge for 3 min at approx 8000g. Combine 10 μL of the supernatant with 15 μL of water and 25 μL of 2X Insert PCR mix. Amplify by PCR using 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min for 40 cycles, followed 7 min at 72°C. Purify the product using the QIAquick PCR purification kit and elute in 50 μL of distilled water.
2. Measure the concentration by UV spectrophotometry and confirm its size by agarose electrophoresis.
3. Use the purified product (approx 25–50 ng of DNA) in an in vitro transcription (IVT) reaction using the Megascript kit (Ambion, Austin, TX) according to the instructions provided for the incorporation of FluoroLink Cy5-UTP. Combine 1 μL (25–50 ng) of DNA with 1X Reaction buffer; 7.5 mM GTP, ATP, and CTP; 2.5 mM UTP; 1.75 mM Cy5-UTP; and 1 μL of enzyme mix in a final volume of 10 μL . Incubate at 37°C for 6 h. After 3 h, add 7.5 U of T7 RNA Polymerase and centrifuge briefly to spin down any condensate. After an additional 3 h add 1 U of RNase-free DNase I and incubate at 37°C for 15 min.
4. Use the clean-up protocol of the RNeasy Mini Kit (Qiagen) to purify the labeled RNA. Elute the RNA in 50 μL of RNase-free water and measure the amount of incorporated label by absorption spectrophotometry at 650 nm. On average, in vitro transcription should produce 8–10 μg of RNA labeled with 400–800 pmol of dye.

3.5. Hybridization of Probes to Vertical Arrays

1. Pre-hybridize, hybridize, and wash vertical arrays following manufacturer's protocol for Ultra GAPS slides. To reduce nonspecific hybridization, use 0.1 mg/mL of Poly dT and 0.1 mg/mL of human Cot-1 DNA, and use 25% formamide during the hybridization. The probe for vertical microarrays comprises 1–1.2 μ g of Cy5-labeled RNA (approx 50–80 pmol of dye) mixed with 10–12 ng (0.7–1 pmol of dye) of Cy3-labeled RAP-pool.
2. Put 100 μ L of pre-hybridization solution in the slide. Cover with a cover slip and place in a Corning hybridization chamber. Incubate at 42°C in a water bath for 45 min.
3. Wash the slide in 400 mL of distilled water shaking gentle for 2 min. Repeat the wash and then dry the slide by centrifugation.
4. Mix Cy5-gene and Cy3-pool of RAPs and add distilled water up to 29 μ L. Mix with 71 μ L of hybridization solution to make a final volume of 100 μ L. Denature the probe by heating at 95°C for 5 min and spin it down to collect condensation. Put 10 μ L of water in the two holes of the hybridization chamber to keep humidity during hybridization. Place the slide in the hybridization chamber. Pipet the probe onto the surface of the slide and carefully place the cover slip. Close the chamber and place it in a 42°C water bath overnight (12–16 h).
5. Disassemble the hybridization chamber right side up.
6. Remove the cover slip by immersing the slide in 50 mL of 2X SSC, 0.1% SDS in a Falcon tube.
7. Wash the slide in the following buffers: 2X SSC, 0.1% SDS at 42°C for 5 min; 0.1X SSC, 0.1% SDS at room temperature for 10 min; 0.1X SSC at room temperature for 1min (repeat this wash four times). Do all the washes in 400 mL of buffer and in a Coplin jar covered with aluminum foil to protect slides from light. Finally rinse the slide in 50 mL of 0.01X SSC in a Falcon tube for 5 s.
8. Dry the slide by centrifugation and keep it protected from the light until scanning.

3.6. Data Acquisition and Analysis

The goal of data acquisition and analysis is to determine the changes in the expression of a gene over the many conditions represented as LCRs on the vertical microarray. Data acquisition will depend on the scanning equipment and software available to the investigator, and analysis must, of course, be guided by the specifics of the experimental design. Next, we list a few general guidelines.

1. Slides can be scanned with any scanner appropriate to normal microarrays. Care must be taken to avoid saturation, and this is usually accomplished by adjusting the laser intensity.
2. The positive controls should show decreasing signal intensity with dilution.
3. Data should be normalized using the signal in the Cy3 channel, which contains a complex mixture that reports the relative amount of DNA placed in the spot by the spotting robot (*see Note 8*).

4. As in any experiment, replicates are essential, and sufficient replicates must be done if meaningful statistical analysis is desired.
5. Differences in intensity can be verified using quantitative RT-PCR.
6. Some transcripts give signals in multiple LCRs. This has the effect of a replicate. When a probe contains a repetitive element, multiple LCRs will be detected. It is a good idea to avoid repetitive elements in probe preparation. RepeatMasker (<http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker>) can be used to identify repeats in potential probes.
7. The sensitivity of the method—that is, how rare an RNA can be before a signal from it can be detected—depends on how many LCRs are prepared whether that particular RNA is sampled robustly, and the extent to which it is amplified relative to other sequences in that LCR. When only a few LCRs are prepared, chances are not very high that a particular RNA will be sampled. We estimate that about 80% of all mRNAs can be detected using 20 RAP-PCR probes on microarrays, and that mRNAs in the least abundant class can be detected in vertical arrays if they are prominent LCR products. A few LCRs can be checked using standard microarrays or RT-PCR to determine whether they have sampled the genes of interest.

4. Notes

1. Arbitrary primers can have some degeneracy, if so desired. Degeneracy tends to increase the number of sequences with which the arbitrary primer interacts. Note, however, that 3' degeneracy can lead to “primer dimer” artifacts, and may interfere with robust PCR owing to competition with perfectly matched primers, and because of lower concentration of each perfectly matched primer. Note also that there is a trade-off between coverage and sensitivity: if the primers sample only a few transcripts, these will be very prominent and easy to detect, but if the primers sample too many, the structure of the amplified population tends toward the original mRNA population structure, and no advantage is gained.
2. Many sequences 10–15 nt in length have been tried and found to work. Avoiding palindromes at the 3' end is advisable, and PCR favors higher G + C contents.
3. M13F and M13R correspond to forward and reverse primers for inserts in IMAGE clones, available from Research Genetics. PCR products generated using these primers include the T7 RNA polymerase promoter, which can be used to make fluorescent probes by *in vitro* transcription.
4. Different primers produce different final total masses. This step presupposes high reproducibility, which is not always obtained. It is a good idea to check for reproducibility on an ongoing basis. Spot-checking is advisable.
5. In a large project, this step can become cumbersome. Once the investigator is confident that their materials and procedures are robust, they may decide that post hoc analysis methods, such as quantitative PCR analysis of interesting changes, adequately addresses reproducibility, and this step may be omitted.
6. We use *Salmonella* LT2 DNA digested with two restriction enzymes in order to produce multiple fragments of DNA that mimic RAP fingerprints.

7. It is usually desirable to place this DNA/DMSO solution in several different places on the plate such that regional variations can be recognized after the scanning step.
8. The mixed LCR control in this protocol used Cy3, but this choice is arbitrary.

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Automated Pattern Ranking in Differential Display Data Analysis

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Summary

Gene expression analysis by differential display (DD) is limited by the labor-intensive visual evaluation of the electrophoretic data traces. We describe a flexible method for computer-assisted ranking of expression patterns in data from DD experiments. The method is based on a pairwise alignment and comparison of the quantitative trace data with respect to specific expression patterns defined by the investigator. The observed patterns are ranked according to a score value that identifies the most potential findings to be confirmed visually instead of the vast amount of original results. This two-step approach, enabled by the efficient computer algorithm for gene expression pattern comparison, will increase the percentage of true-positive findings chosen for the tedious downstream processing, while minimizing the cost and labor involved in large scale DD data analysis.

Key Words: Computer algorithm; electrophoresis; pattern analysis; data mining; differential display; gene expression; pairwise alignment.

1. Introduction

Differential display (DD) is a widely used technique for detecting changes in gene expression levels in two or more tissue or cell samples (1). The technique is rapid, sensitive, and suitable for automation, and has been applied to a variety of biological analysis tasks (2). However, labor-intensive visual evaluation of DD data is often the bottleneck of such investigations.

In DD, the mRNA transcripts present in a sample are divided into subgroups of manageable size by the sequential use of a set of anchored primers in the cDNA synthesis step and a set of arbitrary primers in the differential display polymerase chain reaction (DD-PCR) step. The resulting fragments are separated in gel or capillary electrophoresis and detected by autoradiography or fluorescence, depending on the type of label incorporated during the DD-PCR. In a DD experiment, the task of the investigator is to visually analyze and detect patterns of differential expression, including rare changes in the band intensity or peak area between two or more corresponding fragment pairs in an autoradiogram, the output of a fluorescent scanner, or the trace curves from a capillary electrophoresis apparatus. The selected fragments are then isolated, purified, amplified, and identified by sequencing.

The sample preparation and electrophoretic separation phases can be automated easily by the use of robotics and capillary sequencers. In theory, more than 10,000 DD-PCR traces can be produced in 24 h with a four-color 384-capillary sequencer. However, it is evident that the time-consuming visual evaluation of the resulting expression patterns cannot be performed with similar efficiency. Accurate detection of significant changes among thousands of traces, each containing dozens of peaks, can take several days or even weeks. Moreover, as increasingly complex biological problems are being explored, more complicated expression patterns than clear-cut differences in peak areas between two fragments will be encountered. In such cases, automated data analysis protocols become highly desirable. Significant reduction of tedious visual analysis not only increases throughput, but also minimizes the risks of human errors.

This chapter focuses on recent investigations carried out in our laboratories to improve automation in DD data analysis (3,4). Here we describe a protocol for computer-assisted comparison of expression patterns among multiple electrophoretic data traces. The comparison queries apply flexible rules for searching interesting patterns that can be freely defined by the investigator and are appropriate to the experimental design and the specific questions under the analysis. One may search for partitioning the data into homogenous trace groups, individual peak pairs showing dissimilar intensity in otherwise similar traces, or a gradual up- or downregulation of specific peak complexes in a time series. Biological and technical replicates or various negative and positive controls can also be included. A representative query may contain even combinations of these search rules. The rationale of the queries is that the visual confirmation can be focused on a ranked and highly compressed list of candidate expression patterns instead of the vast amount of original DD results.

In large-scale automated analysis protocols, it becomes necessary to use objective criteria for assessing the significance of the search results. A key component of our trace comparison is a scoring function that evaluates the

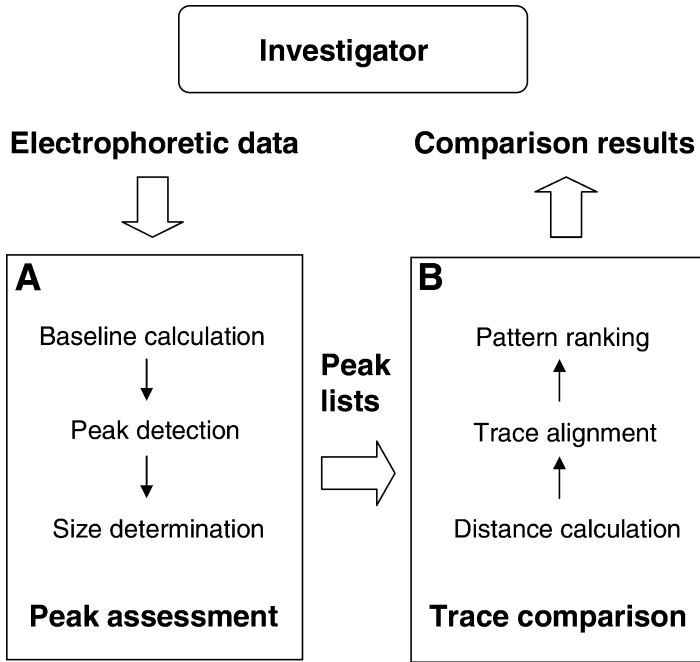


Fig. 1. Schematic representation of the phases included in the current interactive DD data analysis protocol. (A) Peak assessment phase detects and quantifies valid peaks in the given electrophoretic trace data and exports size-ordered peak lists for subsequent computational analysis (see **Note 4**). (B) In the trace comparison phase, peak complexes are modeled and aligned, and score-ranked comparison results are created for confirmation and further analysis (see **Note 13**).

rank of each observed peak pattern. Successful ranking of patterns relies on robust identifications of valid peaks and accurate alignments of the traces with respect to a given distance function on peaks (see **Fig. 1**). Selected candidate patterns are subsequently used to resolve questions related to a particular experiment. The general computational protocol is illustrated here by analyzing data from DD experiments that monitor changes in gene expression patterns in experimental *Escheria coli* infection of mice (5) and human colonic carcinoma (6). We demonstrate the performance of the methodology using specific score functions for characterizing the differences between normal and diseased tissue samples on three levels of increasing complexity.

2. Materials

The focus of this chapter is on the comparison of fragment patterns originating from DD experiments. The data analysis protocol can be applied to any electrophoretic trace data that can be transformed to numeric peak data, where each peak is quantified by its size, area, and height. Below we describe an outline of the specific data acquisition protocol applied to our investigations as an example of material analyzable by this method (*see* also **Notes 1** and **2**).

2.1. Differential Display Analysis

1. cDNA primers: 3 one-base anchored and 12 two-base anchored 5'-tagged dT₁₄ primers of the type TACGACTCACTATAGGGAG(T)₁₄VN (T7-tag common to secondary 3'-primer underlined; V = A, C, or G; N = A, C, G, T, or none).
2. Arbitrary primers: a set (24 or 32) of 18–36 bp primers, originally used as gene-specific amplification primers, but arbitrary in this context.
3. Secondary Cy5-labeled 3'primer: Cy5-GTAATTACGACTCACTATAGGG (T7-tag common to cDNA primer underlined).
4. cDNA synthesis: SuperScriptII kit (Gibco BRL, Rockville, MD).
5. PCR: DynazymeII (Finnzymes, Espoo, Finland).

2.2. Electrophoretic Separation

1. Fluorescent one-color sequencing apparatus (ALFexpressII, Amersham Biosciences, London).
2. 0.5 mm thick denaturing 4%T/3%C polyacrylamide gel (separation distance 20 cm).
3. Standardized electrophoretic conditions (55°C, 1500 V, 60 mA, 50 W) and sampling interval of 2 s.
4. An internal Cy5-labeled size standard series (633 bp, 647 bp, 680 bp).
5. An external molecular weight ladder (ALF Sizer 50–500 bp, Amersham Biosciences).

3. Methods

Automated detection and ranking of interesting expression patterns is organized as a sequence of computational steps. Gaussian curves are first fitted to the complex peak mixtures, and the resulting approximate trace models are then used for peak alignment. Relevant trace pairs sharing identical anchoring and arbitrary primers but derived from different mRNA samples or their replicates are aligned according to the distances computed between the corresponding peak pairs (*see* **Note 3**). The last phase involves the specification and evaluation of a score function that ranks the peak patterns subject to the comparison.

3.1. Distance Calculation

1. Detect and size-call all valid peaks of each trace using a suitable software package. For ALFexpressII, we used the ALFwinTM Fragment Analyser (Amersham,

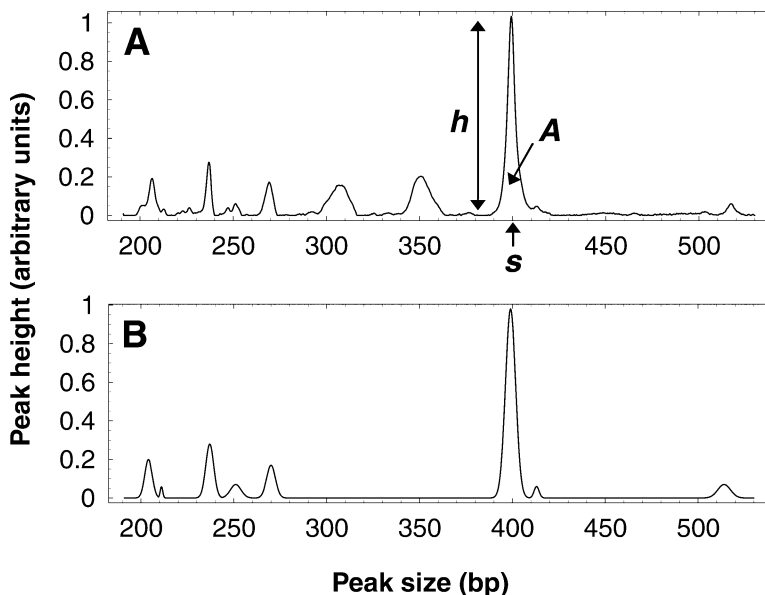


Fig. 2. Trace modeling using the maximum of Gaussian functions. **(A)** Original electrophoretic trace data, where each peak is quantified by its size s in bp, and area A and height h in arbitrary units. The largest peak is characterized by the triple $(s, A, h) = (399, 67, 9.98)$. **(B)** The corresponding continuous approximation $u(t)$, where the $n_u = 8$ peaks were detected by the ALFwin Fragment Analyser software. The artificial Cy5 degradation peaks at 307 bp and 351 bp were excluded from the analysis **(3)**. Reproduced with permission from WILEY-VCH Publishers.

Biosciences) with the following parameter settings: peakshape 10, min height 0.05%, minima detection baseline, and sensitivity 100 (*see Note 4*).

2. Quantify each peak by the three numeric values characterizing its location and intensity: peak size s in bp, area A , and height h in arbitrary units (*see Fig. 2*).
3. Model the intensity of a peak u_k with characteristics (s_k^u, A_k^u, h_k^u) at location t using the Gaussian function:

$$u_k(t) = \frac{A_k^u}{\sigma_k^u \sqrt{2\pi}} \exp \left[-\frac{1}{2} \left(\frac{t - s_k^u}{\sigma_k^u} \right)^2 \right]$$

with the standard deviation:

$$\sigma_k^u = \frac{A_k^u}{h_k^u \sqrt{2\pi}} \quad (\text{see Note 5}).$$

4. Model the intensity $u(t)$ of a trace that contains n_u detected peaks (numbered according to their sizes; *see Note 6*) using the maximum function (*see Fig. 2*):

$$u(t) = \max_{k=1,2 \dots n_u} u_k(t)$$

5. For relevant trace pairs (u, v) , define the Matusita distance d (*see Note 7*) between peaks u_i and u_j by the formula:

$$d(u_i, v_j) = A_i^u + A_j^v - \left[\frac{8A_i^u A_j^v}{\sigma_i^u / \sigma_j^v + \sigma_j^v / \sigma_i^u} \right]^{1/2} \exp \left[- \frac{(s_i^u - s_j^v)^2}{4(\sigma_i^{u2} + \sigma_j^{v2})} \right]$$

3.2. Trace Alignment

1. Insert into each trace u a virtual “zero” peak u_0 characterized by an arbitrary size s_0 and zero-area $A_0^u = 0$ with $\sigma_0^u = 1$ (*see Note 8*).
2. Specify the maximum size difference T in bp allowed for the corresponding peak pairs in separate traces using existing information on the sizing accuracy of the electrophoresis method (*see Note 9*). Forbid the peak pairs with sizes outside the predefined error tolerance:

$$d(u_i, v_j) = \text{MaxDist} \text{ if } |s_i^u - s_j^v| > T \text{ and } i, j > 0$$

3. Compute the total distance $D_{i,j}$ between the first i and j peaks in the lanes u and v ($i = 1, 2, \dots, n_u$ and $j = 1, 2, \dots, n_v$) using the following recurrence relation:

$$D_{i,j} = \min \left\{ D_{i-1,j} + d(u_i, v_0), D_{i-1,j-1} + d(u_i, v_j), D_{i,j-1} + d(u_0, v_j) \right\}$$

with the initial conditions:

$$D_{0,0} = 0, D_{i,0} = \sum_{k=1}^i d(u_k, v_0), D_{0,j} = \sum_{k=1}^j d(u_0, v_k)$$

4. Find the optimum peak pair alignment $(u_{i[k]}, v_{j[k]})_{k=1}^{L_{u,v}}$ by traversing the backward pointers that record at each combination (i, j) the entry $(i-1, j)$, $(i-1, j-1)$ or $(i, j-1)$ which was involved in the optimal selection of the $D_{i,j}$ value above (*see Note 10*). The traversing proceeds from the position (n_u, n_v) to $(0, 0)$, and the $L_{u,v}$ pointers at interpositions define the optimal alignment (*see Fig. 3*).
5. Define the alignment distance D between the traces u and v as $D(u, v) = D_{n_u, n_v}$ (*see Note 11*).

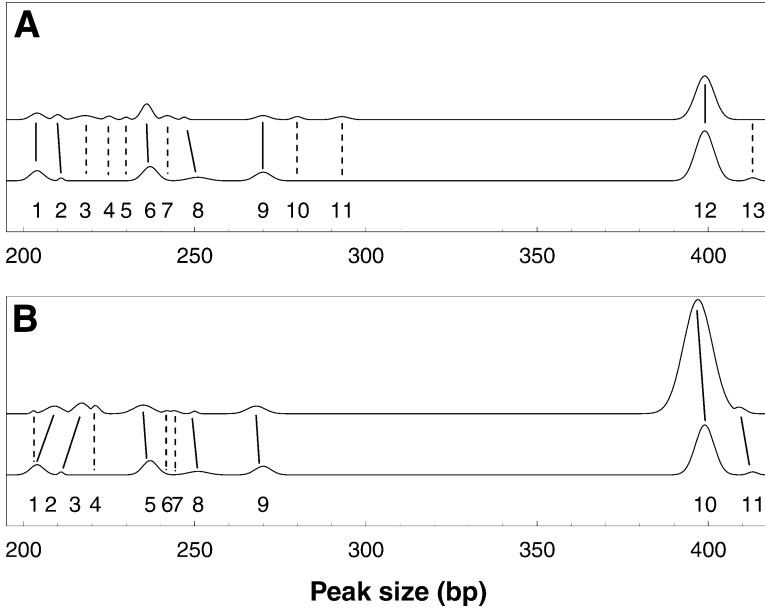


Fig. 3. Two examples of trace alignments from a DD study of acute-phase response in mice in experimental *Escherichia coli* infection (5). Solid lines indicate matches between two real peaks, dashed lines between real and zero peaks. (A) Matching the peaks of trace u (lower trace, $n_u = 7$) with the peaks of a similar trace v (upper trace, $n_v = 12$). The optimal alignment of length $L_{u,v} = 13$ gives the trace distance $D(u,v) = 28.04$. (B) Matching the trace u with a dissimilar trace v with $n_v = 11$ detected peaks. The optimal alignment of length $L_{u,v} = 11$ gives the trace distance $D(u,v) = 118.33$.

3.3. Pattern Ranking

1. Evaluate the trace level scoring function S_1 , which characterizes the average within-group distance in a group G containing m_G traces (see **Note 12**):

$$S_1(G) = \frac{\sum_{(u,v) \in G} D(u,v)}{m_G(m_G - 1)/2}$$

2. Evaluate the peak level score S_2 , which characterizes the extent of which the k^{th} aligned peak pair contributes to the overall trace distance (see **Fig. 4**):

$$S_2(k) = \frac{d(u_i[k], v_j[k])}{D(u,v) / L_{u,v}}$$

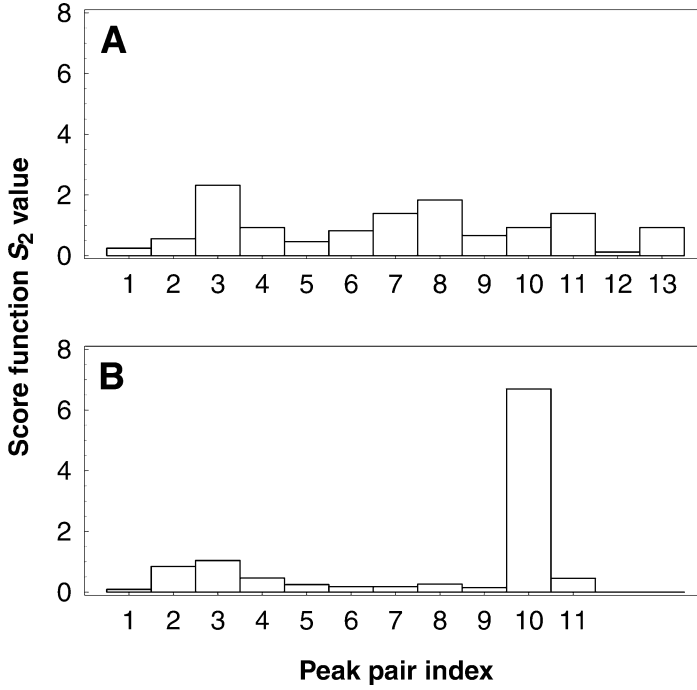


Fig. 4. Detection of differentially displayed peaks with the score function S_2 from the alignments of (u, v) illustrated in **Fig. 3**. Each bar represents the score value originating from a single aligned peak pair $k = 1, 2, \dots, L_{u,v}$. **(A)** The matched peaks of similar traces obtain relatively small score values. The maximum peak pair distance is $d(u_{i[3]}, v_{j[3]}) = 5.0$ which gives the score $S_2(3) = 2.32$. **(B)** The differentially expressed peak pair at $t = 398$ bp gives the distance $d(u_{i[10]}, v_{j[10]}) = 72.05$, and an extreme score value $S_2(10) = 6.70$.

3. Evaluate the multippeak scoring function S_3 , which characterizes the signed comparison among such differences in the trace pairs of G that satisfy a condition K (see **Fig. 5**):

$$S_3(G, K) = \prod_{(u,v) \in G, k \in K} \text{sgn}(A_{i[k]}^u - A_{j[k]}^v) S_2(k)$$

4. Rank the detected patterns according to decreasing score values, and confirm visually a reasonable subset of findings with the highest ranks (see **Note 13**).
5. Purify and reamplify the fragments resulting from the selected patterns, and identify the corresponding genes by nucleic acid sequencing.

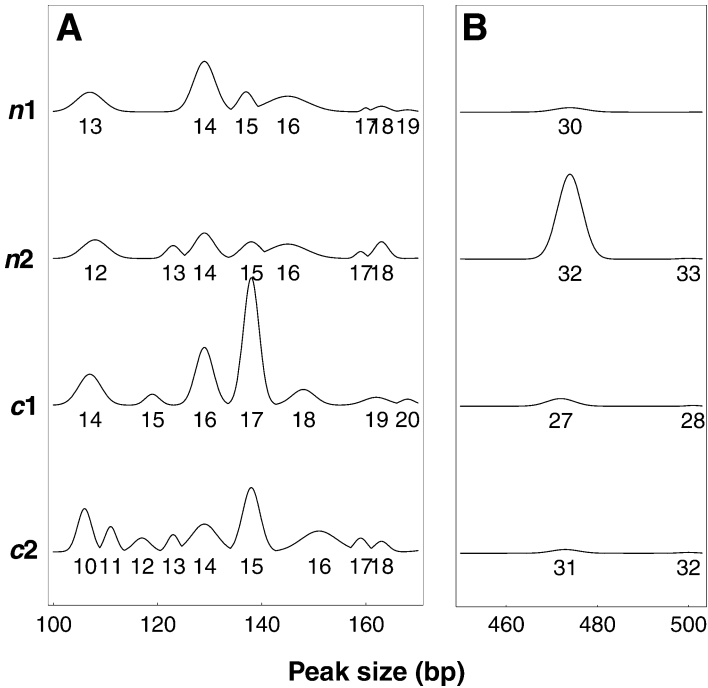


Fig. 5. Evaluation of inter-subject variability with the score function S_3 in a DD experiment monitoring changes in gene expression in human colonic carcinoma (6). The trace pair group G includes both non-neoplastic normal colonic mucosa and samples from carcinomatous tissue from two patients $\{(n1, c1), (n2, c2)\}$. The index group K includes the aligned peak pairs k and l in the two trace pairs whose sizes are within the error range T , that is, $\max\{s_k^{n1}, s_l^{n2}, s_k^{c1}, s_l^{c2}\} - \min\{s_k^{n1}, s_l^{n2}, s_k^{c1}, s_l^{c2}\} \leq T$. (A) Upregulation of the phosphoserine aminotransferase gene at ca. 140 bp. The presence of colonic carcinoma is associated with markedly increased intensity values in both pairs $(n1_{15}, c1_{17})$ and $(n2_{15}, c2_{15})$. (B) Downregulation of the α -tropomyosin gene identifiable from the peak pair $(n2_{32}, c2_{31})$. The cause for the observed inconsistency in the regulation pattern between the two patients in relation to the colonic carcinoma is not known (see Note 14). Both automatically identified differentially expressed genes belonged to the top 0.3% of all expression patterns found in the data set (4). Reprinted with permission from Eaton Publishing.

4. Notes

1. Results from a fluorescent DD (FDD) method can be more easily retrieved in size-called numerical peak data than from autoradiogram, and FDD is therefore preferred.

2. The highest throughput can be achieved in capillary electrophoresis. Because the amount of data will be large, the linking of the results to mRNA-samples and DD-setup is most conveniently handled in database format.
3. The actual setup of the DD-matrix is not constrained by the data analysis. The number of mRNA samples, anchoring primers, and arbitrary primers, are chosen according to the nature of the question under study.
4. The peak extraction step can be done using a variety of analysis software packages that allow the quantification of peaks in electrophoretic trace data, and exporting the resulting peak lists in a standard format for subsequent computational analysis.
5. Standard deviation (SD) of the peak intensity distribution can be used to govern the sharpness of the Gaussian approximation. Scaling σ_k^u by a factor smaller than one shrinks the intensity around the mean s_k^u , whereas factors larger than one broaden the intensity distribution. Optimal scaling is defined by investigating the fit between the original trace and its Gaussian mixture model (see Fig. 2).
6. The size range of interest in the trace modeling can be attached to the protocol by extracting the peaks within the specified range only. With the aforementioned data acquisition protocol, we have noticed that peaks smaller than 200 bp are not easy to purify and sequence in the gene identification phase, whereas peaks larger than 1000 bp are rare and quantitatively unreliable.
7. The distance function between peaks pair is derived from the Matusita distance

$$d(u_i, v_j) = \int \left| \sqrt{u_i(t)} - \sqrt{v_j(t)} \right|^2 dt.$$

Alternative distance functions better suited for ones' needs can be applied as well.

8. Zero peaks are introduced to balance the alignment of traces with unequal number of peaks. Notice that $d(u_i, v_0) = A_i^u$ and $d(u_0, v_j) = A_j^v$ for all the real peaks $i, j > 0$.
9. The threshold T is introduced to prevent the formation of irrelevant peak pairs that cannot have any interesting relationship with each other. In polyacrylamide gel electrophoresis (PAGE) analysis, we have used the threshold value $T = 7$ bp, whereas in capillary electrophoresis $T = 3$ bp because of the better sizing accuracy.
10. If ties occur in the tracebacking phase, the pointer giving a match with a zero peak is followed. The time complexity required for filling the dynamic programming table $D_{i,j}$ is of the order of $n_u \times n_v$, which is the size of the table as well. The upper limit on the time requirement for the traversing step is $n_u + n_v$.
11. Distance D is the solution of optimization problem $D(u, v) = \min \sum_{k=1}^{L_{u,v}} d(u_{i[k]}, v_{j[k]})$ where the minimum is computed over the set of global peak alignments $(u_{i[k]}, v_{j[k]})_{k=1}^{L_{u,v}}$ constructed by matching $L_{u,v}$ real or zero peaks of the traces u and v with each other. Because the total number of such alignments can be extremely large, the optimum alignment is determined by a dynamic programming method similar to the Needleman-Wunsch algorithm (7).

12. If some of the traces are excluded from the comparison characterized by the score function S_1 , then the cardinality m_G is decreased accordingly. The score function S_2 is designated to identify differentially expressed peaks, where the requirement of similar background is considered by normalizing the score with the average trace difference. The scoring function S_3 gives large values when each peak pair k in the index group K is relatively dissimilar and the directions of the differences are the same.
13. The automated pattern ranking is presumed to exclude true negative findings from the laborious visual confirmation step. The primary importance of ranking patterns with respect to differential expression arises from the constraint that only a limited number of findings can be followed up in a typical DD experiment. The biological significance of the confirmed findings must be verified by subsequent testing.
14. Disturbances in amplitude and mobility are the main causes for inadequate comparison efficiency. Whereas the differences in mobility can be corrected in the sizing step by the use of internal molecular standards, substantial variations are expected to remain in trace intensities, even when the same DD experiment is repeated. Some of the systematic variation inherent in the DD itself is a result of electrophoretic conditions, and can be eliminated by careful optimization, but many other sources of noise may still confound the real biological variation corresponding to changes between experimental conditions (e.g., different cell types, tissues, or individuals).
15. If repeated measurements from the same RNA sample are available, reproducibility of the method can be investigated. Statistical significance of the observed differences can be assessed through multiple biological replicates of RNAs.
16. We have previously described an approach to statistical analysis of DD data using the permutation test of trace groups (8). This approach is applicable also under single measurement experiments, and can be applied even for individual peak patterns.
17. Currently, we are evaluating the data analysis method for ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), a 16-capillary 5-color sequencer.

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Linking cDNA-AFLP-Based Gene Expression Patterns and ESTs

Ling Qin, Pjotr Prins, and Johannes Helder

Summary

Massive amounts of DNA sequence data, generated from expressed sequence tag (EST) and genome sequencing projects, require efficient methods to link sequence databases with temporal and spatial expression profiles. To meet this need, we have developed a powerful computer program (GenEST), which links cDNA sequence data (including EST sequences) with transcript profiles revealed by cDNA-amplified fragment length polymorphism (AFLP). cDNA-AFLP is a highly reproducible differential display method based on restriction enzyme digests and selective amplification under high stringency conditions. GenEST predicts the sizes of virtual transcript derived fragments (TDFs) from cDNA sequences digested *in silico*. The resulting virtual TDFs could be traced back among the thousands of TDFs displayed on cDNA-AFLP gels. As a consequence, cDNA sequence databases can be screened very efficiently to identify genes with relevant expression profiles. Vice versa, using the restriction enzyme recognition sites, the primer extensions and the estimated TDF size as identifiers, the DNA sequence(s) corresponding to a TDF with an interesting expression pattern can be identified.

Key Words: cDNA-AFLP; bioinformatics; EST; gene expression display.

1. Introduction

cDNA-amplified fragment length polymorphism (AFLP) is a novel gel-based RNA fingerprinting technique to display differentially expressed genes (1–3), which is derived from the genomic DNA-based AFLP mapping technique (4). In cDNA-AFLP procedure (*see Fig. 1*), cDNAs are digested by two different restriction enzymes and oligonucleotide adapters are ligated to these restricted cDNA fragments. The resulting population of cDNA fragments with

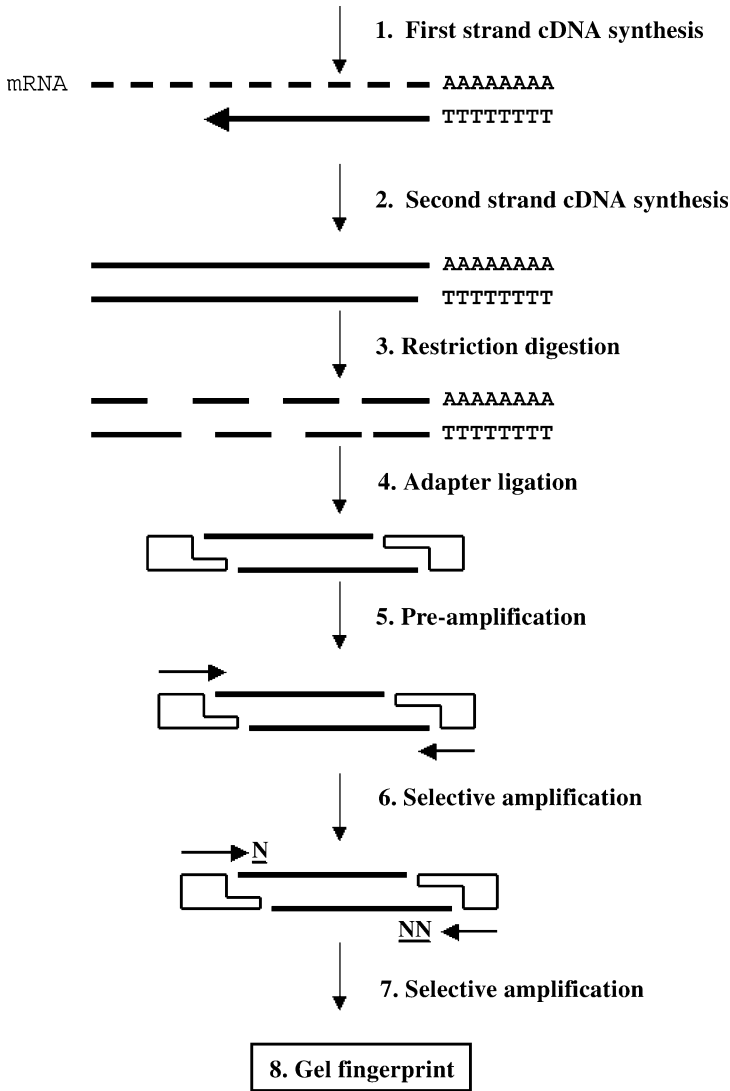


Fig. 1. Schematic drawing of the procedure of cDNA-AFLP.

adapters at their termini serves as a template for two rounds of polymerase chain reaction (PCR) amplification. After a first nonselective amplification with primers corresponding to the adapters, a selected pool of cDNAs is amplified using primers with additional selective nucleotides at the 3' ends and displayed on a denaturing polyacrylamide gel. The complexity of the final fingerprint can be fine-tuned by varying the number of selective nucleotides.

Owing to the specific amplification made possible by the introduced adaptors, no compromise between the quantity of the target molecule and the quality of the target match occurs, making cDNA-AFLP very robust and reproducible. In contrast to hybridization-based techniques, such as cDNA microarrays (5), cDNA-AFLP can distinguish between highly homologous genes from individual gene families. In addition, cDNA-AFLP does not need any pre-existing sequence information, which makes it an excellent tool to identify novel genes. Although oligonucleotide array is potentially discriminating (6), its design requires substantial sequence information, which is not available for most organisms. Serial analysis of gene expression (SAGE), another useful technique in this context can provide quantitative expression data (7). However, it is expensive and labor-intensive when multiple sample points are to be compared, whereas cDNA-AFLP is relatively inexpensive and can be performed in any laboratory.

High-throughput DNA sequencing has accelerated the pace of gene discovery tremendously; many genomes are completely sequenced and many more will be available in the near future (8,9). Furthermore, millions of expressed sequence tags (ESTs)—single-pass cDNA sequences—have been generated and deposited in public and private databases (10). However, this amazing speed of gene discovery far exceeds that of gene function studies; for a significant proportion—40–60% of the newly identified genes—no significant similarities can be found (9,11). Even if a gene of interest shows significant sequence similarity with known genes, functional homology cannot be taken for granted, as in the case of paralogs. Therefore, it is informative to establish a link between raw sequence data to expression data generated by large-scale gene expression display technologies, such as cDNA-AFLP.

With this in mind, we developed a software named GenEST, which link cDNA-AFLP-based gene expression patterns to ESTs *in silico*. This program can be used in two ways. First, GenEST predicts restriction fragments of ESTs for any desired restriction enzyme combinations. These virtual fragments can then be traced back on cDNA-AFLP gels to identify the corresponding bands using the primer extensions and the fragment size as a unique identifier. This enables any researcher to screen databases for genes with interesting expression profiles in an efficient and inexpensive way. Second, using the size and primer extensions of an interesting band shown on a cDNA-AFLP gel, the matching ESTs can be identified from databases. This makes quick gene identification feasible, overcoming one major bottleneck in gel-based RNA fingerprinting techniques.

Contrary to EST approaches, the cDNA-AFLP technique is not biased toward abundant transcripts and does not involve a selection on insert size. Moreover, there is no unwanted selection owing to intolerance of *Escherichia*

coli toward a subset of the inserts. Therefore, cDNA-AFLP can be used in discovering new genes that are difficult to tag by EST approach. Failure to find a good match for a TDF shown on cDNA-AFLP gel in a large-scale EST database suggest that the corresponding gene is presumably a novel gene expressed at a low level or a gene refractory to cloning in *E. coli*.

2. Materials

1. Total RNA Isolation Reagent (TRIzol Reagent, GIBCO BRL, Gaithersburg, MD).
2. Chloroform.
3. Isopropyl alcohol.
4. 75% ethanol.
5. RNase-free, DEPC-treated Milli-Q water.
6. Gene Quant II (Pharmacia, Uppsala, Sweden).
7. Dynabeads Oligo(dT)₂₅ purification kit (Dynal, Oslo, Norway).
8. SuperScript Choice System for cDNA synthesis (Life technology, Breda, the Netherlands).
9. 0.5 M ethylenediaminetetraacetic acid (EDTA).
10. Phenol:chloroform:isoamyl alcohol (25:24:1) (store at 4°C).
11. 7.5 M NH₄OAc.
12. Absolute ethanol (−20°C).
13. Restriction enzymes: *Eco*RI, *Taq*I.
14. 1,4 All buffer (10X) (Pharmacia).
15. dNTPs ATP (10 mM) (Pharmacia).
16. T4 DNA ligase (Pharmacia).
17. 10X PCR-buffer: 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂.
18. PE-9600 thermal cycler (Wellesley, MA).
19. AmpliTaq DNA polymerase (Perkin Elmer).
20. Low DNA mass ladder (Life technology).
21. $\gamma^{33}\text{P}$ -ATP (10 $\mu\text{Ci}/\mu\text{L}$) (Amersham Bioscience, Freiburg, Germany).
22. T4 kinase (10 U/ μL) (Pharmacia).
23. Loading buffer: 98% formamide, 10 mM EDTA, pH 8.0, and 0.1% bromo phenol blue and 0.1% xylene cyanol as tracking dyes, store at −20°C.
24. Bio-Rad Sequi-Gen II gel system (Bio-Rad, Hercules, CA).
25. SequaGel-6 (National diagnostics, Atlanta, CA).
26. Gel dryer model 583 (Bio-Rad).
27. 30–330 bp AFLP DNA ladder (GibcoBRL).
28. SequaMark (Research Genetics, Huntsville, AL).
29. X-ray films, 35 × 43 cm (Konica, Tokyo, Japan).
30. T-reagent (cycle sequencing kit, Amersham).
31. Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany).
32. 100 bp DNA ladder (Life Technologies).

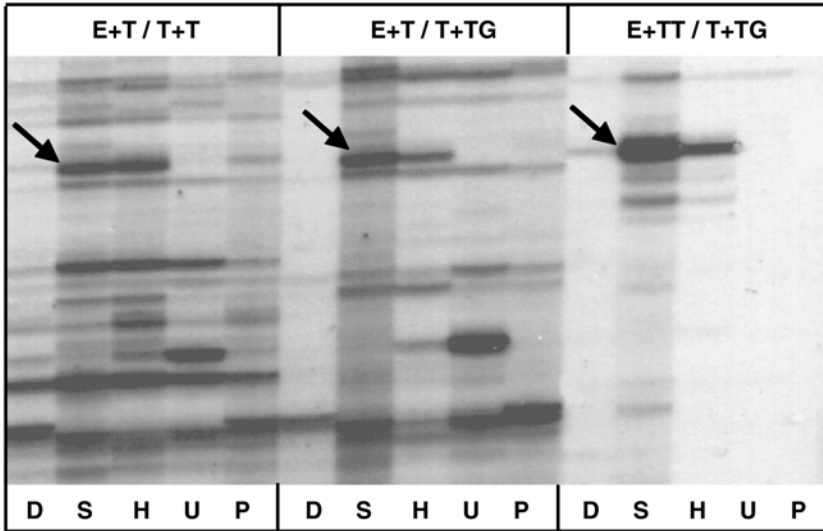


Fig. 2. Displayed on cDNA-AFLP gel, the arrows point to the expression pattern of a cellulase gene from five different developmental stages of the potato cyst nematode. Three different primer extensions were used: E+T/T+T, E+T/T+TG, and E+TT/T+TG. The D, S, H, U, and P refer to the different developmental stages.

3. Methods

3.1. Total RNA Isolation

1. Homogenize sample (in our case 100 mg of nematodes) in 1 mL of Total RNA Isolation Reagent using a power homogenizer.
2. During grinding, the homogenate is frozen a few times in liquid nitrogen to facilitate homogenization.
3. Spin down the debris briefly and add 0.2 mL of chloroform to the supernatant.
4. After shaking and incubation at room temperature for 2–3 min, centrifuge the sample at 12,000g for 15 min at 4°C.
5. Following centrifugation, the sample is separated into three phases: a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. Transfer the aqueous phase, which contains all the RNA, to a new RNase-free tube.
6. Precipitate the RNA by mixing with 0.5 mL isopropyl alcohol per 1 mL TRIzol Reagent used for the initial homogenization.
7. Incubate the sample at room temperature for 10 min and centrifuge at 12,000g for 10 min at 4°C in order to facilitate precipitation of the RNA.
8. After removal of the supernatant, wash the RNA pellet once by mixing with 1 mL of 75% ethanol, followed by centrifugation at 7500g for 5 min at 4°C.

9. Air-dry the RNA pellet for 5–10 min and dissolve in 80 μ L RNase-free water for 20 min at 60°C.
10. Check the quality of the RNA by running a small aliquot on agarose gel. Measure the absorbance of each sample and estimate the RNA concentration using, e.g., the Gene Quant II.

3.2. Purification of Poly A⁺ RNA (mRNA) From Total RNA

1. Adjust the volume of 100 μ g RNA to 100 μ L with RNase-free, DEPC-treated water.
2. Purify the mRNA from total RNA using Dynabeads Oligo(dT)₂₅.
3. To elute the mRNA from the Dynabeads, add 10 μ L Elution solution and incubate at 65°C for 2 min.
4. Transfer the supernatant containing the mRNA to a new RNase-free tube and put it on ice immediately. Use 9 μ L purified mRNA for cDNA synthesis.

3.3. cDNA-Synthesis From Purified mRNA

1. An oligo(dT)₁₂₋₁₈ primer is used for the conversion of mRNA into first strand cDNA (SuperScript Choice System for cDNA synthesis).
2. Add 2 μ L primer to 9 μ L mRNA, heat to 70°C for 10 min and immediately put on ice. The following components are added:

Component:	Volume (μ L):
5X first-strand buffer	4
0.1 M DTT	2
10 mM dNTP mix	1

3. Mix the components by gently vortexing and place at 37°C for 2 min. Then add 2 mL of SuperScript II Reverse Transcriptase (RT) and incubate the mixture (total volume 20 mL) at 37 °C for 1 h. To terminate the reaction, place the tube on ice.
4. Use this first strand reaction mixture for second strand cDNA synthesis. The following reagents are added:

Component:	Volume (μ L):
DEPC-treated water	91
5X second-strand buffer	30
10 mM dNTP mix	3
<i>E. coli</i> DNA ligase (10 U/ μ L)	1
<i>E. coli</i> DNA polymerase (10 U/ μ L)	4
<i>E. coli</i> RNase H (2 U/ μ L)	1

5. Mix gently and incubate for 2 h at 16°C. Then add 10 μ L of 0.5 M EDTA and 150 μ L of phenol:chloroform:isoamyl alcohol (25:24:1). Centrifuged at room temperature for 5 min at 14,000g to separate the phases.
6. Transfer the upper, aqueous layer (around 140 μ L) to a fresh RNase-free 1.5-mL tube.

7. Add 70 μL 7.5 M NH_4OAc and 0.5 mL of absolute ethanol (-20°C) and vortex. Centrifuge the mixture at room temperature for 20 min at 14,000g. Remove the supernatant and overlay the pellet with 0.5 mL of 70% ethanol (-20°C).
8. Centrifuge at 14,000g for 2 min, remove the ethanol. Then dry the cDNA at 37°C for 10 min, and resuspend it in 70 μL Milli-Q water.

3.4. Restriction of cDNA With *TaqI* and *EcoRI*

1. For the digestion of cDNA (see **Note 1**) with *TaqI* (frequent cutter) and *EcoRI* (rare cutter), add the following components:

Component:	Volume (μL):
cDNA	5
1,4 All buffer (10X)	3
<i>TaqI</i>	1
Milli-Q	21
Total	30

2. Mix gently and incubate for 2 h at 65°C .
3. For the digestion with *EcoRI*, add the following components:

Component:	Volume (μL):
1,4 All buffer (10X)	5
<i>EcoRI</i>	1
Milli-Q	4
Total	10

4. Incubate for 2 h at 37°C .

3.5. Preparation of Adapters

The sequences of the designed adapters are listed.

Name	Sequence
<i>TaqI</i> -adapter-upper	GACGATGAGTCCTGAC
<i>TaqI</i> -adapter-lower	CGGTCAGGACTCAT
<i>EcoRI</i> -adapter-upper	CTCGTAGACTGCGTACC
<i>EcoRI</i> -adapter-lower	AATTGGTACGCACTCTAC

1. For the preparation of the *TaqI*-adapter, combine 25 μg of the *TaqI*-adapter-upper with 22 μg of the *TaqI*-adapter-lower and adjust the volume to 100 μL with water.
2. For the *EcoRI*-adapter, combine 1.7 μg of the *EcoRI*-adapter-upper with 1.5 μg of the *EcoRI*-adapter-lower and adjust the volume to 60 μL with water.
3. To optimize the annealing of the upper and lower strand of the adapter, incubate the tubes in a water bath with a temperature of 65°C and let it slowly cool down to room temperature.

3.6. Ligation of *TaqI*- and *EcoRI*-Adapters

For the ligation of the adapters to the sticky ends of the restricted cDNA, add the following components to the digestion mixture from **Subheading 3.1.4.:**

Component:	Volume (μL):
<i>EcoRI</i> -adapter	1
<i>TaqI</i> -adapter	1
ATP (10 mM)	0.5
1,4 All buffer (10X)	0.5
T4 DNA ligase (5U/μL)	0.2
Milli-Q	2
Total	5.2

The final restriction-ligation mixture volume is 45 μL and is incubated for 3 h at 37°C.

The (10X) diluted product of the restriction-ligation reaction is called the primary template.

3.7. Pre-Amplification of the Primary Template

The primary template is pre-amplified using nonselective *TaqI*- and *EcoRI*-primers (see **Note 2**).

Name	Sequence
<i>TaqI</i> +0	GATGAGTCCTGACCGA
<i>EcoRI</i> +0	GACTGCGTACCAATTC

The following components are put together:

Component:	Volume (μL):
Primary template (10X dil.)	20
<i>TaqI</i> +0 primer (50 ng/μL)	2
<i>EcoRI</i> +0 primer (50 ng/μL)	2
PCR-buffer (10X)	5
dNTP (2.5 mM each)	4
Milli-Q	17
AmpliTaq	0.25
Total	50.25

PCR program (see **Note 3**):

- 30 s at 94°C (denaturation)
- 30 s 52°C (annealing)
- 60 s at 72°C (elongation)
- Repeat for 30 cycles

1. Check the products of the pre-amplification (10 μ L) on a 1% agarose gel (20–25 min at 80 V), using 2 μ L low mass ladder as a marker.
2. Dilute the pre-amplification product 30–100 times in water, which is the secondary template.

3.8. cDNA-AFLP

1. The secondary template is then amplified with selective primers (see **Note 4**) and the rare-cutter primer is radioactively labeled with $\gamma^{33}\text{P}$ -ATP.

*Eco*RI +N⁺ GACTGCGTACCAATTCN (N = +A, C, G, or T)

2. The components of the labeling reaction (for 1 PCR sample):

Component:	Volume (mL):
$\gamma^{33}\text{P}$ -ATP (10 mCi/mL)	0.15
Primer 1 (50 ng/mL)	0.2
1,4 All buffer (10X)	0.1
T4 kinase (10 U/mL)	0.02
Milli-Q	0.53
Total	1.0

3. Incubate this reaction mixture for 1 h at 37°C, followed by 10 min at 72°C to stop the labeling reaction (inactivation of T4 kinase).
4. Use the labeled primer in combination with unlabeled *Taq*I +2 primers (primer 2), with the following general sequence: GATGAGTCCTGACCGANN. “+NN” symbolizes the extensions +AA, AC, AG, AT and so forth for all four nucleotides, which gives a total of 16 different *Taq*I +2 primers.
5. Add 1 μ L of labeled primer one to the following primer/dNTP mix (for one PCR sample):

Component:	Volume (mL):
Unlabeled primer 2 (50 ng/mL)	0.6
dNTP (5 mM)	0.8
Milli-Q	2.6
Total	5.0

6. Prepare the *Taq* Polymerase mixture (for 110 PCR samples):

Component:	Volume (μ L):
PCR buffer (10X)	220
AmpliTaq	8.8
Milli-Q	870
Total	1098.8

7. The final mixture (20 μ L) per active PCR sample is composed of: 5 μ L secondary template, 5 μ L primer/dNTP mix, and 10 μ L *Taq* Polymerase mix. The active PCR profile started with a touchdown PCR of 14 cycles: 30 s at 94°C, 30 s at 65–

56°C (a decrease of 0.7°C each cycle), and 60 s at 72°C. This touchdown PCR is continued by 24 cycles with the following PCR-conditions: 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C.

8. After the selective amplification with active PCR, add 20 µL of loading buffer to each sample and the samples store at -20°C.
9. Analyze the amplified fragments on a Bio-Rad Sequi-Gen II gel system, which contains an integrated buffer chamber (IPC), a glass plate, two clamps, two spacers, and two combs (assemble this system according to the manufacturer's instructions).
10. Before loading on a 5% denaturing polyacrylamide gel (SequaGel-6), heat the total mixture (40 µL/sample) for 5 min at 95°C in the PCR machine or heating block, and immediately put on ice to prevent the formation of new secondary structures.
11. For each sample, load 3.5 µL (*see Note 5*). Perform electrophoresis at a constant power of 110 W/gel for 2.5–3 h.
12. After electrophoresis, dismantle the gel system and cover the gel with a piece of Whatmann 3MM paper. Remove parts of the gel not covered by the Whatmann paper. In this way, the gel is attached to the Whatmann paper and can be removed from the glass plate.
13. Cover the gel with Saran Wrap (protection) and dry it in a pre-warmed gel dryer at 80°C for approx 1 h under vacuum.
14. After drying, attach the gel with tape to a standard X-ray film. Mark the gel and the attached X-ray film with a perforator at three sides (*see Note 6*) and place them in a cassette to prevent the exposure to light.
15. Expose the gel to the X-ray film for at least 3 d.
16. Develop the X-ray film (autoradiograph).

3.9. 30–330 bp AFLP DNA Ladder and SequaMark

1. In order to estimate the size of the Transcript Derived Fragments (TDFs), two different labeled DNA ladders are loaded on every AFLP-gel. Label the 30–330 bp AFLP DNA ladder according to the manufacturer's instructions (GibcoBRL). After incubation of 5 min at 70°C, load 3.5 µL of DNA ladder per lane.
2. For the preparation of labeled SequaMark, an active PCR is performed. Add the following components for the labelling of the SequaMark primer:

Component:	Volume (µL):
SequaMark primer	2.5
1,4 All buffer (10X)	0.5
T4 kinase	0.5
γ ³³ P-ATP	1.5
Total	5

3. Incubate this reaction mixture for 90 min at 37°C, followed by 10 min at 72°C to stop the reaction.
4. Use the labeled primer in an active PCR-reaction:

Component:	Volume (μL):
Labeled SequaMark primer	5
Template ssDNA	5
T-reagent	8
Milli-Q	12
Total	30

5. Use the following PCR condition: 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C, cycle 24 times.
6. After PCR, add an equal volume (30 μL) of loading buffer.
7. Before loading (3.5 μL/lane), heat the SequaMark ladder for 5 min at 95°C.

3.10. Excise Fragments From the Polyacrylamide Gel

1. Align the X-ray film to the original gel precisely and mark the band position on the dried gel carefully.
2. Excise the gel piece with a sharp scalpel out of the polyacrylamide gel and transfer it to an Eppendorf tube (*see Note 7*).
3. Overlay with 100 μL Milli-Q and incubate for 20 min at room temperature.
4. Discard the supernatant, add another 100 μL Milli-Q and crush the polyacrylamide fragment using a pipet tip.
5. Incubate for 15 min at 65°C and leave overnight at room temperature.
6. Spin down the debris and transfer the supernatant to a fresh Eppendorf tube.

3.11. Reamplification of Selected TDFs

1. Use High Fidelity (HF) PCR (Expand High Fidelity PCR System) for the reamplification of the excised TDFs to minimize the PCR errors. Use the same primers as in the initial active PCR.

Add the following components (for one PCR-sample):

Component:	Volume (μL):
Expand HF buffer (10X conc., 15 mM MgCl ₂)	5
10 mM dNTP	4
Primer 1 (50 ng/μL)	1.5
Primer 2 (50 ng/μL)	1.5
Expand HF enzyme mix (Taq and Pwo DNA pol.)	0.4
Milli-Q	30.6
Template	7
Total	50

2. Use the same touchdown PCR program as for the active AFLP PCR.
3. Check the PCR-products (15 μL/sample) on a 2.5% agarose gel (120 V for 2 h). Use 1 μL 100 bp DNA ladder as size marker.
4. Sequence the PCR products directly or first clone them into a plasmid (*see Note 8*).

3.12. GenEST and Computer Analysis

GenEST is command line-based and can be run on Unix/Linux and from the MS-DOS prompt under Microsoft Windows. To obtain the most recent version of the GenEST program, please send an e-mail inquiry to Ling.Qin@wur.nl. Here I show step by step how to use GenEST under Windows:

1. Unzip the received program while maintaining the original directory structure (for example, you can name this folder - C:\genest).
2. The latest edition of Ruby can be retrieved from: <ftp://ftp.easynet.be/ruby/ruby/> or check this website for latest development: <http://www.ruby-lang.org/en/>. Follow the instruction and install Ruby (for example, under C:\ruby) (*see Note 9*).
3. To test a working installation of Ruby, type (all the commands typed are shown *italicized*): C:>*ruby -v*.

This command should return the version number. If the program is not found, you may have to update the PATH setting.

4. Change to the root directory of the installed GenEST program (for example, C:\genest) and type:

C:\genest>*ruby src\genest*.

5. The output containing the usage and the options will show up (*see Fig. 3*).
6. Download and make your sequence database in FASTA format (containing EST or cDNA sequences), for example EST.txt.
7. A command file can be created with a text editor, which contains restriction enzyme recognition sites to be used as the begin- and end-tags and the marker length modifier (the marker length modifier is designed to compensate for the additional adapter sequences present in TDFs as they appear on cDNA-AFLP gel, in this case 22 nt). Multiple combinations can be defined in a command file.

For example a command file named NTtag.txt, which looks for *NcoI* and *TaqI* tags:

```
CCATGG TCGA 22
TCGA CCATGG 22
```

8. GenEST uses the begin tag to search for the tag sequence in the cDNA data, which are contained in the input files in FASTA format. If such a tag is found, it will continue its search for a matching end tag. This search action is executed in both directions for all begin/end tag combinations.
9. To execute the search, type in a DOS window:
C:\genest>*ruby src\genest -c NTtag.txt -o TDFout.txt EST.txt*
(-o TDFout.txt - indicates the output file, which will contain a list of the found virtual TDFs ordered by the primer extensions).
10. Another application of GenEST is to use the identifiers of a band on gel (restriction enzyme recognition sequences, primer extensions, and the band size [*see Note 10*]) as a search query to quickly identify corresponding EST(s).

```

C:\genest>ruby src\genest

genest 2.00pre7 by Piotr Prins & Ling Qin GPL (c) 1999-2003

Usage: genest [-v] [--trace] [--sort-sequence] [--version1]
             [-o outputfile]
             -c commandfile estfilename1 [estfilename2..n]

Options:

    -v verbose           Give some verbose information
    -o outputfile        Output file, otherwise console
    -c command file      Commands are read from text file

    --sort-sequence      Sort on sequence name
    --sort-length        Sort on sequence length
    --trace              Trace information on stdout
    --version1           Genest 1.x compatibility mode
    --color              Colors in output

    estfiles             One or more files with (EST) sequence
                        data

```

Fig. 3. The output DOS window showing the usage and the options of GenEST.

11. To search the corresponding ESTs for a TDF digested with *Nco*I and *Taq*I, amplified with N+A/T+CA, size around 72, write the following in a command file named search.txt:
 CCATGGA TGTCGA 22
 Search 70-74
12. To execute the search, type in a DOS window:
 C:\genest>ruby src\genest -c search.txt -o TDFout.txt EST.txt.
13. For additional applications of GenEST (see **Note 11**).

4. Notes

1. It is a good idea to target several control genes with well-documented expression pattern with cDNA-AFLP. Choose genes containing the same set of restriction enzymes used in cDNA-AFLP; determine the primer extensions and the size of the predicted bands based on the known sequences. This can be done easily with GenEST. Beside, to increase the percentage of genes displayed by cDNA-AFLP, enzymes like *Apo*I (RAATTY) and *Bst*YI (RGATCY) can be used, which recognize degenerate hexa-nucleotides.
2. The quality of the template and the whole procedure is checked after pre-amplification. The amplified cDNA smear on agarose gel should be between 50–

500 bp. A successful preamplification will produce enough material for hundreds of selective amplifications later on.

3. Because the adapters ligated to the digested cDNA fragments are not phosphorylated, only one strand of the adapter is ligated to the cDNA. Therefore, NEVER perform hot start PCR in pre-amplification. If you do so, the DNA will be denatured and you will lose your adapter, whereas during a normal PCR process, the 3' recessive end will be filled in by Taq polymerase within seconds.
4. The choice of the number of the selective primer extensions depends on the gene number of the studied organism. Ideally, it should be between 60 and 100 bands displayed per lane. From our own experience, for a nematode genome of about 19,000 genes, +1/+2 primer extensions are appropriate; for tomato with around 25,000 genes, +2/+2 gives a better separation. But when *ApoI* or *BstYI* is used together with *TaqI* to display nematode genes, +2/+2 produces a better resolution.
5. Do not leave an empty lane on gel, because this will distort the banding pattern. Fill it with control sample of the same ionic strength.
6. The asymmetrical perforations made on dried gels and the exposed X-ray films are used to position precisely the developed films back on the gels, which is essential for the correct excisions of the right bands.
7. To check if the excision is performed properly, after the selected fragments are cut out of the gel, another X-ray film can be exposed and developed. The absence of a signal from and only from the excised position is a good indication.
8. Confirmation of the cloned cDNA-AFLP fragments can be done conveniently by adding one additional selective base (based from the sequenced fragment) to the 3'-ends of the E and/or T primers in selective amplifications and run on gel.
9. *Ruby* is well-suited to rapid prototyping and much cleaner than *Perl* (for more details, please read the article written by Pjotr Prins published in Linux Journal: <http://www.linuxjournal.com/article.php?sid=5915>). It is becoming increasingly popular among bioinformaticians. Microsoft Windows users can download the "Pragmatic Programmers" edition of *Ruby* from: <http://www.pragmatic-programmer.com/ruby/downloads/ruby-install.html>. See also <http://www.ruby-lang.org/en/> for more information on *Ruby*.
10. Because band resolution is not uniformly distributed on a polyacrylamide gel (at the top of the gel bands are closer to each other than at the bottom end; the estimation of band size should be adjusted accordingly) allow ± 1 nt for bands smaller than 200 nt and ± 3 for bands larger than 200 nt.
11. Besides generating restriction patterns of sequences, GenEST can also be used to find other sequence motifs in a large data set, a process often too time-consuming to be done manually. For example, GenEST was used to predict the occurrence of *trans*-spliced leader sequences from an EST database of a plant parasitic nematode. In many nematode species, up to 70% of the mature mRNAs are *trans*-spliced with a 22 nt leader sequence to the 5' end of the mRNAs (12). When *EcoRI* recognition sequence in the command file is replaced with the *trans*-spliced leader sequence, all the ESTs containing this sequence can be quickly

identified using GenEST. This information can be used to estimate the fraction of full-length cDNAs present in a library and to check whether the encoded open reading frames start with a peptide signal for secretion. AFLP techniques have been used extensively in genetic mapping in various organisms and a large number of AFLP markers associated with genes of interest have been identified (4,13). Such markers combined with a fully sequenced genome (e.g., *Arabidopsis thaliana* [14]) could facilitate the efficient cloning of target genes. To this purpose, GenEST can be adapted to assist in the identification of the physical locus of an interesting gene by using the identifiers of appropriate AFLP markers.

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Differentially Expressed Genes Associated With Hepatitis B Virus HBx and MHBs Protein Function in Hepatocellular Carcinoma

Dae-Ghon Kim

Summary

HBx and MHBs^t products from hepatitis B virus-DNA (HBV-DNA), which become transcriptional transactivators of cellular and viral genes, are known to play causative roles in the development of hepatocellular carcinoma (HCC). However, the biomolecular mechanism(s) for their roles in hepatocarcinogenesis *in vivo* remain poorly understood. To identify authentic cellular genes involved in HBx and MHBs^t-transactivated carcinogenesis, we used mRNA differential display polymerase chain reaction (DD-PCR). We examined HBx and MHBs-positive or -negative HCC, which had chromosomally integrated HBV DNA, vs nontumor tissues, respectively, and differentially expressed genes in either type of HCC were identified and compared with each other. Using 240 different combinations of three one-base anchored oligo-dT primers and 80 arbitrary 13mers, 16 genes were differentially expressed in the HBx and MHBs-positive HCC including *Ro RNA hY1*, glutamine synthetase, factor H homologue 3' end, voltage-dependent anion channel 3 (*VDAC3*), three ribosomal proteins, four mitochondrial genes, and four novel genes. Unexpectedly, upregulated genes in association with functional HBV proteins were different from those reportedly transactivated by HBV viral proteins *in vitro*. Ten genes were downregulated, including three novel genes. In contrast, 15 genes in HCC tissue negative for HBx and MHBs-expression were preferentially expressed including pancreatic secretory trypsin inhibitor (*PSTI*), *H19*, guanidine nucleotide-binding protein α -1 subunit (*GNAZ*), carbamyl phosphate synthetase I (*CPS I*), insulin-like growth factor (IGF)-II, and 10 ribosomal proteins genes. Eighteen genes were downregulated including acute phase genes, a novel gene, and particularly the retinoblastoma susceptibility gene. Only two genes (ribosomal protein P0 and L37a) were commonly upregulated in both types of HCC tissues. These results suggest that cellular genes involved in the viral protein-transactivation may generally differ from those not associated with transactivation in established HCC, and that the specific oncogenic

coordination through the transactivation by viral proteins which works in experiments in vitro, may play only a potential role in hepatocarcinogenesis in vivo. In addition, the functional analyses of the eight novel genes identified in this study might be valuable to further understand the mechanism(s) of hepatocarcinogenesis.

Key Words: Hepatocellular carcinoma; HBx; MHBs; HBV DNA integration; DD-PCR.

1. Introduction

Chromosomally integrated hepatitis B virus (HBV)-DNA is detectable in more than 90% of HBsAg-positive hepatocellular carcinomas (HCCs) (1), and thus, this molecular finding is considered to be of crucial importance for HCC development. Unlike the woodchuck model, in which specific HBV-DNA integration occurs preferentially in *c-myc* or *N-myc* regulatory or coding sequences (2), specific HBV insertion into cellular genes has been found to be a rare event in humans. DNA integration sites are random and such integration occurs at random times during the course of chronic viral infection (3,4). Thus, the rearrangement of viral sequences following integration into human DNA appears not to be a specific marker for HCC, because non-neoplastic hepatocytes may have similar patterns of integration. The recent discovery of transactivating functions exerted by hepatitis B virus X protein (HBx) (5,6) and truncated HBs proteins (MHBs^t) (7,8) provide an alternative mechanism for HBV-associated hepatocellular carcinogenesis.

The HBx protein has been shown to interact, either directly or indirectly, with various transcriptional activators, including AP1 (9), AP2 (10), CREB/ATF2 (11), C/EBP (12), and NF- κ B (13,14), and with RNA polymerase II and III (15), tumor necrosis factor (TNF)- α (16), and inducible nitrous oxide (NO) synthetase (17) promoters. The MHBs^t protein displays transactivating activity towards the *c-myc* P2 (7,8,18) and *c-fos* (3,19) promoters, and nuclear factor (NF)- κ B (20). These results have generally been derived from in vitro experiments and their biological contributions to hepatocarcinogenesis in vivo need to be further elucidated. HBV *trans*-activator sequences are frequently integrated in DNAs derived from HCC tissues or hepatoma cell lines. Analysis of all HBV-containing tissues revealed that either HBx and/or MHBs^t transactivators were expressed and were functionally active (21). The viral cellular fusion proteins are translated and mutual enhancement was found when both *trans*-activators were coexpressed. However, these *trans*-activator apparently do not have properties usually associated with viral oncogenes in that rapid transformation of primary cultured cells and their expression do not lead to rapid neoplastic transformation. Thus, these *trans*-activators are not complete oncogenes and additional genetic changes seem to be required for progression to HCC.

In this study, we have searched for authentic genes *trans*-activated by hepatitis viral proteins that are simultaneously involved in hepatocellular carcinogenesis. The mRNA differential display method was applied to HCC tissues that had chromosomally integrated HBV-DNA and either expressed HBx and/or MHBs proteins or did not express them. Because liver cancer tissue is easily delineated from nontumor tissue, we could identify differentially expressed cellular genes in association with (or without) transactivation by functional viral proteins and could compare them with each other in this study. Subsequently, we isolated novel genes that are, causally or subsequently, involved in hepatocarcinogenesis, and these genes seem to be excellent candidates for the further study of hepatocellular carcinogenesis.

2. Materials

1. Cobra Core Enzyme Immunoassay (EIA) kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland).
2. A liquid hybridization assay (Abbott Laboratories, North Chicago, IL).
3. Microparticle Enzyme Immunoassay (Abbott).
4. TRI Reagent (Molecular Research Center Inc., Cincinnati, OH).
5. Primary antibody, including those recognizing HBs, HBc, or α -FP (Novocastra Laboratories, Newcastle, UK), or HBx (Dr. Y. D. Yoon, Mok-Am Res. Inst. Suwon, Korea).
6. 0.4N NaOH.
7. α -³²P dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA).
8. Hybridization buffer: 0.5 M Na₂H₂PO₄, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 1 mM ethylenediaminetetraacetic acid (EDTA).
9. 2X saline sodium citrate (SSC) 0.1% SDS: 150 mM NaCl, 15 mM SDS.
10. 0.2X SSC, 0.1% SDS.
11. 1% agarose gels containing 2.2% formaldehyde and 50 mM MOPS.
12. UV cross-linker (Stratagene, La Jolla, CA).
13. Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).
14. SuperScript II RNase H Reverse transcriptase (Invitrogen, Carlsbad, CA).
15. DyNAzyme[®] II DNA Polymerase (Finnzyme OY, Espoo, Finland).
16. MessageClean[®] Kit (GenHunter Corporation, Nashville, TN).
17. RNAimage[®] Kit (GenHunter).
18. H-AP primer sets 1-10 (GenHunter).
19. GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT).
20. Whatman 3MM.
21. pGEM-T vector using the TA cloning system (pGemT; Promega, Madison, WI).
22. T7 and SP6 primers.
23. ABI PRISM 377 DNA sequencer (Applied Biosystems).
24. 1 M Tris-buffer, pH 7.5.
25. 3,3'-diaminobenzidine.
26. Meyer's hematoxylin.

27. The cDNA probe for HBx and middle HBs (pre S2 and S).
28. Envision-labeled polymer reagent) (Dakopatts, Denmark).
29. Marathon cDNA Amplification kit (Clontech Laboratories Inc., Palo Alto, CA).

3. Methods

3.1. Serological Assays and Tissue Acquisition

1. Two patients were selected and tested for HBV or HCV markers in their sera using a Cobra Core EIA kit. HBV DNA was quantitated by a liquid hybridization assay with a detection limit of 1.7 pg/mL. Patient A was positive for HBs, anti-HBe Ab, and was negative for anti-HBs, HBe Ag, HBV DNA, and HCV Ab. Patient B was positive for HBs Ag, HBe Ag, HBV DNA (at 570 pg/mL), and was negative for anti-HBs Ab, anti-HBe Ab, and HCV Ab (*see Table 1*). These results are indicative of the integrated stage of HBV in patient A and of the replicated stage of HBV in patient B.
2. The level of α -fetoprotein (α -FP) was quantitatively determined by a Microparticle Enzyme Immunoassay. Patient A has a normal range of serum α -FP (7 ng/mL) and patient B has a high serum level of α -FP (1750 ng/mL).
3. Primary HCCs and surrounding nontumor liver tissues were obtained from both patients undergoing surgical resection of primary HCC. Written informed consent was obtained from each patient.
4. HCC and nontumor tissues were histologically confirmed by pathologists. After resection, the tissues were rinsed in sterile phosphate-buffered saline (PBS) and were immediately stored in liquid nitrogen until used for RNA isolation.
5. Total cellular RNA was extracted using the TRI reagent.
6. This protocol conformed to the ethical guidelines of the Institutional Review Board (IRB).

3.2. Liver Histology and Immunohistochemistry

1. Formalin-fixed, paraffin embedded specimens, including HCCs and nontumor tissues, were sectioned at 4 μ m thickness. Deparaffinization and rehydration were performed using xylene and alcohol.
2. Liver disease of each patient was rated according to the histology activity index (HAI) as previously described (22).
3. Sections were treated with 0.3% hydrogen peroxide for 3 min and with blocking antibody for 30 min. The sections were incubated with primary antibody, including those recognizing HBs, HBc, or α -FP, or diluted 1:100 to 1:1000 in 1 M Tris-buffer, pH 7.5, for 1 h.
4. Detection was performed using the avidin-biotin-peroxidase complex method, i.e., peroxidase-labeled goat anti-rabbit and anti-mouse immunoglobulin (envision-labeled polymer reagent) were applied for 60 min as the secondary antibodies.
5. 3,3'-diaminobenzidine was used as the chromogen. Counter-staining was performed with Meyer's hematoxylin.

Table 1
Serological and Immunohistochemical Characteristics of Tumor and Nontumor Tissues in HCC
With or Without HBx and MHBs Expression (Patient A or Patient B, respectively)

		Patient A		Patient B	
Serology	HBs Ag	+		+	
	HBe	-		+	
	Anti-HBe Ab	+		-	
	α -FP (ng/mL)	7		1750	
	HBV DNA (pg/mL)	0		720	
Immuno- histochemistry		Nontumor ^a	Tumor	Nontumor	Tumor
	HBs Ag	-	+	+	-
	HBc	-	-	+	-
	HBx	$\pm$	+	+	-
	α -FP	-	-	$\pm$	+

^a $\pm$ some nontumor tissue adjacent to tumor tissue was positive, but the majority of nontumor tissue was negative.

6. Tumors were evaluated for the percentage of positive cells and the intensity of staining. Negative controls included samples incubated with PBS, or with mouse *IgG1* instead of the primary antibody.

3.3. Southern Blot Analysis

1. Ten-microgram aliquots of genomic DNA were digested with the restriction enzyme *Bam* HI, electrophoresed on 1% agarose gels.
2. Transferred onto nylon membranes in 0.4N NaOH.
3. Hybridized with ^{32}P -labeled HBx DNA (subtype *adr* 4) in hybridization buffer at 65°C overnight.
4. The membranes were then washed in 2X SSC, 0.1% SDS at 65°C for 20 min twice, then washed in 0.2X SSC, 0.1% SDS at 65°C for 20 min (*see* **Fig. 1**).
5. The blots were then exposed to X-ray film at -70°C.

3.4. Northern Blot Analysis

1. Samples containing 20 µg of total nontumor or tumor RNAs were fractionated on 1% agarose gels containing 2.2% formaldehyde and 50 mM MOPS.
2. Samples were transferred to a nylon membrane.
3. The membrane was cross-linked using as UV cross-linker
4. The membrane was individually hybridized with each cDNA probe generated from a digested cDNA insert labeled with α - ^{32}P dCTP (3000 Ci/mmol) by random priming.
5. Hybridization and washing conditions were the same as described previously (**23**).
6. The blots were then exposed to X-ray film at -70°C (*see* **Fig. 2**).
7. Autoradiographs of Northern hybridization were scanned using a Personal Densitometer SI.

3.5. DD-PCR

1. For the mRNA DD it is crucial to clean up RNA samples, i.e., complete removal of the DNA contamination (**24**). We used a GenHunter MessageClean® kit routinely for this purpose.
2. Modified DD-PCR was performed using a GenHunter RNAimage®. Based on an improved method described by Liang et al. (**25**), 4 µg of total RNA from tumor or nontumor tissues were reverse-transcribed with 200 U SuperScript II RT enzyme in the presence of 1 µmol/L one-base anchored oligo(dT) primers for 1 h at 42°C in a total volume of 20 µL. 0.1 µL of α -[^{32}P]dCTP (3000 Ci/mmol) was used instead of α -[^{32}P]dATP or α -[^{35}S]dATP for the PCR labeling.
3. The reaction was terminated by incubation at 75°C for 10 min.
4. Two microliters of the reaction mixture was PCR amplified with DyNAzyme in 1 µM H-AP, a 13mer (5' end primers; H-AP1 ~ H-AP80) and one base anchored oligo(dT)₁₁ primers (3' end primers; H-T₁₁A, H-T₁₁G, or H-T₁₁C), using a GeneAmp PCR system 9600. 240 PCR reactions were carried out on each sample.

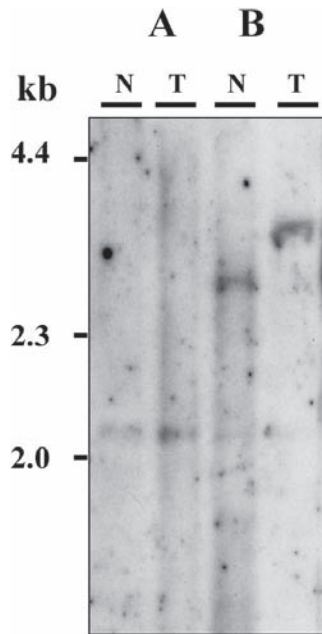


Fig. 1. Southern blot analysis of HCC DNA in two patients, A and B. Nontumor tissue DNAs were in lanes 1 and 3, respectively. Tumor tissue DNAs were in lanes 2 and 4, respectively. Ten-microgram aliquots of genomic DNA were digested with *Bam*HI, electrophoresed on 1% agarose gels, and transferred onto nylon membranes. The blot was hybridized with ^{32} P-labeled HBx DNA (subtype *adr* 4), then washed and exposed to X-ray film.

5. The cycling conditions were as follows: 94°C for 30 s, 42°C for 1 min, 72°C for 30 s for 40 cycles followed by an extension at 72°C for 5 min.
6. Each PCR reaction was stopped by the addition of stop solution and was heated at 94°C for 2 min and displayed on a denaturing 6% polyacrylamide gel.
7. To avoid the possibility of losing rarer mRNAs and to minimize errors in the PCR procedure that would generate spurious bands, duplicate samples were analyzed with different amounts of RNA (*see* Fig. 3).

3.6. Recovery and Cloning of Reamplified cDNA

1. The polyacrylamide gel was blotted on a piece of Whatman 3MM paper and dried without fixing.
2. An autoradiogram was made and cDNA bands of interest from the dried gel were marked through the film.
3. Gel slices were submerged in 100 μ L distilled water and boiled for 15 min to elute cDNAs. cDNAs were recovered by ethanol precipitation and were dissolved in 10 μ L distilled water.

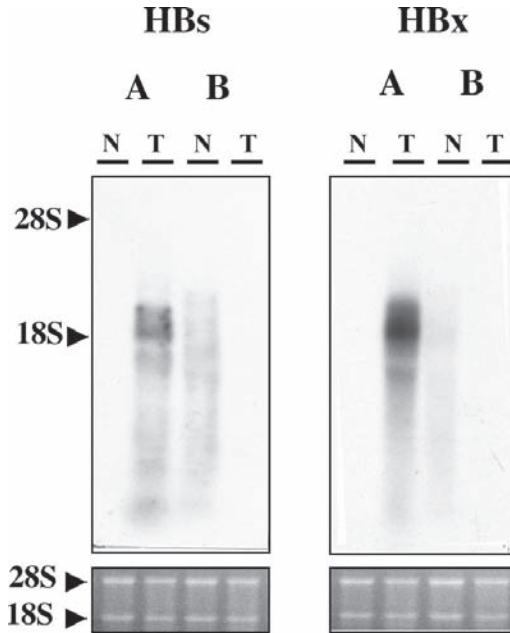


Fig. 2. The expression of HBx and Middle HBs mRNAs on Northern blot. Aliquots of 20 μ g of total nontumor or tumor RNAs were fractionated on 1% agarose gels containing 2.2% formaldehyde and 50 mM MOPS and transferred to nylon membranes. The blots were individually hybridized with each cDNA probe, middle HBs (pre S2 and S) or HBx, generated from a digested cDNA insert labeled with α - ^{32} P dCTP (3000 ci/mmol) by random priming. Hybridization and washing conditions were the same as described in Materials and Methods, and blots were then exposed to X-ray film at -70°C .

4. Four microliters of eluted cDNA was reamplified using the same primer set and with the same PCR conditions as used in the initial mRNA display.
5. PCR products were resolved on a 2% agarose gel and were extracted from the gels. The amplified cDNAs were cloned into pGEM-T vector using the TA cloning system (pGemT).
6. To eliminate false clones, clones were prescreened by dot-blot hybridization with the original amplified cDNA (optional).
7. Verify clones (probe) by Northern blot analysis. The cloned cDNA was excised by *Hind*III or *Sac*I and *Sac*II, and used as a probe.
8. Expression levels of each gene were normalized against the level of *GAPDH* and the hybridized signals were then calculated as fold induction from nontumor or HCC tissues, or vice versa (see Fig. 4).

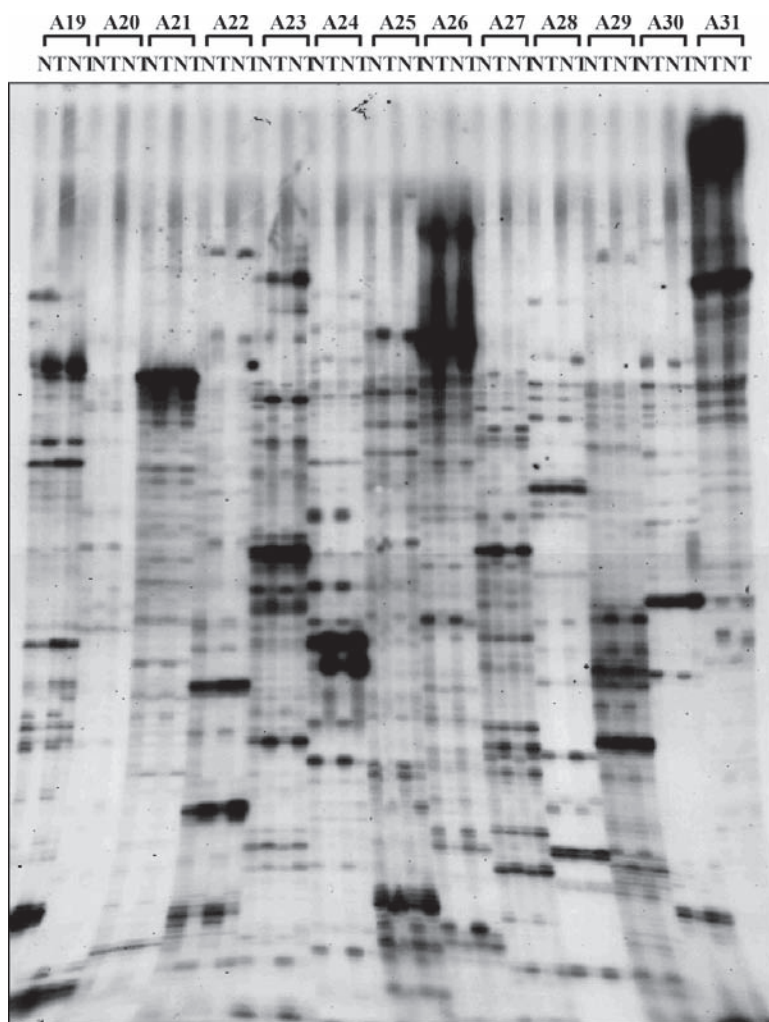


Fig. 3. DD-PCR gels showing differentially expressed bands/genes. Representative patterns of differentially expressed genes were obtained from DD-PCR analysis using duplicated nontumor (N) and tumor (T) liver samples. cDNA fractions obtained by reverse transcription with the 3' primer H-T11A and the 5' primers H-AP 33 to H-AP 67 primers were used to amplify the cDNA with annealing at 42°C and samples were run on a standard sequencing gel.

3.7. Sequencing of Cloned cDNAs and Data Analysis

1. Sequence analysis was performed in both directions using T7 and SP6 primers with a fluorescent automated ABI PRISM 377 DNA sequencer.

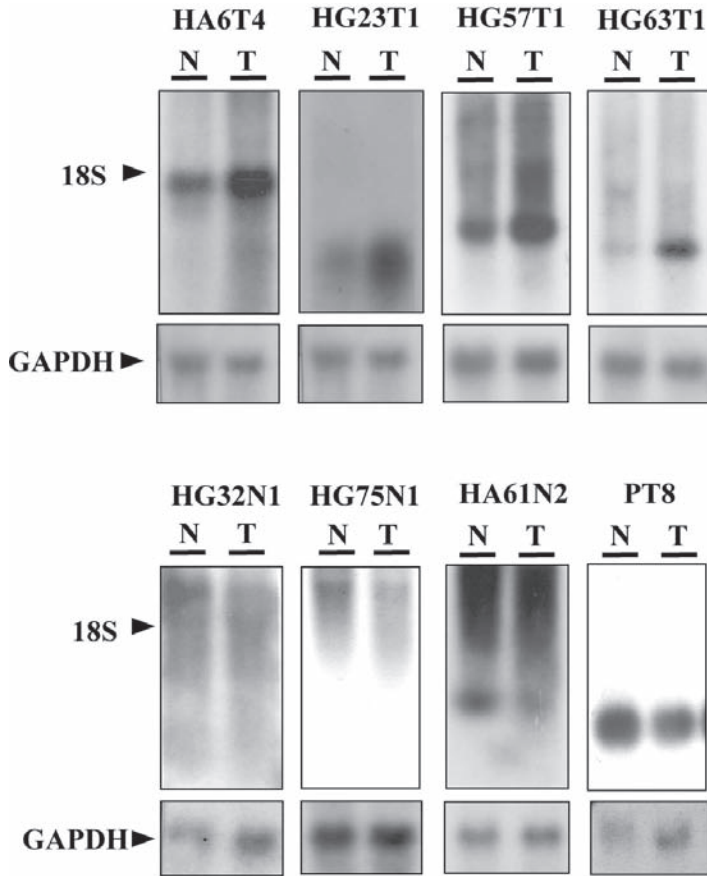


Fig. 4. Pattern of respective mRNA expression hybridized with eight putative novel genes (fragment) in nontumor (N) and tumor (T) tissue from HCC in patient A or B. Preparation of Northern blots, hybridization, and washing conditions were the same as described in Materials and Methods, and blots were then exposed to X-ray film at -70°C . The membrane was stripped and subsequently hybridized with a probe for *GAPDH* as a loading control.

2. The sequenced cDNAs were analyzed via the BLAST program for matches in the GeneBank database and were compared with each other via FASTA analysis.

4. Notes

1. The other advantage of DD-PCR analysis is the ability to identify increased or decreased mRNA levels simultaneously (24). Generation of more than 20,000

transcripts from DD-PCR gels and isolation of 74 cDNAs that correspond with increased or decreased mRNAs in both types of HCC tissues compared with nontumor tissues with chronic HBV infection, some of which were identical genes (*see Table 2*).

2. The successful use of three one-base anchored oligo-dT primers and rationally designed arbitrary 13mers in differential display further cuts down the number of reverse transcription reactions needed for each RNA sample, and minimizes the redundancy and under-representation of certain RNA species owing to the degeneracy of the primers (25).
3. To avoid the possibility of losing rarer mRNAs and to minimize errors in the PCR procedure that would generate spurious bands, duplicate samples were analyzed with different amounts of RNA (26).
4. For the PCR labeling 0.1 μ L of α -[^{32} P]dCTP (3000 Ci/mmol) was used instead of α -[^{32} P]dATP or α -[^{35} S]dATP (26). Adjust exposure time of autoradiogram according to radioactivity (26).
5. The amplified cDNAs were cloned into pGEM-T vector using the TA cloning system. To eliminate false clones, clones were prescreened by dot-blot hybridization with the original amplified cDNA (27).
6. To isolated novel genes or to confirm known genes, an antisense primer was synthesized, based on the 3' untranslated region (UTR) sequence of a single cDNA fragment. Rapid amplification of the 5' cDNA end (5'-RACE) was performed using the antisense primer following the protocol supplied with the Marathon cDNA Amplification kit (28).
7. One major difficulty of the DD-PCR method is to distinguish truly differentially expressed mRNAs from false positives produced by PCR artifacts. Furthermore, it is a challenging task to verify corresponding mRNA expression by Northern blot analysis. However, the mRNA expression of selected 60 unique kinds of cDNA clones was confirmed by respective Northern blot analysis (26).
8. There is no overlap in differentially expressed genes between nontumor tissues of patients A and B. We suggest that this may result from the different background of inflammatory reactions in those nontumor tissues. Thus, the expression of acute-phase protein genes may be relevant to the more serious inflammatory reactions with HBV replication in patient B than in patient A. From the aforementioned results, the expression of cellular genes involved in hepatocarcinogenesis is different according to whether HBx and MHBS⁺-transactivation exists or not. Thus, we propose that oncogenic coordination of cellular genes may depend on causative initiation by a specific carcinogen or by a carcinogenic mechanism. In addition, the novel genes identified in this study should be further analyzed by characterization of their full-length cDNA and a determination of their functional role(s) should improve our understanding of the molecular mechanism(s) of hepatocarcinogenesis.

Table 2
Isolation of Genes Differentially Expressed in HCC Tissues
(A) Upregulated Genes in Association With the Functional HBV Protein Expression.

Clone no.	Accession no.	Description	Fold induction by Northern
HA6T4			2.4
HA19T1, HA22T1	X62996	Mitochondrial gene (ATPase 8/6)	2.4
HA25T1	M17885	Acidic ribosomal phosphoprotein P0	4.6
HA29T1	AF042507	Mitochondrial gene (cytochrome b)	2.0
HA30N1	K01562	Ro RNA (scRNA) hY1	8.1
HA60T1	LO6498	Ribosomal protein S20	20.5
HA65T1	S70290	Glutamine synthetase	140.7
HA65T3	J01415	Mitochondrial gene (ND1)	3.5
HA78T1	M65294	Factor H homologue 3' end	6.8
HA78T0-1	NM005662.1	Voltage-dependent anion channel 3 (VDAC3)	4.3
HC33T1	M31520	Ribosomal protein S24	8.6
HC37T2	AJ223473	Transcription elongation factor TFSII	2.2
HC64T1, HG25T3, HG44T1	X66699	Ribosomal protein L37a	5.7
HG23T1			3.2
HG38T3	X62996	Mitochondrial gene (ND3)	4.0
HG57T1			2.4
HG63T1			2.5

(B) Downregulated Genes in Association With the Functional HBV Protein Expression.

Clone no.	Accession no.	Description	Fold induction by Northern
HC23N1	M11309	Coagulation factor IX	11.3
HC56T1	Z34975	LDLC	4.4
HC56N1	J00307	Prothrombin (phii-3 clones)	3.5
HG32N1			3.1
HG32T1	AF0027	RAC3 mRNA	2.1
HG75N1			2.2
HA5N2	101415	Mitochondrial gene (tRNA)	9.1
HA7T1	NM000971	Ribosomal protein L7	3.5
HA61N2			4.9
HA70N1	Y17172	HIV-associated non-Hodgkin's lymphoma homolog 3'end	2.2

(C) Differentially Upregulated Genes in Association Without the Functional HBV Protein Expression. (continued)

Clone no.	Accession no.	Description	Fold induction by Northern
KHC14T2	M777234	Ribosomal protein S3a	5.7
KHG3T1, KHG3T2	NM000990.1	Ribosomal protein L27a	16.4
KHG41T1	NM000973.1	Ribosomal protein L8	9.9
KHG54N2, KHA2T1	U14967	Ribosomal protein L21	8.2
KHG58N3	M11949	Pancreatic secretory trypsin inhibitor (PSTI)	7.0
KHG59T2	AF125183.1	H19 gene	765
KHG64T1	NM002954	Ribosomal protein S27a	8.3
KHG67T2	AF055013	Guanine nucleotide-binding protein alpha-1 subunit (GNAZ)	6.8
KHG69T1	NM001012.1	Ribosomal protein S8	6.7
KHG73T1	X69181	Ribosomal protein L31	8.9
KHA8T2	NM000996.1	Ribosomal protein L35a	8.0
KHA19N1	AF154830.1	Carbamyl phosphate synthetase I	963
KHA25T2	NM001002.1	Ribosomal protein large P0 (RPLP0)	21
KHA29T1	X03562	Insulin-like growth factor II (IGF II)	51
KHA44T1	L22154	Ribosomal protein L37a	8.7

(D) Differentially Downregulated Genes in Association Without the Functional HBV Protein Expression

Clone no.	Accession no.	Description	Fold induction by Northern
KHC15N2	J03048	Hemopexin mRNA, 3'end	8.5
KHC20N1	M11725	C-reactive protein gene	59
KHC20N2	M64982	Fibrinogen alpha chain gene	2.7
KHC26N2, KHC26N5	X97260	Metallothionein isoform 2	Over 1000
KHC31N1	AL024458	Keratin, type II cytoskeletal psuedogene	2.1
KHC33N2, KHC33N3, KHC35N1	L05920	Serum amyloid A protein	83
KHC74N1	NM006744.1	Retinol binding protein	5.2
KHG27N1, KHG27N2, KHG64N1	M15656	Aldolase B (ALDOB)	483
KHG36N1, KHG54N3, KHA20T3	D000096	Prealbumin	31.5
PT8			4.8
KHG45N1, KHG45N2	V00494	Serum albumin	9.0
KHG56T4	L11910	Retinoblastoma susceptibility gene	4.9
KHG58N1	D10040	Long-chain acyl-CoA synthetase	7.8
KHG58N2	U08021	Nicotinamide N-methyltransferase (NNMT)	49
KHA20T4, KHA54N1, KHA54N2	M13692	Alpha-1 acid glycoprotein	43.3
KHA28N1	NM005651.1	Tryptophan 2,3-dioxygenase (TOD2)	1.9
KHA53N3	NM000669.1	Alcohol dehydrogenase	3.4
KHA75N1	AF004340	Mitochondrial gene (ATPase 8/6)	2.9

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Identification of Disease Markers by Differential Display

Prion Disease

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Summary

In order to identify molecular markers of prion disease in peripheral tissues, we used the differential display reverse-transcriptase polymerase chain reaction (DDRT-PCR) procedure to compare gene expression in spleens of infected and uninfected mice. In this study, we identified a novel erythroid-specific gene that was differentially expressed as a result of prion infection. We were able to demonstrate that a decrease in the expression levels of this transcript in hematopoietic tissues was a common feature of prion diseases. Our findings suggest a previously unknown role for the blood erythroid lineage in the development of prion diseases and should provide a new focus for research into diagnostic and therapeutic strategies.

Key Words: Prion disease; EDRF; AHSP; erythroid-specific; SSCP; BSE; differential display.

1. Introduction

Prion diseases are a group of fatal transmissible neurodegenerative disorders that affect a wide variety of mammalian species. Research to identify the infectious agent led to the novel concept that the infectious agent is composed solely of a misfolded protein isoform (PrP^{Sc}) with an identical primary amino acid sequence to the host-encoded cellular Prion protein (PrP^C). In recent years, these disorders have gained particular prominence owing to the epidemic of bovine spongiform encephalopathy (BSE/mad cow disease) in UK cattle, the

resulting appearance of new variant Creutzfeldt-Jakob disease (vCJD) in humans, and the outbreak of Chronic Wasting Disease (CWD) in the North American deer population. The public health concerns surrounding these diseases are likely to be exacerbated by the recent reports of infectivity in blood and muscle (*1,2*). The pathological effects of disease occur predominantly in the central nervous system (CNS), where common hallmarks include vacuolation, gliosis, accumulation of disease-specific PrP^{Sc}, and neuronal cell death (*3,4*). Whereas the pathological effects of disease on the CNS are morphologically well-defined, the underlying molecular events are poorly characterized, and there is little information available regarding the molecular effects of these diseases in tissues other than the CNS. However, the lymphoreticular system, and the spleen in particular, has been shown to have a key role in replication of the prion agent in rodent models (*5–7*), although the precise mechanisms are unclear (*8,9*). In order to identify molecular markers of prion disease in non-CNS tissues, we used the differential display reverse-transcriptase polymerase chain reaction (DDRT-PCR) procedure to specifically identify genes differentially expressed as a result of transmissible spongiform encephalopathy (TSE) infection. We used the original DD procedure as described by Liang and Pardee (*10*) with the addition of a modified single-strand conformational polymorphism (SSCP) procedure to purify the cDNA of interest from any co-migrating, nondifferentially expressed cDNAs (*11*). In addition, to ensure that isolated clones represent the original differentially expressed transcript, we routinely screen cloned cDNAs by hybridization to nylon membranes containing duplicate DDRT-PCR reactions (*11,12*) (see **Fig. 1** and **Note 1**).

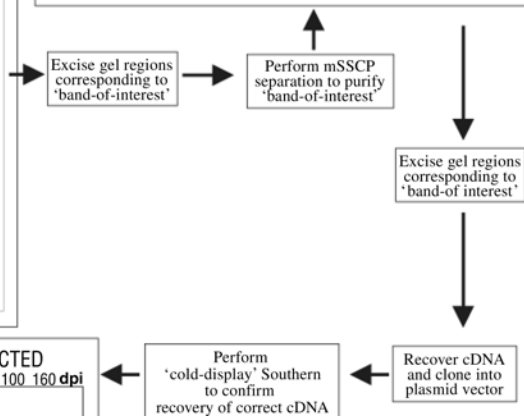
1.1. Experimental Design

Age- and sex-matched mice from an inbred line (C57 BL/6J) were inoculated intra-cerebrally with 20 μ L of a 10% brain homogenate from healthy C57 BL/6J mice (control), or from C57 BL/6J mice at the terminal stage of a scrapie (Me7) infection (infected). Six control and six infected mice were sacrificed at 10, 20, 30, 40, 60, 80, 100, and 160 (terminal) days-post-infection (dpi) (see **Notes 2** and **3**).

2. Materials

1. RNABee (Ambion).
2. Double-autoclaved water.
3. Polytron Homogenizer (Radleys).
4. Spectrophotometer for RNA/DNA measurement.
5. First-strand cDNA synthesis kit (Amersham Biosciences).
6. Primers (dT)₁₂-VC, (dT)₁₂-VG, (dT)₁₂-VT, (dT)₁₂-VA.
7. Primers (random 10mers) $\times$ 20.
8. PCR block (Biometra GmbH, Maidstone, Kent, UK).

9. dNTPs (dATP, dGTP, dCTP, and dTTP).
10. Taq polymerase.
11. 10X Taq polymerase buffer: 100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3.
12. Genomix LR DNA Sequencer (Beckman-Genomix).
13. [α -³⁵S-dATP](1000 Ci/mmol; Amersham Biosciences).
14. Mineral oil.
15. HR1000 Acrylamide (Beckman Genomix).
16. Gel loading tips.
17. 3MM blotting paper (Whatman).
18. Sterile disposable scalpels.
19. Biomax MR film (33 × 62 cm).
20. Glogos™ II autorad markers (Stratagene).
21. See-DNA (Amersham Biosciences).
22. [α -³³P]-dATP (2500 Ci/mmol), Amersham Biosciences.
23. SSCP loading buffer: 80% formamide, 1mM ethylenediamine tetraacetic acid (EDTA), 10 mM NaOH, 0.01% bromophenol blue, 0.01% xylene cyanol.
24. MDE gel solution (Flowgen, Staffs, England, UK).
25. Extended primers containing *Eco*RI restriction sites, e.g., (GTCAGAATTC + random 10mer).
26. Phenol/Chloroform/Isoamyl alcohol (25:24:1), pH 8.0.
27. pBluescript II KS (Stratagene).
28. Calf Intestinal Alkaline Phosphatase.
29. T4 DNA Ligase (Roche).
30. 10X Ligase Buffer: 20 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 60 mM KCl, 50% glycerol (v/v), pH 7.5.
31. Ampicillin.
32. 5'-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) 10 mg/mL in dimethylformamide.
33. IPTG: Isopropyl- β -D-thiogalactoside.
34. 15 mL Falcon 2059 polypropylene tubes.
35. XL10 Gold ultracompetent cells (Stratagene).
36. NZY+ Broth: 1% NZ amine (casein hydrolysate), 0.5% yeast extract, 0.5% NaCl. Adjust to pH 7.5 with NaOH. Autoclave, then add (per liter) 12.5 mL of 1 M MgCl₂, 12.5 mL of MgSO₄, and 20 mL of 20% (w/v) glucose. Filter-sterilize.
37. RNA sample buffer: 50% formamide, 18% formaldehyde, 10% 10X MOPS; 22% double-autoclaved water.
38. MOPS : 0.2 M 3[n-morpholino] propane sulphonic acid, 0.05 M sodium acetate, 0.01 M EDTA, pH to 7.0 with acetic acid.
39. Hybond-N (Amersham Biosciences).
40. 1X SSC buffer: 0.15 M sodium chloride, 0.015 M sodium citrate, adjust to pH 7.0 with sodium hydroxide.
41. Salmon sperm DNA.
42. Transfer RNA.



Western blot analysis showing p150 expression. The top row is labeled 'CONTROL' and 'INFECTED'. Below each label are time points: 60, 80, 100, 160 dpi. The blot shows a single band for p150. In the CONTROL lanes, the band is present at all time points. In the INFECTED lanes, the band is absent at 60 and 80 dpi, and appears at 100 and 160 dpi.

Perform
'cold-display' Southern
to confirm
recovery of correct cDNA

If 'cold-display' Southern replicates original DDRT-PCR profile, perform Northern analysis to confirm differential expression

CONTROL

INFECTED

10 20 30 40 60 80 100 160 10 20 30 40 60 80 100 160 dpi

Fig. 1. Schematic illustration of overall differential display (DD) strategy. In order to circumvent problems associated with unrelated cDNAs co-migrating with the “band of interest” (*see Note 1*), we have modified the original DD strategy to include two further steps. Following DD-polymerase chain reaction (PCR) and visualization of product by gel electrophoresis (I), the area corresponding to the band of interest is excised from all gel lanes. The cDNAs recovered from these gel slices are subjected to a further five rounds of PCR in the presence of ^{33}P -dATP. The resulting products are

43. [α - ^{32} P] dCTP (3000 Ci/mmol), Amersham Biosciences.
44. RediprimeTM II Random Prime Labeling System (Amersham Biosciences).
45. TE buffer: 0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0.
46. 6X Type III loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol.
47. 1X TBE buffer: 0.054 M Tris-borate, 0.001 M EDTA.

3. Methods

3.1. Tissue Collection

Animals were sacrificed humanely, spleens collected, flash-frozen in liquid nitrogen within 5 min of sacrifice and then stored at -80°C until required. Frozen tissue was transferred from -80°C to liquid nitrogen and directly from liquid nitrogen to RNABee for RNA extraction.

Fig. 1 (*continued from opposite page*) denatured, allowed to self-reanneal, and then resolved by MDE electrophoresis, which separates nucleic acids of identical size on the basis of sequence-dependent conformation (II). Co-migrating sequences are visualized as bands of equivalent intensity in control and infected samples, while the “band of interest” maintains the expression profile observed on the original differential display gel (*). Gel slices corresponding to this region are excised, cDNA recovered and subjected to a further five rounds of PCR amplification, and cloned into Bluescript plasmid. To ensure that the cloned inserts correspond to the original band identified by DD rather than some contaminating cDNA still present, we have introduced a “cold-display Southern” step. Here the original DDRT-PCR reactions are repeated in the presence of unlabeled nucleotides and the resulting products resolved by agarose electrophoresis before transfer to nylon membranes. These membranes are then hybridized with radiolabeled inserts from candidate clones. If cDNA corresponding to the original band of interest has been successfully isolated, the cold-display Southern produces a strong signal that corresponds to the original expression profile in a matter of hours, by autoradiography (III). If probed with an insert cloned from a contaminating/co-migrating sequence, the cold-display Southern produces a weak signal that does not replicate the original expression profile even after autoradiography for 1–2 d. Following the successful isolation of cDNAs (I, II, III), it is necessary to confirm that the expression profile observed on the original DD is genuine rather than an artifact of the sample material or the extraction procedures. We routinely confirm differential expression by Northern analysis of RNA extracted from samples obtained from a separate experiment (IV).

3.2. RNA Extraction

RNA used to prepare cDNA for DD-RT-PCR should be extracted as a single batch to minimize variation in RNA quality caused by subtle day-to-day differences in extraction procedures (*see Note 4*).

1. Homogenize the tissue samples in RNA-Bee (1 mL/50 mg tissue) on ice using a Polytron homogenizer (3×15 s at full speed).
2. Add 0.2 mL of chloroform per 1 mL of RNA-Bee and shake vigorously for 30 s.
3. Place the samples on ice for 5 min, then centrifuge the homogenate at 12,000g for 15 min at 4°C.
4. Transfer 80% of the aqueous phase to a clean tube, add 0.5 mL of isopropanol, mix, and then store the samples for 10 min at room temperature.
5. Centrifuge the samples at 12,000g for 5 min at room temperature.
6. Remove the supernatant and wash the RNA pellet with 1 mL of 75% ethanol.
7. Centrifuge for 5 min at 7500g at room temperature.
8. Remove the supernatant and air dry the pellet for 5–10 min on ice.
9. Dissolve the RNA pellet in double-autoclaved water.
10. Take a sample of the RNA for quantitation and store the remaining RNA as an ethanolic precipitate using 0.1 vol of 3 M sodium acetate pH 5.2 and 2.5 vol of 100% ethanol.

3.3. cDNA Synthesis

The steps below describe the synthesis of first-strand cDNA from total RNA and its storage for use in the DDRT-PCR reaction.

1. Centrifuge a volume containing 8 µg of RNA at 10,000g for 25 min at 4°C.
2. Discard the supernatant and wash the pellet with 250 µL of 85% ethanol.
3. Centrifuge the sample at 10,000g for 5 min, discard the supernatant, and dry the pellet at 45°C for 2 min, and then resuspend the RNA in 7 µL of RNase-free water.
4. Take 2 µL of this sample, dilute to 100 µL with water, and quantitate it in a spectrophotometer at 260 nm wavelength.
5. 5 µg of this RNA in a 5 µL vol should be used to synthesize first-strand cDNA (first-strand cDNA synthesis kit). Add the 5 µg of RNA to 1 µL of 200 mM DTT, 5 µL of bulk first-strand mixture, and 4 µL of either (dT)₁₂ VA, (dT)₁₂ VG, (dT)₁₂ VC; or (dT)₁₂ VT primer (25 µM: V = A, G, or C).
6. Incubate the reactions at 37°C for 1 h and then heat at 95°C for 10 min to inactivate the reverse transcriptase.
7. Dispense the reactions into 1 µL aliquots and then store at –20°C.

3.4. DDRT-PCR

The next steps in the procedure outline the set-up of the DDRT-PCR reaction and display of the reaction products on a native polyacrylamide gel. All steps performed on ice unless otherwise indicated (*see Note 3*).

1. Take 1 µL of cDNA and dilute to 133 µL in sterile distilled water.

2. Remove 10 μL of the diluted cDNA (equivalent to the amount of cDNA produced from 25 ng RNA) and add this to 2 μL of random primer (5 μM).
3. Overlay this mixture with 30 μL of mineral oil.
4. Place 8 μL of master mix containing 2 μL 10X PCR buffer (100 mM Tris-HCl, 15 mM MgCl_2 , 200 mM KCl, pH 8.3), 2 μL dNTPs (20 μM), 0.3 μL Taq DNA polymerase (1.5 U), 2 μL (dT)₁₂-VN (25 μM), 1 μL of [α -³⁵S] dATP (1000 Ci/mmol), and 0.7 μL H₂O in each tube and centrifuge briefly.
5. Incubate reaction tubes in a Uno Thermoblock™ at 94°C (2 min) followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 2 min and extension at 72°C for 30 s. A final extension step at 72°C for 5 min should be carried out.
6. Add 4 μL of loading dye to each reaction tube.
7. Load 8 μL of each sample onto a 6% nondenaturing HR1000 Genomix LR™ Polyacrylamide gel.
8. Run the sample for 2.5 h at 2700 V (50°C) on a Genomix LR DNA sequencer.
9. Transfer the gel to 3MM blotting paper, dry, place Glogos™ on the edges of filter paper to allow alignment of the dried gel with the autorad, and expose overnight to BIOMAX MR film.

3.5. Modified SSCP (mSSCP)

The following steps describe the procedures used to purify the cDNA of interest from co-migrating cDNA species (*see* **Note 1** and **Fig. 1**). This procedure is a modification of standard SSCP protocols (*II*).

3.5.1. Recovery of DNA

1. Align the differential display gel with the autoradiograph using the Glogos.
2. From selected “control” and “infected” lanes excise gel regions corresponding to bands representing candidate cDNAs using sterile disposable scalpels (*see* example of a differential display gel; **Fig. 2A**).
3. Rehydrate the gel fragments by incubation at room temperature for 15 min in 100 μL of water.
4. Elute the cDNA by heating to 99°C for 15 min.
5. Transfer the aqueous phase to a fresh 0.5-mL microcentrifuge tube.
6. Precipitate the DNA by adding 0.5 μL See-DNA, 0.1 vol of 3 M sodium acetate, pH 5.2, 2.5 vol of ethanol, and store on dry ice for 1 h.
7. Centrifuge the DNA at 10,000g for 25 min at 4°C.
8. Wash the DNA pellet with 250 μL 75% ethanol, dry at 45°C for 2 min and then resuspend in 4 μL of distilled H₂O.

3.5.2. mSSCP-PCR

1. To the 4 μL of DNA recovered from the DD gel (*see* **Subheading 3.5.1.**) add 4 μL of 10X PCR buffer, 3.2 μL dNTPs (2.5 mM dGTP, dCTP, dTTP, 0.025 mM dATP), 2.5 μL modified oligo (dT) primer (20 μM), 2.5 μL random primer (20 μM), 0.3 μL Taq DNA Polymerase (1.5 U), and 0.5 μL [α -³³P] dATP (1000 Ci/mmol), then adjust the reaction volume to 40 μL with sterile water.

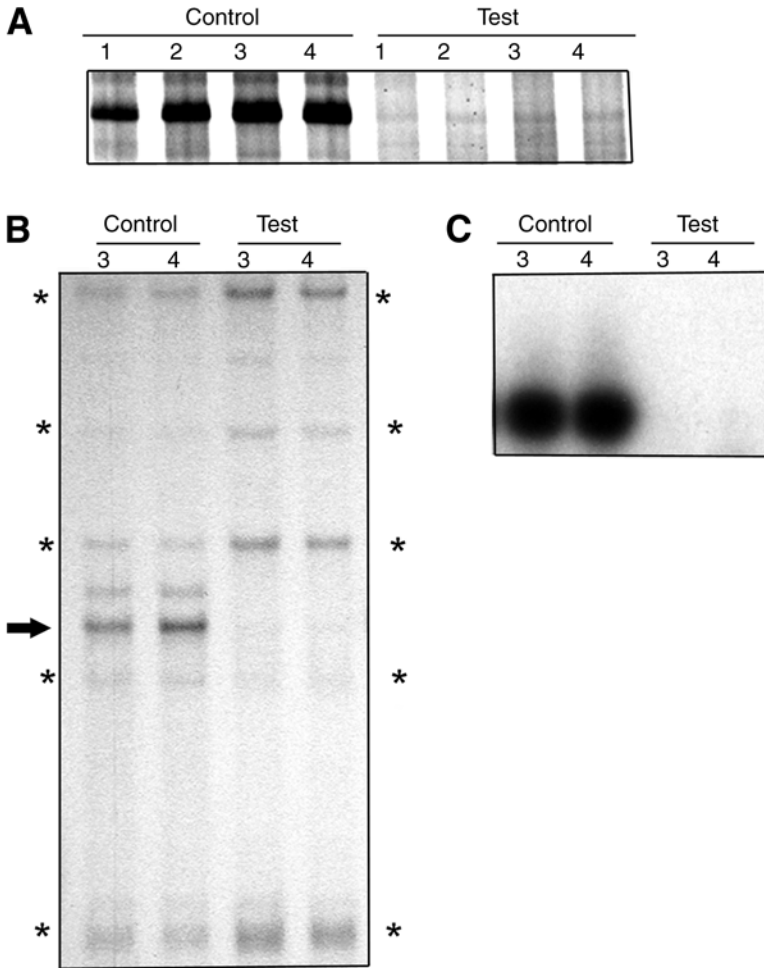


Fig. 2. Example of differential display reverse-transcriptase polymerase chain reaction (DDRT-PCR) modifications. Cloning of differentially expressed transcripts identified by DDRT-PCR. (A) Shows a section of a DDRT-PCR gel containing a band clearly differentially expressed between control and infected samples (equivalent to step I in Fig. 1). Gel slices corresponding to this band are excised from lanes 3 and 4 of both control and infected time-courses. Following recovery from gel slices, cDNA samples are reamplified and resolved by mSSCP electrophoresis. (B) Shows a section of an mSSCP gel containing the band of interest (arrow) and a number of co-migrating cDNAs indicated by asterisks (equivalent to step II in Fig. 1). cDNA representing the band of interest is recovered from the mSSCP gel and cloned into a plasmid vector. Cloned cDNA insert is gel-purified and used as a probe in hybridization to repeat reactions of original DDRT-PCR. (C) Shows hybridization of cloned cDNA to cold-display Southern (equivalent to step III in Fig. 1). Replication of the original differentially expressed profile confirms successful cloning of cDNA of interest.

2. Incubate the reaction tubes in a Uno Thermoblock™ at 94°C (2 min) followed by five cycles of denaturation at 94°C for 30 s, annealing at 40°C for 2 min and extension at 72°C for 1 min. A final extension step at 72°C for 5 min should be carried out.
3. Remove the mineral oil from the reaction and precipitate the PCR product for 1 h on dry ice by adding 0.5 µL See-DNA, 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of 100% ethanol.
4. Wash the pellets with 250 µL of 75% ethanol.
5. Centrifuge at 10,000g for 5 min at 4°C.
6. Aspirate the supernatant and air dry the pellets.
7. Resuspend the pellets in 8 µL of SSCP loading buffer.
8. Denature the sample at 95°C for 10 min then load samples onto a 0.5X MDE™ gel.
9. Electrophorese the samples for 18 h at 300 V, 8W, 25°C in 0.6X TBE buffer.
10. Transfer the gel to 3MM filter paper dry, fix Glogos™ to the edge of the filter paper, and expose to BIOMAX MR film.
11. Align gel and autoradiograph and excise areas of the gel corresponding to the band of interest.
12. Elute cDNA and precipitate as described in **Subheading 3.5.1.** (example of mSSCP shown in **Fig. 2B**).

3.6. Reamplification Using Extended Primers

The next steps describe the reamplification of cDNAs that have been recovered from the mSSCP gel. This involves reamplification for five cycles in the presence of a modified version of the random 10mer and (dT)₁₂ primers used in the original display reaction. Primers have been modified to include *EcoRI* restriction sites to facilitate cloning of the amplified fragment.

1. Spin the precipitated cDNA recovered from the mSSCP gel (**Subheading 3.5.2.**) at 10,000g for 25 min at 4°C.
2. Wash the pellet with 250 µL of cold 75% ethanol.
3. Centrifuge at 10,000g for 10 min.
4. Aspirate the supernatant and air dry the pellet.
5. Resuspend the pellet in 4 µL of sterile distilled H₂O.
6. To the 4 µL of cDNA, add 4 µL of 10X PCR buffer, 3.2 µL of dNTPs (10 mM), 2.5 µL of extended oligo (dT) primer (20 µM), 2.5 µL of extended random primer (20 µM), 0.3 µL of Taq DNA Polymerase (1.5 U), then adjust the volume to 40 µL with sterile dH₂O.
7. Overlay with 30 µL mineral oil.
8. Centrifuge the tubes briefly then incubate in a UNO Thermoblock™ at 94°C for 2 min followed by five cycles of denaturation at 94°C for 30 s, annealing at 58°C for 2 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min.
9. After amplification remove the mineral oil.
10. Make up the aqueous volume to 100 µL with sterile water.
11. Add 100 µL of Phenol/Chloroform/IAA to the sample and mix for 15–30 s.
12. Centrifuge at 10,000g for 10 min at room temperature.

13. Remove the aqueous phase into a fresh tube, taking care to avoid the interphase.
14. Add an equal volume of chloroform and shake the tube for 15–30 s.
15. Centrifuge at 10,000g for 10 min at room temperature.
16. Remove the aqueous phase into a fresh tube.
17. Precipitate the PCR products with 0.5 μ L of See-DNA, 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of ethanol, and store on dry-ice for 1 h.
18. Centrifuge the samples at 10,000g for 25 min at 4°C.
19. Aspirate the supernatant and wash the pellet with 250 μ L cold 75% ethanol.
20. Centrifuge the samples at 10,000g for 10 min at 4°C.
21. Aspirate the supernatant, air dry the pellet, then resuspend in 4 μ L sterile water.

3.7. Cloning SSCP-Purified, Reamplified DNA

The next steps describe the cloning of reamplified DNA into Bluescript KS II plasmid and transformation into XL10-Gold ultracompetent cells.

3.7.1. Digestion of Reamplified DNA With EcoRI

1. To the 4 μ L of DNA (*see Subheading 3.6.*), add 5 μ L of 10X restriction enzyme buffer, 1 μ L of *EcoRI* (10 U/ μ L) and adjust the volume to 100 μ L.
2. Incubate at 37°C for 1 h.
3. Add 100 μ L of Phenol/Chloroform/IAA to the sample and vortex for 5–10 s.
4. Centrifuge at 10,000g for 10 min at room temperature.
5. Remove the aqueous phase into a fresh tube taking care to avoid the interphase.
6. Add an equal volume of chloroform and vortex for 15–30 s.
7. Centrifuge at 10,000g for 10 min at room temperature.
8. Remove the aqueous phase into a fresh tube.
9. Precipitate the digestion reaction with 0.5 μ L See-DNA, 0.1 vol of 3 M sodium acetate, pH 5.2, 2.5 vol ethanol, and store on dry ice for 1 h.

3.7.2. Ligation Into pBluescript II KS

1. Centrifuge the precipitated DNA at 10,000g for 25 min at 4°C.
2. Remove the supernatant, then wash the pellet with 250 μ L of cold 75% ethanol.
3. Centrifuge at 10,000g for 10 min and aspirate the supernatant.
4. Air dry the pellet and reconstitute in 7 μ L of sterile water.
5. To the 7 μ L of DNA, add 1 μ L (10 ng) of *EcoRI*-digested, CIAP-treated, pBS II KS 1 μ L of T4 DNA ligase, and 1 μ L of 10X ligase buffer.
6. Incubate the reaction overnight at 16°C.

3.7.3. Transformation Into XL10-Gold Ultracompetent Cells (see Note 5)

1. Thaw the XL10-Gold ultracompetent cells on ice.
2. Gently mix the cells by hand.
3. Aliquot 100 μ L of the cells into a pre-chilled 15-mL Falcon 2059 polypropylene tube.

4. Add 4 μL of the β -mercaptoethanol mix provided with the cells.
5. Swirl the contents of the tube and incubate on ice for 10 min, swirling gently every 2 min.
6. Add 2 μL of the ligation reaction (*see Subheading 3.7.2.*) to 100 μL of cells.
7. Incubate on ice for 30 min.
8. Pre-heat NZY + broth in a 42°C water bath.
9. Heat-shock the tubes at 42°C for 30 s.
10. Incubate on ice for 2 min.
11. Add 0.9 mL of pre-heated (42°C) NZY + broth to the tubes and incubate at 37°C for 1 h shaking at 225–250 rpm.
12. Plate out the whole of the transformation reaction on LB-ampicillin, X-gal, IPTG, agar plates.
13. Incubate the plates overnight at 37°C.

3.8. Cold-Display Southern Blot Analysis

3.8.1. Southern Blotting of DDRT-PCR Products

The following procedure allows the rapid screening and validation of clones generated by the DDRT-PCR method.

1. Perform DDRT-PCR reactions as described in **Subheading 3.4.** except that radioactive nucleotide is replaced with unlabeled dATP.
2. To the completed DDRT-PCR reaction add 4 μL of gel loading dye and centrifuge briefly.
3. Load 6 μL of this reaction onto a 1% TBE-agarose gel.
4. Electrophorese at 80 V until the bromophenol-blue dye has run about three-fourths of the gel length.
5. Transfer the DNA to nylon membrane by capillary action, overnight, in 20X SSC.
6. Bake the nylon membrane at 80°C for 2 h.

3.8.2. Preparation of Probes (for Cold-Display Southern and for Northern Analysis)

1. Take 25 ng of gel purified DNA insert and adjust to 45 μL volume in TE buffer.
2. Denature the DNA at 99°C for 10 min and then snap-cool on ice for 5 min.
3. Add 5 μL of [α -³²P] dCTP (3000 Ci/mmol) to a Rediprime vial followed by 45 μL of the denatured DNA.
4. Mix the contents.
5. Incubate the labeling reaction at 37°C for 15 min.
6. Purify the labeled probe by Sephadex[®] G-50 Nick[™] columns.

3.8.3. Cold-Display Southern Hybridization

1. Pre-hybridize the cold-display membrane in 20 mL of 0.5 M sodium phosphate pH 7.2/7% SDS containing 100 $\mu\text{g/mL}$ of denatured salmon sperm DNA and 40 μL of 10 mg/mL tRNA for 1 h at 65°C.

2. Add 1.5×10^6 cpm/mL of denatured radiolabeled probe to the hybridization solution and hybridize overnight at 65°C.
3. Wash the membrane for 2×15 min in 50 mM sodium phosphate pH 7.2/0.1% SDS.
4. Expose for 1 h at -80°C to BIOMAX-MS film (for example of cold-display Southern; see **Fig. 2C**).

3.9. Northern Blot Analysis

3.9.1. Preparation of Sample

1. Centrifuge 10 µg of RNA per sample at 10,000g for 25 min at 4°C.
2. Aspirate the supernatant and wash the pellet with 500 µL of cold 85% ethanol.
3. Centrifuge at 10,000g for 10 min at 4°C.
4. Remove the supernatant and dry the pellet at 45°C for 2 min.
5. Resuspend the pellet in 15 µL of RNA sample buffer.
6. Heat the RNA to 65°C for 10 min then place on ice.
7. Add 4 µL of 6X Type III loading dye and 1 µL of ethidium bromide (10 mg/mL) to the RNA.

3.9.2. Preparation of 1% Agarose Formaldehyde Gel

1. To 1 g of agarose add 10 mL 10X MOPS and 73 mL of double-autoclaved water.
2. Melt the agarose mixture in a microwave, then allow it to cool to 60°C in a fume hood.
3. Add 17 mL of formaldehyde to the molten agarose and mix thoroughly.
4. Rinse the gel tank and comb in 0.2 M sodium hydroxide, then double-autoclaved water.
5. Pour the gel.

3.9.3. Gel Electrophoresis

1. Load the denatured RNA samples (see **Subheading 3.9.1.**) and run the gel at 80 V for 3 h.
2. Rinse the gel in distilled H₂O for 1×30 min and then 10X SSC for 1×30 min.
3. Transfer the RNA onto Hybond®-N membrane overnight by capillary action, in 10X SSC buffer.
4. After transfer, bake the membrane at 80°C for 2 h.

3.9.4. Northern Hybridization

1. Pre-hybridize the membrane in 20 mL of 0.5 M sodium phosphate, pH 7.2/7% SDS containing 100 µg/mL of denatured salmon sperm DNA and 25 µg/mL of denatured tRNA for 1 h at 65°C.
2. Add 1.5×10^6 cpm/mL of denatured radiolabeled probe to the hybridization solution and hybridize overnight at 65°C.
3. Wash the blot for 2×15 min in 50 mM sodium phosphate, pH 7.2/0.1% SDS.
4. Expose overnight at -80°C to BIOMAX-MS film.

3.10. Application Example

In this study, we used DDRT-PCR to compare the expression profiles of approx 10,000 transcripts in control and infected spleens at each of eight timepoints during the development of prion disease. With a single exception, the expression profiles of all spleen transcripts appeared identical between control and infected animals. The single exception represented a band that clearly showed a decrease in intensity level in the spleens of prion-infected mice, compared with control mice. The difference in intensity of this band was most pronounced at the later stages of disease (*see Fig. 3*). Gel slices representing this band were excised from both control and infected lanes and the cDNA recovered and subjected to mSSCP purification. cDNA was then recovered from the mSSCP gel, reamplified, and cloned into Bluescript plasmid. Sequence analysis of this cDNA revealed 100% homology to an unpublished sequence deposited in the public nucleotide databases, and designated erythroid differentiation related factor (*Edrf*). The original DD reactions were repeated with unlabeled nucleotides and the PCR products separated on a standard 1% agarose gel and capillary transferred to nylon membrane. Hybridization of radiolabeled *Edrf* cDNA to these DD products showed the expected expression profile and confirmed the successful isolation of the “band of interest.”

Northern blot analysis of RNA isolated from spleens of prion-infected and control mice confirmed that levels of the *Edrf* transcript are dramatically decreased at the terminal stages of disease (**Fig. 4**). Average *Edrf* RNA expression levels in spleens of infected mice compared with spleens of control mice were 75% over the 40–60 dpi period, 55% over the 60–80 dpi period, and 35% over the 80–100 dpi period. By the terminal stages of disease (162 dpi); *Edrf* levels in spleens of infected mice were only approx 3% of the levels in spleens of control mice. In addition, Northern blot analysis of RNA from spleens from both mice and hamsters infected with a number of different strains of prion agents confirms the substantial decrease in levels of the *Edrf* transcript (**Fig. 5**). Thus, differential expression of *Edrf* in spleen is clearly a feature common to experimentally induced rodent prion diseases. In order to determine the normal tissue distribution of *Edrf* expression, we performed Northern blot analysis of RNA from multiple murine tissue sources (**Fig. 6**). From this analysis, *Edrf* clearly shows a severely restricted pattern of expression, with transcripts present only in spleen, bone marrow, and blood, and with highest levels in bone marrow. A similar analysis on human tissue RNAs revealed an even more restricted pattern of *Edrf* expression. In humans, *Edrf* expression was confined to blood and bone marrow, with no detectable expression in spleen RNA (data not shown). These analyses revealed that normal expression of *Edrf* correlates

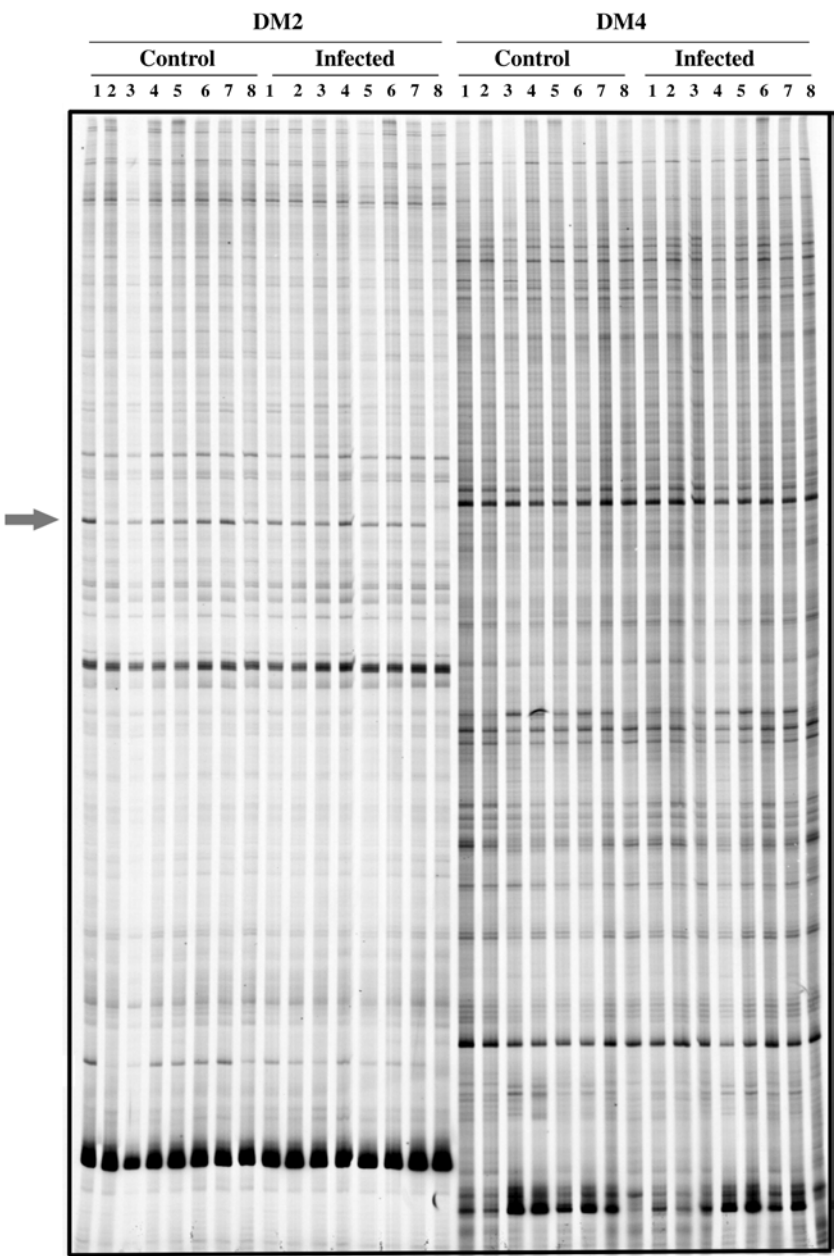


Fig. 3

closely with sites of hematopoiesis in both these species (**13,14**). To determine whether the effect of prion disease on *Edrf* expression seen in spleen was common to all *Edrf*-expressing tissues, we compared *Edrf* expression in RNA isolated from bone marrow and whole blood of prion-infected and control mice (see **Fig. 7**). As with spleen, the level of the *Edrf* transcript is significantly decreased in bone marrow and whole blood of prion-infected mice compared with uninfected control animals. Having established that reduced levels of *Edrf* expression in hematopoietic tissues is a common feature of prion diseases in rodents, we extended these findings to other species and to natural prion diseases, specifically BSE in cattle and scrapie in sheep. Using a degenerate PCR primer strategy, we amplified and cloned a partial cDNA for bovine *Edrf*. Using the bovine *Edrf* cDNA as a probe, we performed Northern blot analyses to determine the expression levels of *Edrf* in hematopoietic tissues of cattle with BSE and sheep with scrapie. This analysis revealed that, compared with healthy animals, expression levels of *Edrf* are significantly decreased in the bone marrow of cattle displaying initial clinical signs of BSE (see **Fig. 8**) and in whole blood of sheep displaying clinical signs of scrapie (data not shown). We performed further studies in order to determine which hematopoietic cells express *Edrf* and, consequently, which of these cells are functionally compromised as a result of prion disease (**15**) (data not shown). This analysis confirmed that *Edrf* expression is restricted to cells of the erythroid lineage. The earliest stage of hematopoietic development at which *Edrf* is expressed is the bi-potential progenitor cell capable of developing along either the megakaryocyte or erythroid lineages. Thereafter, *Edrf* expression is confined to the erythroid lineage with higher levels of expression in blast-forming (BFU-E), colony-forming (CFU-E), and maturing erythroid cells.

In this study, we observed a previously unrecognized effect of prion disease on erythroid tissues. The levels of *Edrf*, expressed specifically in cells of the

Fig. 3. (*opposite page*) Differential display reverse-transcriptase polymerase chain reaction (DDRT-PCR) analysis of gene expression in spleens of control and infected mice during the development of prion disease. DDRT-PCR gel shows "RNA fingerprints" from two different primer combinations (DM 2 and DM 4) for eight time points from control and infected mice (1, 2, 3, 4, 5, 6, 7, 8 represent 10, 20, 30, 40, 60, 80, 100, and 160 dpi, respectively). There are no differences between the control and infected profiles obtained using the DM 4 primer combination. The DM 2 primer combination showed a single difference in banding pattern between control and infected samples (arrow). This difference is evident as a decrease in intensity in the lanes representing the later stages of prion infection (compare lanes 5–8 control with lanes 5–8 infected).

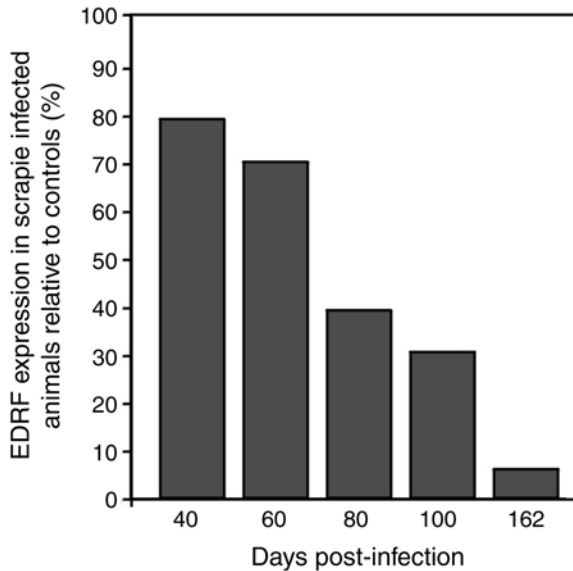


Fig. 4. *Edrf* expression in spleen during prion infection. Expression levels of *Edrf* RNA in spleens of scrapie-infected (Me7 strain) C57BL mice relative to controls throughout disease pathogenesis (normalized for variations in RNA loading). Histograms represent phosphor imager quantitation of signal obtained by Northern analysis. Levels represent the average of values obtained from two experiments. 162 days-post-infection (dpi) represents final stage of disease. (Reprinted with permission of Nature Publishing Group.)

erythroid lineage, are clearly affected in animals during prion pathogenesis. Although the precise involvement of the erythroid lineage is unclear, it is possible that dysfunctional erythroid activity may contribute to the etiology of prion disease. Our data implicating a role for erythroid cells in the progression of prion diseases provides an entirely novel area on which to focus research aimed at therapeutic intervention and non-PrP^{Sc}-based preclinical diagnosis. The involvement of cells of the erythroid lineage may be relevant to recent reports of detectable infectivity in bone marrow from cattle at the clinical stages of BSE (16) and in whole blood sampled from preclinical BSE-infected sheep (1).

Since the original publication of these findings, M. Weiss and colleagues in Philadelphia have demonstrated that the primary function of *Edrf* is to act as a stabilizing protein/chaperone for α -globin during erythrocyte development (17,18).

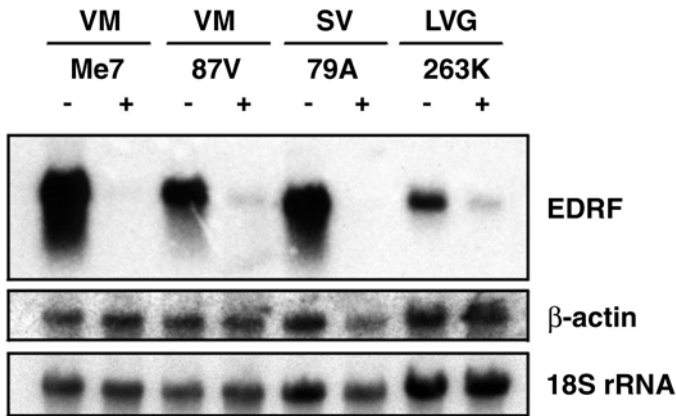


Fig. 5. Northern blot analysis of *Edrf* expression at the terminal stage of prion disease. Spleen *Edrf* RNA-expression levels at the final stages of disease pathogenesis (+) from various rodent models of TSE disease, and controls (–). β -actin is expressed at equivalent levels in all samples and 18S rRNA levels indicate equivalent loading of RNA in all lanes. VM and SV are inbred lines of mice, LVG is a hamster line; and Me7, 87V, 79A, and 263K are rodent experimental scrapie strains. (Reprinted with permission from Nature Publishing Group).

4. Notes

1. Expression studies carried out using clones isolated from DDRT-PCR gels sometimes fail to replicate the differential expression patterns seen on the original display gels. The frequency of “failures” can be quite high and has led some groups to question the validity of the original DDRT-PCR profiles. We believe that, in the majority of cases, the original DDRT-PCR profiles are genuine and that these “failures” represent the isolation of an unrelated cDNA. We believe that these unrelated cDNAs are identical-sized DNA fragments that co-migrate with the band of interest on the display gels and contaminate cDNA recovered from gel slices. In the original DDRT-PCR reactions, these contaminants are present at lower levels than the band of interest and do not incorporate sufficient radionucleotide to be visualized. Under standard DDRT-PCR protocols, 40 cycles of PCR are sufficient to amplify these contaminating cDNAs to levels equivalent to those of the cDNA of interest. These contaminants can then be cloned with equal efficiency to the cDNA representing the original band of interest, and naturally do not replicate the original expression profile when used in downstream expression studies.

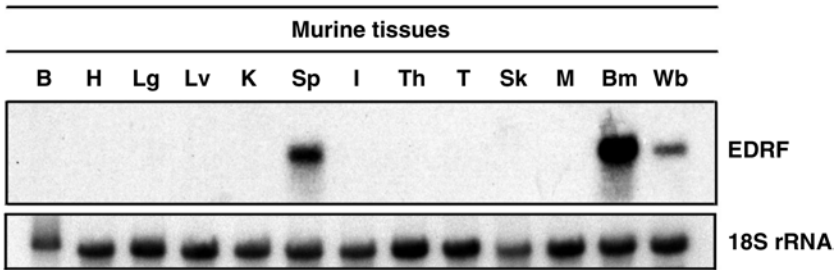


Fig. 6. Tissue distribution of murine *Edrf* expression. Northern analysis of *Edrf* expression in multiple mouse tissues. Abbreviations: B, brain; H, heart; Lg, lung; K, kidney; Sp, spleen; I, intestine; Th, thymus; T, testis; Sk, skin; M, muscle (skeletal); Bm, bone marrow; Wb, whole blood. 18S rRNA levels indicate equivalent loading of RNA in all lanes. (Reprinted with permission from Nature Publishing Group.)

Under our procedures, all recovered cDNA species can be visualized following radiolabeling with ^{33}P and resolution by mSSCP. This allows the cDNA species replicating the original expression profile to be identified and purified from contaminating cDNA of identical length.

2. A major problem with the DDRT-PCR studies is the isolation of differentially expressed transcripts that are irrelevant to the biological process under study. These false positives are not the result of technical difficulties but rather reflect genuine variation in the samples analyzed. Strenuous efforts are necessary to minimize variation in the biological samples analyzed, to ensure that any differences in gene expression observed are related to the biological process being studied. For example, in studies investigating the incubation times of different strains of prion agent, it is not uncommon to inject control animals with saline and test animals with prion brain homogenate, and then simply monitor animals and record the time taken to develop disease. Although appropriate for these studies, such an approach could lead to problems in a DDRT-PCR study. In our DDRT-PCR analysis, control animals were injected with homogenate from healthy brains and test animals with prion brain homogenate. Had we not adopted this approach, it is possible that any differential expression we observed could be owing to differences in the responses to saline and to brain homogenate, rather than owing to the development of disease. Genuine differences in gene expression can result from apparently trivial differences in the treatment of samples; metabolic studies can be affected by the time of day animals are sacrificed, cell culture experiments can be affected by the position of the culture vessel within the incubator. DDRT-PCR users must also be aware of natural variation in gene expression levels in the starting material: inbred lines of mice show very little variation in expression levels of particular genes between individuals, whereas non-inbred species can show considerable variation in the normal levels of a specific gene product between individuals. When using inbred lines of mice, we

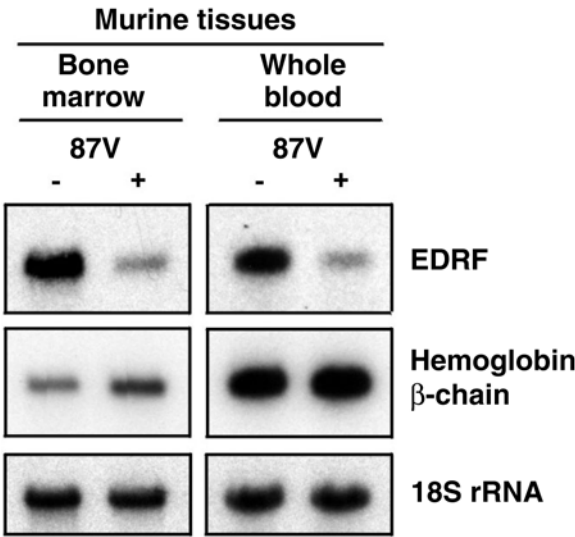


Fig. 7. Northern analysis of *Edrf* expression in blood and bone marrow of mice with experimentally induced prion disease. *Edrf* RNA levels in infected mice (+) are decreased relative to controls (–) in both bone marrow and whole blood of mice at the terminal stages of prion disease pathogenesis following infection with the 87V scrapie strain. Hemoglobin β -chain is expressed at similar levels in bone marrow and blood of control and scrapie-infected mice. 18S rRNA hybridization confirms equivalent loading of RNA in all lanes. (Reprinted with permission from Nature Publishing Group.)

pool material from six individuals for each sample, whereas for non-inbred species, we pool material from 25 to 50 individuals in an attempt to minimize this natural variation. We also ensure that all animals included in our study are sourced from a tight age group and are all of a single sex. We believe that in a properly designed DDRT-PCR study, there should be a very small number of genuine differences in expression between control and test samples and that it is necessary to display a large number of bands in order to identify these differences. If a screen of 1000 bands produces 5–10 differences, it is unlikely that these are relevant to a specific biological process.

- 3. In DDRT-PCR, differences in band intensity between samples may be artifactual as a result of technical problems in RNA extraction (see **Note 4**), cDNA synthesis or the PCR reaction itself. Naturally processing such bands will be time-consuming and ultimately will be unrewarding. We believe that displaying single (or pairs of) test and control samples is likely to lead to the identification of artifactual differences; however, we also believe that the majority of such artifacts can be identified and ignored with appropriate experimental design. For instance, we routinely design experiments to contain multiple timepoints at which samples are collected. By processing these samples in parallel, all bands of con-

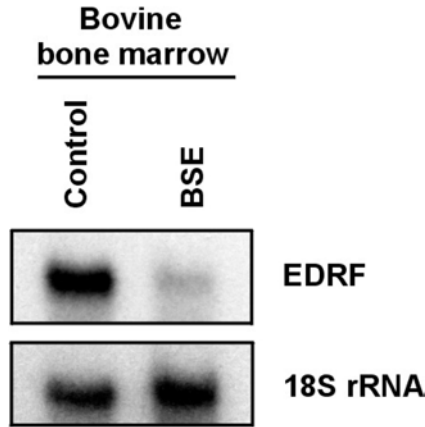


Fig. 8. *Edrf* expression in BSE. Northern analysis of *Edrf* expression in the bone marrow of healthy and BSE-infected cattle. 18S rRNA levels indicate equivalent loading of RNA in all lanes. (Reprinted with permission of Nature Publishing Group.)

stant intensity effectively become controls for the equivalent bands in other lanes, and technical artifacts are easily identified. For example, in **Fig. 3**, Control lane 3 of the DM 2 primer combination is clearly different than all other control lanes, suggesting a slight difference in efficiency between this PCR reaction and the others displayed here. If a particular sample is consistently different with different primer combinations, it suggests a problem with the efficiency of cDNA synthesis and new cDNA should be generated from all RNA samples. If the same problem is observed with the new cDNA, this suggests an artifactual difference introduced in the RNA extraction step. Although obvious in the example shown here, artifacts of this type can be fairly subtle and may manifest as a decrease or increase in intensity of a small numbers of bands. In such cases, differences should be evaluated in the context of the banding patterns of all other gel lanes. In instances where the collection of timed samples is inappropriate, we routinely display a minimum of four test and four control reactions simultaneously (all samples processed independently from tissue extraction step).

4. RNA used in DDRT-PCR analyses should be of the highest quality that is *practically* possible to obtain. However, we believe that reproducibility is more important than absolute quality, i.e., even if RNA samples are not of the highest quality, a DDRT-PCR analysis can still be performed if all samples are of similar quality. We find that subtle differences in the RNA extraction procedure, when carried out on different days (even by the same individual) can result in RNA samples of different quality that generate apparent differences in DDRT-PCR banding patterns; these are, of course, artifacts. For this reason, all RNA samples used for a particular DDRT-PCR analysis are extracted in a single session by one individual. cDNA for DDRT-PCR analysis is also synthesized in a single session, dispensed into single-use aliquots, and stored frozen.

5. Following recovery from mSSCP gels, it is necessary to amplify the cDNA of interest prior to cloning. Even following mSSCP, it is theoretically possible that small quantities of co-migrating cDNA species remain, and, as with all PCR reactions, it is also possible to introduce DNA contamination at this stage. To minimize the risks associated with these possibilities, we routinely perform only five cycles of PCR to achieve incorporation of *EcoRI*-modified primers. Unlike the standard 40 cycles of PCR, this reaction results in very small quantities of PCR product. In order to maximize the efficiency of our cloning process, we routinely use XL10 Gold Ultracompetent cells with a transformation efficiency of 5×10^9 cfu/ μ g.

Acknowledgments

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Saturation Screening for p53 Target Genes by Digital Fluorescent Differential Display

Yong-jig Cho, Susanne Stein, Roger S. Jackson II, and Peng Liang

Summary

Differential display (DD) is one of the most commonly used approaches for identifying differentially expressed genes. Despite the great impact of the method on biomedical research, there has been a lack of automation of DD technology to increase its throughput and accuracy for a systematic gene expression analysis. Most of previous DD work has taken a “shotgun” approach of identifying one gene at a time, with limited polymerase chain reaction (PCR) reactions set up manually, giving DD a low-technology and low-throughput image. With our newly created DD mathematical model, which has been validated by computer simulations, global analysis of gene expression by DD technology is no longer a shot in the dark. After identifying the “rate-limiting” factors that contribute to the “noise” level of DD method, we have optimized the DD process with a new platform that incorporates fluorescent digital readout and automated liquid handling. The resulting streamlined fluorescent DD (FDD) technology offers an unprecedented accuracy, sensitivity, and throughput in comprehensive and quantitative analysis of gene expression. We are using this newly integrated FDD technology to conduct a systematic and comprehensive screening for p53 tumor-suppressor gene targets.

Key Words: Differential display; p53 target genes; apoptosis.

1. Introduction

Cancer is a disease state caused by multiple genetic alterations, which lead to unregulated cell proliferation. The most frequently mutated gene among all genes known to be involved in human cancers is the tumor suppressor p53. A major outcome of such mutations is inactivation of the biochemical and biological functions of the wild-type (wt) p53 protein. Among the biological effects elicited by wtp53, the best documented are cell-cycle arrest and programmed

cell death (apoptosis). p53 is a transcription factor that can mediate many downstream effects by the activation or repression of target genes. The tumor suppressor p53 is activated by a variety of cellular stresses such as heat shock, hypoxia, osmotic shock, and DNA damage (e.g., UV), which in turn leads to growth arrest and/or apoptosis. Apoptosis is likely to be the most important function of p53 in suppressing tumor formation. However, the mechanism by which p53 actually induces apoptosis remains to be determined. p53 can induce expression of proteins that target both the mitochondrial and the death-receptor-induced apoptotic pathways. Presently, particular interest has focused on identifying target genes that mediate p53-induced apoptosis, because the induction of programmed cell death appears to be critical component of p53-mediated tumor suppression and because of the therapeutic potential of reactivation of this response in tumors. Recently, several p53 target genes were reported, which appear to contribute to p53-dependent apoptosis pathways (1–4). Remarkably, many of these p53 target genes were found by differential display (DD) method (4). However, the identification of additional, if not all, p53 target genes is of great importance, because this could provide the missing link between p53-mediated apoptosis and tumor suppression.

In this chapter, we present the procedure of DD and also discuss some critical factors affecting the accuracy of the method. DD methodology was invented in 1992 (5). Traditionally, DD is based on ^{33}P radioactive labeling of cDNA bands. This is the most commonly used DD technology, because of its sensitivity, simplicity, versatility, and reproducibility. Since its creation, numerous differentially expressed genes have successfully been identified in diverse biological fields such as cancer research, developmental biology, neuroscience, plant physiology, and many other areas. Recently, a very similarly sensitive DD method was established by fluorescent labeling (6). This is the first nonradioactive DD system with sensitivity equivalent to that of the ^{33}P isotopic labeling method. Fluorescent labeling utilizes automation, which can greatly increase the throughput and accuracy of DD.

The general strategy of fluorescence differential display (FDD), which is very similar to radioactive DD, is outlined in **Fig. 1**. Total RNA is used for DD comparison in gene expression. Removal of all chromosomal DNA from the RNA samples with DNase I is essential before carrying out DD, so that interfering bands amplified from genomic DNA can be unmasked. The principle of the DD and FDD method is to detect differential gene expression patterns by reverse transcription polymerase chain reaction (RT-PCR) using one of the three individual one-base anchored oligo-dT primers that anneals to the beginning of a subpopulation of the poly(A) tails of mRNAs. The anchored oligo-dT (H-T₁₁V) primers consist of 11 Ts (T₁₁) with a 5' *Hind*III (AAGCTT) site, plus one additional 3' base V (V may be dG, dA, or dC), which provides

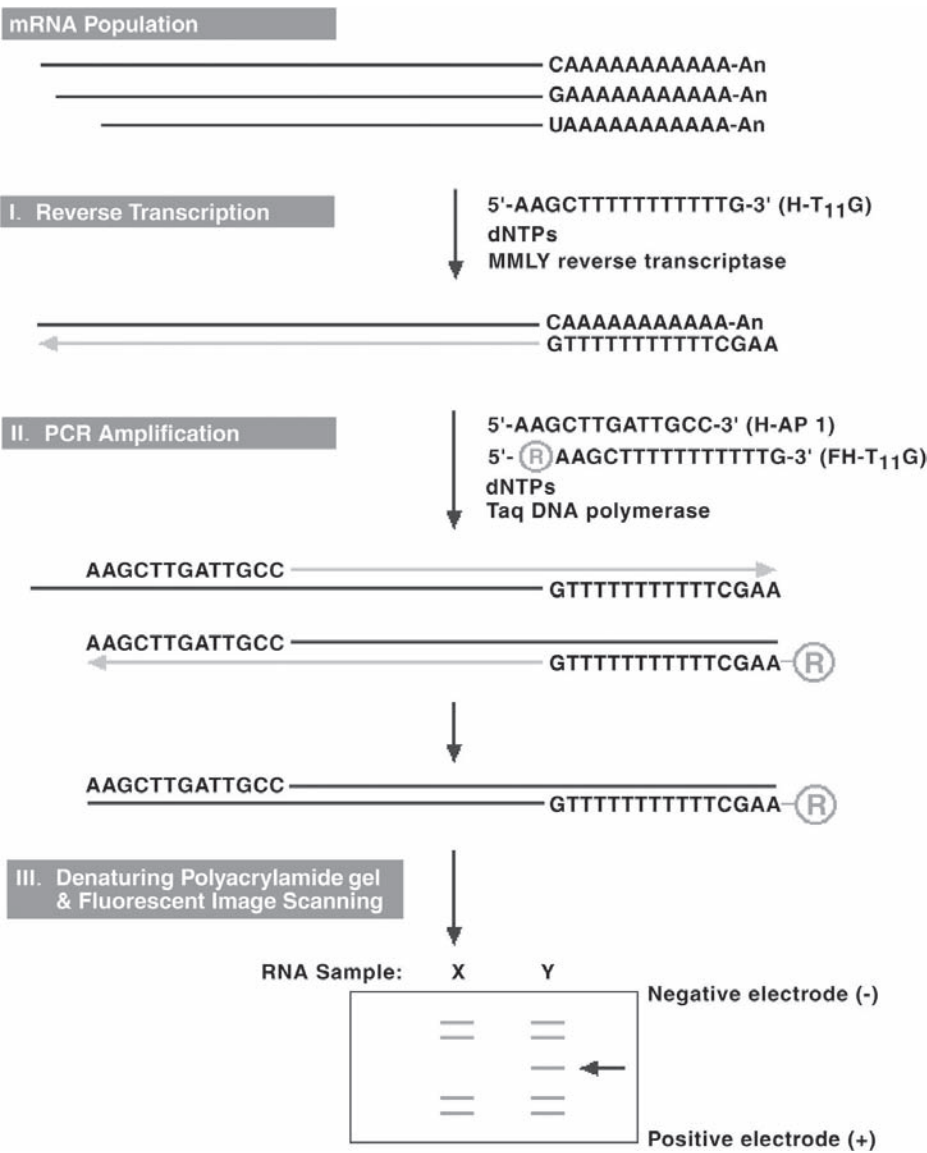


Fig. 1. Schematic representation of fluorescent differential display (FDD) method. (Illustration courtesy of GenHunter.)

specificity. For FDD, fluorescent (Rhodamine, red)-labeled anchored R-H-T₁₁V primers are combined with various arbitrary primers (13mer, also containing a 5' *Hind*III site, H-AP primers) in the PCR steps. The amplified PCR

products up to 700 bp can be separated on a denaturing polyacrylamide gel. FDD image can be obtained using a fluorescent laser scanner. Side-by-side comparisons of cDNA patterns between or among relevant RNA samples reveal differences in gene expression. The cDNA fragments of interest can be retrieved from the gel, purified, and reamplified with the same set of primers (but without fluorescent labeling of H-T₁₁V primer) under the same PCR conditions as in the initial FDD-PCR reactions. For further molecular characterization, the obtained reamplified PCR fragments can be sequenced directly with arbitrary primers, or cloned first before sequencing if a reamplified cDNA band contains more than one mRNA species. DNA sequence analysis of these cDNA fragments by Blast Search of the Genbank (<http://www.ncbi.nlm.nih.gov/BLAST/>) may provide bioinformatics information on whether a gene identified by DD is known, homologous to known, or novel.

The final step of the DD procedure is to confirm the differential expression of the mRNA identified by DD through Northern blot analysis using the same RNA samples as used for DD screenings. Such analysis provides not only confirmation by a method independent of DD, but also information about the size and relative abundance of the gene. After confirmation by Northern blot, the cloned cDNA probe can be used to screen a cDNA library for full-length clones, which can be used for functional characterization of the gene.

2. Materials

2.1. RNA Isolation and RNA Purification

1. RNApure[®] Reagent (GenHunter, Nashville, TN).
2. MessageClean[®] Kit (GenHunter), 10X Reaction buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂.
3. DEPC-treated dH₂O (GenHunter).
4. RNA Loading Mix (GenHunter).
5. 10X MOPS buffer: 0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 0.01 M ethylenediamine tetraacetic acid (EDTA) (store in dark).

2.2. RT Reaction and FDD-PCR

1. 5X Reverse Transcription (RT) buffer: 125 mM Tris-HCl, pH 8.3, 188 mM KCl, 7.5 mM MgCl₂, 25 mM DTT (GenHunter) (*see Note 1*).
2. MMLV Reverse Transcriptase (100 U/μL) (GenHunter) (*see Note 1*).
3. dNTP Mix (2.5 mM) (GenHunter) (*see Note 1*).
4. H-T₁₁V anchor primer (V = A, C, G) (2 μM) (GenHunter) (*see Note 1*).
5. R-H-T₁₁V anchor primer (V = A, C, G) (2 μM) (GenHunter) (*see Note 1*) (Rhodamine-labeled primers are light sensitive).
6. H-AP 13mer primers (1–160) with 50–70% GC content (2 μM) (GenHunter) (*see Note 1*).

7. 10X PCR buffer: 100 mM Tris-HCl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin (GenHunter) (see **Note 1**).
8. FDD Loading dye: 99% formamide, 1 mM EDTA, pH 8.0, 0.009% xylene cyanol FF, 0.009% bromophenol blue (GenHunter) (see **Note 1**).
9. Rhodamine Locator Dye (GenHunter) (see **Note 1**).
10. 10X TBE buffer (for 1 L): 108 g Trizma base, 55 g boric acid, 3.7 g EDTA.
11. Autoclaved double-distilled H₂O [dH₂O] (GenHunter).
12. Taq DNA polymerase (5 U/μL) (Qiagen).

2.3. Northern Blot Analysis

1. 20X SSC buffer: 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0.
2. 100X Denhardt's solution (for 500 mL): 10 g Ficoll 400, 10 g polyvinylpyrrolidone MW 360,000, 10 g bovine serum albumin (BSA) fraction V (store at -20°C).
3. Hybridization buffer: 5X SSC, 50% formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and fresh 100 μg/mL heat-denatured, sheared, nonhomologous salmon sperm DNA (store at -20°C).
4. HotPrime[®] DNA labeling Kit (GenHunter).

3. Methods

3.1. RNA Isolation From Cell Cultures

Total RNA can be isolated with one-step acid-phenol extraction method by using of RNeasy Reagent.

1. After removal of the cell culture medium, and wash step with 10 mL cold phosphate-buffered saline (PBS), set the flask or plate on ice. Add 2 mL RNeasy Reagent per culture flask to lyse the cells spread the solution by shaking the plate, and let sit on ice for 10 min. Pipet the cell lysate (using a sterile cell scraper) into sterile Eppendorf tubes, and add 150 μL chloroform per milliliter of cell lysate. Finally, mix very well by vortexing for about 10 s. Freeze the tubes at -80°C or proceed to **step 2**.
2. Spin the tubes in an Eppendorf centrifuge at 4°C for 10 min with maximal speed (14,000 U/min).
3. Carefully remove the upper (aqueous) phase and save this phase, which contains the RNA, into a clean new sterile tube.
4. For RNA precipitation, add an equal volume of isopropanol to the aqueous phase, mix well by vortexing, and let sit on ice for 10 min. Spin down the RNA pellet for 10 min at 4°C. Rinse the RNA pellet with 0.5–1 mL cold 70% ethanol (treated with DEPC-dH₂O). Spin down again for 10 min at 4°C. Remove the ethanol and resuspend the RNA pellet in 20–50 μL DEPC-dH₂O. Make RNA aliquots and store the RNA at -80°C.
5. Before treatment with DNase I (see **Subheading 3.2.**), measure the RNA concentration at OD₂₆₀ with a spectrometer, and check the integrity (18S and 28S rRNA bands) of the RNA samples by running about 2 μg each RNA on a 1% agarose gel with 7% formaldehyde.

3.2. DNase I Treatment of Total RNA

Removal of all contaminating chromosomal DNA from the RNA sample is absolutely essential for successful DD. The MessageClean Kit is specifically designed for the complete digestion of single- and double-stranded DNA.

1. Incubate 50 μL (10–50 μg) of total cellular RNA (use DEPC-dH₂O when diluting RNA) with 10 U (1 μL) of DNase I (RNase-free) in 5.7 μL 10X Reaction buffer for 30 min at 37°C.
2. Inactivate DNase I by adding an equal volume of phenol:chloroform (3:1) to the sample. Mix by vortexing and leave the sample on ice for 10 min. Centrifuge the sample for 5 min at 4°C in an Eppendorf centrifuge.
3. Save the supernatant and ethanol precipitate the RNA by adding 3 vol of ethanol in the presence of 0.3 M sodium acetate.
4. After incubation at –80°C for 1 h (overnight to a few days at –80°C is recommended), pellet the RNA by centrifuging at 4°C for 10 min. Rinse the RNA pellet with 0.5 mL of 70% ethanol (made with DEPC-dH₂O) and dissolve the RNA in 20 μL of DEPC-treated dH₂O.
5. Measure the RNA concentration at OD₂₆₀ with a spectrophotometer. Check the integrity of the RNA samples before and after cleaning with DNase I by running 2–3 μg of each RNA on a 1% agarose gel with 7% formaldehyde. It is recommended to store the RNA samples as 1–2 μg aliquots at –80°C before using for DD.

3.3. Reverse Transcription of mRNA

The success of the DD technique depends on the integrity of the RNA and that it is free of chromosomal DNA contamination (*see Subheading 3.2.* and **Note 1**).

1. Set up three RT reactions for each RNA sample in three PCR tubes (0.2–0.5 mL size). Each should contain one of the three different one-base-anchored H-T₁₁V primers (where V may be A, C, or G), for 20 μL final volume: dH₂O 9.4 μL , 5X RT buffer 4 μL , dNTP Mix (2.5 mM) 1.6 μL , total RNA 2 μL (0.1 $\mu\text{g}/\mu\text{L}$ freshly diluted), and H-T₁₁V primer (2 μM) 2 μL . To minimize pipetting errors, it is recommended that a core mix without an RNA template is made for each anchored oligo-dT primer if two or more RNA samples are to be compared.
2. Program the thermocycler to 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, and 4°C for 5 min.
3. 1 μL MMLV Reverse Transcriptase is added to each tube 10 min after at 37°C incubation to initiate the RT reaction. At the end of the reaction, spin the tube briefly to collect condensation. Set tubes on ice for FDD-RT-PCR or store at –20°C for later use.

3.4. Fluorescent Differential Display

The RNAspectra™ Red Kit can be used for this step as well as the aforementioned RT reactions (*see also Notes 1–3*). This step can be automated with

a robotic liquid handling workstation such as BioMek 2000, which can greatly increase throughput and accuracy.

1. Set up on ice (in dim light) a 20 μ L PCR reaction in thin-walled reaction tubes. For each primer set combination, use the following formula: dH₂O 4.2 μ L, 10X PCR buffer 2 μ L, dNTP Mix (2.5 mM) 1.6 μ L, H-AP-primer (2 μ M) 8 μ L, R-H-T₁₁V (2 μ M) 2 μ L RT-mix from **Subheading 3.3**. (it has to contain the same H-T₁₁V primer used for FDD-PCR) 2 μ L, and Taq DNA polymerase 0.2 μ L. Make core mixes as much as possible to avoid pipetting errors.
2. Mix well by pipetting up and down. PCR conditions for 40 cycles are following: 94°C for 20 s, 40°C for 2 min, 72°C for 1 min. Followed by 72°C for 5 min and 4°C for 5 min. Keep samples in the dark after running the PCR reaction and store at -20°C until the gel run.
3. Prepare a 6% denaturing polyacrylamide gel in 1X TBE buffer. Let the gel polymerize for about 2 h before using. It is recommended that one glass plate be treated with Sigmacote® (Sigma) to facilitate the separation of the plates after running. Pre-run the gel for 30 min. Urea in the wells is very critical and must be completely flushed just before loading of the samples.
4. Mix each PCR reaction with 8 μ L FDD loading dye and incubate at 80°C for 2–3 min immediately before loading onto the 6% DNA polyacrylamide gel.
5. Electrophoresis for 2 h at 60 W constant power until the xylene dye (the slower-moving dye) reaches the bottom.
6. After the gel run, clean the outsides of the plates very well with dH₂O and ethanol.
7. Scan the gel on a Fluorescence Imager using a 585 nm filter (for Rhodamine).
8. Cut out the bands of interest, after carefully separating the glass plates. For orientation of the lanes, a very helpful hint is to use the Rhodamine Locator Dye.

3.5. Purification and Reamplification of cDNA Bands From FDD

1. Soak the gel slice (*see Subheading 3.4., step 8*) in 1 mL dH₂O for 30 min mixing gently by finger-tipping.
2. Remove the water without taking the gel slice, and again add new 200 μ L dH₂O. Boil the tube with tightly closed cap for 15 min to elute the DNA template from gel. Spin for 2 min to collect condensation and pellet the gel. Transfer the supernatant to a new tube, and keep as cDNA template for reamplification reaction. The tube with the gel slice must be also saved for the reamplification PCR.
3. Re-amplification should be carried out in a total volume of 40 μ L using the same primer combination and concentration (4 μ L of each 2 μ M primer), but without fluorescent-labeled anchor primer. The PCR conditions should be also the same except the dNTP concentrations is changed: use 1 μ L of a dNTP mix (250 μ M). As DNA template can be used: (1) 4–5 μ L from supernatant **step 2**, and/or (2) the gel slice, which contains still little trace of the removed DNA (**step 2**).
4. Check 30 μ L of each PCR sample on a 1.5–2% agarose gel (depends on the expected cDNA length) stained with ethidium bromide. Save the remaining PCR samples at -20°C for future experiments (e.g., cloning, Northern blotting). Compare the size of the reamplified PCR products with the originally found length on

the FDD gel. Extract the reamplified cDNA fragments from the agarose gel with a Qiaex II Gel Extraction Kit (Qiagen).

3.6. Sequencing and Cloning of PCR Products

One crucial advantage of FDD is the rapid identification of the cDNA sequence by direct sequencing of the PCR products without subcloning of these fragments. Despite this, after gel purification from **step 4** of **Subheading 3.5.**, reamplified cDNA probes could be ligated into various cloning vector systems, and then subjected to DNA sequence analysis.

3.7. Confirmation of Differential Gene Expression by Northern Blot

The confirmation of the differential gene expression found by the FDD procedure by Northern blotting is very important because the method is extremely sensitive and details can be obtained about the exactly mRNA size of the screened cDNA fragments. The Northern blot can be performed by using the HotPrime DNA labeling kit, following the standard procedure (7). The HotPrime DNA labeling Kit is up to 10 times better in labeling efficiency than traditional Random-Prime kit. This high labeling efficiency is attributed to the use of random decamers instead of hexamers, as well as anchored oligo-dT primers, which ensure that the full-length cDNA probe is made from DD cDNA fragments.

One should remember that the DD method is unlikely to be able to detect mutations at the DNA level directly. For diseases caused by single gene mutations that have clear a genetic component, chromosome mapping of the mutation locus should be the method of choice. It should be emphasized that the method is only a simple screening tool. However, after confirmation by Northern blot, the differentially expressed cDNA probe(s) can trigger a series of molecular studies to understand complex pathways.

3.8. Specific Applications of Fluorescence Differential Display

Decades of research have clearly demonstrated that p53 tumor suppressor plays a pivotal role in curtailing cancer formation in mammals. Loss of functional mutations in p53 have been found in a variety of human cancers (including breast, ovary, colon, and lung). p53 is usually expressed at low level in the nuclei of normal cells. However, many cellular insults (e.g., DNA damage, hypoxia, stress, and so on) result in elevated levels of this protein through post-translational modifications by phosphorylation. The increased expression of the p53 protein protects the genome from accumulating excessive mutations. p53 is well-known as a cell-cycle check-point regulator, promoting cell-cycle arrest and apoptosis in response to DNA damage. The human p53 contains an N-terminal transcriptional activation domain, a central DNA-binding domain,

and a tetramerization domain on the C-terminal region. The central region binds to a consensus DNA sequence and allows p53 to regulate the transcription of a series of genes, the best-characterized of which are *mdm2* and *p21^{WAF/CIP}* (8). Murine double minute 2 *Mdm2* is a proto-oncogene and a negative feedback regulator of the tumor suppressor gene p53. *Mdm2* binds to the N-terminus of p53, represses p53-dependent transactivation of target genes, and is also able to promote the rapid degradation of p53 through the ubiquitin-proteasome pathway. The transcriptional activity of p53 leads to increased expression of *p21^{WAF/CIP}* (cyclin-dependent kinase inhibitor, a universal cell-cycle inhibitor). *p21^{WAF/CIP}* is a direct p53 target gene and deletion of this gene significantly reduces the cell-cycle arrest response to p53 (9).

An intense search for additional p53 target genes, which are involved in the p53-dependent apoptosis pathways, has been carried out by numerous laboratories over the past few years. Many target genes of p53 have been identified that play a role in the p53-dependent as well as p53-independent apoptosis. The DD methodology has played a major role in this effort, because it has uncovered more well-characterized p53 target genes than any other methods, including SAGE and DNA microarrays (1–4). However, at the moment there is still considerable uncertainty about how exactly p53 expression is able to trigger apoptosis, although there is convincing evidence that p53 is necessary for the induction of apoptosis. Therefore, it is necessary to search for additional p53 target genes, which could be integrated in the p53-mediated programmed cell death.

3.8.1. Identification of p53-Induced and p53-Repressed Genes by Fluorescent Differential Display

In an attempt to identify p53 target genes, we used a tetracycline-regulated (“tet-off” system) p53 expression in a colon cancer cell line, DLD-1 (10). This cell line contains an endogenous mutant p53 gene. The p53 protein in this system is induced rapidly after removal of tetracycline. The induction of wild-type p53 leads to the expression of the two well-known p53 target genes: *p21* and *mdm2*, which were confirmed by Western blot analysis (Fig. 2). Some of our FDD results from a comprehensive FDD screening for p53 target genes are shown in Fig. 3. In this screening, total RNA was extracted from the cells at the same time points at 8 h and 12 h either with tet (+tet, no p53 induction), or after tet removal (–tet, increased p53 expression). Two related inducible p53 cell lines, clone A2 (quicker p53 induction starting at 3 h after tet removal) and A4 (slower p53 induction starting at 6 h after tet removal) were compared with different combinations of FDD primers. Excellent reproducibility in the FDD gene expression pattern was obtained that readily revealed good candidate p53 target genes as indicated by arrows (Fig. 3). Our comprehensive FDD screen-

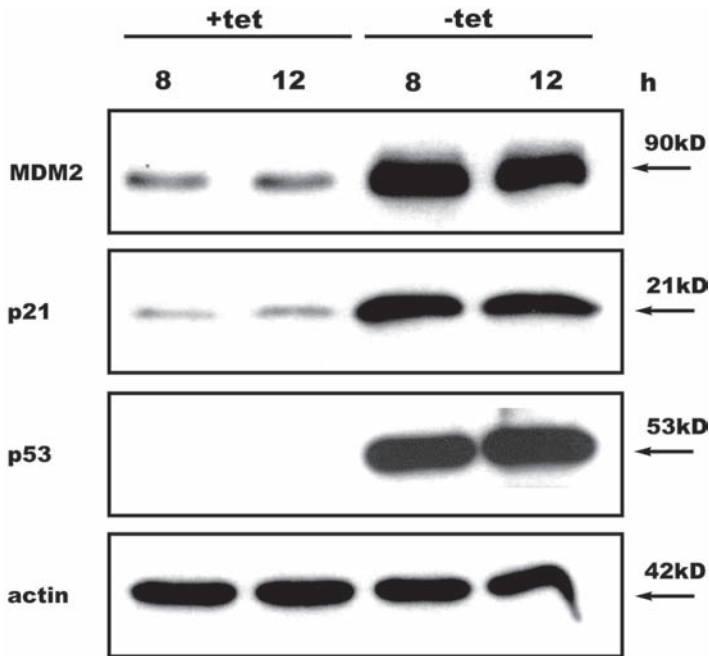


Fig. 2. Western blot analysis of p53 regulated expression of p21 and MDM2 in the tet-regulated DLD-1 cell system. After removal of tetracycline at different time points (8 h-tet and 12 h-tet), protein levels for p53, p21, and MDM-2 were analyzed. The polyclonal antibody (pAb 1801) against p53 was used. The antibodies against MDM2 (SMP-14) and p21 (C-19) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). As control for equivalent protein loading, anti-actin was used (cat. no. A2066, Sigma-Aldrich).

ing using hundreds of FDD primer combinations yielded over two dozen p53-regulated genes (results to be published elsewhere). Among them are p53 itself, displayed by R-H-T₁₁G with arbitrary primer H-AP20, and mdm-2, displayed by R-H-T₁₁A with arbitrary primer H-AP10. This provides a strong validation of our nonbiased and exhaustive screening for p53 target genes by DD strategy. Furthermore, FDD also allows the analysis of digital gene expression profiling and precise quantification of gene expression differences (**Fig. 4**).

The cell system used in our study also showed apoptotic features 16 h after removal of tet. Furthermore, the expression of apoptotic genes such as PIGs, Bax, and GADD45 could be confirmed in the cell system used here (**10**). We have identified numerous p53 target genes (results to be published elsewhere), which are either induced or repressed by p53 using comprehensive FDD screen-

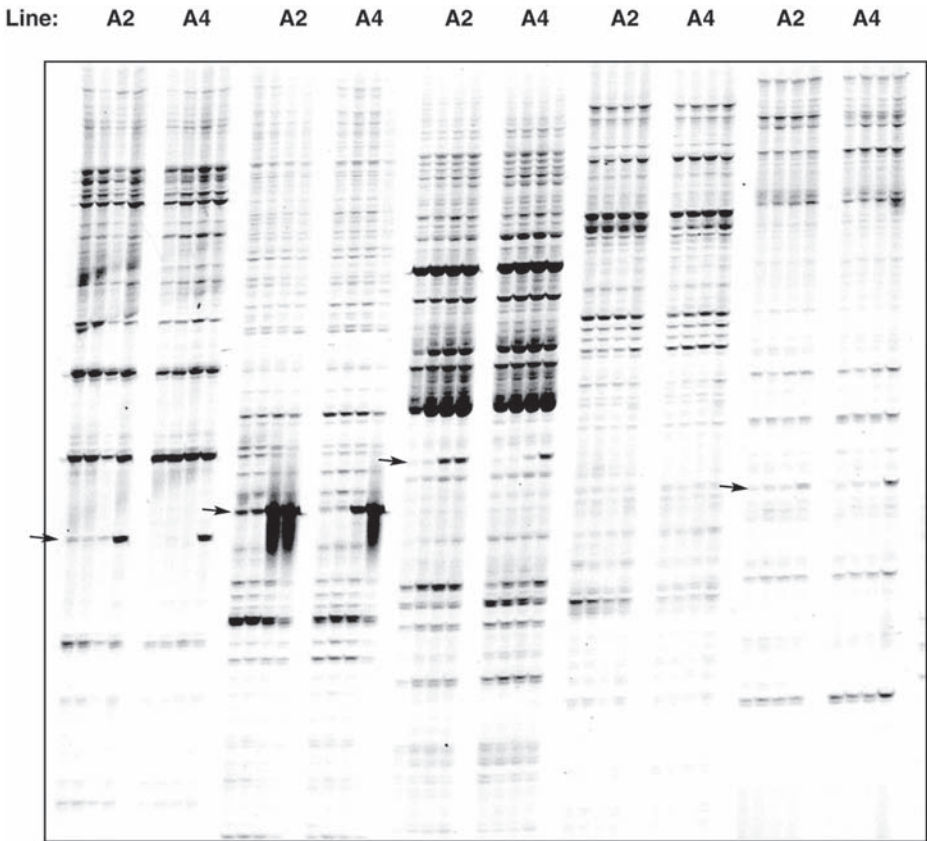


Fig. 3. Representative highly reproducible fluorescent differential display (FDD) image of saturation screening of p53 regulated genes in two related inducible p53 cell lines, A2 and A4 (10). All FDD polymerase chain reaction (PCR) samples were prepared by Beckman Biomek 2000 robot and analyzed by digital FDD fluorescent-labeled anchored primers. Four RNA samples from 4 and 8 h with tetracycline (no p53 induction) and without (p53 induced) were compared side-by-side with different arbitrary 13mers in combinations with G-anchored oligo-dT primer. Note that the induction of p53 did not cause a global change in gene expression. The p53-perturbed gene expression in both inducible cell lines is marked by arrows.

ing. Interestingly, over 50% of these genes represent novel and previously uncharacterized genes. This is in contrast to methodology of DNA microarrays, which can only sample known gene sequences (4). Other advantages of DD over DNA microarrays are the requirement of much less RNA, and the ability

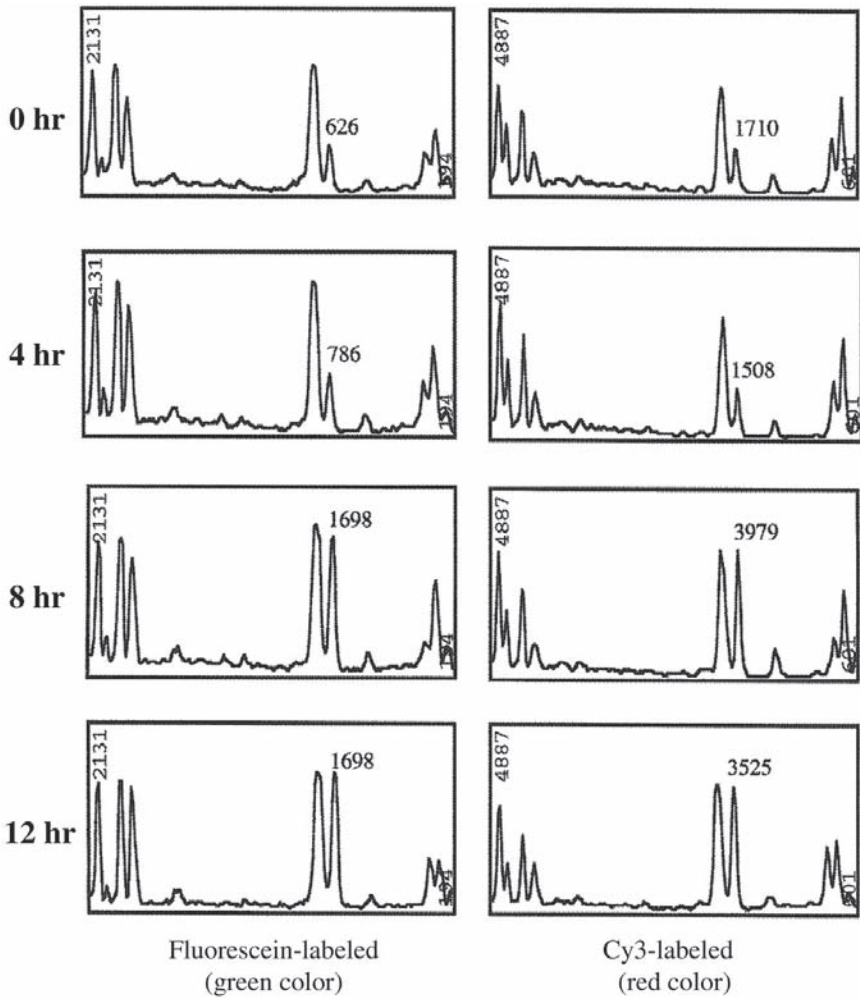


Fig. 4. Digital quantification of differentially expressed genes by fluorescent differential display (FDD). The region of the multicolor FDD image where p21 was located was quantified in either green or red fluorescence using Hitachi FMBIO Analysis software. The peak heights in fluorescent intensity (arbitrary unit) for p21 message before and after 4, 8, and 12 h of p53 induction were obtained. After subtracting the background signal (lower right figure of each window of analysis), the fold of induction of p21 could be derived. The upper left figure indicates the scale of the fluorescence intensity. The maximum induction of p21 was 3.5- and 3.7-fold measured by the green and red fluorescent labels, respectively. (For more detail, *see* ref. 6.)

to compare more than two different RNA samples simultaneously with the capability of detecting rare mRNAs.

4. Notes

1. All materials or products for DD and FDD technology are commercially available from GenHunter. The company also offers automated FDD services (from RT reaction to FDD results).
2. DD is widely used for the identification and isolation of differentially expressed genes, and now the description of the FDD method can open a big area for automated screening for much more differential expressed genes. At the moment, some problems could occur in the technical equipment. For example, check the glass plates for evenness; this is very critical for correct scanning. The comb must fit well between the two glass plates to avoid leaking between lanes. The major problem of FDD is that a fluorescent laser scanner is very expensive.
3. Information to minimize extrinsic and intrinsic factors can be found in recently published papers or reviews (*11,12*).

Acknowledgments

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Identification of p53-Regulated Genes by the Method of Differential Display

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Summary

The p53 tumor-suppressor gene is mutated in a wide range of human cancers. The ability of p53 to control passage through the cell cycle (in G1 and in G2) and to control apoptosis in response to abnormal proliferative signals and stress, including DNA damage, is considered to be important for its tumor-suppression function. p53 is a transcription factor that binds to DNA in a sequence-specific manner to activate transcription of target genes. In this chapter, we describe the application of differential display to identify p53-regulated genes.

Key Words: p53 gene/protein; gene expression; transcription regulation; cDNA cloning; Northern blotting; ubiquitin.

1. Introduction

The p53 tumor-suppressor gene is mutated in a wide range of human cancers (1). The ability of p53 to control passage through the cell cycle (in G1 and in G2) and to control apoptosis in response to abnormal proliferative signals and stress, including DNA damage, is considered to be important for its tumor-suppression function (2). p53 is a transcription factor that binds to DNA in a sequence-specific manner to activate transcription of target genes. The consensus DNA binding sequence for p53 consists of two repeats of the 10 bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by 0–13 bp (3). Mutated p53 alleles typically found in tumors encode defective products no longer capable of binding to DNA or activating transcription. There is now compelling evidence that the transcriptional activity of p53 is required for its growth-suppressing and tumor-suppressing activities (4,5). p53 has also been

implicated as a transcriptional repressor (6,7); however, neither the physiological significance nor the mechanism of p53-mediated repression is known.

Friend virus-transformed mouse erythroleukemia cells that lack endogenous p53 protein expression and express a transfected temperature-sensitive (ts) p53 mutant allele provide a well-characterized model to investigate the role of p53 in regulating G1 arrest and apoptosis. The p53ts protein contains Val instead of Ala at amino acid position 135, and behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C (8). The DP16.1 erythroleukemia cell line expressing p53ts protein (DP16.1/p53ts) grows well at 37°C. At 32°C, however, these cells undergo apoptosis following expression of the wild-type p53 conformation (9,10). To identify endogenous genes regulated by p53, we prepared RNA from DP16.1/p53ts cells grown at 37°C and 8 h after incubation at 32°C, and analyzed the RNA using differential display (DD) (11).

2. Materials

2.1. RNA Preparation

1. GTC lysis buffer: 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl. Adjust pH to 7.0 with 0.1 N NaOH, filter, and store at room temperature (12). For each 1 mL GTC buffer, add 7 µL β-mercaptoethanol just before use for cell lysis.
2. Phenol: add equal volume of diethyl pyrocarbonate (DEPC)-treated water to solid phenol and leave at room temperature overnight to dissolve. Add 8-hydroxyquinoline to a final 0.1%. Aliquot and store the treated phenol at -20°C.
3. Chloroform/isoamyl alcohol 49:1 (v/v).
4. 2 M sodium acetate, pH 4.0.
5. Isopropanol.
6. DEPC-treated water.
7. 70% ethanol in DEPC-treated water, stored at -20°C.

2.2. Differential Display

1. RNAimage® Kits (GenHunter, Nashville, TN). The kits provide primers and buffers for reverse transcription and PCR amplification. Two additional reagents are needed but not supplied in the kits: α-[³³P] dATP and Taq DNA polymerase.
2. Reagents and apparatus for denaturing sequencing gels.
3. X-ray film (e.g., BioMax-MR).

2.3. PCR Reamplification and Northern Blotting

1. QIAEX II gel extraction silica particles (Qiagen, Valencia, CA), or GENECLAN (Qbiogene, Carlsbad, CA).
2. Polymerase chain reaction (PCR) reagents (with Taq DNA polymerase).
3. Random priming DNA labeling kit.
4. Positively charged nylon membrane.

5. 20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
6. Pre-hybridization buffer: 5X SSC, 5% sodium dodecyl sulfate (SDS), 50% formamide, and 10X Denhardt's. (10X Denhardt's: 0.2% bovine serum albumin [BSA], 0.2% Ficoll 400, and 0.2% polyvinylpyrrolidone.) SDS and Denhardt's are prepared separately and then added to the other components of the buffer. Add SDS last to prevent precipitation by the concentrated SSC.
7. Hybridization buffer: 5X SSC, 5% SDS, 50% formamide, 100 µg/mL sonicated and boiled salmon sperm DNA. Aliquot both buffers and store at -20°C.
8. Northern blot wash Buffer I: 2X SSC, 1% SDS.
9. Northern blot wash Buffer II: 0.2X SSC, 0.1% SDS.
10. PhosphorImage screen (Amersham Biosciences, Piscataway, NJ), and/or X-ray film.

2.4. cDNA Cloning

1. DNA agarose gel extraction kit.
2. Reagents to clone PCR-amplified DNA.
3. LB agar plates.
4. Plasmid mini-prep kit.
5. 5' RACE reagents, including gene-specific primers (GSP), MAP, and MAPT adaptor primers (*see Note 1.*), reverse transcriptase, RNase inhibitor, terminal transferase, DNA cleanup/desalting columns, and PCR reagents (with DNA polymerase, e.g., *pfx* by Invitrogen, Carlsbad, CA, or *Advantage* by Clontech, Palo Alto, CA).

3. Methods

The methods described in **Subheadings 3.1.–3.4.** outline: (1) the preparation of RNA; (2) the method of DD and resolution of the DNA fragments by polyacrylamide gel electrophoresis (PAGE); (3) the extraction of DNA fragments from the gels, reamplification of cDNA fragments, and Northern blotting; and (4) the cloning of full-length cDNA (**Fig. 1**).

3.1. RNA Preparation

1. Prepare RNA from DP16.1/p53ts cells grown at 37°C and 8 h after incubation at 32°C. 1×10^7 cells are centrifuged at 230g for 5 min. After the culture medium is removed, briefly vortex the pellet and add the following solutions: 1 mL GTC, 0.1 mL 2 M sodium acetate, 1 mL phenol, and 0.2 mL chloroform/isoamyl alcohol. Mix by inverting the tube a few times after each solution is added.
2. Transfer the lysate into two 1.5-mL Eppendorf tubes and spin in a microcentrifuge at >12,000g for 10 min at 4°C. Transfer the upper aqueous phase to new Eppendorf tubes and avoid any DNA and protein at the interface. Add an equal volume of isopropanol, mix by vortexing, and store at -20°C overnight. Centrifuge at maximum speed for 20 min at 4°C. After most of the solution is removed, centrifuge the tube for 5 s and remove the residual fluid.

Murine Friend erythroleukemia cells
expressing temperature sensitive p53 (DP16.1/p53ts)

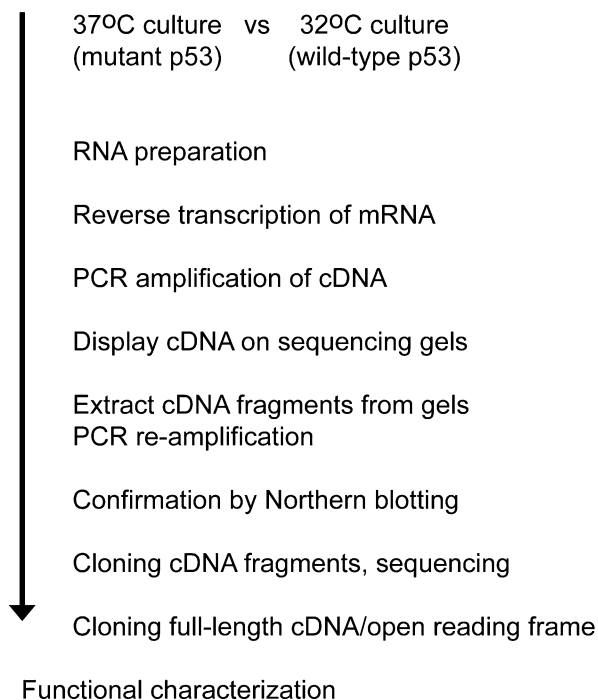


Fig. 1. Flow chart of procedures used in the differential display to isolate p53-regulated genes.

3. (This optional repeat of the extraction will make RNA easier to dissolve in water in the final step.) Resuspend the pellet in 500 μ L GTC buffer. Add 50 μ L sodium acetate, 500 μ L phenol, and 100 μ L chloroform/isoamyl alcohol. Vortex for 10 s and centrifuge at $>12,000g$ for 5 min at 4°C. Transfer the upper aqueous phase to a new tube, mix with an equal volume of isopropanol, and store at -20°C. Finally, precipitate the RNA by centrifugation at maximum speed ($>12,000g$).
4. Rinse the RNA once with 70% ethanol and centrifuge at maximum speed for 5 min at 4°C. Carefully pour off the ethanol, centrifuge the tube for 5 s, and remove the residual fluid. Air dry the RNA by leaving the cap open on bench for 10 min and resuspend the RNA in 50 μ L DEPC-treated water. Store RNA samples at -70°C.
5. Determine RNA concentration by UV spectrophotometry. Examine RNA by agarose gel electrophoresis. Load equal amounts (1–5 μ g) of the RNA on a 1% agarose gel and examine ethidium bromide (EtBr) staining of the RNA. The intact

ribosomal 18S and 28S RNAs indicate good quality of the preparation (*see Note 2*). Make aliquots of the RNA so that each aliquot provides sufficient RNA for a single reverse transcription (RT) reaction, and store these at -70°C . RNA yield varies with cell types, but the general expectation is 10 μg for one million cells.

3.2. Differential Display

DD is carried out with GenHunter RNAImage kits. Because detailed protocols are supplied with the kits, we encourage readers to refer to the supplier's manual for specific information. We will describe the methods briefly with details for some modifications.

3.2.1. Reverse Transcription of mRNA to Produce the First-Strand cDNA

The RNAImage kit provides three oligo-dT primers with one-base specificity (either A, G, or C) anchored to the dT₁₁ sequence, thus producing three different cDNA populations for each RNA sample (*see Note 3*).

3.2.2. PCR Amplification of the cDNA

The three cDNA populations are used directly as templates in the PCR reactions. Each of the original oligo-dT primer is paired with one of 80 arbitrary primers and the total number of PCR reactions is 480 for the comparison of each pair of control and test RNA samples.

3.2.3. Display of the PCR-Amplified cDNA Fragments on 6% Denaturing Sequencing Gels

Dry the sequencing gel onto a sheet of 3M filter paper, which is subsequently taped onto a second sheet of paper to increase rigidity. In the darkroom, place a Kodak BioMax-MR film on the dried gel and tape the film together with the gel on four corners and sides and store in a film cassette overnight at room temperature. Before the film is removed for processing, use a 15-gage needle to puncture through both the film and gel at three spots. These measures help to ensure precise alignment when positive bands are cut from the gel. After the film is processed, cover the dry gel in plastic wrap and store it between two heavy plates.

3.3. Reamplification of cDNA Fragments and Northern Blotting

3.3.1. Extraction of cDNA Fragments From the Dry Sequencing Gel

Select positive bands based on difference between the control and test samples (*see Note 4*). Align the film with the dried gel on the three needle holes. Place small clips along the edge of the film and gel to keep the film in place. Use a 23-gage needle to puncture through both the film and the gel at four corners of the positive bands. After the bands are excised from the gel, cut

the gel fragments into smaller pieces before they are placed into Eppendorf tubes containing 100 μ L of water.

Boil the gel fragments for 15 min and centrifuge for 1 min at maximum speed. Transfer the fluid to a new tube and purify the DNA according to instructions in the QIAEX II system manual for the purification process. Use 10 μ L of silica particles for each DNA fragment and elute DNA in 20 μ L 5 mM Tris-HCl buffer, pH 8.5. Use 10 μ L for PCR re-amplification and store the remainder at -20°C .

3.3.2. PCR Reamplification of the cDNA

Primers for the reamplification are the same as those used in the original PCR. Using the DNA extraction method described in **Subheading 3.3.1.**, we successfully reamplified 90% of the cDNA fragments excised from the gels. A small number of fragments may require an additional round of PCR. The re-amplified cDNA fragments can be used as templates to prepare hybridization probes for Northern blotting and as inserts in cDNA cloning.

3.3.3. Cloning cDNA Fragments

Assess the quality of the reamplified PCR products by subjecting 10% of the PCR reaction to electrophoresis on a 1.5% TBE agarose gel. Those fragments with clean single bands of correct sizes can be used directly for cloning. PCR products with multiple bands or smaller nonspecific fragments need to be separated on an agarose gel and the fragments of correct sizes are then extracted from the agarose gel for cloning. Although many different cloning systems are available today for PCR products, T/A cloning strategy is simple and has worked well in our lab in cloning cDNA fragments isolated from the DD.

3.3.4. Northern Blotting to Verify Differential Expression

We use Northern blotting as the standard method to confirm that the cDNA isolated from the DD is indeed responsive to p53 activation. This confirmation is based on mRNA increase or decrease when p53 is activated after the cell culture temperature is shifted to 32°C . Temperature-dependent changes in gene expression unrelated to p53 are identified by comparing the p53-negative parental cells under the same conditions (**Fig. 2**). We performed Northern blotting twice: first, using PCR reamplified cDNA as probe and second, using the cDNA fragment cloned into a vector as probe. Since the initial cDNA fragments isolated from the dried gel may contain multiple species, the second Northern blot serves to verify that the correct cDNA has been cloned (*see Note 5* for Northern blotting).

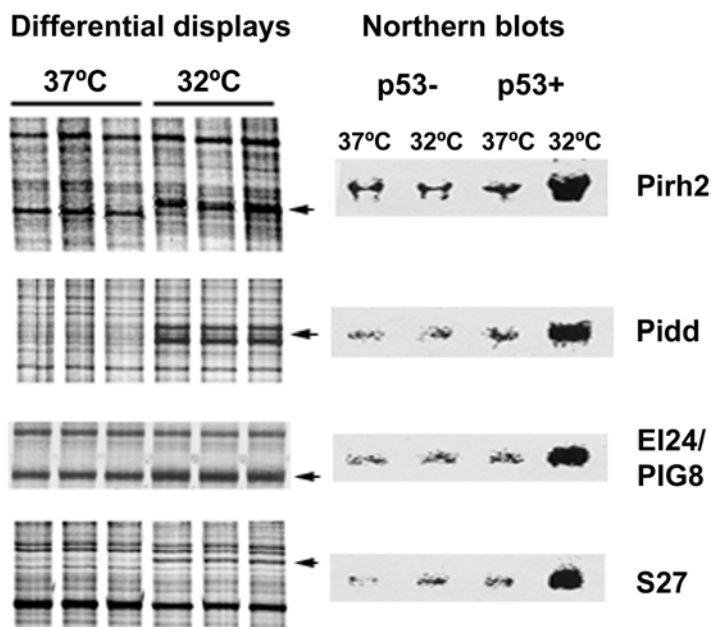


Fig. 2. Isolation of cDNA fragments from sequencing gels and confirmation by Northern blotting. Left column: sequencing gel images of differentially expressed genes. PCR-amplified cDNAs from cells either expressing mutant p53 (37°C) or wild-type p53 (32°C) were displayed side by side as triplicate reactions/lanes. Right column: phosphorimages of Northern blots with cDNA probes isolated from the sequencing gels. p53-negative (p53-) cells were used as control for the temperature shift from 37°C to 32°C. Gene names are shown on the right.

3.4. Cloning Full-Length cDNA

After the cDNA fragments were cloned and sequenced, we found that the sequences could be grouped into a number of categories: (1) known p53 transcriptional targets; (2) known genes, but unknown relation to p53; (3) unknown genes with full-length cDNA sequences in the databases; (4) unknown genes with matches to ESTs in the Unigene database; and (5) unknown gene with no match in the databases. Full-length cDNAs in categories (1) to (3) can be cloned by a number of methods including a simple end-to-end PCR from various cDNA sources. With the rapid increase of cDNA databases, it is very likely that most cDNA isolated from the DD will match EST sequences. The next step is to clone the full-length cDNA. Although library screening remains a

reliable method to obtain full-length cDNA, other faster methods can be considered.

In this section, we describe a standard protocol known as 5' RACE (rapid amplification of cDNA ends) to obtain 5' unknown sequence based on known 3' cDNA fragment (**13**). A 3' gene-specific primer is used to generate the first-strand cDNA by reverse transcription. A poly(dA) chain is added to the 3' end of the first-strand cDNA by terminal transferase and an adaptor poly(dT) primer will anneal to the poly(dA) tail and extend from the 5' end to generate the second strand. 5' cDNA is then generated in two rounds of PCR using gene-specific nested primers and 5' adaptor primers. The PCR reaction is run on an agarose gel and DNA fragments are extracted, cloned, and sequenced (**Fig. 3**; see **Note 1**).

3.4.1. Reverse Transcription

Set up an RT reaction with 1–5 μg total RNA, 0.5 μg gene-specific primer (GSP1, 25 nt) and water to 10.5 μL . Incubate at 65°C for 5 min and 42°C for 5 min. Add 4 μL 5X first-strand buffer, 2 μL 0.1 M dithiothreitol (DTT), 0.5 μL RNase inhibitor, 2 μL 10 mM dNTP, and 1 μL RT (e.g., Superscript II). Incubate at 42°C for 60 min and inactivate the enzyme at 75°C for 10 min.

3.4.2. dA Tailing

Purify the RT reaction with any cleanup/desalting column that can selectively remove the primer. Set up a 20 μL dA-tailing reaction: 20% cDNA from RT, 4 μL 5X buffer, 1.2 μL 25 mM CoCl_2 , 1 μL 30 mM dATP, and 1 μL terminal transferase. Heat the cDNA in water first at 95°C for 2 min, quickly cool on ice, and add the remaining reagents. Terminal transferase is believed to work more efficiently on single-stranded DNA. Incubate at 37°C for 20 min and inactivate the enzyme at 65°C for 10 min. Purify the DNA with any desalting spin column and use the entire eluate for PCR.

3.4.3. PCR Amplification of 5' cDNA With Mapping Adaptor Primers (MAPT and MAP) and 3' Gene-Specific Primers (GSP1 and GSP2, 25 nt)

This step consists of three rounds of PCR reactions: 5' MAPT primer extension, first-round PCR with GSP1 and MAP primers, and second-round PCR with GSP2 and MAP primers.

1. 5' MAPT primer extension. Set up reaction: cDNA from dA tailing reaction, 5 μL 10X PCR buffer, 1 μL dNTP mix (10 mM), 2 μL MAPT primer (10 μM), 2 μL GSP1 primer (10 μM), 1 μL DNA polymerase. Add water to total 50 μL . Set up a PCR program to run two cycles with the following parameters: 94°C, 5 min, 42°C, 2 min and 72°C, 10 min.

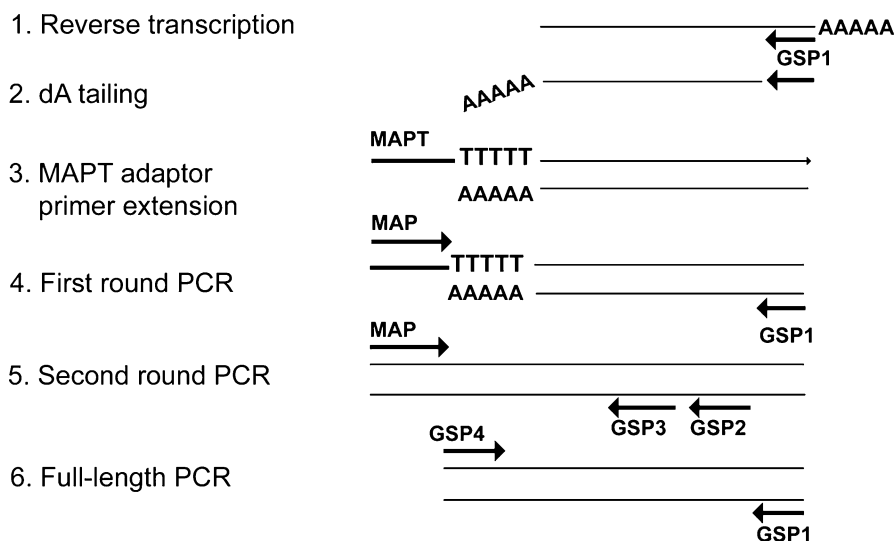


Fig. 3. Diagram of 5' RACE to isolate full-length cDNA. GSP: gene specific primer. MAPT: oligo(dT) anchored primer. MAP: primer identical to MAPT except that it lacks the oligo(dT) portion (*see Note 1*).

2. First-round PCR. Purify the reaction with a column to remove the MAPT primer and use 5 μ L of the diluted eluate (1:50) for the first round of PCR. Set up a PCR reaction the same as MAPT primer extension but replace MAPT with MAP primer, which is identical to MAPT except that it lacks the poly(dT) sequence. Run 35 cycles of PCR with the following parameters: 94°C, 5 min; 5 cycles with 94°C, 15 s, 45°C, 30 s, and 72°C, 3 min; 30 cycles with 94°C, 15 s, 58°C, 30 s, and 72°C, 3 min.
3. Second-round PCR. Repeat the PCR reaction with MAP and GSP2 primers and 5 μ L of 1:100 dilution of the first-round PCR. Run 5 μ L (1/10) of the PCR reaction on a 1% agarose gel along with a DNA marker (e.g., 1 kb ladder) and take a photograph of the gel with a fluorescent ruler beside the DNA marker. If the PCR shows a clear single band, excise it from the gel for cloning. If there are multiple bands, prepare Southern blotting of the agarose gel and hybridize with an end-labeled gene-specific primer (GSP3, >30 nt long) that is upstream of GSP2. Select the positive bands for cloning.

3.5. Functional Characterization of Novel p53-Regulated Genes

After the confirmation by Northern blotting, cloning, and sequencing, we directed our efforts to the functional characterization of potential p53 targets. Methods used for functional gene analysis exceed the scope of this chapter.

We describe here fundamental strategies that we have used to characterize two new p53-regulated genes: Pidd/Lrdd (**14**) and Pirh2/Zfp363 (**15**).

3.5.1. p53-Dependent Gene Expression in Various Experimental Models

After we confirmed p53-dependent expression of the genes in the same cells from which the RNA was isolated for DD, we tested different cells exposed to various genotoxic agents that are known to activate endogenous p53. Confirmation of p53-dependent expression in different cell models reduces the probability of artifacts owing to p53 overexpression in the DP16.1/p53ts cells.

3.5.2. Presence of a Genuine p53-Binding Site

We compared the full-length cDNA to genomic DNA sequences and searched for a consensus p53 response element on promoter as well as intron sequences. Potential p53 binding sites are examined for their interaction with p53 protein in an in vitro electrophoresis mobility shift assay (EMSA). The binding of p53 protein to p53 response elements in vivo can be investigated using chromatin immunoprecipitation (ChIP) assays. Conservation of p53 response elements in orthologous genes from human and mouse provides additional assurance that the genes represent p53 target genes.

3.5.3. Protein Domains Associated With Biological and Biochemical Activities

We searched for functional domains on Pidd and found a C-terminal “death domain” that is similar to death domains found on other proteins such as FADD. Death domains are sites of protein–protein interaction and are found commonly on proteins involved in death receptor-mediated apoptosis. Consequently, we asked if Pidd was a pro-apoptotic protein. Ectopic overexpression of Pidd caused cell death and inhibited cell growth. Transfection experiments provide an indication of function but need to be interpreted cautiously because the phenotypes observed are often the result of nonphysiological levels of expression. More comprehensive studies are required to reveal the precise roles that these proteins play in specific signaling pathways.

Sequence analysis of multiple Pirh2 cDNAs revealed an open reading frame of 261 amino acids corresponding to a predicted translation product of 32 kDa. Pirh2 protein contains a cysteine-rich zinc-binding motif known as a RING motif defined by the consensus sequence **CXXCX**(9,39)**CX**(1,3)**HX**(2,3)**C/HXXCX**(4,48)**CXXC** with eight cysteines and histidines that coordinate two zinc ions (**16**). Structural, biochemical, and biological studies of these motifs reveal that they are involved in protein–protein interactions. Recently, RING-containing proteins have been shown to play a role in ubiquitin-mediated protein degradation (**17**). To determine if Pirh2 has intrinsic ubiquitin protein ligase activity, we used an in vitro ubiquitination assay. Affinity purified GST-

Pirh2 or GST alone were added to bacterial extracts containing recombinant E1 and E2 (UbcH5b), and his-tagged ubiquitin. Our data indicate that Pirh2 has E3 ubiquitin protein ligase activity in vitro, and this E3 ligase activity is dependent on the presence of E1 and E2 (**Fig. 4**). It is of considerable interest that p53 protein can serve as a substrate for Pirh2-dependent ubiquitination in vitro and that Pirh2 can promote p53 ubiquitination in vivo (**15**).

3.5.4. Disruption of Protein Expression and Function

Gene knockout in mouse remains a powerful tool to confirm the putative protein functions and discover new signaling networks. However, mouse knockout may not necessarily result in observable phenotype and alternative approaches should be used to disable the gene. We have experimented with antisense oligonucleotides with some success. Recently a new technology, RNAi (RNA interference) or sRNA (small interfering RNA), has showed promise of higher efficiency in gene silencing.

4. Notes

1. Notes on 5' RACE.

- a. MAPT adaptor consists of a poly (dT) anchor and a primer sequence identical to MAP (**Fig. 3**). Design of the MAP primer depends on the cloning method for PCR-amplified fragments. For blunt-end ligation or T/A cloning, the MAP primer sequence can be random sequence that follows general rules for PCR primers. MAP primer and gene-specific primers may contain restriction sites for convenient directional cloning. Here is an example of the MAP primer in which *XhoI* and *Sall* restriction sites are inserted:

MAPT 5'- GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT -3'

MAP 5'- GACTCGAGTCGACATCGA

- b. We used the 5' RACE protocol to clone the full-length cDNA of a number of p53-responsive genes including Pidd and Pirh2. The main limitation of this standard protocol is that it often yields cDNA fragments smaller than 1 kb, thus making it necessary to do a "walking RACE" for large cDNA. An alternative is to screen a cDNA library first and carry out 5' RACE to obtain the remaining 5' end sequence. Recent developments in 5' RACE try to resolve this problem by adding RNA oligo to the 5' end of the messenger RNA instead of the first-strand cDNA so that during PCR with 3' gene-specific primer and 5' universal primer, cDNA with intact 5' end is selected for amplification (**18**).
- c. Sometimes the 5' end of a cDNA contains GC rich region and it becomes difficult to get through under normal conditions of reverse transcription. The extension temperature can be increased to 55–58°C in the presence of 1.2 M betaine and a thermostable reverse transcriptase (e.g., DuraScript by Sigma).
- d. The DNA polymerases that are efficient for the 5' RACE may not have stringent proofreading activity. Mutations do occur, particularly in cDNAs longer than 2 kb. Verify sequence from at least three clones for each cDNA frag-

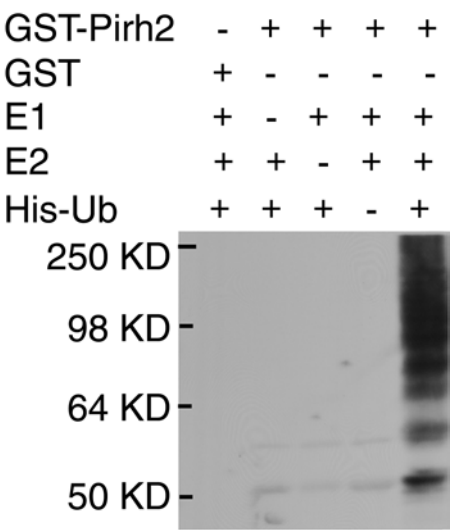


Fig. 4. In vitro ubiquitination assay. GST-Pirh2 was evaluated for E3 activity in the presence of recombinant E1, E2 (UbcH5b), and His-tagged Ub as indicated. Following the ubiquitination reaction, the samples were subjected to SDS-PAGE and immunoblotting with a His antibody to reveal ubiquitinated products.

- ment. Once the full length sequence has been determined, use a proofreading enzyme to amplify the cDNA from end to end.
- e. Whenever possible, use only 365 nm UV for both agarose gel imaging and excision for cloning. Even a short exposure to either 254 nm or 302 nm UV will severely reduce cloning efficiency. For large cDNA, consider alternative methods of DNA staining. For instance, use crystal violet staining under visible light. Incorporate crystal violet (final 10 µg/mL) in both the agarose gel and running buffer and load DNA samples without dyes. The drawback of crystal violet staining is that it 10× less sensitive than EtBr.
2. RNA quality (intactness, free of contaminants) is absolutely critical in order to achieve consistency of DNA band patterns in DD. Partially degraded RNA can lead to false-positive bands. Whenever possible, examine RNA quality through gel electrophoresis and use the 28S and 18S ribosomal RNA as markers of integrity. In any case, if the ratio of 28S over 18S is below one or the two RNA samples for comparison have different 28S/18S ratios, new RNA preparation should be made. Good RNA isolation kits can also be used to make RNA for DD.
 3. RNase inhibitors are commonly included in RT reactions to prevent RNA degradation. However, not all RNase inhibitors work well with the MMLV reverse transcriptase used in the RNAimage kits. Certain RNase inhibitors may actually inhibit reverse reverse transcription in the DD. The precise reason is not known, but it calls for caution when an RNase inhibitor is included in the reaction.

4. We identified all DNA fragments that showed a difference in signal intensity between the two starting RNA preparations from cells expressing mutant p53 (37°C cultures) and cells expressing “wild-type” p53 (32°C cultures) (**Fig. 2**). Importantly, all RT-PCR reactions for the DD were performed in triplicate. We consider a cDNA fragment to be differentially expressed only if it is different in two or three of the triplicate PCR reactions. The 40-cycle PCR may have narrowed the difference in the original mRNA abundance (**Fig. 2**) and the signal intensity of cDNA fragments on the film images may not have quantitative value. All differentially expressed fragments regardless of signal intensity were considered as potential targets.
5. Specific conditions used in the Northern blotting.
 - a. Prepare a 1% RNA agarose gel (minimum 15 cm in length) according to standard method, transfer the RNA to positively charged nylon membrane and fix the RNA in a UV cross-linker.
 - b. Make radioactive DNA probes with any random priming labeling kit that can produce high specific activity of 10^9 cpm/ μ g template. Up to 5 cDNA fragments (25 ng each) isolated from the display can be pooled in a single labeling reaction. Use the probes within 3 d after labeling to prevent significant decay of the DNA probes.
 - c. Incubate the blot in 10 mL prehybridization buffer at 42°C for 30 min. The high concentration of SDS (5%) is very effective at suppressing the background radioactive signal bound to the entire membrane. The high concentration of radioactivity used in this protocol can darken the blot at lower concentrations of SDS. The hybridization solution is quite thick and needs to be warmed up at 60°C just before use.
 - d. Transfer an aliquot of radioactive probe with a total of 1×10^7 cpm to a screw-cap Eppendorf tube and adjust the volume to 100 μ L with TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Boil the probe for 5 min and quickly cool on ice for 5 min.
 - e. Remove the prehybridization buffer. Mix the probe with 10 mL hybridization buffer and add to the blot immediately. Allow hybridization at 42°C overnight.
 - f. Remove the hybridization buffer and wash the blot three times at room temperature with Buffer I, 10 min each. Then wash the blot with Buffer II at 55°C, 10 min each.
 - g. Wrap the blot in plastic membrane and expose to the PhosphorImage screen for 24 h at room temperature. Although the PhosphorImage screen is best suited for this application, X-ray film can also be used with multiple exposures.

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Identification by Differential Display of IL-24 Autocrine Loop Activated by Ras Oncogenes

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Summary

Ras signaling pathway is thought to control the expression of a subset of yet-to-be-defined genes that are crucial for cell growth and differentiation. Here we have identified by differential display a novel oncogenic Ras target gene encoding a new cytokine. Biochemical studies reveal that this cytokine, which we named IL-24, is a member of IL-10 family of cytokines, and it signals through two heterodimeric receptors, whose expression is also upregulated by ras oncogenes. Thus, IL-24 and its receptors may represent a novel autocrine loop coordinately activated by ras oncogenes.

Key Words: Ras oncogene; differential display; IL-24; APtag; AP-TAG.

1. Introduction

Oncogenic conversion of a normal cell into a tumor cell requires multiple genetic alterations. Of particular interest is the fact that mutations in ras oncogenes cooperate with several other proto-oncogenes or mutant p53 tumor-suppressor genes to transform mammalian cells. Mutations in the ras oncogenes have been found at high frequency in a variety of human cancers, including those of gastrointestinal origin, such as pancreas and colon. Oncogenic ras mutations lock the RAS protein into a constitutively activate state, rendering the downstream signaling pathway unregulated. In tissue culture, ras has been known as one of the most potent oncogenes capable of causing dramatic transformation of the cells, which include morphological changes, increased growth rate, loss of contact inhibition, the ability to grow in soft-agar, and tumor formation in nude mice. It is known that Ras proteins function in part, whether directly or through other signaling molecules, to control expression of genes

that are important for cell growth and differentiation. Much progress has been made towards the understanding of the Ras signaling pathway from growth factor receptor through activation of a cascade of protein kinases. In contrast, much less is known about the downstream genes transcriptionally modulated by ras, and only a few candidate genes have been implicated. These genes could not completely account for the complex transformation phenotypes mediated by ras oncogenes.

Through systematic screenings by differential display (DD) strategy (*1–3*), we have identified many ras target genes under stringent experimental conditions to ensure the accuracy of the screenings (*4–6*). Some of these genes are known ras targets, but many are novel ones. The majority of these ras target genes, whether induced or repressed by oncogenic ras, encode secreted proteins that fall into two categories implicated in cell matrix functions, including collagen and lysyl oxidase (Liang et al., unpublished data), rCop-1 (*7*), stromolysin-1 (*8*), and Pai-2 (*9*); and in cellular immune functions, including Mob-1/IP-10, MGSA/Gro, and Mob-5 (*4,10,11*). Our discovery of the link, at the molecular level, between ras oncogenes and the deregulation of cellular immune responses is of great importance, because cancer has been long dubbed as “a wound that never heals.” Further supporting evidence came when we recently demonstrated that nuclear factor (NF)- κ B is required for Ha-ras oncogene-mediated abnormal cell proliferation and tumorigenesis (*12*).

This chapter describes our journey in identifying and in-depth, functionally characterizing one of the ras target genes, mob-5 (IL-24) (*10,11*).

2. Materials

1. RNApure[®] RNA isolation reagent (GenHunter Corporation, Nashville, TN).
2. MessageClean[®] kit (GenHunter) including 10X Reaction buffer, RNase free DNase I, 3 M NaOAc, RNA Loading Mix, and DEPC-H₂O.
3. Phenol:chloroform (3:1).
4. Ethanol (100, 85, and 70%).
5. Spectrophotometer.
6. RNAimage[®] kits (GenHunter) including 5X RT buffer, dNTP 250 μ M, HT₁₁M anchor primers, MMLV Reverse transcriptase, 10X PCR Buffer, dNTP 25 μ M, glycogen, and H-AP arbitrary primers.
7. Taq DNA polymerase, 5 U/mL (Qiagen, Valencia, CA).
8. α -[³³P]dATP (>2000 Ci/mmol) (NEN-Perkin Elmer).
9. α -[³²P]dATP (>3000 Ci/mmol) (NEN-Perkin Elmer).
10. Thermocycler.
11. Whatmann 3MM paper.
12. Qiaex Kit (Qiagen).
13. PCR-TRAP[®] polymerase chain reaction (PCR) cloning kit (GenHunter).

14. HotPrime[®] kit for radioactive labeling of cDNA (GenHunter).
15. DNA sequencing apparatus.
16. AP-TAG[®] vector system for making alkaline phosphatase fusion proteins (GenHunter).
17. cos-1 cells (GenHunter).
18. 1X saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS).
19. 0.25X SSC, 0.1% SDS.

3. Methods

3.1. RNA Purification

Purification of polyadenylated RNAs is neither necessary nor helpful for DD (*see Note 1*). The major pitfalls of using the polyadenylated mRNAs are the frequent contamination of the oligo-dT primers, which give high background smearing in the display and the difficulty in assessing the integrity of the mRNAs templates. Total cellular RNAs can be easily purified with one-step acid-phenol extraction method using RNeasy Reagent.

3.2. DNase I Treatment of Total RNA

Regardless of what method is used for the total RNA purification, trace amount chromosomal DNA contamination in the RNA sample will be present and could be amplified along with mRNAs, thereby complicating the pattern of displayed bands. Therefore removal of all contaminating chromosomal DNA from RNA samples is essential before carrying out DD.

The protocol next is adopted from that of MessageClean kit.

1. Incubate 10–100 µg of total cellular RNA with 10 U of DNase I (RNase free) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ for 30 min at 37°C.
2. Inactivate DNase I by adding an equal volume of phenol:chloroform (3:1) to the sample.
3. Mix by vortexing and leave the sample on ice for 10 min.
4. Centrifuge the sample for 5 min at 4°C in an Eppendorf centrifuge.
5. Save the supernatant, and ethanol precipitate the RNA by adding 3 vol of ethanol in the presence of 0.3 M NaOAc, and incubate at –80°C for 30 min.
6. Pellet the RNA by centrifuging at 4°C for 10 min.
7. Rinse the RNA pellet with 0.5 mL of 70% ethanol (made with DEPC-H₂O) and redissolve the RNA in 20 µL of DEPC-treated H₂O.
8. Measure the RNA concentration at OD₂₆₀ with a spectrophotometer by diluting 1 mL of the RNA sample in 1 mL of H₂O.
9. Check the integrity of the RNA samples before and after cleaning with DNase I by running 1–3 µg of each RNA on a 7% formaldehyde agarose gel with RNA Loading Mix.
10. Store the RNA sample at a concentration higher than 1 µg/mL at –80°C before using for DD.

3.3. Reverse Transcription of mRNA

1. Find components from a RNAlmage kit.
2. Set up three reverse transcription (RT) reactions for each RNA sample in three microfuge tubes (0.5 mL size), each containing one of the three different anchored oligo-dT primers as follows.
3. For 20 μL final volume, add 9.4 μL of dH_2O , 4 μL of 5X RT buffer, 1.6 μL of dNTP (250 μM), 2 μL of DNA-free total RNA (freshly diluted to 0.1 $\mu\text{g}/\mu\text{L}$ with DEPC-treated H_2O), and 2 μL of AAGCT₁₁V anchored primer (2 μM) (V can be either G, A, or C).
4. Program the thermocycler to 65°C, 5 min $\rightarrow$ 37°C, 60 min $\rightarrow$ 75°C, 5 min $\rightarrow$ 4°C. (see **Note 3**).
5. 1 mL MMLV reverse transcriptase is added to each tube 10 min after at 37°C and mixed well quickly by finger tipping.
6. Continue incubation and at the end of the RT reaction, spin the tube briefly to collect condensation.
7. Set tubes on ice for PCR or store at -80°C for later use.

3.4. PCR Amplification

1. Set up PCR reactions at room temperature as follows: 20 μL final volume for each primer set combination: 10 μL of dH_2O , 2 μL of 10X PCR buffer, 1.6 μL of dNTP (25 μM), 2 μL of arbitrary 13mer (2 μM), 2 μL of AAGCT₁₁V (2 μM), 2 μL of RT-mix from **Subheading 3.3.**, 0.2 μL of α -[^{33}P]-dATP (see **Note 2**), 0.2 μL of Taq DNA polymerase.
2. Mix well by pipetting up and down (see **Note 4**).
3. Add 25 μL mineral oil if needed.
4. PCR as 94°C, 30 s $\geq$ 40°C, 2 min $\geq$ 72°C, 30 s for 40 cycles $\geq$ 72°C, 5 min $\geq$ 4°C.
5. For Perkin-Elmer's 9600 thermocycler, it is recommend that the denaturation temperature be shortened to 15 s and the rest of parameters kept the same.

3.5. 6% Denaturing Polyacrylamide Gel Electrophoresis

1. Prepare a 6% denaturing polyacrylamide gel in TBE buffer.
2. Let it polymerize at least for more than 2 h before using.
3. Pre-run the gel for 30 min.
4. Mix 3.5 μL of each sample with 2 μL of loading dye and incubate at 80°C for 2 min immediately before loading onto a 6% DNA sequencing gel (see **Note 5**).
5. Electrophorese for about 3.5 h at 60 W constant power (with Voltage not to exceed 1700 V) until the xylene dye (the slower moving dye) reaches the bottom.
6. Turn off the power supply and blot the gel onto a piece of 3 MM paper.
7. Cover the gel with a plastic wrap and dry it at 80°C for 1 h. Do not fix the gel with methanol/acetic acid (see **Note 6**).

8. Orient the autoradiogram and dried gel with radioactive ink or needle punches before exposing to a X-ray film.

3.6. Reamplification of cDNA Probe

1. After developing the film (overnight to 72-h exposure), orient the autoradiogram with the gel.
2. Locate bands of interest (*see Note 7*) either by marking with a clean pencil from underneath of the film or punching through the film with a needle at the four corners of each band of interest.
3. Handle the dried gel with gloves and save it between two sheets of clean paper.
4. Cut out the located band with a clean razor blade.
5. Soak the gel slice along with the 3MM paper in 100 μ L dH₂O for 10 min.
6. Boil the tube with tightly closed cap (e.g., with parafilm) for 15 min.
7. Spin for 2 min to collect condensation and pellet the gel and paper debris.
8. Transfer the supernatant to a new microfuge tube.
9. Add in 10 μ L of 3 M NaOAc, 5 μ L of glycogen (10 mg/mL), and 450 μ L of 100% ethanol.
10. Let sit for 30 min on dry ice or in a -80°C freezer.
11. Spin for 10 min at 4°C to pellet DNA.
12. Remove supernatant and rinse the pellet with 200 μ L ice-cold 85% ethanol (you will lose your DNA if less concentrated ethanol is used).
13. Spin briefly and remove the residual ethanol. Dissolve the pellet in 10 μ L of dH₂O and use 4 μ L for re-amplification. Save the rest at -20°C in case of mishaps. Re-amplification should be done using the same primer set and PCR conditions except the dNTP concentrations are at 20 μM (use 250 μM dNTP stock) instead of 2–4 mM and no isotopes added. A 40 μ L reaction is recommended for each reaction.
14. Run 30 μ L of the PCR sample on a 1.5% agarose gel stained with ethidium bromide (EtBr). Save the remaining PCR samples at -20°C for subcloning.
15. Check to see if the size of your re-amplified PCR products are consistent with their size on the denaturing polyacrylamide gel.

3.7. Confirmation of Differential Gene Expression

1. Extract the reamplified cDNA probe from the agarose gel using QIAEX kit.
2. Use the extracted cDNA as a probe labeled with HotPrime kit for Northern blot confirmation following the standard protocol (*see Note 8*).
3. Clone the cDNA probe using the PCR-TRAP cloning system (*see Note 9*).
4. Confirmation of differentially expressed cDNA probes can be also carried out more efficiently by “Reverse Northern” dot blot or differential screening of cloned cDNA probes by colony hybridization.

5. Clone the full-length cDNA by screening a cDNA library following the standard procedure.

3.8. Results

3.8.1. Identification of IL-24 as an Immediate Target Gene of Oncogenic h-ras

Using DD screening with rational primer designs (3), two paradigms, one with Rat-1:iRas cells containing an IPTG inducible oncogenic h-ras, the other with oncogenic h-ras transformed Rat-1 cells before and after treatment of a MAP kinase kinase inhibitor PD98059, were set up for the systematic screening of oncogenic ras target genes. After screening through 90 combinations of primers, a total of 14 ras inducible genes and 7 ras repressible genes were identified (4–10). Four of these ras inducible genes, transin/stromelysin-1 (8), osteopontin, Cox-2, and Pai-2, were also identified by other methods in previous studies as ras targets. One of the remaining ras inducible genes, mob-5 (now designated IL-24) (10), appeared to be novel and the gene was identified in both screenings using either inducible ras oncogene or inhibitor, which blocks ras signaling (Fig. 1A,B). The 394 bp IL-24 cDNA was reamplified from the DD gels, cloned, and used as a probe to successfully verify the h-ras induction of the gene (Fig. 1C). Mob-1, another ras target gene that we identified previously (4,5) was used as a control for ras induction. The IPTG treatment of Rat-1:iRas led to the appearance of oncogenic H-Ras protein 4 h post IPTG induction, with concomitant rapid induction of IL-24 mRNA. This result suggests that IL-24 is an early target gene of oncogenic h-ras.

3.8.2. Overexpression of IL-24 in Human Colon Cancer

To determine the relevance of IL-24 expression in human cancer is of great importance for our model system for ras-mediated cell transformation. To evaluate the expression of human IL-24 in colorectal cancer, which has frequent mutations in ki-ras proto-oncogene, we carried out a quantitative RT-PCR analysis with RNA isolated from the tumors and the adjacent normal tissues from five colorectal cancer patients (Fig. 2, top panel). The quantitative RT-PCR results were also confirmed by regular RT-PCR of the entire coding region of IL-24 followed by Southern blot using human IL-24 cDNA as a probe (Fig. 2, middle panel). These results showed that IL-24 expression was confined to cancer tissues from five out five patients, with patient No. 5 expressing IL-24 at a much higher level.

3.8.3. Identification of Human IL-24 Receptors

Because IL-24 shares significant homology to IL-10, we predicted that IL-24 is a member of the IL-10 family of cytokines. Supporting this hypothesis is

our finding that not only IL-24, but also its putative cell surface receptor, appears to be induced by ras oncogenes (**10**). Shortly after our initial description of IL-24, two more IL-10 family of cytokines, IL-20 and IL-22, and their receptors were reported. Sequence alignment indicated that IL-24 and the other members of the IL-10 family of cytokines share an overall homology ranging from 24 to 33% among each other, further supporting the existence of IL-24 receptor(s).

To help dissect the biological functions of IL-24 in normal, as well as in pathological cellular conditions such as cancer, one must first identify and characterize its receptor. Based on the published data, as well as a database search in GenBank (NCBI), only three R1 and two R2 types of IL-10 family of receptors from humans could be found. The three R1 type of receptor subunits are IL-10R1, IL-20R1, and IL-22R1, whereas the two R2 type of receptor subunits are IL-20R2 and IL-10R2, the latter of which is also the second receptor subunit for IL-22. To determine if IL-24 is the ligand for one of the new combinations of these known R1 and R2 receptor heterodimers, we carried out receptor binding assays using either alkaline phosphatase (AP) or human IL-24-AP fusion protein as probes (**Fig. 3A**). The quantitative cell surface binding assays were carried out after transfecting Cos cells with plasmids overexpressing human IL-10R2, IL-20R2, IL-20R1, and IL-22R1 individually or in all four R1/R2 combinations (**Fig. 3B**). The results clearly indicated that IL-24-AP, but not AP, exhibited significant binding to Cos cells transfected with IL-20R2 alone, and the binding was further dramatically increased when IL-20R2 was co-transfected with either IL-20-R1 or IL-22R1. Neither IL-20-R1 nor IL-22R1 alone was able to bind to IL-24-AP. These data plus subsequent confirmation of STAT activation by IL-24/IL-24R signaling support that IL-24 is a new cytokine of IL-10 family, and that ras oncogenes can activate the IL-24 autocrine loop (**11**).

4. Notes

1. The initial choice of using two-base anchored oligo-dT primers (**1**) instead of one-base anchored primers (**3**) was owing to a historical rather than scientific reason. The cloned murine thymidine kinase (TK) cDNA originally used as a control cDNA template had only 11 As in its poly(A) tail. It was found that one-base anchored primer T11C failed to amplify the TK 3' terminus in combination with an upstream primer specific to TK. Extension of one more base from the 3' end instead of the 5' end of the anchored primer was a logical. Interestingly, T11CA started to work successfully in PCR to amplify the expected TK cDNA template (**1**). Later, longer one-base anchored primers that had mismatches at the 5' ends of the primers were shown to be much more efficient for DD in subdividing the mRNA populations into three groups (**3**). One-base anchored primers have significant advantages over the two-base anchored primers in that the former cuts down the redundancy of priming, eliminates the high background smearing

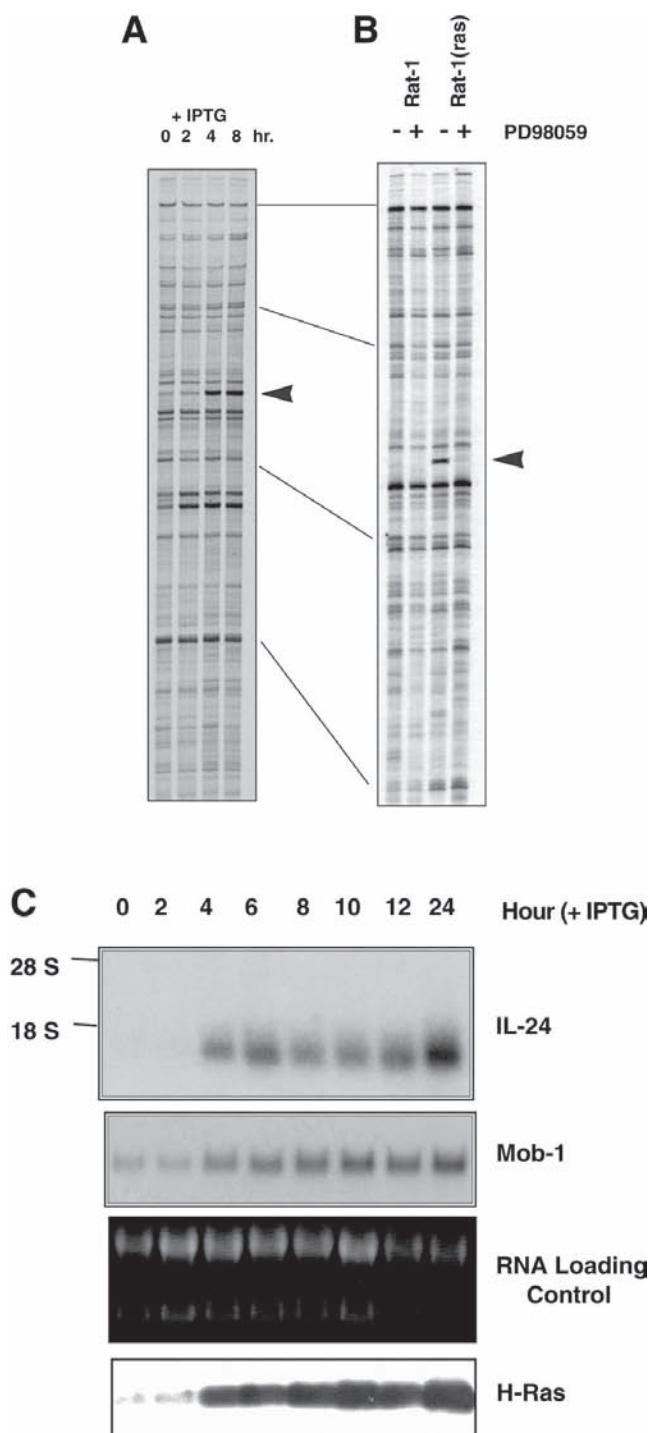


Fig. 1

- problem for two-base anchored primers ending with the 3' "T," and reduces the number of reverse transcription reactions from 12 to 3 per RNA sample.
2. It has been observed that the ^{35}S -labeled nucleotide originally used for DD would leak through PCR reaction tubes (especially when thin-walled tubes are used) and ^{33}P labeled nucleotide was recommended as the best alternative (9). ^{33}P is not only safer to use, but also gives better sensitivity as compared to ^{35}S .
 3. For the RT reaction, the initial 65°C incubation is intended to denature the RNA secondary structure. The final incubation at 75°C for 5 min is to inactivate the reverse transcriptase without denaturing the cDNA/mRNA duplexes. Therefore "hot start" PCR is neither necessary nor helpful for the subsequent PCR reactions using cDNAs as templates.
 4. Make core mixes as much as possible to avoid pipeting errors (e.g., aliquot RT-mix and AP-primer individually. Otherwise it is difficult to pipet $0.2\ \mu\text{L}$ of Taq Polymerase. Mix well by pipetting up and down.
 5. It is crucial that the urea in the wells be completely flushed out right before loading the samples. For best resolution, flush every four to six wells each time during sample loading while trying not to disturb the samples that have been already loaded.
 6. Because DNA is acid-labile, especially at high temperature when the gel is dried, you do not want to fix the gel. This will affect the subsequent PCR during the reamplification of the cDNA fragments to be analyzed further.
 7. First, tentatively identify those bands that appear to be differentially expressed on the initial display gel. Then repeat the RT step and the PCR reactions for these lanes and see if these differences are reproducible before pursuing further. It is recommended that bands bigger than 100 bp be selected. It has been generally

Fig. 1. (*opposite page*) Identification of IL-24 as an oncogenic ras target gene by differential display. (A) Identification of IL-24 as an oncogenic h-ras inducible gene. Rat-1:iRas cells were induced by IPTG. (B) Identification of IL-24 activated by oncogenic h-ras but inactivated by MAP kinase kinase inhibitor, PD98059. Rat-1 and Rat-1(ras) cells were treated with or without 10 mM of PD98059 for 24 h. The arrows indicate IL-24. (C) Northern blot confirmation of IL-24 as an oncogenic h-ras specific target gene. Another ras target gene, mob-1, was also analyzed as a positive control. The induction of oncogenic h-Ras protein was confirmed by Western blot.

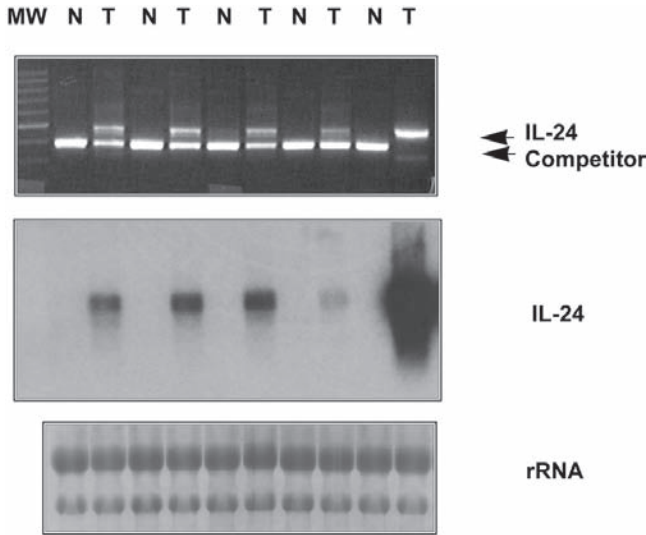


Fig. 2. Differential expression of the IL-24 mRNA in human colorectal cancer. Total RNA from pair-wise matched cancer tissues and their adjacent normal tissues from five patients were analyzed by either quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (top panel) or regular RT-PCR followed by Southern blot using a human IL-24 cDNA probe (middle panel). The image from rRNAs used for the analysis was shown as a control for equal sample loading.

observed that shorter cDNA probes have higher probability of failing to detect any signals on the Northern blot.

8. It is recommended that the standard prehybridization and hybridization condition at 42°C be used. Wash with 1X SSC, 0.1% SDS at room temperature for 15 min twice followed by washing with 0.25X SSC, 0.1% SDS at 55–60°C for 15–30 min. Do not go over 60°C. Expose with intensifying screen at –80°C overnight up to 1 wk.
9. PCR-TRAP cloning system is by far the most efficient cloning method for PCR products that we have tested. The PCR-TRAP cloning system utilizes the third-generation cloning vector that features positive-selection for DNA inserts. Only the recombinant plasmids confer the antibiotic resistance. The principle of this unique cloning system is based on the fact that the phage Lambda repressor gene *cI* cloned on the PCR-TRAP vector codes for a repressor protein. The repressor protein binds to the Lambda right operators *Or1* to *Or3* of the *cro* gene, thereby turning off the promoter that drives the *Tet^R* gene on the plasmid. Therefore, cloning of the PCR products directly, without any post-PCR purification, into the *cI* gene leads to the inactivation of the repressor gene, thus turning on the *Tet^R* gene. The cloned PCR insert can then be readily sequenced or retrieved as a probe by PCR using primers flanking the cloning site of the vector.

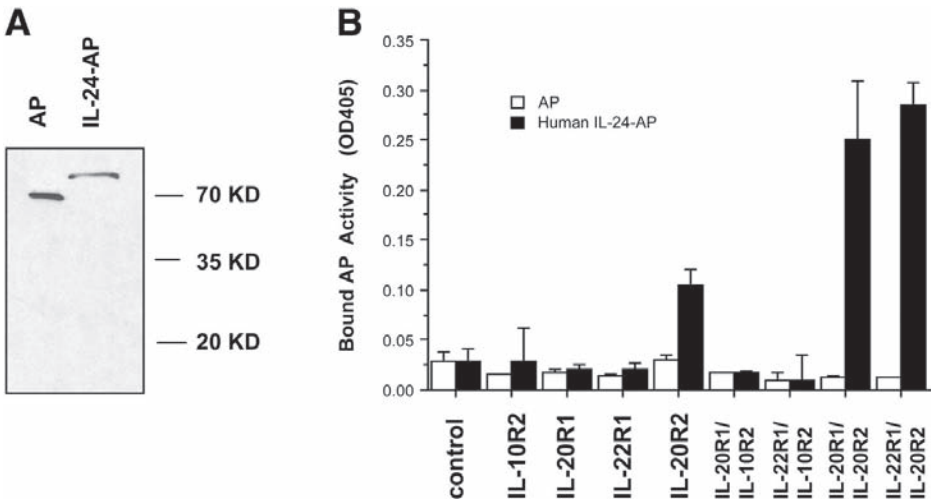


Fig. 3. Receptor binding analysis for IL-24. (A) Western blot analysis of conditioned media containing AP or human IL-24-AP, using polyclonal antibody against AP. These media were used to conduct the IL-24 receptor binding studies. (B) Receptor binding analysis for IL-24. Cos-E5 cells (a clonally purified Cos-1) were transiently transfected with expression vectors (individually or in pairs) encoding the corresponding receptor subunits as indicated. The cells with and without transfection were subsequently assessed for their ability to bind IL-24-AP (solid bars) vs AP control (open bars). None of the receptor subunit alone, except IL-20R2, exhibited appreciable IL-24-AP specific binding, which was substantially potentiated when either IL-20R1 or IL-22R1 was co-transfected with IL-20R2.

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Comprehensive Analysis of Ovarian Gene Expression During Ovulation Using Differential Display

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Summary

Mammalian ovulation is a normal biological process that is initiated when a gonadotrophic hormone stimulates G protein-coupled receptors in the plasma membrane of cells in ovarian follicles. This article outlines differential display (DD) protocols and associated methods that have been used to discover more than 30 genes that are expressed in the rat ovary during the ovulatory process. Details are provided regarding the methods for total RNA extraction, reverse transcription (RT), DD-polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE), Northern analysis of the differentially expressed cDNA fragments, cloning of the cDNA fragments, sequencing of the cDNA, and *in situ* hybridization of the cDNA fragments with sections of ovarian tissue. These methods provide clear evidence of the temporal and spatial patterns of expression of ovulation-specific genes in the ovary. Most of the genes that have been discovered to date have been associated previously with cascades of gene expression in acute inflammatory reactions. Therefore, the data support the working hypothesis that the ovary becomes inflamed at the time of ovulation, and this acute condition softens local connective tissues and causes ovarian follicles to rupture and release fertile eggs.

Key Words: Differential display; ovary; ovulation; Graafian follicle; granulosa cell; gonadotropin; luteinizing hormone.

1. Introduction

Mammalian ovulation is a fundamental biological process that is initiated when mature ovarian follicles are stimulated by a surge in luteinizing hormone (LH) secretion from the anterior pituitary gland (for review, *see refs. 1,2*). The length of time between stimulation of the ovary and rupture of the mature follicle(s) varies among different species. For example, the durations of the ovulatory process in rabbits, rats, pigs, and humans are 10, 12, 22, and 35 h, respectively. Nevertheless, it is generally thought that the molecular events

leading to rupture of ovarian follicles are essentially the same in all mammals. The present report describes the use of DD to detect more than 30 ovulation-specific genes in rat ovaries in order to expand the existing knowledge about the molecular events of ovulation (*see Table 1*).

For the past 26 yr, the ovulatory process has been likened to an acute inflammatory reaction in the ovary (for review, *see refs. 3,4*). The ovulation-specific genes that have been discovered by DD provide substantial support for this hypothesis that the ovary becomes acutely inflamed at the time of ovulation (for review, *see refs. 5–7*). The data also suggest that the ovulatory surge in gonadotropin induces a cascade of gene expression in the ovary (*see Table 2*) (5). Genes such as the one for the zinc-finger transcription factor known as early growth response protein-1 are expressed in ovulatory follicles within an hour after they have been stimulated by gonadotropin (8). Other genes, such as the one for epiregulin, are transiently expressed for a brief period around 4 h into the ovulatory process (**Fig. 1**) (5). However genes such as carbonyl reductase are expressed continuously up to the time of follicular rupture (**Fig. 1**) (9). Yet, genes such as metallothionein-1 are not upregulated until after rupture, when the ovarian follicles are transforming into corpora lutea (**Fig. 1**) (10). Thus, the random nature of the arbitrary primers used in the DD method have allowed the detection of a number of novel genes and their protein products that have not heretofore been associated with the ovulatory process (7).

Of special interest has been the discovery of expression of the gene for a disintegrin and metalloproteinase with thrombospondin motifs-1 (ADAMTS-1) (**Fig. 1**) (11). Such a special collagenolytic enzyme was first hypothesized as a component of the ovulatory process over 40 yr ago (12). Now, after many fruitless years of attempting to find such an enzyme in the ovary, the DD procedure has yielded ADAMTS-1. This unusual metalloproteinase has basically all of the properties a collagenolytic enzyme should possess in order to decompose the connective tissue in an ovarian follicle and cause rupture. Therefore, the discovery of this gene, alone, has made the years of investment in the DD procedure a worthwhile endeavor.

The results summarized in this report have been obtained by means of the arbitrary (i.e., random) primers and the anchor (i.e., poly-T) primers in 10 RNAimage Kits (G501-G510) from GenHunter Corporation. Of the 41 DD cDNAs that have been tracked by this method, several of the ovulation-specific genes were discovered multiple times. For example, of the first six cDNAs to be detected, sequencing revealed that two were fragments of the gene for carbonyl reductase, and two were fragments of the LINE gene (*see Table 1*). On the other hand, there was not any duplication of genes among the last 20 genes that were discovered (**Table 1**). Thus, the results, to date, suggest a high probability of finding additional ovulation-specific genes by using additional primers

Table 1
Genes Isolated by Differential Display and Chronological Order of Their Discovery

1. Carbonyl reductase	22. Carbonyl reductase
2. Unknown	23. Steroidogenic acute regulatory protein
3. LINE ^a	24. Vimentin
4. Early growth response protein-1	25. Pancreatitis-associated polypeptide-III
5. LINE	26. Seminal vesicle protein-5
6. Carbonyl reductase	27. ADAMTS-1 ^b
7. Niehmann-Pick C-1	28. Unknown
8. Unknown	29. Glutamate transporter protein
9. c-Ha-ras proto-oncogene	30. TNF-stimulated gene 6
10. G protein-coupled receptor	31. Unknown
11. 3 α -hydroxysteroid dehydrogenase	32. Tissue inhibitor of metalloproteinase-1
12. CD63 (surface antigen)	33. Adrenodoxin
13. Unknown	34. Cytochrome P450 aromatase
14. Unknown	35. PACAP ^c
15. Helix-loop-helix protein	36. 5-aminolevulinate synthase
16. Unknown	37. Metallothionein-1
17. Unknown	38. cAMP-specific PDE
18. Unknown	39. Glutathione-S-transferase T-1
19. Regulator of G protein-signaling protein	40. Glutamylcysteine synthetase
20. Cyclooxygenase-2	41. Epiregulin (of EGF family)
21. Carbonyl reductase	

^aLINE = long interspersed nucleotide element.

^bADAMTS-1 = a disintegrin and metalloproteinase with thrombospondin motifs-1.

^cPACAP = pituitary adenylate cyclase-activating polypeptide.

Table 2
Composite View of the Temporal and Spatial Patterns of Expression of 20 mRNAs

Spatial distribution of mRNAs (in ovarian tissue)	Pattern of signal from Northern blots (Intensity at hours after hCG)					
	0 h	2 h	4 h	8 h	12 h	24 h
Granulosa Layer						
5-aminolevulinate synthase	• ^a	•••	•	•	o ^b	•
Early growth response protein-1	o	••••	•••••	•••	••	o
Glutamylcysteine synthetase	•	••	•••••	•••	•	o
Cyclo-oxygenase-2	o	o	•••••	o	o	o
Epiregulin	o	o	•••••	o	o	o
PACAP ^c	o	o	•••••	•••••	o	o
Tumor necrosis factor-stimulated gene-6	o	•	•••••	•••••	•	o
Regulator of G protein-signaling protein-2	•	••	•••••	••••	••	•
Adrenodoxin	•	••	•••••	••••	••	•
Steroidogenic acute regulatory protein	o	••	•••••	••••	•••	••
cAMP-specific phosphodiesterase	•	•••	••••	•••••	•••	•
3 α -hydroxysteroid dehydrogenase	o	•	••	•••••	••••	•
CD63 (surface antigen)	••	••	••	•••••	•••	••
ADAMTS-1 ^d	o	o	•	••••	•••••	•••
Theca and Stroma Tissue						
Tissue inhibitor of metalloproteinase-1	•	••	••••	••••	•••••	••••
Carbonyl reductase	o	•	••••	•••••	•••	o
G protein-coupled receptor	o	o	••	•••••	••	o
Vascular Endothelium						
Pancreatitis-associated protein-III	o	o	••	•••••	••	•
Corpus Luteum						
Glutathione-S-transferase	•	o	o	o	o	•
Metallothionein-1	o	o	o	o	o	•••

^a• = each solid dot represents approx 20% of maximum signal detected on Northern blot.

^bo = each open dot indicates negligible signal on Northern blot.

^cPACAP = pituitary adenylate cyclase-activating polypeptide.

^dADAMTS-1 = a disintegrin and metalloproteinase with thrombospondin motifs-1.

beyond those present in the first 10 RNAimage Kits. Lastly, it might be worth noting that the project has been carried out by a physiologist who did not have any experience with molecular methods prior to inception of this study.

2. Materials (see Note 1)

2.1. Extraction and Quantitation of Total RNA

1. Guanidine isothiocyanate (GITC) solution: 59 g GITC, 0.24 g Tris-HCl, 0.34 g ethylenediamine tetraacetic acid (EDTA), 50 mL sterile H₂O, heated to 60°C to dissolve GITC, then cool, and dilute to 100 mL with H₂O. Then filter through a 0.45- μ m nylon filter into a sterile bottle. Add 5 mL 2-mercaptoethanol (Fisher, cat. no. BP176-100) prior to use.
2. Cesium chloride (CsCl) solution: 96 g CsCl, 1.68 g EDTA, and 50 mL sterile H₂O. Then autoclave and use H₂O to adjust the final volume to 100 mL.
3. Ultra-low freezer (-80°C).
4. Dry ice.
5. Liquid nitrogen.
6. Six autoclaved mortars and pestles.
7. Six autoclaved spatulae.
8. Six 12-mL round-bottomed polypropylene tubes for homogenization of samples.
9. Tissue-Tearor homogenizer Model 985-370, Biospec Products, Inc. (Fisher, cat. no. 15-338-55).
10. Microcentrifuge.
11. Ultracentrifuge.
12. Six ultracentrifuge tubes.
13. TE buffer, pH 8.0 (Ambion, cat. no. 9858).
14. 10% sodium dodecyl sulfate (SDS) (Fisher, cat. no. BP166-500).
15. Re-suspension solution for RNA: mix 9.9 mL TE buffer and 0.1 mL 10% SDS.
16. 3 M NaOAc, pH 5.2 (Sigma, cat. no. S7899).
17. Ethanol (EtOH).
18. Phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma, cat. no. P-3803).
19. Spectrophotometer.

2.2. Removal of DNA Contamination From Total RNA

1. MessageClean[®] Kit (GenHunter, cat. no. M601).

2.3. Reverse Transcription of mRNA

1. RNAimage[®] Kit (GenHunter, cat. no. G501-G510).
2. Thermocycler (MJ Research, cat. no. PTC-1160).

2.4. PCR Amplification of cDNA

1. ³⁵S-dATP (1200 Ci/mM) (PerkinElmer, cat. no. NEG-034H).
2. Taq DNA Polymerase (Qiagen, cat. no. 201205).

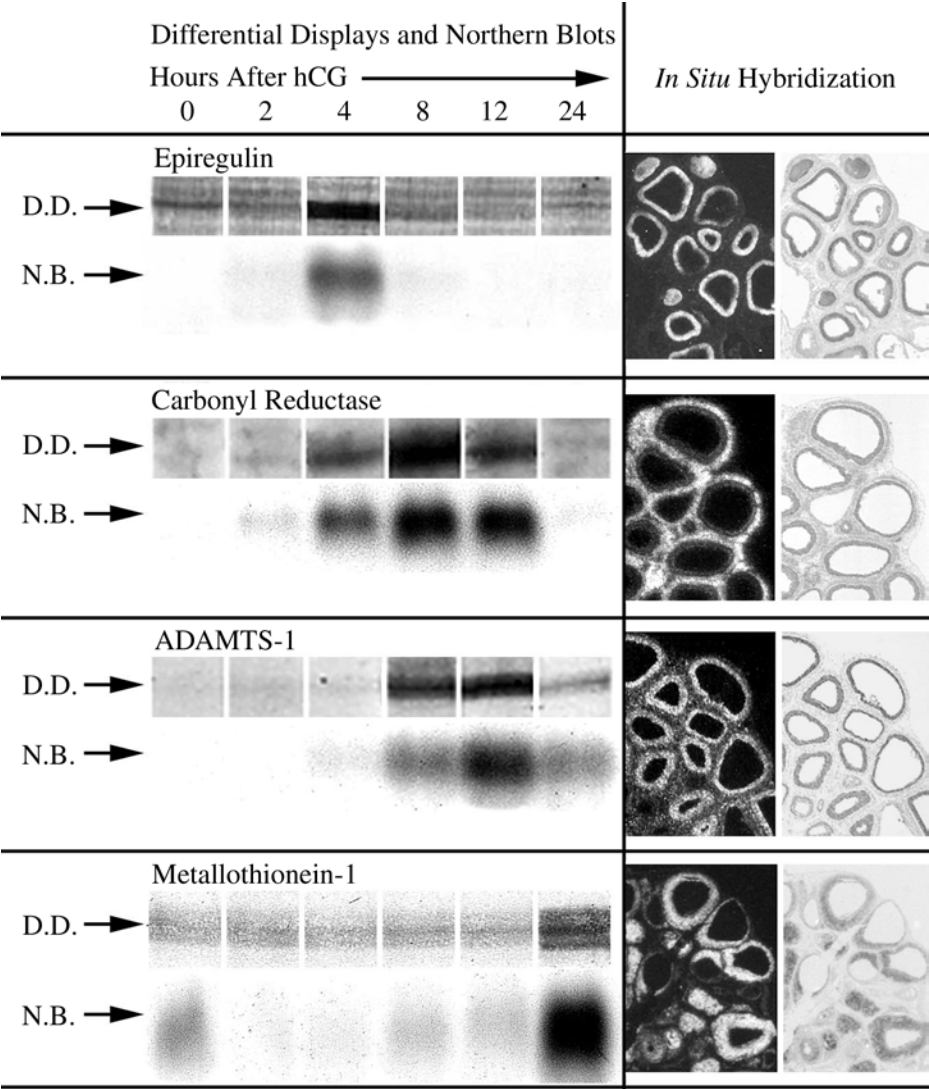


Fig. 1. Variations in the temporal and spatial patterns of expression of ovulation-specific genes. Pattern of differentially expressed cDNAs on differential display (D.D.) autoradiographs usually corresponded with the pattern of the signal from the Northern blots (N.B.). However, note that different genes were expressed at different intervals of time after induction of the ovulatory process with human chorionic gonadotropin (hCG). Also, note that *in situ* hybridization revealed different spatial patterns of expression of the different genes. Epiregulin was expressed only in the granulosa layer of follicles, carbonyl reductase was expressed in the thecal connective tissue surrounding the granu-

2.5. PAGE of Amplified cDNA

1. Sigmacote (Sigma, cat. no. SL2).
2. Glass plates for electrophoresis (Owl, cat. no. S1S-45R, S1S-45G).
3. Gel casting tape (Owl, TAPE).
4. 30% acrylamide stock solution: 28.5 g acrylamide (Promega, cat. no. V3111), 1.5 g bisacrylamide (Promega, cat. no. V314A), and dilute to 100 mL with sterile H₂O.
5. 10X TBE buffer (USB, cat. no. 70454).
6. Ammonium persulfate (USB, cat. no. 76322).
7. TEMED (USB, cat. no. 32825).
8. Urea (USB, cat. no. 75826).
9. Vertical electrophoresis system (i.e., sequencing system, with 0.4 mm spacer strips and shark-toothed comb) (Fisher, cat. no. FB-SEQ-2045).
10. Adhesive temperature indicators (Owl, TEMP-5).
11. Electrophoresis power supply (Fisher, cat. no. FB-EC-4000P).
12. Chromatography paper, Whatman 3MM Chr (Fisher, cat. no. 05-714-5).
13. Dry bath incubator (Fisher, cat. no. 11-718).
14. Gel dryer (Fisher, cat. no. FB-GD-45-10).
15. Autoradiography film, Kodak BioMax[®] MR Film (Fisher, cat. no. 05-728-27).
16. Film cassettes (Fisher, cat. no. FB-XC-810).
17. Kodak GBX developer.
18. Kodak GBX fixer.

2.6. Extraction and Reamplification of Differentially Expressed cDNA

1. Single-edge razor blades.
2. 25-gage hypodermic needles.

2.7. Estimation of Size of Extracted cDNA

1. Horizontal electrophoresis system for 7 × 10 cm minigel (Fisher, cat. no. FB-SB-710).
2. Power supply (Fisher, cat. no. FB-105).
3. Agarose (USB, cat. no. 32829).
4. Ethidium bromide (Sigma, cat. no. E-1510).
5. PCR markers (Promega, cat. no. G316A).
6. Loading dye, 6X (Promega, cat. no. G190A).

Fig. 1. (continued from opposite page) *losa*, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) was expressed mainly in the granulosa, but also in the thecal connective tissue, and metallothionein-1 was expressed in the luteinizing granulosa layer after the follicles had ruptured.

7. Photodocumentation system, Polaroid/Fisher (Fisher, cat. nos. 04-441-43 and 04-441-55).

2.8. Northern Analysis of Differentially Expressed cDNA

1. Agarose (USB, cat. no. 75817).
2. HEPES 1 M buffer (Fisher, cat. no. BP299-500).
3. 1 M sodium phosphate buffer, pH 7.0: mix approx 42 mL 1 M sodium monophosphate and approx 58 mL 1 M sodium diphosphate, and adjust pH with either 1 N HCl, or 1 N NaOH.
4. 1 M sodium phosphate buffer, pH 6.5: mix approx 67 mL 1 M sodium monophosphate and approx 33 mL 1 M sodium diphosphate, and adjust pH as in **step 3**.
5. Formaldehyde.
6. Running buffer for Northern: mix 20 mL of 1 M sodium phosphate buffer, pH 7.0, 50 mL HEPES, and 930 mL sterile H₂O.
7. RNA loading mix (GenHunter, cat. no. R104).
8. 20X sodium chloride/sodium citrate (SSC): weigh 175 g NaCl, 88 g sodium citrate, dilute to 1 L with sterile H₂O, and adjust pH to 7.0 with 1 N HCl.
9. 6X SSC: dilute 45 mL 20X SSC with 105 mL sterile H₂O.
10. Nitrocellulose transfer membrane, Osmonics/MSI brand (Fisher, cat. no. EP4HYB0010).
11. Saran wrap (or equal quality wrap).
12. SpectroLinker XL-1000 UV Crosslinker, Spectronics (Fisher, cat. no. 11-992-89).
13. 50X Denhardt's solution (USB, cat. no. 70468).
14. Deionized formamide (Ambion, cat. no. 9342).
15. Salmon testes DNA (Sigma, cat. no. D7656).
16. Hybridization incubator (Fisher, cat. no. 13-247-20).
17. Hybridization tubes (Fisher, cat. no. 13-247-100).
18. Hybridization stock solution: mix 4 mL sterile H₂O, 12.5 mL 20X SSC, 5 mL 50X Denhardt's, 2.5 mL 1 M sodium phosphate buffer, pH 6.5, 0.5 mL 10% SDS, 25 mL deionized formamide, and store at 4°C.
19. Prime-a-Gene® Labeling kit (Promega, cat. no. U1100).
20. ³²P-dCTP (3000 Ci/mM) (Perkin-Elmer, cat. no. NEG-013H).
21. Quick-Spin Columns (Roche, cat. no. 1523023).
22. Table-top centrifuge.
23. Rinse solution for radioactive Northern blots: mix 100 mL 20X SSC, 890 mL sterile H₂O, and 10 mL 10% SDS (mix in indicated order to avoid formation of precipitate).

2.9. Cloning of the Differentially Expressed cDNA

1. PCR-TRAP® Cloning Kit (GenHunter, cat. no. P404).
2. LB agar tablets (Sigma, cat. no. L-7025).
3. LB broth base tablets (Sigma, cat. no. L-7275).

4. 1% tetracycline (tet) solution: add 10 mg tet (Sigma, cat. no. T-7660) to 1.0 mL of a solution consisting of 50% EtOH and 50% MtOH, and wrap storage tube in foil to avoid exposure to light.
5. Disposable Petri dishes (Fisher, cat. no. 08-757-12).
6. Flint glass Pasteur pipet (Fisher, cat. no. 13-678-7C).

2.10. Northern Analysis of cDNA in Individual Cloning Colonies

1. Same as in **Subheading 2.8**.

2.11. Further Cloning and Storage of Ovulation-Specific cDNAs

1. 15-mL screw-cap culture tubes.
2. Shaker bath, Precision (Fisher, cat. no. 15-453-205).
3. 1.5-mL screw-cap microfuge tubes.
4. Sterile glycerol.

2.12. Sequencing of Differentially Expressed cDNA

1. Wizard *Plus*[®] Minipreps DNA Purification System (Promega, cat. no. A7500).
2. 40% isopropanol/4.2 M guanidine HCl solution: dissolve 66.9 g guanidine HCL (Promega, H5381) in 100 mL sterile H₂O by warming to <37°C, and then mix 30 mL of this 7 M guanidine HCl solution with 20 mL of isopropanol (store at room temperature).
3. Sequenase[®] T7 Sequencing kit (USB, cat. no. 70770).
4. Lseq and Rseq sequencing primers (GenHunter, cat. no. L402, L403).

2.13. Use of NCBI Database to Identify the Differentially Expressed Genes

1. Access to the internet is necessary for this step.

3. Methods

Sexually immature Wistar rats were the source of the RNA for this study because, in this experimental model, it is easy to induce super-ovulation and extirpate ovaries that consist mainly of gonadotropin-responsive follicular tissue. The immature rats were weaned at approx 23 d of age, and the next day they were injected with 10 IU sc of equine chorionic gonadotropin (eCG) (Sigma). Two days later, the ovulatory process was initiated by 10 IU of human chorionic gonadotropin (hCG), which acts as well as LH for induction of ovulation. Total RNA was extracted from whole ovaries that were extirpated at 0, 2, 4, 8, 12, and 24 h after the ovulatory process had been initiated by the hCG. These six intervals during the peri-ovulatory period represent the six basic experimental groups for DD analysis.

3.1. Extraction and Quantitation of Total RNA (see Note 2)

1. The extirpated ovaries are placed immediately into 1.5-mL microfuge tubes on dry ice.
2. Add 1–2 mL GITC solution to each homogenization tube.
3. Rinse homogenizer in 1.0 *N* NaOH for 2X 10 s, then in sterile H₂O for 3X 10 s, and then in GITC solution for 10 s.
4. Before homogenizing, freeze a mortar and pestle by pouring ample liquid nitrogen into mortar, and then grind frozen ovaries (up to 2 g maximum) into small granules (*see Note 3*).
5. Add ovarian tissue (approx 0.5 g/mL GITC) to homogenizing tube, and homogenize for approx 30 s.
6. Layer homogenate onto 1.5 mL CsCl solution in ultracentrifuge tube.
7. Use GITC to balance matched tubes and spin at 30,000–32,000 rpm at 20°C for 12–15 h.
8. Use sterile Pasteur pipet to remove supernatant fluid to below start of CsCl cushion.
9. Quickly invert each tube and drain contents onto paper towel.
10. Flame a scalpel blade (or a hemostat-held razor blade) and cut homogenizing tube in two about 1 cm from bottom.
11. Briefly rinse the RNA pellet in bottom portion of tube with 70% EtOH at room temperature.
12. Re-suspend pellet in approx 300 μ L re-suspension solution and incubate at 45°C (or higher) to facilitate solubilization.
13. Transfer dissolved RNA to 1.5-mL microfuge tubes.
14. To 300 μ L RNA solution, add 30 μ L 3 *M* NaOAc and 900 μ L cold 100% EtOH.
15. Shake and then incubate at –80°C for 15 min.
16. Centrifuge at 13,000g for 10 min at 4°C and discard supernatant fluid.
17. Wash pellet with 500 μ L 70% EtOH, centrifuge 5 min, decant supernatant fluid, and allow microfuge tube to dry.
18. Re-suspend RNA in 330 μ L TE, pH 7.6, and add an equal volume of phenol:chloroform:isoamyl alcohol solution.
19. Vortex, “bump” centrifuge, and pipet 300 μ L of upper phase into a fresh microfuge tube.
20. Repeat all steps listed under **steps 14–17**.
21. Re-suspend RNA in 100 μ L sterile H₂O per 1.0 g of original ovarian tissue by heating to at least 45°C.
22. Quantitate RNA by adding 2 μ L of RNA solution to 998 μ L ddH₂O and measuring optical density (O.D.) at 260 nm. (O.D. 1.0 = 40 μ g RNA/mL of diluted solution, i.e., 40 μ g RNA/2 mL re-suspended RNA solution if one uses 2.0 μ L for the quantitation.)
23. Store remaining RNA suspension at –80°C.
24. This procedure has consistently provided high-quality RNA for both Northern blotting and for RT-PCR for DD work (*see Note 4*).

3.2. Removal of DNA Contamination From Total RNA

1. As per instructions in GenHunter MessageClean Kit, calculate the volume of each RNA extract that represents 50 μg RNA, and dilute this up to 50 μL with sterile H_2O .
2. Mix 50 μL (i.e., 50 μg) RNA, 5.7 μL 10X Reaction Buffer, 1.0 μL DNase I, and incubate this mixture for 30 min at 37°C.
3. Add 40 μL of phenol:chloroform:isoamyl alcohol: 25:24:1 to the DNA digest, vortex for 30 s, and allow to sit for 10 min.
4. Microfuge at room temperature at approx 10,000g for 5 min, collect 50 μL of the supernatant fluid, and transfer it to a 0.5-mL microfuge tube.
5. Add 5.0 μL of 3 M NaOAc and 200 μL 95% EtOH, vortex, and keep at -80°C for 15 min.
6. Microfuge at 13,000g at 4°C for 10 min and pipet off the supernatant.
7. Wash the RNA pellet with 300 μL of 70% EtOH.
8. Microfuge for 5 min at approx 10,000g at 4°C for 5 min and remove the EtOH.
9. Microfuge briefly, pipet out any residual liquid, and allow to air dry.
10. Re-dissolve the RNA in 50 μL of sterile H_2O by “pump” pipetting.
11. Take 2 μL of this RNA solution and add 998 μL of dd H_2O to quantitate at OD₂₆₀.
12. Store the remaining 48 μL of RNA solution at -80°C.

3.3. Reverse Transcription of mRNA

1. As per the instructions in the GenHunter RNAimage Kit, prepare ample master mix consisting of designated amounts of sterile H_2O , 5X RT buffer, and dNTP (250 μM).
2. Aliquot 15.0 μL of this reaction mixture into the desired number of 0.5-mL PCR tubes (in this case, six PCR tubes).
3. Add 2.0 μL of one of the appropriate HT₁₁N anchor primers to each of the PCR tubes.
4. Dilute an appropriate amount of each of the six RNA extracts from **Subheading 3.2., step 12** to a final concentration of 0.1 $\mu\text{g}/\mu\text{L}$ with sterile H_2O , and add 2.0 μL of each diluted RNA extract to the corresponding PCR tube (*see Note 5*).
5. Program a thermocycler to 65°C for 5 min, 37°C for 60 min, 75°C for 5 min (*see Note 6*), followed by a 4°C dwell.
6. Pause the thermocycler at 10 min into the 37°C step and add 1.0 μL of MMLV RT to each reaction tube.
7. At the end of RT, store the cDNA products at -20°C until use in **Subheading 3.4., step 5**.

3.4. PCR Amplification of cDNA

1. Prepare ample master mix (depending on number of arbitrary primers to be used to amplify the cDNA preparations) consisting of sterile H_2O , dNTP (25 μM), HT₁₁N anchor primer, 10X PCR buffer (*see Note 6*), and *Taq* DNA polymerase as per instructions in RNAimage Kit (*see Note 7*).

2. Separate this master mix into approx 102 μL submasters for each arbitrary primer to be used.
3. Add 12 μL of a selected arbitrary primer individually to each submaster tube.
4. Aliquot 18 μL of each submaster into each of a set of six 0.5-mL PCR tubes.
5. Add 2 μL each of the 0-, 2-, 4-, 8-, 12-, and 24-h cDNA preparations from **Subheading 3.3., step 7** to a set of PCR tubes containing one of the submaster mixtures.
6. PCR at 94°C for 30 s, 38°C for 2 min, and 72°C for 30 s and repeat these three steps for a total of 40 cycles.
7. End the PCR reaction with a step at 72°C for 5 min followed by a 4°C dwell.
8. Store products at -20°C for use in **Subheading 3.5., step 12**.

3.5. Electrophoresis of Amplified cDNA

1. Thoroughly wash and rinse the inside surface of both glass plates (*see Note 8*).
2. Create a space between the plates using 0.4-mm Teflon strips.
3. Clamp the plates together and tape them to seal against acrylamide leaks.
4. Prepare a 6% acrylamide solution consisting of 24 g urea, 16 mL deionized H_2O , 5 mL TBE stock, and 10 mL acrylamide stock.
5. Heat this solution to 40°C to dissolve the urea, dilute to 50 mL using deionized H_2O , and allow to cool to 25°C before pouring (*see Note 9*).
6. Just before pouring acrylamide solution between the glass plates, add 400 μL of 10% ammonium persulfate and 20 μL of TEMED, swirl-mix the acrylamide, and immediately pour the solution.
7. Lift the open end of the plates up about 20°, and use a plastic squeeze-bottle to fill the space between the glass plates with the acrylamide solution.
8. Insert the straight side of a shark-toothed comb into top of gel.
9. Cover the open end of the plates with Saran wrap to prevent drying, and allow acrylamide to polymerize for at least 2 h (or overnight) before electrophoresis.
10. Remove tape from bottom, place plates in electrophoresis system (*see Note 10*), add 1X TBE to system reservoirs, remove shark-toothed comb from top of gel, rinse space where comb will be re-inserted, and re-insert comb with teeth about 0.5 mm into gel.
11. Set the electrophoresis power supply at 70 W, 32 mAMP, and 4000 V and allow gel to heat to 45°C (but not higher than 50°C).
12. Mix 3.5 μL of each PCR sample from **Subheading 3.4., step 8**, mix with 2.0 μL of loading dye (provided in RNAimage Kit), and heat on dry-bath incubator at 80°C for 2 min just before loading.
13. Rinse wells with 1X TBE, load 3.0 μL of PCR mixtures into wells, and run the system at 50 W, 23 mAMP, and 4000 V until xylene dye is 10 cm from bottom of glass plates.
14. Remove plates from electrophoresis system, place on flat surface, allow cooling to <30°C, and gently pry plates apart.
15. Place a properly sized Whatman paper over the exposed gel, and press it with a plastic roller (*see Note 11*).

16. Slowly peel the paper off the glass plate, so that the gel adheres to the paper.
17. Turn the Whatman paper over to expose the gel, and cover this side with Saran wrap.
18. Insert the paper, with gel face up, in a gel dryer set at 80°C and allow drying under vacuum for 60–80 min.
19. Remove the Saran wrap, place the dried gel against X-ray film, and staple the gel paper and film together (*see Note 12*).
20. After the film has been exposed to the radioactivity in the gel for approx 48 h, remove the staples, and develop the film according to Kodak instructions.

3.6. Extraction and Reamplification of Differentially Expressed cDNA

1. Orient the dried gel (from **Subheading 3.5., step 19**) with the autoradiograph (from **Subheading 3.5., step 20**) by inserting hypodermic needles through the staple holes into a small cork.
2. Locate cDNA band(s) of interest on autoradiograph, and poke four corners of each band with a hypodermic needle to distinguish the site of the cDNA(s) on the dried gel.
3. Use a clean razor blade to cut the cDNA band(s) from the dried gel.
4. Place each gel slice in 50 μ L sterile H₂O in a 0.5-mL tube and boil for 10 min (*see Note 13*).
5. For each cDNA extract, prepare a PCR reaction mixture consisting of 20.4 μ L sterile H₂O, 3.2 μ L 250 μ M dNTP, 4.0 μ L 10X PCR buffer, 4.0 μ L appropriate HT₁₁N anchor primer, 4.0 μ L appropriate arbitrary primer, 4.0 μ L of boiled solution from **Subheading 3.6., step 4**, and 0.4 μ L *Taq* DNA polymerase.
6. Hot-start at 94°C for 2 min, and then PCR at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s and repeat these three steps for a total of 40 cycles.
7. End the PCR reaction with a step at 72°C for 5 min followed by a 4°C dwell.
8. Store products at –20°C.

3.7. Estimation of Size of Extracted cDNA

1. Dissolve 1.0 g agarose in 50 mL of 0.5X TBE by briefly boiling in a microwave oven.
2. Allow agarose to cool to 65°C, add 5 μ L ethidium bromide, and swirl by hand.
3. Immediately pour warm agarose into electrophoresis minigel and insert small-toothed comb (*see Note 14*).
4. After agarose has gelled, carefully remove the comb, re-position gel in electrophoresis system (*see Note 15*), and fill chamber with 0.5X TBE until top surface of gel is submerged approx 2 mm.
5. Mix 5.0 μ L PCR Marker (i.e., DNA ladder) with 1.0 μ L loading dye, and mix 5.0 μ L PCR product from **Subheading 3.6., step 8** with 1.0 μ L loading dye.
6. Load these mixes into the gel (*see Note 16*), and run the electrophoresis system at approx 120 V for approx 30 min.
7. Remove the gel from the system, place under a UV trans-illuminator, and take a picture of the illuminated gel.

8. Analyze the photograph to confirm each cDNA was amplified and to estimate the length of each cDNA.

3.8. Northern Analysis of Differentially Expressed cDNA

1. Add 0.6 g agarose (*see Note 17*) to 37 mL of sterile H₂O, 2.5 mL HEPES, 1.0 mL 1 M sodium phosphate buffer, pH 7.0, heat this mixture to a boil, and then swirl vigorously by hand for 1 min (*see Note 18*).
2. Allow agarose to cool to approx 70°C, and add 9.0 mL 37% formaldehyde under the hood.
3. Immediately pour warm agarose into electrophoresis minigel and insert eight-toothed comb.
4. After the gel is solid (after approx 1 h), remove comb and add running buffer to about 4–5 mm above the surface of the gel (*see Note 19*).
5. Mix 1–10 µL (containing 20 µg) RNA (from **Subheading 3.1., step 24**) with 18 µL RNA loading mix, heat at 65°C for 10 min, and ice for 5 min. (If loading an RNA marker along with the ovarian RNA, mix 5 µL marker with 18 µL RNA loading mix.)
6. Run the electrophoresis system at 30–35 V (*see Note 20*) for 5 h.
7. Use a single-edged razor to trim the wells from the gel, and cut a notch at the corner of the gel that is closest to the well that was first loaded with RNA (in this case 0-h RNA).
8. Transfer gel to a small plastic tray (*see Note 21*), and rinse 4 × 5 min in 10 mM sodium phosphate buffer, pH 6.5, to rid the gel of most of the formaldehyde.
9. Visualize and photograph the gel under UV light to confirm equal amounts of RNA in the different lanes of the gel.
10. In preparation for transfer of RNA from the gel to a nylon membrane, turn a 7 × 10 cm plastic gel-tray upside down in a larger plastic dish and lay two sheets of Whatman paper on top in a folded fashion to form a wick.
11. Pour 20X SSC on top of the Whatman paper until the plastic dish is approx 1 cm full, and use a plastic pipet as a “rolling pin” to eliminate any air bubbles trapped between the paper and the upside down gel tray.
12. Cut a nylon membrane about 1 mm larger than the gel in both dimensions, cut a notch out of the top corner to match the cut corner of gel, immerse membrane in sterile H₂O for 1 min (*see Note 22*), and then immerse membrane in 20X SSC for 5 min.
13. Simultaneously rinse gel for 3 min in 20X SSC and invert it onto the moist Whatman paper.
14. Align the moistened nylon membrane (from **Subheading 3.8., step 12**) on top of gel; moisten two pieces of Whatman paper (the same size as the membrane) with 20X SSC; use blunt forceps to lay the paper on top of the membrane; and then eliminate air bubbles trapped between paper, membrane, and gel (as per **Subheading 3.8., step 11**).
15. On top of this, layer 2.5–3.0 in. of paper towel (or other absorbent paper) that is exactly the same dimensions as the nylon membrane (*see Note 23*), and weight the towel down with something like a new box of microscope slides.

16. Cover the entire preparation with Saran wrap, and allow 10–15 h for the RNA in the gel to blot onto the nylon membrane during movement of the SSC solution into the paper towel.
17. Place the fresh Northern blot in 6X SSC for 5 min, and allow to drip dry for 2–3 min.
18. Use a pen with permanent ink to mark the membrane, and then firmly affix the RNA to the nylon by placing the membrane in cross-linker under optimum cross-linking conditions (*see Note 24*).
19. Put Northern blot in a hybridization jar and add 6 mL hybridization solution.
20. Boil salmon testes DNA for 10 min, quick-chill it, and add 60 μ L to hybridization jar.
21. Cap the jar, place it in hybridization system set at 42°C for at least 1 h (*see Note 25*).
22. Label the cDNA probe (*see Subheading 3.6., step 8*) according to instructions in the Prime-a-Gene Labeling kit (*see Notes 26–29*).
23. Incubate the labeling mix at 28°C for 1–2 h.
24. After following Roche instructions for preparation of quick-spin column(s), dilute the 50 μ L radioactively labeled cDNA probe with 50 μ L TE buffer, and slowly apply 100 μ L of each labeled cDNA to the top-center of the sephadex in the quick-spin column(s).
25. Insert the column tip into a 1.5-mL screw-cap microfuge tube already placed inside a table-top centrifuge tube, centrifuge at approx 1200g for 2 min, retrieve, and cap the microfuge tube.
26. Determine amount of radioactivity in 1.0 μ L of cDNA probe, heat the probe to approx 98°C for 10 min, quick chill on ice, and add appropriate amount of probe (*see Note 30*) to hybridization tube containing a Northern blot (*see Note 31*).
27. After 4–48 h in the hybridization chamber at 42°C, remove the hybridization tube, and pour the radioactive cDNA solution into a plastic storage receptacle for ^{32} P waste.
28. Put 10 mL of rinse solution into the hybridization tube as a preliminary wash of the Northern blot, and then pour wash into the ^{32} P waste receptacle.
29. Put 25 mL of rinse solution into the hybridization tube, cap the tube, and rotate it in the hybridization chamber at room temperature for 10 min.
30. Repeat three times the rinse in **Subheading 3.8., step 29**, place the Northern blot on a paper towel to air dry for 5 min, and then transfer this membrane to a small, thin plastic bag (*see Note 32*).
31. Staple the bagged membrane to autoradiography film (the same as in **Subheading 2.5.**).
32. Develop the film and determine whether the temporal pattern of the Northern blot signal is comparable to the temporal pattern of the complimentary cDNA bands on the DD autoradiograph in **Subheading 3.5., step 20**.

3.9. Cloning of the Differentially Expressed cDNA

1. As modified from PCR-TRAP Cloning kit instructions (*see Note 33*), mix 13 μ L sterile H₂O, 2.0 μ L PCR-TRAP vector, 2.0 μ L 10X ligation buffer, 2.0 μ L PCR

- product from **Subheading 3.6., step 8**, and 1.0 μL T₄ DNA ligase, and incubate 15–24 h in thermocycler at 16°C.
2. Follow PCR-TRAP Cloning kit instructions for transformation of competent cells provided in the kit.
 3. Use four LB broth base tablets to make 200 mL LB medium (without tet), autoclave, and store at 4°C.
 4. Use four LB agar tablets to make 200 mL LB agar, autoclave, allow to cool to approx 50°C (*see Note 34*), add 400 μL tet solution (making 20 μg tet/1.0 mL agar), pour 20 mL of the agar solution into each of 10 sterile Petri dishes, and allow to gel.
 5. To the 110 μL mix of transformed cells prepared in **Subheading 3.9., step 2**, add 400 μL of LB medium (without tet), tap-mix the microfuge tube, and incubate the cells at 37°C for 1 h.
 6. Mark the agar plates to designate specific cDNAs, and plate 250 μL of the incubated cells onto an agar dish (*see Note 35*).
 7. Incubate the agar plates upside down at 37°C for 36–48 h.
 8. Sequentially number (i.e., score) the bacterial colonies on each agar plate that is to be analyzed in search of a plasmid vector that contains a cDNA that corresponds to a differentially expressed cDNA that was positively identified by Northern analysis in **Subheading 3.8., step 32** (*see Note 36*).
 9. Aliquot 25 μL of colony lysis buffer from the PCR-TRAP cloning kit into labeled 0.5-mL microfuge tubes, use clean 200- μL pipet tips to pick up a speck of bacteria from at least four of the marked colonies on a single agar plate (*see Note 37*), “pump” these samples into separate tubes containing the lysis buffer, and incubate the tubes at 95°C for 10 min.
 10. Centrifuge the tubes at 10,000g for 2 min, and use the supernatant solution for PCR re-amplification of the cloned cDNA in subsequent steps.
 11. As per GenHunter instructions in the PCR-TRAP Cloning kit, for each colony extract prepare a PCR mix consisting of 10.2 μL sterile H₂O; 1.6 μL dNTP (250 μM); 2.0 μL Lgh primer; 2.0 μL Rgh primer; 2.0 μL 10X PCR buffer; 2.0 μL supernatant fluid containing plasmid vector (*see Subheading 3.9., step 10*); 0.2 μL *Taq* DNA polymerase; and PCR at 94°C for 30 s, 52°C for 40 s, and 72°C for 60 s for a total of 30 cycles, followed by a 5 min extension at 72°C and a dwell at 4°C.
 12. Confirm amplification and estimate the length of each PCR product (*see Note 38*) by electrophoresis on an agarose minigel exactly as described in **Subheading 3.7.**

3.10. Northern Analysis of cDNA in Individual Cloning Colonies

1. Use 10 μL of each PCR product (obtained in **Subheading 3.9., step 11** and meeting the length requirement described in **Note 38**) to perform a secondary Northern analysis to confirm that a given cloning colony contains the correct cDNA fragment (i.e., a gene fragment that is differentially expressed in the ovary during the ovulatory process) (*see Note 39*).

2. Follow the exact procedure for Northern analysis described in **Subheading 3.8.**

3.11. Further Cloning and Storage of Ovulation-Specific cDNAs

1. After a secondary Northern analysis has confirmed that a specific cloning colony contains the ovulation-related cDNA that was initially targeted by the original DD autoradiograph and by the preliminary Northern analysis, relocate the corresponding colony on the agar plate(s), probe the colony with a pipet tip as described in **Subheading 3.9., step 9**, pump the speck of cells into 5–10 mL of LB broth containing 10 μ g tet/mL broth in a 15 mL culture tube (*see Note 40*), and incubate in a shaker bath at 37°C for 15–24 h.
2. Put 850 μ L aliquots of the bacterial culture from **Subheading 3.11., step 1** in 1.5-mL screw-cap microfuge tubes, add 150 μ L sterile glycerol to each tube, shake the contents by repeated inversion, snap-freeze the contents by placing tubes in EtOH/dry ice, and immediately store the tubes of cloned cells at –80°C.

3.12. Sequencing of Differentially Expressed cDNA

1. Thaw (on ice) a tube of the stored clones from **Subheading 3.11., step 2**, transfer 100 μ L of the clones to two 15-mL culture tubes containing 5–6 mL of LB broth without tet, and incubate at 37°C for 15–24 h. (Quick-freeze the remaining clone stock, and store at –80°C.)
2. Use a spectrophotometer to confirm adequate growth of the clone colony by the detection of an A_{600} value between 1.5–4.0.
3. Pipet 1.5 mL of the cultured clone into a microfuge tube and centrifuge at 12,000g at 4°C for 1 min, decant the supernatant fluid from the pellet of cells, add another 1.5 mL of the cell culture, and repeat the centrifugation and decantation (*see Note 41*).
4. Follow the instructions in a Wizard *Plus* Minipreps DNA purification kit to obtain a clean plasmid preparation for sequencing (*see Note 42*).
5. After the plasmid cDNA has been extracted, precipitated, rinsed, and dried, resuspend this cDNA in 8.0 μ L of sterile H₂O, and store at –20°C.
6. Denature and sequence the plasmid cDNA as per instructions in a Sequenase T7 Sequencing kit (USB), using Lseq and Rseq primers (GenHunter), and ³⁵S-dATP (PerkinElmer, cat. no. NEG-034H).

3.13. Use of NCBI Database to Identify the Differentially Expressed Genes

1. Go to <http://www.ncbi.nlm.nih.gov/BLAST> and click on “Standard nucleotide-nucleotide BLAST [blastn].”
2. Enter the sequence of the newly discovered gene fragment into the “Search” window, click on “BLAST!” and then on “FORMAT!”
3. Now, sit back and smile, if the BLAST search reveals that the differentially displayed cDNA fragment is homologous to an exciting gene in the NCBI database.

3.14. Consideration of In Situ Hybridization as a Supplementary Experiment

Considering the fact that the RNA extracted in **Subheading 3.1.** was from homogenates of whole ovaries, *in situ* hybridization can provide valuable information about the specific sites of gene expression in the ovary. Parallel groups of ovaries can be fixed and sectioned by standard histological methods, and then probed by standard *in situ* methods. As a matter of routine, the cDNA fragments discovered in this project have been subjected to *in situ* analyses in order to establish the spatial pattern of their expression in the ovary (e.g., see **Fig. 1** and **Table 2**, and refs. 8–11, 13–17). The *in situ* method for this project was adapted from the procedure originally outlined by (18), but the more current protocols outlined by (19) are also worthy of consideration.

The real value to *in situ* hybridization is that the information it provides about the spatial distribution of gene expression nicely complements the data about the temporal pattern of gene expression that is determined by the DD autoradiographs and by the Northern analyses. Furthermore, if *in situ* hybridization is performed on ovaries taken at each of the six basic intervals during the periovulatory period, the intensity of the signal from the probed tissue provides further confirmation of the temporal pattern of expression of a given gene.

4. Notes

1. Materials already listed in an earlier step will not be re-listed in subsequent steps.
2. The extraction procedure is a modification of the original method described by Chomczynski and Sacchi (20).
3. It is necessary to continue adding liquid nitrogen to mortar during grinding. Immediately transfer the frozen granules of ovarian tissue to the homogenizer tube before they have a chance to melt and become soggy.
4. Although this method of RNA extraction has consistently yielded high quality RNA, the extraction requires 2 d, the procedures are tedious, an ultracentrifuge is necessary, and the materials are relatively expensive. Therefore, in recent months, we have been testing RNAPure Reagent (GenHunter, cat. no. P502), as a convenient, less expensive method for RNA extraction. It is not yet clear whether this simplified method yields an equivalent quality of RNA.
5. In this study, there are six different experimental groups, representing six different RNA extractions. Therefore, for any given HT₁₁N anchor primer, there must be six RT reaction tubes (i.e., PCR tubes) to which one would add 2.0 μ L of a different, diluted RNA extract to each tube.
6. If *Taq* DNA polymerase from Qiagen is used, it is suggested that one use the 10X PCR buffer (containing 15 mM MgCl₂) that is provided with the polymerase from this source.
7. It is important to keep in mind that there can be evaporation of some of the solution or retention in the pipet tips during preparation of the reaction mixtures. There-

- fore, to avoid running out of reaction mixture during aliquoting, it is necessary to prepare approx 10% more mixture than the exact mathematical calculation.
8. Successful pouring of the acrylamide solution into the glass plates and successful removal of the solidified gel at the end of electrophoresis is highly dependent on adequate preparation of the inner surfaces of the glass plates. Use paper towels to thoroughly scrub the plates with a laboratory detergent. Rinse the plates with deionized water and dry them with paper towels. Do not allow the plates to air-dry and never touch the plates with bare hands or oily gloves. Pour 1–2 mL of Silicote on the inner surface of each glass plate and spread this solution uniformly over the surface using Kimwipes. Allow at least 5 min for the Silicote to dry and then buff the plates with Kimwipes. Spread 70% ethanol over the inner surface and dry with Kimwipes. In this condition, the plates can be stored for weeks before use. If one of the two plates (usually the notched plate) is re-silicoted and treated with 70% ethanol just before use, this will facilitate separation of the plates after electrophoresis and retention of the entire acrylamide gel (without tearing or distortion) on a single plate (usually the unnotched plate).
 9. Discard the acrylamide solution, and start over, if temperature rises above 50°C.
 10. It is useful to apply two temperature strips—one toward the top of the system and one toward the bottom of the system—to the aluminum backplate of the electrophoresis system before using the system for the first time. This allows accurate monitoring of the temperature of the acrylamide gel during electrophoresis.
 11. At the end of this step, it is important to date and mark the corner of the Whatman paper in the corner closest to the well where the first PCR mixture was loaded.
 12. The staple holes in the Whatman paper and the film serve as convenient marks to properly align the paper and film back together after the film has been developed and after cDNAs that might be differentially expressed have been identified on the autoradiograph.
 13. Instructions in the GenHunter RNAimage Kit state that one should use sodium acetate to precipitate and clean the dissolved cDNA at this stage. However, it is possible to consistently obtain good PCR product by directly using 4 μ L of this crude gel extract in the amplification step that follows.
 14. Time required for the agarose to gel can be cut in half by placing the gel chamber at 4°C.
 15. Make sure the end of the gel with the loading wells is oriented toward the negative electrode.
 16. Delineation of the wells in the gel can be facilitated by placing black paper beneath the electrophoresis system.
 17. Do not confuse this agarose (USB, cat. no. 75817) with the agarose used in **Subheading 3.7., step 1**.
 18. Thorough mixing of this agarose solution is essential to avoid clumping of the gel.
 19. More buffer is better than less in order to avoid overheating (and melting) of the gel.
 20. Do not run at higher voltage, or the agarose will melt.
 21. Use a large flat spatula, and take precautions to avoid cracking the fragile gel.

22. As simple as the brief soaking in H₂O might seem, this is essential for good transfer of RNA to the nylon membrane.
23. The absorbent paper must not droop in a way that would allow the SSC solution in the plastic reservoir to short-circuit the gel and the membrane, and migrate directly to the absorbent paper.
24. Alternatively, the RNA can be cross-linked to the nylon membrane by placing membrane between Whatman paper in a gel dryer at 80°C for 80 min.
25. Although the Northern blot can stay under these prehybridization conditions for as much as 1 wk, no more than 24 h is recommended.
26. Most protocols recommend quantitation of the cDNA product before labeling, but such a step is not really necessary if a strong cDNA band is detectable in the agarose minigel at **Subheading 3.7., step 8**.
27. GenHunter has recommended that the appropriate HT₁₁N anchor primer be added to the labeling mix, but strong radioactive labeling of the cDNA can be obtained without this step. However, the addition of HT₁₁N certainly will not be of any harm and may give more full-length products.
28. Expensive ³²P-dCTP can be conserved at this step by adding only 3 μL of the radioisotope instead of the recommended 5 μL, and then exposing the Northern blot to film for a longer period of time.
29. Be sure to boil the cDNA fragment for 5 min, and quick-chill before adding to labeling mix.
30. Although various protocols specify certain amounts of radioactivity (in CPM) that should be used per unit volume of hybridization solution, the ultimate intensity of the signal from a probed Northern blot will be determined in large part by the amount of parent RNA for a given cDNA that was present in the original RNA extract. As a rule of thumb, one can use approx 25 μL of probe if the radioactivity in 1.0 μL is >300,000 CPM, approx 40 μL if 200,000–300,000 CPM, approx 60 μL if 100,000–200,000 CPM, and all (approx 90 μL) if 50,000–100,000 CPM in 1.0 μL.
31. Some protocols call for replacement of the pre-hybridization solution with fresh hybridization solution before addition of the radioactively labeled cDNA to the hybridization tube, but good results can be obtained without this additional measure.
32. Some protocols recommend additional rinsing in 0.5X SSC at 55°C, but these more stringent conditions reduce the ultimate strength of the signal on the autoradiograph.
33. PCR-TRAP Cloning kit instructions call for a 2–10X dilution of the PCR product before adding 5.0 μL to the ligation mix. This can be achieved simply by adjusting the H₂O volume to 13 μL and by adding only 2.0 μL of undiluted PCR product directly to the ligation mix.
34. To avoid denaturing the tetracycline, do not add this antibiotic to LB broth at temperatures above 50°C.

35. Uniform distribution of a lawn of transformed cells onto the agar can be achieved by simply dipping the tip of a Pasteur pipet in 100% EtOH, flaming the glass, heating the glass to make a 90° bend about 4 cm from the tip, and using this as an inoculating loop to spread an even lawn of cells on the agar.
36. The agar plates can be sealed with Parafilm (or Saran wrap) and stored at 4°C until further analysis.
37. Sometimes it is necessary to analyze 10 or more cloning colonies before finding a colony with a vector containing the cDNA that was differentially expressed.
38. The Lgh and Rgh primers both hybridize at a position on the cloning vector that is 62 bp flanking either side of the insertion site of the cDNA. Therefore, in this case, the target length of a PCR product that would be equivalent to the original cDNA identified in **Subheading 3.7., step 8** would be the estimated length of that original cDNA + 62 bp + 62 bp.
39. It is important to keep in mind that each cDNA band extracted from the DD gel at **Subheading 3.6., step 2** and re-amplified at **Subheading 3.6., step 5**, can be contaminated with uninteresting “housekeeping” cDNAs (albeit the same length as the ovulation-specific cDNA) that might also be incorporated into some of the cloning vectors. Therefore, this secondary Northern analysis is necessary to clearly identify cloning colonies that contain ovulation-specific gene fragments.
40. Some procedures recommend that a sampling of cells from the original colony be re-suspended in 100 µL LB broth and cultured on a fresh agar plate. However, the direct growth of a sample of the original colony in 5–10 mL of LB broth and subsequent storage at –80°C appears to be satisfactory for studies such as this analysis of ovarian gene expression.
41. If the A_{600} value was 2.0–3.0, collect cells from a total of 4.5 mL of the cultured cells, and if A_{600} was less than 2.0, collect from 6.0 mL of cultured cells.
42. The *Escherichia coli* competent cells provided with GenHunter PCR-TRAP cloning kits are end-A positive. Therefore, it is necessary to wash the minicolumn with 2 mL of isopropanol/guanidine HCl solution.

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Functional Analysis of Nocturnin

A Circadian Clock-Regulated Gene Identified by Differential Display

Julie E. Baggs and Carla B. Green

Summary

Within the retina there is a circadian clock that controls the 24-h timing of processes such as hormone release, cell movement, and gene transcription. In an effort to better understand the molecular nature of this retinal clock, a differential display (DD) screen was performed to isolate a gene with high amplitude circadian rhythmicity in the *Xenopus* retina. A novel gene expressed in the early evening in photoreceptor cells was isolated and named nocturnin for night factor. This article outlines the steps we took to study a protein of unknown function, particularly highlighting the analyses one can perform when little more than the primary sequence of a gene is known. In addition, we describe the results of sequence analysis that assisted in predicting the function of nocturnin. We have shown that nocturnin acts as a deadenylase in vitro, removing the poly(A) tail from a mature messenger RNA in a process that either leads to degradation or translational silencing of a message. Although the role of nocturnin in the retina is unknown, future studies to identify target mRNAs that are deadenylated by nocturnin will assist in elucidating its physiological role in this tissue.

Key Words: Circadian; nocturnin; deadenylation; PCE II; retina; photoreceptor.

1. Introduction

The circadian clock is a 24-h timekeeper that controls daily events from sleep/wake cycles to rhythmic gene expression. Circadian clocks exist in organisms ranging from cyanobacteria to humans, and clocks are found in many tissues throughout the body of higher organisms (**1**), including in the retina of

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the African clawed frog *Xenopus laevis* (2). Circadian fluctuations in the retina occur at the physiological, morphological, and molecular levels, with rhythms in the release of the hormone melatonin, the movement of rod and cone photoreceptor cells, and the expression of genes, respectively (3). The persistence of these rhythms under constant conditions (e.g., constant darkness) is a hallmark of circadian clocks, as the underlying clock continues to drive rhythmicity in the absence of exogenous timing cues.

In an effort to better understand the molecular nature of this retinal clock, we performed a differential display (DD) screen to isolate a gene with high amplitude circadian rhythmicity in the *Xenopus* retina. Briefly, retinal RNA was isolated at 6-h intervals over a 2-d period, and DD was performed on these samples to identify a gene that fluctuated over a 24-h cycle (for more details see refs. 4,5). A novel gene was identified that has peak mRNA levels in the early night, and was thus named *nocturnin* for night factor (6).

At the time it was cloned, the only information about *nocturnin* came from its primary sequence: *nocturnin* was novel and was similar in sequence only to a portion of a transcriptional activator protein in *Saccharomyces cerevisiae* and contained a leucine zipper-like protein interaction motif (6). Therefore, we were initially limited to experimental approaches that required little knowledge about the function of the protein. Furthermore, we did not have many tools, such as antibodies, with which to work. Therefore, we began by investigating things that could be studied with the primary sequence. First, we examined the control of the unique spatial and temporal expression of *nocturnin* by analyzing the promoter, and also investigated the location of *nocturnin* within the cell. Finally, careful analysis of the sequence led us to several hypotheses about the function of the protein, eventually leading us to deduce its likely in vivo function. This chapter will focus on describing the methods we used to investigate *nocturnin* since its isolation in a DD screen, particularly highlighting the general approaches we took to investigate a novel sequence and strategies we employed to identify its function.

2. Expression Profiles

Given a sequence of unknown function, investigating the expression profile is one method to gather more information about the protein product. In the case of *nocturnin*, the temporal expression profile was already known, because it was the basis for identification of this gene. Nighttime expression of *nocturnin* mRNA was verified by Northern blot on retinal extracts collected over a 24-h day (6). As expected, *nocturnin* mRNA expression is highest in the early night, and barely detectable at other times of day (6). In addition, *nocturnin* mRNA is rhythmic in animals kept in constant darkness or constant dim light, indicating that *nocturnin* expression is controlled by the circadian clock (6). In order to

look at the location of *nocturnin* mRNA in the retina, *in situ* hybridization was done on retinas collected at various time points. *Nocturnin* mRNA is expressed primarily in the rod and cone photoreceptor cells, with high levels at night and nondetectable levels during the day (5,7). In *X. laevis*, a circadian clock sufficient to drive the release of melatonin resides in the photoreceptor cell layer (8,9). Furthermore, homologs of known circadian clock genes are expressed in these cells (10,11). Because *nocturnin* expression is confined to the clock-containing photoreceptor cells and *nocturnin* mRNA fluctuates over a circadian cycle, a hallmark of many clock genes, we hypothesized that *nocturnin* plays a role in the retinal circadian clock.

2.1. Promoter Analysis

Given the unique spatial and temporal expression pattern of *nocturnin*, we set out to identify the sequences that control both the photoreceptor-specific expression and the nighttime expression of *nocturnin*. The upstream regulatory region of *nocturnin* was isolated from a genomic library and sequenced. To investigate the photoreceptor-specific expression of *nocturnin*, we utilized the transgenic technique in *X. laevis*, a widely used procedure in which several dozen transgenic tadpoles can be generated in 1 d (12,13). The green fluorescent protein (GFP) cDNA was cloned downstream of the *nocturnin* promoter, and transgenic animals were made that express these constructs. With a 2 kb portion of the 5' flanking sequence, GFP expression was found in both the rod and cone photoreceptor cells, suggesting that the sequence(s) important for driving photoreceptor-specific expression of *nocturnin* are located within this fragment (14). To narrow down the promoter piece responsible for targeting *nocturnin* to the rods and cones, promoter sequences of decreasing size were cloned upstream of the GFP coding sequence and transgenic animals were produced. The photoreceptor-specific expression of various promoter sequences in transgenic frogs (Fig. 1) in combination with electromobility shift assays (EMSA) using retinal nuclear extracts led to the identification of a novel protein binding motif we named photoreceptor conserved element II (PCE II), which consists of three perfect repeats of a 14 basepair element sufficient to drive GFP expression in the photoreceptor cells (14).

Nuclear run-on assays showed that the rhythmic expression of *nocturnin* mRNA was controlled at the transcriptional level (6). Therefore, we set out to identify elements in the promoter region that control the circadian expression of *nocturnin* transcription. Sequence analysis of the promoter identified an E-box-like element, a sequence that has been implicated in the rhythmic transcription of several genes, and EMSAs confirmed this region is indeed a protein-binding motif (7). This sequence looks like an intermediate between the cyclic AMP response element (CRE), which is bound by the CRE-binding

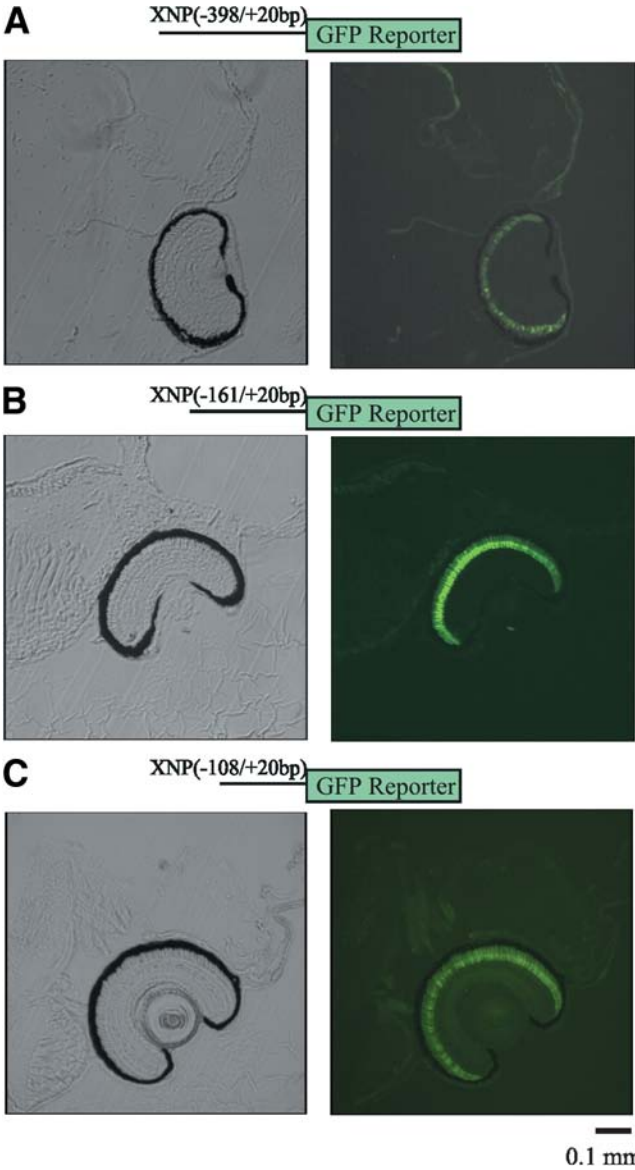


Fig. 1. GFP Expression directed by shorter nocturnin promoter reporter constructs is restricted to photoreceptor cells. Transgenic tadpoles were produced using decreasing sizes of the *Xenopus* nocturnin promoter (XNP) fused to GFP. Phase-contrast images are shown on the left, and corresponding fluorescent images are shown on the right. (Figure reprinted with permission from ref. 14.)

protein (CREB), and a circadian E-box, which is found in regulatory regions of clock genes and bound by the circadian clock proteins CLOCK and BMAL1. Therefore, we called this novel promoter sequence the nocturnin element (NE). EMSAs and transcriptional activation assays in a heterologous cell system with the NE showed that the NE is bound by phosphorylated CREB and not by CLOCK/BMAL (7). Because the levels of phosphorylated CREB are rhythmic in the retinal photoreceptor cells with a phase similar to the peak of *nocturnin* transcription, we concluded that phosphorylated CREB likely drives the rhythmic, nighttime expression of *nocturnin*.

The analysis of the 5' regulatory region of *nocturnin* is an example of experiments that can be done on a gene sequence of unknown function. The regulatory regions responsible for the spatial and temporal expression of *nocturnin* were identified in these studies. Furthermore, we identified two promoter elements: the novel PCE II element responsible for photoreceptor-specific expression and the NE (CRE), which drives the circadian expression of *nocturnin*. It will be interesting to see if these regulatory regions are functionally conserved in promoters of other genes.

2.2. Intracellular Localization of Nocturnin

At the time of its discovery, database analysis showed that nocturnin was similar in sequence to a transcriptional activator protein in *S. cerevisiae* called CCR4 (6). Because we had no other information about its function, we hypothesized that nocturnin was also a transcription factor. Therefore, we set out to investigate its intracellular localization, expecting to find nocturnin in the nucleus.

Because there were no antibodies against nocturnin at that time, we needed to find an alternate way to visualize the protein. Therefore, we cloned nocturnin in-frame with GFP under the control of a strong cytomegalovirus (CMV) promoter and followed the fusion protein in transiently transfected COS-7 cells by fluorescence microscopy. The nocturnin-GFP fusion proteins were found exclusively in the cytoplasm of transfected cells, regardless of whether the GFP tag was at the N- or C-terminus of nocturnin (Noc-GFP and GFP-Noc) (Fig. 2A). To exclude the possibility that the addition of the large GFP protein causes mislocalization of nocturnin, smaller peptide tags (V5 and FLAG) were cloned in-frame with nocturnin and expressed in COS-7 cells. Immunocytochemistry using antibodies against the V5 and FLAG tags confirmed the cytoplasmic localization of the nocturnin-GFP fusion proteins. With the FLAG tag located on either the amino terminus or the carboxy terminus of nocturnin, we observed greater than 90% of cells with nocturnin in the cytoplasm only (Noc-FLAG, 91%, $n = 300$; FLAG-Noc, 92%, $n = 250$). Similarly, nocturnin

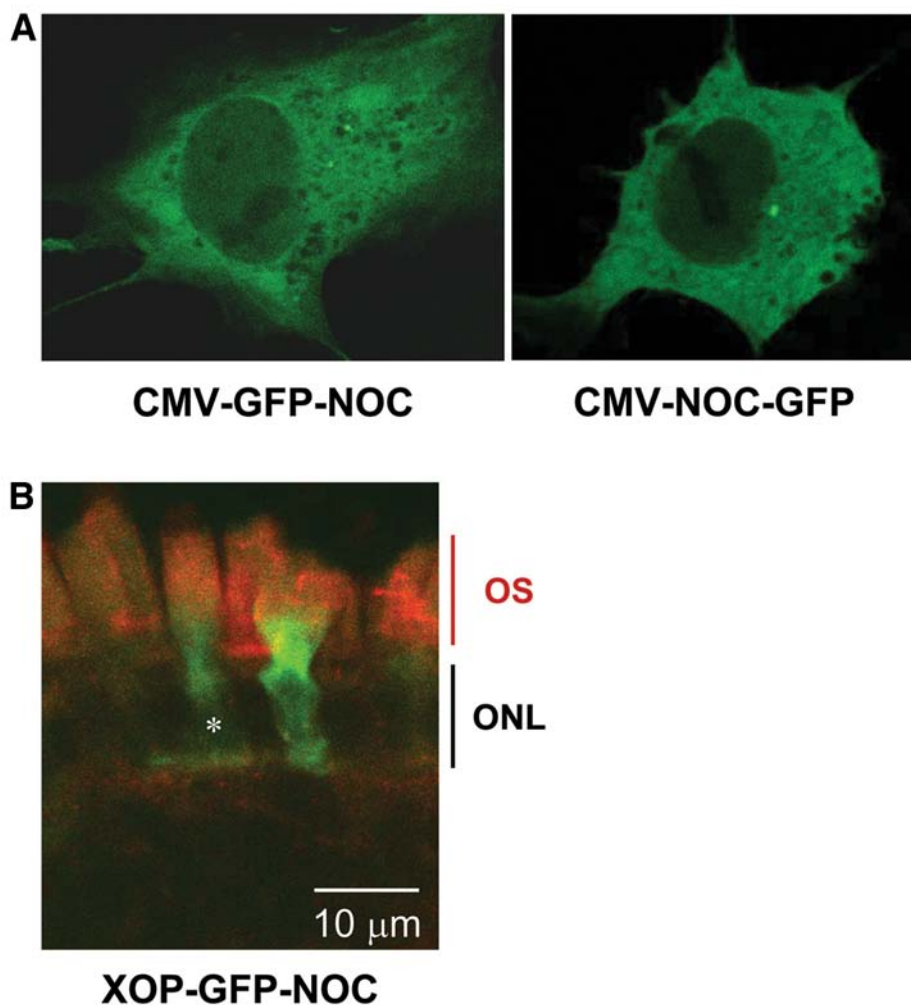


Fig. 2. Nocturnin localizes primarily in the cytosol when overexpressed in *Xenopus laevis* retina and COS-7 cells. (A) Confocal images of COS-7 cells expressing nocturnin-GFP fusion proteins by transient transfection under the control of the CMV promoter. (B) Using the *Xenopus* transgenic technique, nocturnin-GFP fusion proteins were expressed in rod photoreceptor cells under the control of the *Xenopus* rod opsin promoter (XOP). Outer segments of all photoreceptor cells were stained using the B630N anti-opsin primary antibody (29) and a rhodamine-conjugated secondary antibody. Photoreceptor cell nucleus is denoted by an asterisk. OS, photoreceptor outer segment; ONL, outer nuclear layer.

with a V5 tag at the carboxy terminus accumulates solely in the cytoplasm in 82% of cells ($n = 200$).

To verify that the cytosolic localization was not an artifact of using a heterologous cell line, localization of nocturnin protein within the photoreceptor cells was investigated by making transgenic tadpoles that overexpress nocturnin fusion proteins in the retina. The two nocturnin-GFP fusion proteins (Noc-GFP and GFP-Noc) were targeted to the rod photoreceptor cells under the control of the *Xenopus* opsin promoter (XOP) (15). Expression of nocturnin-GFP fusion protein was found exclusively in the cytoplasm of sectioned retinas at all times of day examined (Fig. 2B).

The cytosolic localization of nocturnin has since been confirmed in the *X. laevis* retina using antibodies generated against nocturnin. Therefore, making fusion proteins with a tag such as GFP can be a useful technique to visualize a gene product, even when no functional data is available.

3. Functional Analyses

3.1. Sequence Analysis

The cytoplasmic localization of nocturnin led us to reevaluate our hypothesis that nocturnin is a transcription factor acting in the nucleus of the photoreceptor cells. We continued to search sequence databases to try to find more information about the potential function of nocturnin. The development of new BLAST search algorithms, specifically the Position Specific Iterative BLAST (PSI-BLAST) (16), provided new insight into the potential function of nocturnin. The PSI-BLAST search often reveals proteins with weak, although biologically significant, homology. When a sequence is entered, this program begins by performing gapped-BLAST searches and generates a multiple alignment with the results. The alignment is then used to re-search the database, and this process is continued for a specified number of times or until convergence (16).

Using the PSI-BLAST search program, we discovered that nocturnin belonged to a larger family of proteins with nuclease activity that include inositol polyphosphate 5-phosphatases and apurinic/apyrimidinic endonucleases involved in DNA repair (17–21). Although nocturnin shares low sequence similarity with the members of this family, there are several domains of high sequence similarity. The conserved domains are important for the catalytic function of these proteins, and include a magnesium-binding residue (17). We tested nocturnin for phosphatase and AP endonuclease function, and found that it did not have these nuclease functions (data not shown). Therefore, we hypothesized that nocturnin was a nuclease that shared a conserved catalytic mechanism with this family of proteins, but probably cleaved a substrate we had not yet tested.

3.2. *In Vitro* Activity

Although the amino terminus of CCR4 was known to function as a transcriptional activator (22), the function of the carboxy terminus, the region similar in sequence with nocturnin and other members of the magnesium-dependent nuclease family, was unknown. The similarity with CCR4 eventually led us to the likely substrate of nocturnin nuclease activity. Recently, the C-terminus of CCR4 was shown to possess nuclease activity—specifically, the removal of the poly(A) tail of a mature mRNA in a process called deadenylation (23). Deadenylation is important in the control of gene expression, because removal of the poly(A) tail leads to either the degradation or translational silencing of a message (24,25).

Although the deadenylase function of CCR4 was demonstrated in yeast, we hypothesized, based on our sequence analysis, that the functionality of the protein was conserved in the *Xenopus* protein nocturnin. Furthermore, the almost exclusive cytoplasmic localization of nocturnin is consistent with it functioning as a cytoplasmic deadenylase. Therefore, we set out to test whether nocturnin could cleave the poly(A) tail from a synthetic mRNA in vitro. After cloning a GST-nocturnin construct that placed GST in-frame with the amino-terminus of nocturnin, we expressed and purified the GST-nocturnin fusion protein in bacteria. The deadenylase function of GST-nocturnin was tested using a radiolabeled mRNA with a 100 nucleotide poly(A) tail at its 3' end as a substrate. When nocturnin was added to the poly(A)-containing mRNA, we saw a time-dependent reduction in the size of the synthetic substrate, consistent in size with the removal of the poly(A) tail (Fig. 3) (26). Therefore, bacterially expressed nocturnin has deadenylase activity in vitro. Because the predicted magnesium-binding residue is conserved between nocturnin and other members of the magnesium-dependent nuclease family, we tested the requirement of magnesium in the deadenylase activity of nocturnin. When EDTA, a divalent cation chelator, was added to the reaction, GST-nocturnin no longer deadenylated the mRNA substrate (Fig. 3, lane 6). Furthermore, deadenylase activity is lost in a mutated form of GST-nocturnin that has a point mutation in the residue predicted to bind magnesium (26). In addition, we characterized the deadenylase activity of GST-nocturnin further, demonstrating that nocturnin is an exonuclease that specifically degrades the 3' poly(A) tail (26).

4. Summary

Although we are in the early stages of understanding the function of nocturnin in vivo, we have some hypotheses as to its role in the retina. Because removing the poly(A) tail leads to either degradation or translational silencing of mRNAs

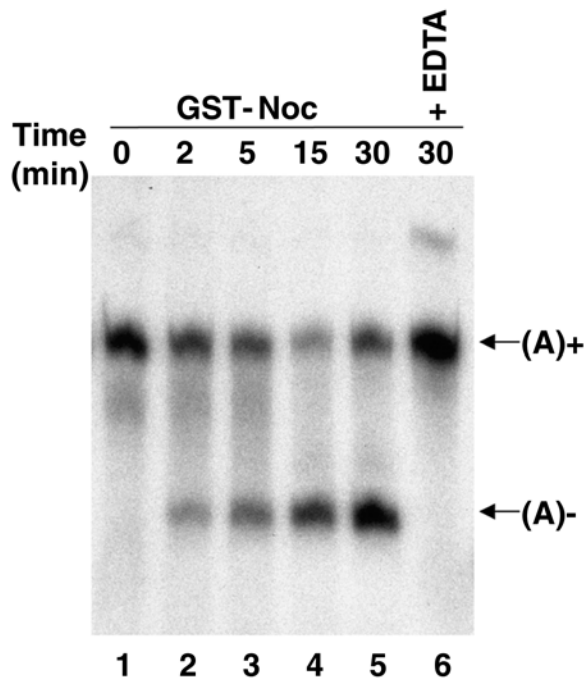


Fig. 3. Nocturnin exhibits deadenylase activity in vitro. Nocturnin deadenylates a synthetic mRNA substrate in a time-dependent, EDTA-sensitive manner. Purified nocturnin protein (GST-noc, 1 μ g) was incubated with radiolabeled poly(A)+ mRNA and deadenylation was measured at time points up to 30 min (lanes 1–5). Decrease of activity was observed with the addition of EDTA (lane 6). Arrows indicate poly(A)+ and poly(A)– sizes.

(24), nocturnin is likely involved in turning genes off at the posttranscriptional level. In a tissue like the retina, where very dramatic changes occur day vs night, the regulation of gene transcription at a particular time of day is an important process. Similarly, genes involved in the circadian clock are expressed throughout a 24-h cycle, which means that turning genes on and off at the correct time is an integral process in maintaining proper clock function. Although we do not yet know whether nocturnin plays a role in the circadian clock, the rhythmic, photoreceptor-specific expression of nocturnin suggests that it may. Furthermore, there are several lines of evidence suggesting that regulation at the posttranscriptional level, regulation such as deadenylation, is important and essential for a properly timed circadian clock (27,28). At this time, no candidates other than nocturnin are known that could fill this role in the clock. We are currently working to identify target mRNAs that are

deadenylated by nocturnin in an attempt to understand its role in the retina and its potential role in posttranscriptional regulation in the circadian clock.

Acknowledgments

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Isolation and Characterization of Anthocyanin 5-*O*-Glucosyltransferase in *Perilla frutescens* var. *crispa* by Differential Display

Mami Yamazaki and Kazuki Saito

Summary

The glucosylation of 5-position of anthocyanin molecules is one of most important modification step, which is responsible for the stability and color hue of anthocyanin molecules. However, anthocyanin 5-*O*-glucosyltransferase (5-GT) is unstable and it had been difficult to purify this enzyme by biochemical methods. The isolation of cDNA encoding 5-GT was succeeded first by mRNA differential display (DD) of red and green chemotypes of *Perilla frutescens* var. *crispa*. The full-length cDNA was isolated by the screening of perilla cDNA library with cDNA fragments, which was displayed specifically in red perilla. The biological characters of this enzyme were investigated by using the recombinant protein expressed in yeast. We have shown an example of the application of DD technique to the molecular biology in plant secondary metabolisms.

Key Words: Anthocyanin; glucosyltransferase; differential display; *Perilla frutescens*.

1. Introduction

Recently, the genes regulating plant secondary metabolisms have been isolated by the mRNA differential display (DD) technique (*1*). Such a genetic molecular approach has led to novel findings on the specific proteins that are difficult to purify by biochemical methods. We applied the DD technique to isolate the cDNA encoding UDP-Glucose: anthocyanin 5-*O*-glucosyltransferase (5-GT). 5-GT is one of the most important enzymes for anthocyanin pigmentation, which affects the stability of anthocyanin molecules and their co-pigmentation. However, the biochemical purification of 5-GT protein has

not succeeded because of low quantity and/or instability of the enzyme activity. The gene fragments related to anthocyanin production and/or accumulation were isolated by DD between red and green forms of *Perilla frutescens* var. *crispa*, a pair of chemotypes differing in the anthocyanin production (2). The full length cDNAs corresponding to these fragments were further isolated by library screening. The deduced amino acid sequence of the two obtained cDNA clones, 3R4 and 3R6, exhibited low but significant homology with those of UDP-glucose:anthocyanidin 3-*O*-glucosyltransferase genes from various plants. The recombinant protein of clone 3R4 expressed in yeast exhibited the catalytic activity of 5-GT. Using this recombinant protein, the substrate specificity was studied and the order of catalytic steps in the late stage of anthocyanin modification was investigated (Fig. 1).

2. Materials

1. Young plant of red or green form of *Perilla frutescens* var. *crispa*.
2. Liquid nitrogen.
3. A mortar and a pestle.
4. QuickPrep Micro mRNA Purification Kit (Amersham), which consists of the Extraction Buffer containing guanidinium thiocyanate and *N*-laurylsarcosine, oligo(dT)-cellulose, the Elution Buffer: 10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA); the High-Salt Buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl; and the Low-Salt Buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl; and a MicroSpin Column.
5. RNase-free water.
6. First-strand cDNA synthesis kit (Amersham) containing the bulk first-strand cDNA reaction mix: 4.4–5.5 U/ μ L M-MuLV Reverse transcriptase, 2.7 U/ μ L RNAGuard, 240 μ g/mL bovine serum albumin (BSA), 5.5 mM dNTP, 135 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM dithiothreitol (DTT), 30 mM MgCl₂; and 200 mM DTT.
7. One-base anchored oligo(dT) primers, H-T11G, H-T11A, or H-T11C, (GenHunter) and arbitrary primers, H-AP1 to H-AP8 (GenHunter).
8. *Taq* DNA polymerase (Toyobo).
9. 10X *Taq* buffer: 100 mM Tris-HCl, pH 9.0, 500 mM KCl, 0.1% Triton X-100, 12.5 mM MgCl₂; 1.2 mM dNTPs; [³²P]dCTP.
10. Polymerase chain reaction (PCR) equipment.
11. Agarose and DNA sequencing gel equipments.
12. X-ray film and equipments for film developing.
13. pT7Blue-T (Novagen), pBluescriptII SK (Stratagene), and pYE22m (yeast expression vector [5]).
14. *Saccharomyces cerevisiae* G1315 (*trp1*).
15. Yeast complete medium (YPD medium): 1% yeast extract, 2% polypepton, 2% glucose.

16. LA medium: 100 mM lithium acetate, 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA.
17. LAG medium: 100 mM lithium acetate, 15% glycerol, 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA.
18. 50% polyethylene glycol 4000 (PEG).
19. 20X dropout solution: 0.03% isoleucine, 0.015% valine, 0.02% adenine hemisulfate, 0.02% arginine hydrochloride, 0.02% histidine hydrochloride monohydrate, 0.03% lysine hydrochloride, 0.02% methionine, 0.05% phenylalanine, 0.02% threonine, 0.03% tyrosine, 0.02% uracil (*see Note 1*).
20. SD minimal medium: 6.7 g/L yeast nitrogen base without amino acids (YNBD, Difco), 1X dropout solution, 0.1% leucine, 2% glucose (*see Note 2*).
21. Suspension buffer: 100 mM potassium phosphate buffer, pH 8.5, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF).
22. Glass beads, for disintegrating yeast (Sigma).
23. Microcentrifuge and vortex mixer.
24. Reagents for enzyme assay: standard reaction mixture: 100 mM phosphate buffer, pH 8.5, 200–250 mM anthocyanin substrates, 1 mM UDP-glucose, and 100 mg protein of crude enzyme extract; 1% HCl in CH₃OH.
25. High-performance liquid chromatography (HPLC) equipment.
26. Reverse-phase column, Asahipak ODA-50, 4.6 mm ϕ $\times$ 250 mm (Showa-Denko).
27. HPLC buffer for gradient elution: 10–50% acetonitrile with 0.5% trifluoroacetic acid (TFA).
28. HPLC buffer for isocratic elution: 50% acetonitrile with 0.5% trifluoroacetic acid.
29. 0.1X SSPE, 0.1% sodium dodecylsulfate (SDS).

3. Methods

The methods described in **Subheadings 3.1–3.4.**, outline: (1) mRNA DD; (2) the screening of cDNA library; (3) the construction of the expression vector in yeast; (4) protein expression yeast; and (5) the characterization of the protein.

3.1. mRNA Differential Display

The mRNA DD between red and green forms of perilla was performed basically according to the original method (3). Using 3 oligo(dT) primers with A, G, or C at the 3'-position and 8 different arbitrary primers, 36 fragments were differentially displayed as specifically expressed cDNAs in leaves of red form but not in green form out of about 2600 amplified fragments in total (**Fig. 2**). The differentially expressed fragments were isolated and reamplified using the same primer combinations as in the first PCR, and subsequently cloned into the plasmid vector. Among the 33 subcloned cDNA fragments, the red form-specific expression of the five cDNA fragments was confirmed by RNA blot analysis. These red form specifically expressed cDNA fragments were selected to be used in library screening as probes. The details of DD method are described in **Subheadings 3.1.1.–3.1.4.** This includes: (1) preparation of

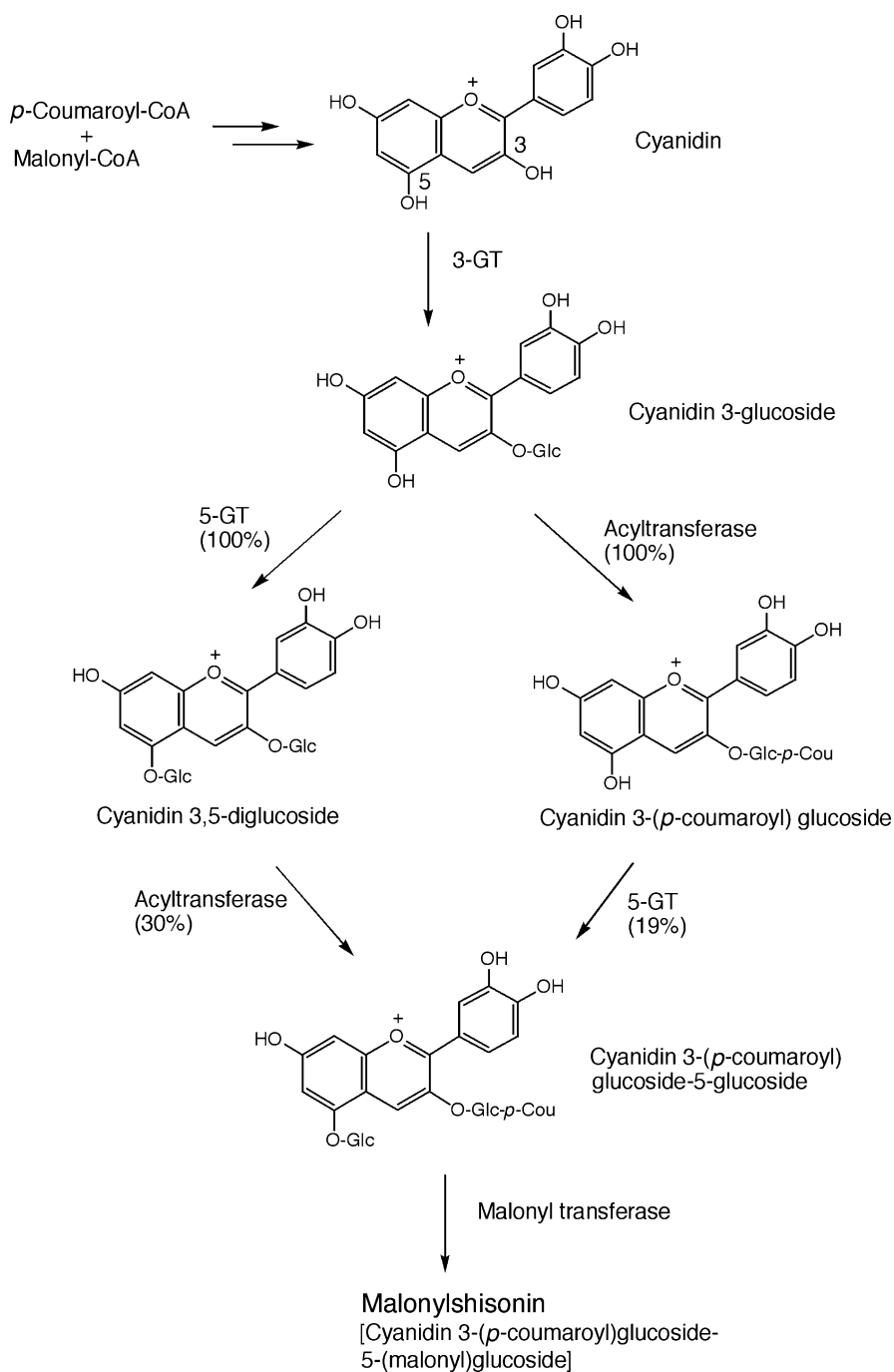


Fig. 1

poly(A)⁺ RNA, (2) cDNA synthesis, (3) PCR, and (4) isolation of differentially displayed DNA fragments.

3.1.1. Preparation of Poly(A)⁺ RNA

Poly(A)⁺RNA is directly purified from leaves of red- or green-form perilla by using Quick Prep Micro mRNA purification Kit (Amersham) containing oligo(dT)-cellulose.

1. Pulverized 100 mg of leaves of red or green form in a mortar containing liquid nitrogen using a pestle.
2. Add 0.4 mL of the Extraction buffer and mix well (*see Note 3*), and then transfer into a 2-mL microtube.
3. Add 0.8 mL of the Elution buffer and mix using a vortex mixer.
4. Centrifuge the mixture at 15,000g for 1 min to make homogenate clear.
5. Take 1 mL of the oligo(dT)-cellulose slurry in a microtube and collect oligo(dT)-cellulose pellet by centrifugation at 3000g for 1 min and pipet off the storage buffer.
6. Add the cleared homogenate to oligo(dT)-cellulose pellet and mix for 3 min.
7. Centrifuge at 15,000g for 10 s and pipet off the supernatant.
8. Wash the pellet five times with 1 mL of the High-Salt Buffer and twice with 1 mL of the Low-Salt Buffer.
9. Resuspend the washed oligo(dT)-cellulose in 0.3 mL of the Low-Salt Buffer and transfer into Micro Spin Column.
10. Wash oligo(dT)-cellulose column three times with 1 mL of the Low-Salt Buffer.
11. Elute RNA with 0.2 mL of the Elution Buffer at 65°C. Repeat elution and combine the elute as purified poly(A)⁺RNA.

3.1.2. cDNA Synthesis Using One-Base Anchored Oligo(dT) Primers

The cDNA is synthesized from poly(A)⁺RNA by using First-Strand cDNA Synthesis kit (Amersham) and one-base anchored oligo(dT) primers (*see Note 4*).

1. Place the 1–5 µg poly(A)⁺RNA in a microtube and add RNase-free water, if necessary, to make the RNA solution to 20 µL.
2. Heat poly(A)⁺RNA at 65°C for 10 min to denature, then chill on ice for 2 min.
3. Place 11 µL of the bulk first-strand cDNA reaction mix in a microtube, and add 1 µL of 200 mM DTT, 1 µL of 50 µM one-base anchored oligo(dT) primers (one of H-T11G, H-T11A, and H-T11C, GenHunter) and 20 µL of the heat-denatured poly(A)⁺RNA in final volume 33 µL, and mix by pipetting.

Fig. 1. The postulated late-stage pathway of anthocyanin biosynthesis in *Perilla frutescens* (2). The numbers in parentheses indicate relative activities of the recombinant 5-GT and the acyltransferase in crude extracts of red perilla leaves.

4. Incubate the reaction mixture at 37°C for 1 h.
5. Heat the completed reaction mixture at 90°C for 5 min to denature RNA-DNA complex and to inactivate reverse transcriptase, and chill on ice (*see Note 5*).

3.1.3. PCR Using One-Base Anchored Oligo(dT) Primers and Arbitrary Primers

The cDNA is amplified with a combination of an arbitrary primer and the same one-base anchored oligo(dT) primer used in the reverse transcription by PCR.

1. Make 20 μ L PCR reaction mixture: mix 2 μ L 10X *Taq* buffer, 2 μ L of 2 μ M one-base anchored oligo(dT) primer, 2 μ L of 2 μ M arbitrary primer, 2 μ L of 1.2 μ M dNTPs, 2 μ L of cDNA solution, 370 kBq [³²P]dCTP, appropriate amount of water and 1 U of *Taq* DNA polymerase.
2. Perform PCR in the condition as follows: a 20-s initial heating at 72°C, followed by 40 three-step cycles of a 30-s denaturation at 94°C, a 2-min annealing at 40°C, and 30-s elongation at 72°C, and then a final 5-min elongation at 72°C in a thermal cycler.

3.1.4. Isolation of Differentially Displayed Fragments

1. Fractionate amplified DNA by electrophoresis in a 5% polyacrylamide sequencing gel.
2. Dry the gel on filter paper in vacuo by heating in a gel drier and expose to X-ray film (**Fig. 1**).
3. Comparing the developed X-ray film and the dried gel, cut out the differentially appeared band from the gel and put it in a microtube.
4. Add 100 μ L water to the gel piece and heat at 100°C for 10 min to elute DNA.
5. Add 98% ethanol to precipitate the DNA. Repeat ethanol precipitation twice to remove impurity from DNA.
6. Wash DNA pellet with 85% ethanol, dry in air, and dissolve in 20 μ L distilled water.
7. Take 10 μ L of DNA solution in PCR tube as the template for re-amplification. The PCR conditions for reamplification are the same as in the first PCR.
8. Apply the reamplified DNA on electrophoresis in 1.5% agarose gel.
9. Cut out the band of the size estimated from the first PCR product and purify by spin column.
10. Clone the DNA fragment into a TA-cloning vector such as pT7Blue-T vector (Novagen).
11. Confirm the specific expression of obtained clones by Northern blot analysis according to a standard method (**4**).

3.2. The Screening of cDNA Library With Differentially Displayed Fragments

The cDNA library of red form leaves was screened with differentially displayed red form specific fragments to isolate corresponding full-length cDNA clones. The method of library screening is described in **Subheadings 3.2.1.– 3.2.2.** This includes: (1) the construction of cDNA library and (2) the screening of cDNA library with the differentially displayed fragments.

3.2.1. The Construction of cDNA Library

The cDNA library was constructed from leaves of red-form perilla (5). The detail of library construction is not described here for the limitation of space but the outline is as follows. The total RNA was extracted from 5 g of young leaves of red perilla by the hot-phenol method (5) and poly(A)⁺ RNA was concentrated from the total RNA by oligo(dT)-cellulose chromatography. From 3 mg of poly(A)⁺ RNA, the first-strand cDNA was reverse transcribed using AMV reverse transcriptase and the second-strand cDNA was synthesized by using ribonuclease H and DNA polymerase I. The obtained double-strand cDNA was ligated to *Eco*RI adaptors and then cloned into the *Eco*RI site of λ gt10, packaged and plated on selective *Escherichia coli* NM514 cells using cDNA cloning system, λ gt10 (Amersham).

3.2.2. The Screening of cDNA Library With Differentially Displayed Fragments

The screening of the cDNA library with differentially displayed fragments was performed by using a standard method (4). The 1×10^5 plaque-forming units (pfu) of the cDNA library derived from red perilla leaves were plated and transferred onto nylon membrane. The membrane was hybridized with ³²P-labeled cDNA fragments. The hybridization was done at 65°C for 16 h and the final wash of membrane was done with 0.1X SSPE, 0.1% SDS at 65°C for 15 min. The positive clones were detected by radioautography and the positive bacteriophage clones were picked up and amplified. The insert cDNA was released by digestion with *Eco*RI (see **Note 4**) and subcloned into the *Eco*RI site of pBluescriptII SK (Stratagene) and sequenced.

3.2.3. The cDNA Encoding Glucosyltransferase Homolog Isolated by Differential Display and Library Screening

Among the five clones isolated by library screening, the two cDNA clones, 3R4 and 3R6, exhibited low but significant homologies with those of UDP-

glucose: anthocyanidin 3-*O*-glucosyltransferase (3-GT) in the deduced amino acid sequences. The similarities between the amino acid sequences of these clones and 3-GTs were less than 40%. However, the 44 amino acids in C-terminal region, which exhibits the signature motif commonly found in UDP-glycosyltransferases, is conserved in these predicted polypeptides, suggesting identity of predicted proteins as members of glycosyltransferase family. The nucleotide sequences of 3R4 and 3R6 were of 1507 bp- and 1474 bp-length, respectively, which contained open reading frames of 1380 bp and 1329 bp encoding 460 amino acids of 50,973 Da and 443 amino acids of 49,109 Da polypeptides, respectively. The identity of deduced amino acid sequence between 3R4 and 3R6 is 92%. The 18 amino acids were substituted between 3R4 and 3R6, and 19 amino acids at C-terminus present in those of 3R4 were deleted in 3R6. It was eventually confirmed that only 3R4 possesses 5-GT activity, but 3R6 does not, from the enzyme assay (2).

3.3. Heterologous Expression of the Recombinant Protein in *S. cerevisiae*

The expression of red form-specific glucosyltransferase cDNA in yeast is described in **Subheadings 3.3.1.–3.3.3.** This includes: (1) the construction of yeast expression system, (2) yeast transformation, and (3) harvest of yeast and protein extraction.

3.3.1. The Construction of Yeast Expression System

An yeast shuttle vector pYE22m (7) was derived from YE_p type plasmid and composed of the ampicillin resistance gene for selection in *E. coli* and the *TRP1* gene from for selection in *S. cerevisiae*. This plasmid carries the promoter and terminator regions derived from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *S. cerevisiae* flanking a multi-cloning site, which consist of unique restriction sites of *Sal*I, *Bam*HI, *Kpn*I, and *Eco*RI. The gene expression controlled by GAPDH promoter is constitutive.

For the cloning of 3R4, which contains an *Eco*RI restriction site inside, the cDNA was digested with *Bst*XI and blunted at the 5' end, and then digested with *Bam*HI at the 3' end. pYE22m vector was digested firstly with *Eco*RI and blunted, then digested with *Bam*HI. The released cDNA insert with a blunt end and a *Bam*HI end were ligated to the digested pYE22m vector to give expression vector pY3R4.

3.3.2. Yeast Transformation

Transform *S. cerevisiae* G1315 (*trp1*) cells with pY3R4 by the lithium acetate procedure (8) as follows.

1. Culture *S. cerevisiae* in 5 mL YPD liquid medium overnight.

2. Inoculated the 0.5 mL of pre-cultured cells 50 mL YPD medium and incubated at 30°C for several hours until the culture medium reached to A_{600} 1.5–2.
3. Collect the cells by centrifugation at 3000g for 5 min and re-suspend in 5 mL LA medium and incubate at 30°C for 1 h.
4. Collect the cells by centrifugation at 3000g at room temperature for 5 min and re-suspended in 5 mL LAG medium.
5. To 0.3 mL competent cells in a microtube, add 0.1–10 μ g of plasmid, pY3R4 or pY3R6, and 0.7 mL 50% PEG 4000 were added and mix by converting the tube.
6. Treat competent cells at 42°C for 5 min.
7. Collect transformed cells by centrifugation and re-suspended in 0.1 mL sterile H₂O.
8. Plated yeast cells onto SD minimal agar plate and incubate at 30°C.

3.3.3. Harvest of Yeast Cells and Protein Extraction

1. Inoculate the transformed yeast cells into 10 mL of SD minimal liquid medium and incubate at 30°C for 24 h with shaking.
2. Collect cells by centrifugation at 3000g at 4°C for 5 min and discard the supernatant.
3. Wash the cells with 10 mL of the suspension buffer by mixing with vortex mixer.
4. Collect cells by centrifugation at 3000g at 4°C for 5 min and discard the supernatant.
5. Add 1 mL of the suspension buffer to the cells and suspend with vortex mixer.
6. Transfer the cell suspension into a microcentrifuge tube.
7. Collect cells by centrifugation at 15,000g at 4°C for 5 min and discard the supernatant.
8. Suspend the yeast cells in 0.4 mL of suspension buffer and using vortex mixer.
9. Add 200 mg glass beads (Sigma) to the cell suspension.
10. Repeat 10–15 cycles of chilling on ice for 30 s and vortex mixing for 30 s and disrupt the yeast cells.
11. Centrifuge the homogenate at 15,000g for 20 min.
12. Transfer the cleared supernatant into a fresh microtube and use as crude extract.

3.4. The Characterization of the Recombinant Protein Expressed in Yeast

1. Make the reaction mixture: 100 mM potassium phosphate buffer, pH 8.5, 1 mM UDP-glucose, 30–100 mM substrate (see **Note 10**), 50 μ g crude protein, and appropriate volume of water in total volume 50 μ L.
2. Incubate the reaction mixture at 30°C for 5 min.
3. Stop the reaction by adding 50 mL of 1% HCl in methanol.
4. Centrifugation at 15,000 rpm for 5 min or filtrate the reaction mixture with DISMIC-3CP filter.
5. Apply 20 μ L of reaction mixture HPLC on a reverse-phase column with a gradient elution (10–50% acetonitrile with 0.5% TFA acid) for 20 min followed by an isocratic elution (50% acetonitrile with 0.5% TFA) at a flow rate of 0.6 mL/min

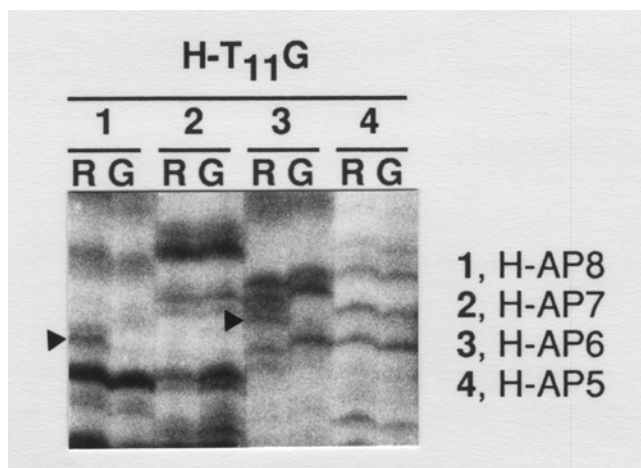


Fig. 2. Differential display of mRNA from red (R) and green (G) forms of *Perilla frutescens*. The one-base anchored cDNAs were amplified in the presence of ^{32}P -dCTP using primer combinations of a one-base anchored oligo d(T) primer (H-T11G) and an arbitrary primer (one of H-AP8 [1], H-AP7 [2], H-AP6 [3], and H-AP5 [4]). Differentially displayed fragments are indicated by arrow heads.

at 40°C with monitoring absorbance at 520 nm. Identification of anthocyanins is done by comparison of retention times (**Fig. 3**) and spectral patterns (250–600 nm) of standard compounds.

DNA sequencing and nucleic acid hybridization analysis were carried out by standard methods (**4**).

4. Notes

1. 20X dropout solution can be autoclaved and stored at 4°C.
2. Glucose must be autoclaved separately as 40% solution and added to SD medium after autoclaving.
3. RNA is unstable on cell disruption without RNase inhibitor such as guanidinium thiocyanate. Add the Extraction Buffer as quickly as possible after pulverization of plant tissue before the powder starts to melt to prevent RNA degradation.
4. It is a critical point to use one-base anchored oligo(dT) primers and divide transcript population for good separation of PCR products by sequencing gel electrophoresis.
5. The cDNA can be stored in -20°C.
6. Some clones containing *Eco*RI restriction site should be amplified by PCR and cloned into a TA-cloning vector.
7. We used 24 combinations of primers: 3 oligo(dT) primers and 8 arbitrary primers. The number of arbitrary primers can be increased if necessary.

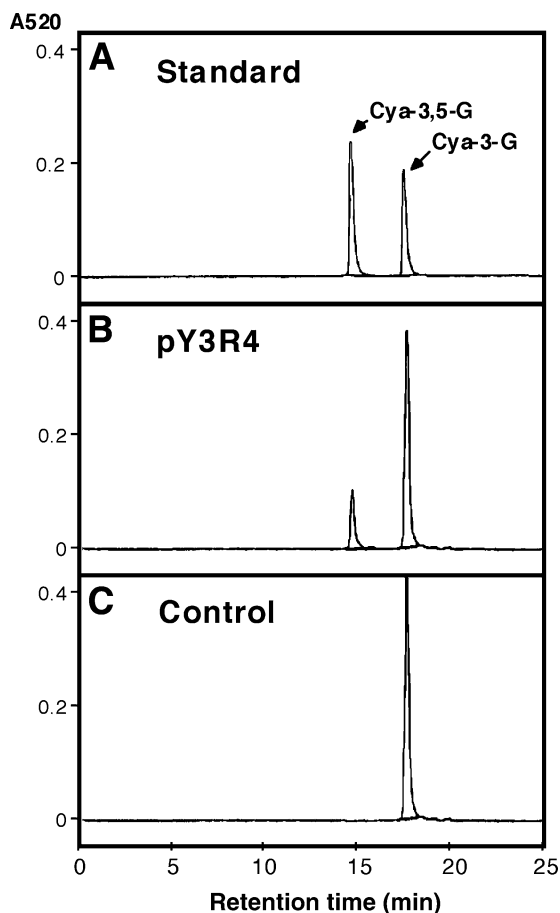


Fig. 3. High-pressure liquid chromatography (HPLC) analysis of the reaction product of 5-GT (2). (A) The elution profile of standard cyanidin 3-*O*-glucoside (Cya-3-G) and cyanidin 3,5-*O*-diglucoside (Cya-3,5-G) on reverse-phase HPLC monitored at 520 nm. (B) The reaction product formed with extracts of yeast expressing 5-GT cDNA (pY3R4). The 5-GT reaction was carried out in the reaction mixture (100 mM phosphate buffer, pH 8.5, 200 mM cyanidin 3-*O*-glucoside, 1 mM UDP-glucose, and 100 mg crude protein) at 30°C for 5 min. (C) The reaction product with extracts of yeast containing the empty vector.

8. Nonspecific genes could be also amplified by PCR. To reduce the number of useless probes, the specific expression of the differentially displayed fragments should be confirmed by northern blot analysis or RT-PCR.
9. The competent cells of *S. cerevisiae* in LAG medium can be stored at -70°C. The transformed *S. cerevisiae* can be stored in YPD medium containing 15% glycerol

at -70°C . In both cases, the cells should be divided in portions in microtubes to prevent the cell damage by the repetitive freeze and thawing.

10. The substrate specificity is quite different depending plant species (2,9). The perilla 5-GT has broad substrate specificity and can glucosylate various anthocyanin 3-*O*-glucosides. Cyanindin 3-*O*-glucoside is the best substrate for perilla 5-GT. Petunia 5-GT has strict substrate-specificity and requires acylated anthocyanin. To determine K_m values, the concentrations of anthocyanin and UDP-glucose were varied; K_m values were obtained by double-reciprocal plots.

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Identification of Target Genes of a Yeast Transcriptional Repressor

Bernard Mai and Linda L. Breeden

Summary

Xbp1 is a transcriptional repressor with homology to the DNA binding domains of two cell-cycle regulatory transcription factors, Swi4 and Mbp1. Target genes bound and regulated by Xbp1, have been identified using differential display (DD). Because little was known about the regulation or function of Xbp1, we identified genes that were underrepresented in RNAs purified from cells in which Xbp1 was ectopically expressed. Four target genes, including three cyclin genes, were identified by DD and then confirmed by binding studies and Northern analysis. The binding site for Xbp1, previously identified by site selection, was present in these target genes and is conserved phylogenetically.

Key Words: Xbp1; yeast; cyclins; transcription; differential; meiosis.

1. Introduction

The regulation of the cell cycle is a complex biological process in which at least 1 in every 10 genes is transcriptionally regulated (**1–3**). A set of transcription factors is responsible for generating a pattern of differential gene expression. These factors are often also transcriptionally regulated during the cell cycle and produced just before they are required to act upon their target genes (**4,5**). Characterizing these proteins and identifying their target genes is of prime interest to understand cell-cycle control.

Swi4 and Mbp1 are related DNA-binding proteins that play a role in the regulation of genes at the G1/S boundary of the *Saccharomyces cerevisiae* cell cycle (**6,7**). YIL101C was an uncharacterized open reading frame in budding yeast with homology to the DNA binding domains of Swi4 and Mbp1. To see

if this third protein played a related role in the cell cycle, we characterized its activity, its regulation, and its target genes. Xbp1 acts as transcriptional repressor and is induced by a variety of stress conditions (8). Its binding consensus sequence is related to that of Swi4 and Mbp1, but it differs at the second position (8).

Using the method of mRNA differential display (DD), a comparative transcriptome analysis was launched to identify Xbp1 target genes (9). Without knowing the physiological function of Xbp1, we could not anticipate the conditions under which Xbp1 exerts its role as a repressor. Moreover, Xbp1 levels are very low in normally growing cells, but they increase under many conditions that stress the cells. For this reason, we compared the expression level of all yeast genes in wild type cells to that of yeast cells lacking Xbp1 in stress conditions. We also looked in cells ectopically expressing Xbp1 to high levels under the control of the GAL1 promoter. It was expected that potential target genes of Xbp1 would be upregulated in the absence of Xbp1 and downregulated when Xbp1 was overproduced. Using 80 different primer combinations, which were theoretically sufficient to detect all 6000 yeast genes (10), we were unable to identify Xbp1 target genes from the comparison of *xbp1* mutant and wild type cells under stress. However, with the same set of primers, we were able to identify several potential Xbp1 target genes that were repressed in cells that ectopically produced Xbp1 (Fig. 1). These putative targets were then confirmed to be Xbp1 targets by Northern blotting (Fig. 2) and binding experiments (11).

From these and other results, we infer that comparison of *xbp1* mutant to wild-type cells yielded no Xbp1 targets because we were not reproducing the physiological conditions under which Xbp1 normally serves to repress its target genes, or because Xbp1's activity is redundant with another repressor, or both. However, when we used ectopic expression of this transcriptional repressor, its target genes were readily identified. A later analysis of Xbp1 targets by DNA microarray experiments agreed, in detail, with the results we obtained with DD (data not shown). Furthermore, each of the potential target genes identified had at least one potential Xbp1 binding site located in its promoter (Fig. 3) that was conserved in other *Saccharomyces* species (12). Once potential targets were identified, we were able to verify that these genes were *in vivo* targets by other means. One of the Xbp1 targets we identified, *CLN1*, is repressed during meiosis and this helps promote an efficient sporulation (11). A second target, *CLB2*, is repressed during nitrogen-limited growth, which gives rise to pseudohyphae formation (13).

Using DD to identify targets of a yeast transcription factor is simplified by the fact that there are only about 6000 genes in *S. cerevisiae* and only 5% of them contain introns (12). Thus, even a small number of affected RNAs can be easily detected. Moreover, after the initial expense of the primers, the method

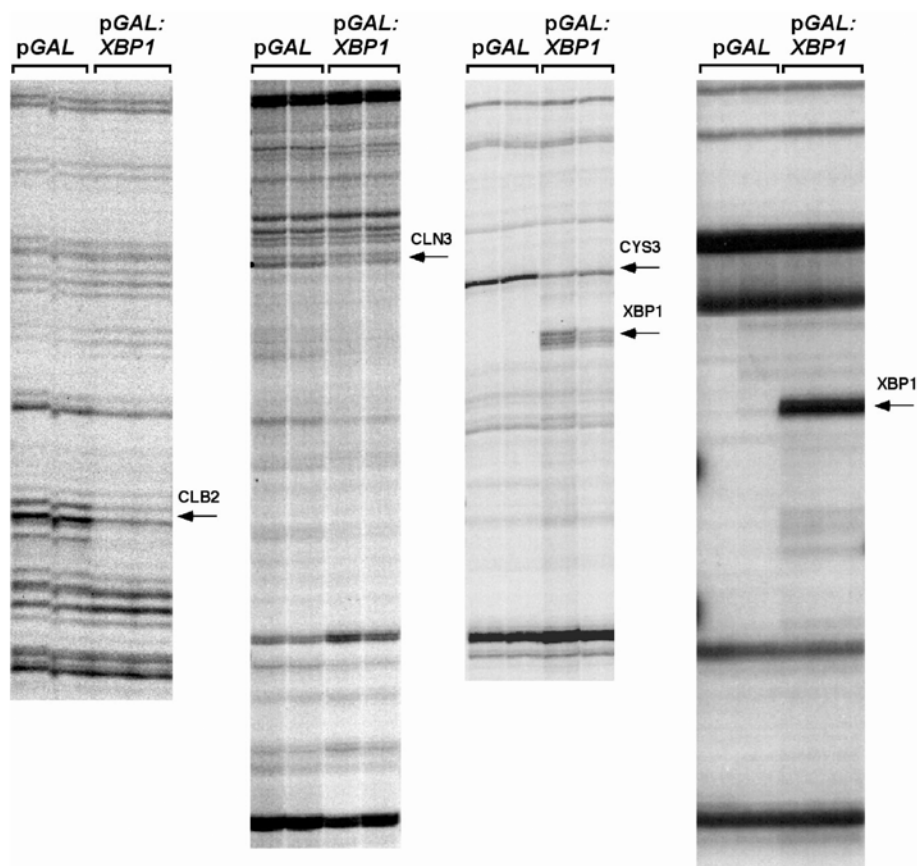


Fig. 1. Identification of Xbp1 target genes. Total RNA from yeast cells lacking XBP1 (pGAL) and cells overexpressing Xbp1 (pGAL:Xbp1) were subjected to DD analysis. Display patterns using different primer combinations are depicted as parts of different denaturing gels. For each condition, duplicate PCR reactions were performed and loaded adjacent to each other onto a 6% sequencing gel. cDNA fragments of interest are indicated by arrows. The right most gel shows the identification of Xbp1 cDNA in RNA from cells overexpressing Xbp1. This was the only RNA upregulated under these circumstances.

is relatively inexpensive and can be carried out with many samples. The first steps are extraction of RNA (*see Subheading 3.1.*) and DNase treatment (*see Subheading 3.2.*). These methods are tailored for working with yeast, but all subsequent steps of reverse transcription (*see Subheading 3.3.*), PCR amplification (*see Subheading 3.4.*) and resolving the differences on polyacrylamide gels (*see Subheading 3.5.*) can be applied to any system. Identifying the

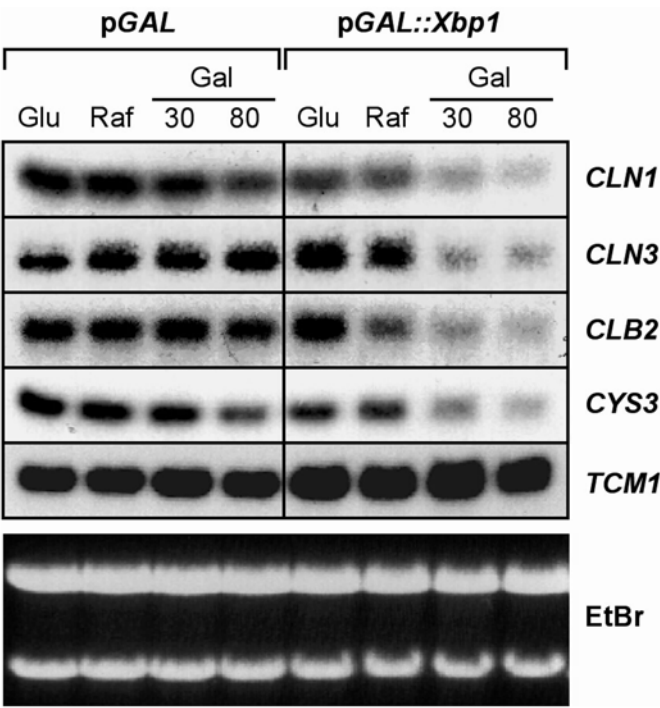


Fig. 2. Confirmation of Xbp1 target genes by Northern blot analysis. Total RNA used in the differential display analysis was subjected to denaturing agarose gel electrophoresis. Northern blotting was carried out with radiolabeled probes containing the complete ORF of the indicated genes, and transcripts were detected by autoradiography. A *TCM1* probe (encoding the ribosomal protein L3) was used as the internal control in each lane. In addition, a picture of the EtBr stained gel is shown that shows stable RNAs.

affected RNAs involves eluting the cDNA from the bands of interest, reamplifying, and sequencing (see **Subheading 3.6.**). This is essentially the same, although with yeast it is likely that each RNA is present at a higher starting concentration than would be expected for a more complex genome. Confirmation of the results is important in any case. We use either Northern blotting (see **Subheading 3.7.** and **Fig. 2**) or oligonucleotide S1 protection (**14,15**).

2. Materials

- 1. RNA buffer: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA).
- 2. RNA SDS buffer: 1.3% sodium dodecyl sulfate (SDS) in RNA-buffer.

CLN3

<i>S. cerevisiae</i>	A	C	T	T	T	C	C	T	C	G	A	G	C	T	T	T	T
<i>S. mikatae</i>	G	C	T	T	T	C	C	T	C	G	A	G	C	T	C	T	C
<i>S. kudriavzevii</i>	A	C	T	T	T	C	C	T	C	G	A	G	G	A	T	A	T
<i>S. bayanus</i>	A	G	T	G	T	T	C	T	C	G	A	G	A	C	T	T	T

CLN1

<i>S. cerevisiae</i>	G	A	C	T	A	A	C	T	C	G	A	A	A	G	T	G	A
	C	G	A	A	G	A	C	T	C	G	A	A	A	A	T	T	A
<i>S. mikatae</i>	G	A	C	T	A	T	C	T	C	G	A	G	A	G	T	G	A
	G	G	A	A	G	A	C	T	C	G	A	A	A	A	T	A	A
<i>S. kudriavzevii</i>	A	A	C	T	A	T	C	T	C	G	A	G	A	G	T	G	A
	G	A	A	A	G	A	C	T	C	G	A	A	A	A	T	A	A

CLB2

<i>S. cerevisiae</i>	T	A	T	T	T	C	C	T	C	G	A	G	G	G	A	C	A
<i>S. mikatae</i>	T	A	A	T	T	C	C	T	C	G	A	G	G	G	A	C	A

Xbp1 consensus site:

site selection	g	c	C	T	C	G	A	R	g	m	g	r
phylogenetic footprinting	t	C	T	C	T	A	R	R	A			

Fig. 3. Conservation of Xbp1 binding sites in the promoters of genes identified by DD. Below are alignments of the *CLN3*, *CLN1*, and *CLB2* promoters from yeast species highly related to *Saccharomyces cerevisiae*: *Saccharomyces bayanus*, *Saccharomyces castellii*, *Saccharomyces kudriavzevii* and+ *Saccharomyces mikatae*. Conserved positions of the Xbp1 binding site consensus are depicted in black boxes; positions conserved among more than 50% of the listed binding sites are shown in gray boxes. The Xbp1 consensus binding site experimentally defined by site selection (8) is shown below, along with the Xbp1 site deduced from phylogenetic comparison (12).

3. RNA buffer equilibrated phenol: 500 g of 99% pure crystallized phenol (EM Industries) was dissolved in dH₂O. 0.5 g hydroxyquinoline hemisulfate (Sigma) was added, then the phenol was washed once with 300 mL of 1 M Tris-HCl, pH 7.4, then twice with 300 mL of 0.01 M Tris-HCl, pH 7.4. 50 mL aliquots were stored at -20°C. Aliquots were thawed as needed and equilibrated with three washes of 10 mL RNA buffer.
4. Glass beads, acid-washed, 425–600 μ (Sigma).
5. FastPrep FP120 disruptor (Bio101, ThermoSolvant).
6. Distilled water (dH₂O).
7. Diethyl pyrocarbonate (DEPC)-treated dH₂O.

8. RNase inhibitor (10 U/ μ L, Promega).
9. RNase-free DNase I (10 U/ μ L, Promega).
10. 10X RNase treatment buffer: 100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM MgCl_2 .
11. Superscript II reverse transcriptase (200 U/ μ L, Invitrogen).
12. dNTPs (250 μ M, Invitrogen).
13. 5X RT buffer: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl_2 , 50 mM dithiothreitol (DTT).
14. Anchored primer set (1 μ M): T_{12}MA , T_{12}MC , T_{12}MG , T_{12}MT (where M can be A, C, or T).
15. Arbitrary decamer primer (0.2 μ M) (kit OPA-G, Operon Technologies Inc.).
16. *Taq* polymerase (5 U/ μ L, Fisher Scientific).
17. dNTPs (25 μ M).
18. α -[^{33}P]dCTP (>2000 Ci/mmol) (Dupont, NEN).
19. Formamide loading dye: 80% formamide, 10 mM EDTA, 1 mg/mL bromphenol blue, 1 mg/mL xylene cyanol.
20. 6% denaturing polyacrylamide gel: 7 M urea, 1X TBE or 0.09 M Tris-base, 0.09 M boric acid, 2 mM EDTA.
21. DNA sequencing apparatus.
22. Glycogen (10 mg/mL, Boehringer Mannheim).
23. QIAEXTM DNA extraction kit (Qiagen, Valencia, CA).
24. 2X RNA loading-buffer: 1X MOPS, 18.5% formaldehyde, 50% formamide, 4% Ficoll400, bromphenol blue (store at -20°C).

3. Methods

3.1. Extraction of Total RNA From Yeast

Because only few yeast genes possess introns, the purification of mRNA is not necessary. Instead, total RNA was extracted from 1 to 2×10^8 cells, exponentially grown in yeast synthetic complete medium complemented with appropriate amino acids and carbon sources. Until RNA was extracted with ethanol, all steps were performed at 4°C .

1. Dissolve cell pellet in 120 μ L ice-cold RNA buffer.
2. Add glass beads to liquid level.
3. Vortex for 30 s in FastPrep disruptor (setting 4.5).
4. Add 450 μ L RNA SDS buffer, mix briefly by hand.
5. Add 450 μ L RNA buffer equilibrated phenol.
6. Vortex for 30 s in FastPrep disruptor (setting 4.5).
7. Spin in microfuge (5 min, 4°C , 10,000g).
8. Transfer upper phase to fresh tube.
9. Add 300 μ L RNA-buffer equilibrated phenol, vortex briefly.
10. Add 450 μ L chloroform, vortex briefly.
11. Centrifuge for 2 min and extract upper phase again with chloroform.
12. Add 20 μ L 4 M NaCl and 1 mL cold ethanol.

13. Incubate 30 min at -20°C .
14. Pellet RNA by centrifugation (5 min, 10,000g).
15. Wash pellet with 150 μL 70% ethanol.
16. Air-dry pellet.
17. Dissolve RNA in DEPC-treated dH_2O to 5–10 $\mu\text{g}/\mu\text{L}$.
18. Check quality of RNA on 1% agarose-formaldehyde gels.

3.2. DNase I Treatment of Total RNA

Because most yeast genes do not contain introns, it is especially important to remove any residual chromosomal DNA from the prepared total RNA. Trace amounts of chromosomal DNA would be amplified in the subsequent PCR amplifications along with the cDNAs, thereby giving rise to high background smearing in the display and complicating the pattern of displayed bands.

1. Mix and incubate for 30 min at 37°C : 50 μg total yeast RNA, 10 U RNase inhibitor, 10 U RNase-free DNase I, 1X RNase treatment buffer.
2. Inactivate DNase I by one phenol-chloroform extraction.
3. Precipitate RNA by adding 0.1 vol 3 M NaOAc and 3 vol ethanol.
4. Incubate for at least 30 min at -80°C .
5. Pellet RNA by centrifugation at 4°C for 10 min.
6. Dissolve RNA in 20 μL DEPC-treated H_2O , check integrity again on a 1% agarose-formaldehyde gel.
7. Store DNA-free RNA at -80°C until used for DD (*see Note 2*).

3.3. Reverse Transcription of Total RNA

1. Dilute DNA-free RNA with DEPC-treated dH_2O to 100 $\text{ng}/\mu\text{L}$, keep on ice.
2. For each RNA sample set up four reactions (one tube for each degenerate anchored oligo(dT) primer set: T_{12}MA , T_{12}MC , T_{12}MG , T_{12}MT ; where M is A, C, or G). For 20 μL final volume, use: 2 μL RNA (100 $\text{ng}/\mu\text{L}$), 4 μL 5X RT buffer, 1.6 μL dNTPs (250 μM), 2 μL T_{12}MN primer (10 $\text{pmol}/\mu\text{L}$), 9.4 μL 1 DEPC-treated H_2O (*see Note 1*).
3. Incubate for 5 min at 65°C , following 10 min at 37°C .
4. Add 1 μL Superscript II reverse transcriptase (200 U/ μL).
5. Incubate for 50 min at 37°C .
6. Inactivate the RT by incubation for 5 min at 95°C .
7. Use cDNA immediately for PCR amplifications or store at -20°C .

3.4. PCR Amplification

1. All PCR reactions should be done in duplicate and run in the same PCR machine to minimize variations (*see Note 3*).
2. Prepare a master mix for all PCR reactions that contains the same T_{12}MN primer. Aliquot 18 μL into each PCR tube and then add one arbitrary decamer primer.
3. Mix for each 20 μL PCR reaction: 2 μL 10X PCR reaction buffer, 1.6 μL dNTP mix (25 μM), 2 μL T_{12}MN primer, 2 μL cDNA (generated using the correspond-

ing T₁₂MN primer), 0.2 μ L *Taq* polymerase (5 U/ μ L), 1 μ L α -[³³P]dCTP (>2000 Ci/mmol), 9.2 μ L H₂O.

4. Aliquot and add 2 μ L arbitrary primer (2 pmol/ μ L).
5. Carry out PCR amplification in 40 cycles as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s, followed by a 5-min extension at 72°C.
6. Store PCR reactions at -20°C.

3.5. 6% Denaturing Polyacrylamide Gel Electrophoresis

1. Prepare 6% denaturing polyacrylamide gel (7 M urea, 1X TBE). Prerun gel for 30 min at 60 W.
2. Mix 3.5 μ L of the PCR reaction with 2 μ L formamide loading buffer, incubate for 3 min at 95°C, chill on ice until loading onto a 6% DNA sequencing gel.
3. Load samples (together with a labeled size marker) and run gel for about 3 h at 60 W constant current until the xylene cyanol dye reaches the bottom.
4. Place gel without fixation on Whatman 3MM paper and dry on a gel dryer for 1 h at 80°C.
5. Autoradiograph overnight at -80°C. Place fluorescent stickers onto gel to identify orientation. **Figure 2** shows a representative DD (*see Note 4*).

3.6. Isolation and Reamplification of cDNA Probe

1. Place film in correct orientation over the dried gel. Mark bands of interest by punching through the film with a needle.
2. Cut out band of interest with a clean razor blade.
3. Soak the dried gel slice in 100 μ L H₂O for 10 min at room temperature in a microfuge tube.
4. Incubate the tube for 15 min at 95°C. Make sure that tube is tightly closed.
5. Centrifuge tube for 2 min in microfuge at maximum speed to pellet debris. Transfer supernatant into a fresh tube.
6. Precipitate DNA by adding 10 μ L 3 M NaOAc, 5 μ L glycogen (10 mg/mL) and 400 μ L ethanol. Incubate at -80°C for at least 1 h.
7. Pellet DNA by centrifugation for 10 min at 4°C (microfuge, maximum speed).
8. Wash pellet with 85% ethanol and dissolve in 10 μ L H₂O.
9. Reamplify fragments in a 20 μ L PCR reaction using the appropriate T₁₂MN—arbitrary primer combination for each fragment (*see Note 5*).
10. Mix 4 μ L of the isolated DNA (store rest at -20°C), 2 μ L 10X PCR reaction buffer, 1.6 μ L dNTPs (250 μ M), 2 μ L T₁₂MN primer (2 pmol/ μ L), 2 μ L arbitrary primer (2 pmol/ μ L), 0.2 μ L *Taq* polymerase (5 U/ μ L).
11. Run PCR using the conditions as described under **Subheading 3.4**.
12. Load 18 μ L of the PCR reaction onto a 1.5% agarose gel, stain with ethidium bromide (EtBr), and isolate the fragment of expected size using the QIAEX DNA extraction kit.

13. If there is no PCR product visible, take 1 μL of the PCR reaction and reamplify again.
14. The isolated DNA can be used directly for cycle sequencing, subcloning, or as probe for Northern blots.

3.7. Confirmation by Northern Blotting

1. Heat denature 5–10 μg of each RNA used for the DD in 1X RNA loading buffer, chill on ice, and add 0.5 μL EtBr (0.5 $\mu\text{g}/\mu\text{L}$).
2. Run several samples of each RNA on a 1% agarose gel containing 0.6 M formaldehyde and 1X MOPS-buffer.
3. Run gel until bromophenol blue reaches the end.
4. Wash gel three times for 5 min in distilled water, photograph, and blot to GeneScreen membrane (Dupont, NEN) using a standard protocol (16).
5. Cut the blot into strips, with each strip containing RNAs of the conditions compared in the DD. Hybridize each strip to a different probe isolated and amplified from the DD sequencing gel (*see Note 6*).

4. Notes

1. We used four different oligo(dT) primer pools, each in combination with 20 different arbitrary decamer primers. This resulted in 80 primer combinations for the DD PCRs, which should have enabled us to detect nearly every expressed yeast gene.
2. The removal of contaminating chromosomal DNA from the RNA samples was checked by PCRs using several sets of primers, each being able to produce signals only in the presence of genomic DNA. RNA samples were tested before and after RNase-free DNase I treatment. A nearly complete loss of PCR products should be visible in the treated sample.
3. We always performed PCR reactions in duplicates per cDNA sample. Sometimes differential expression was observed only in one of the two PCR reactions. In this case, PCR reactions were repeated. In general, PCR reactions producing a differential banding pattern were repeated using an independently generated cDNA pool as probe. Only fragments showing up reproducibly were considered for further analyses. It is important to perform all PCR reactions in the same thermal cycler, because slight differences during the heating and cooling phases of the PCR machine can have a great influence in the yield of PCR fragments. We always used a thermal cycler with a heated lid.
4. In general, only fragments between 100 and 500 nucleotides in length were considered. Fragments below 100 nucleotides could not be visualized owing to the conditions of the DD sequencing gel. For fragments above 500 nucleotides in length, it was found to be difficult to isolate single fragments and reamplify them successfully. Sequencing of these fragments often produces heterogeneous

sequences because it is more likely that several fragments will co-migrate with the band of interest.

5. Most cDNAs had to be amplified twice using 1 μ L of the first amplification as template.
6. Using the reamplified cDNA probe for confirmation in Northern blots generally produced a signal. We found that about 50% of the isolated fragments were false positives, that is, the signal observed in the Northern blot did not show any differential expression. Only the confirmed, reamplified cDNAs were used for cycle sequencing using the appropriate decamer primer as sequencing primer (around one-fifth of the isolated DNA was used for the sequencing reaction). Using the sequence information obtained, primers were synthesized to PCR the ORF of the corresponding yeast gene. Subsequently, this fragment was used as probe for Northern blots to confirm the differential gene expression.

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Detection of an mRNA Polymorphism by Differential Display

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Summary

Differential display technology was utilized to compare programs of gene expression in primary cultures of human skin fibroblasts from normal volunteers and patients diagnosed with melancholic depression. Polymorphic transcripts of a single gene differing by one tandem repeat sequence of four nucleotides (TGAT) in the 3' noncoding region were detected.

Key Words: Differential display; PCR; mRNA polymorphism; variable number tandem repeats (VNTRs).

1. Introduction

Differential display (DD) was introduced in 1992 as a means for amplifying and visualizing most of the mRNA in a cell and for cloning differentially expressed genes (1). The method utilizes three different one-base anchored oligo-dT primers in combination with 80 rationally designed upstream 13mer primers to detect and display approx 96% of expressed genes (2). We applied DD to primary cultures of human skin fibroblasts from two normal volunteers and two patients diagnosed with melancholic depression in order to compare programs of gene expression in these two cell populations. Previously we had demonstrated a highly significant (and consistent) decrease in the β -adrenoceptor-mediated phosphorylation of the nuclear transcription factor CREB in the fibroblasts of depressed patients vs normal controls (3). Because it is the phosphorylation state of CREB rather than the total amount of its steady-state mRNA that regulates CREB-CRE directed transcription (4), the

reduction of CREB-P in patient's cells predicted differential programs of gene expression.

DD has been predominantly utilized to detect the presence or absence of gene transcripts (cDNA species). Accordingly, we initially assumed that the display by a pair of primers shown in **Fig. 1** clearly revealed two genes, one up- and the other downregulated in melancholic depressed patients vs normal controls. However, when the cDNAs were cloned and sequenced (**Fig. 2**), we were surprised to learn that they were in fact polymorphic transcripts from a single gene, and furthermore that the two cDNAs differed by a single-tandem repeat sequence of four nucleotides (TGAT) in the 3' non-coding region amplified by the DD PCR primers used.

2. Materials

2.1. Extraction and Purification of Total RNA

1. TRI Reagent (Molecular Research, Inc., MA).
2. Isopropanol.
3. 75% Ethanol.

2.2. Removal of DNA Contamination

1. MessageClean® Kit (GenHunter Corporation, Nashville, TN), including RNase-free DNase I, 10X Reaction Buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, and 15 mM MgCl₂, and 0.01% gelatin).
2. Phenol:chloroform (3:1).
3. 7% Formaldehyde/agarose gel.

2.3. Reverse Transcription

1. RNImage® Kit (GenHunter, Nashville, TN), including dH₂O, 5X RT Buffer, dNTP (250 μM), HT₁₁M primers, MMLV Reverse Transcriptase.
2. 0.5-mL microfuge tubes (GenHunter).
3. Thermocycler (PTC-100™, MJ Research, Inc, Watertown, MA).

2.4. Differential Display

1. RNImage Kit (GenHunter) including dH₂O, 10X polymerase chain reaction (PCR) Buffer, dNTP (25 μM), arbitrary H-AP primers, HT₁₁M primers.
2. Amplitaq Taq Polymerase, 5 U/μL (PerkinElmer).
3. α³³P dATP, 2000 ci/mmol (NEN).
4. Sequagel-6 Acrylamide Mix (National Diagnostics, Atlanta, GA).
5. Whatman 3MM paper.
6. Saran wrap.
7. X-ray film, Kodak Biomax MR (Kodak).
8. Razor blade.

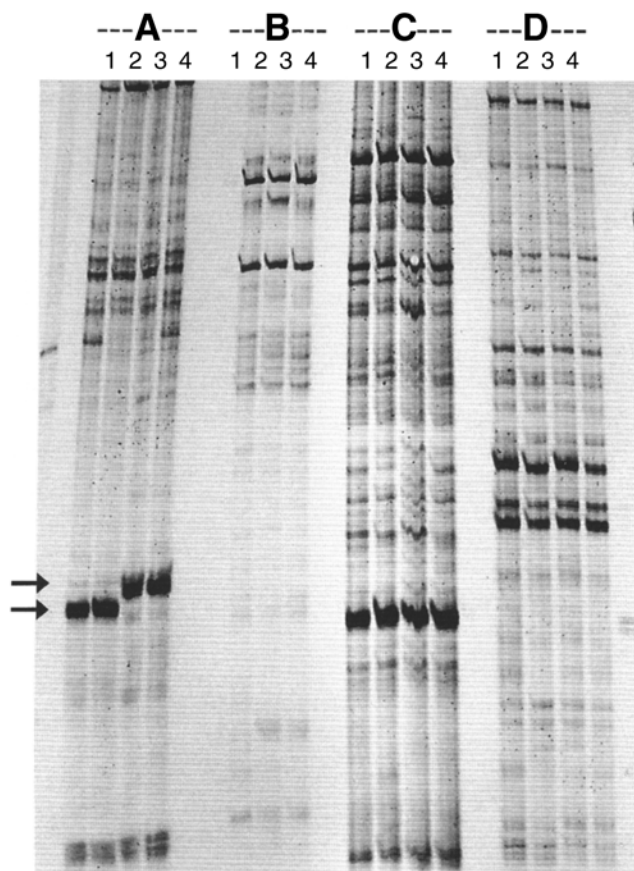


Fig. 1. Differential display of total messenger RNA (reverse transcribed to cDNA) in human skin fibroblasts from two normal volunteers (1,2) and two patients diagnosed with melancholic depression (3,4). The cDNAs were amplified by PCR with primer sets HAP₆₄/HT₁₁C (A); HAP₆₅/HT₁₁A (B); HAP₆₅/HT₁₁G (C); and HAP₆₅/HT₁₁C (D). Primer sets were from GenHunter. Primer set HAP₆₄/HT₁₁C displayed two differentially expressed genes that are similar in size, one up- and the other downregulated in melancholic depressed patients vs normal controls (indicated by arrows). The bands were subsequently excised and cloned into the PCR-Trap vector (GenHunter).

9. 100% ethanol.
10. 85% ethanol.
11. PCR-TRAP[®] Cloning System (GenHunter).

Patients (four TGAT repeats)

AAGCTTCATATGCAAATTCATCATTTGGGCTCACTTCTAATAACTGCAGTGTTTCCCGCC
 TTGGGCTTGCAGCAGAAAAACCTGACAACATAGTGTGTTGCTAAGGCAGTATTTGACTTT
 ACCTTATTTGTGATTACTGTAGTTGATTGATTGATTGATTTACTATTAACTACAAGGTATA
 TTTACTATCACCTTATTTAAATTTTATGAATTAATTTGAATGTTTTTTTACACTAACTAA
 CTTTTCCCAATAAAGTCCACTATGAAACCACGAAAAAAAAAAGCTT

Normal (three TGAT repeats)

AAGCTTCATATGCAAATTCATCATTTGGGCTCACTTCTAATAACTGCAGTGTTTCCCGCC
 TTGGGCTTGCAGCAGAAAAACCTGACAACATAGTGTGTTGCTAAGGCAGTATTTGACTTT
 ACCTTATTTGTGATTACTGTAGTTGATTGATTGATTTACTATTAACTACAAGGTATATTTA
 CTATCACCTTATTTAAATTTTATGAATTAATTTGAATGTTTTTTTACACTAACTAACTTT
 TCCCAATAAAGTCCACTATGAAACCACGAAAAAAAAAAGCTT

Fig. 2. Sequencing of the cloned cDNAs revealed that the two apparently differentially expressed genes were in fact polymorphic transcripts from a single gene. Interestingly, the transcripts differed by a single tandem repeat sequence of four nucleotides (TGAT, underlined) in the 3' noncoding region amplified by the HAP₆₄ and HT₁₁C primers flanking the PCR products (underlined).

3. Methods

3.1. Extraction and Purification of Total RNA

Monolayer cultures of human skin fibroblasts from two normal volunteers and two patients diagnosed with melancholic depression were lysed in the culture dishes by applying 1 mL of TRI reagent per 10 cm² for 5–10 min. The homogenate was supplemented with 0.1 mL BCP per 1 mL of TRI reagent and the resultant mixture was shaken vigorously for 15 s, incubated at room temperature for 15 min, and then centrifuged at 12,000g for 15 min at 4°C (see **Note 1**). The RNA was precipitated in isopropanol (0.5 mL/mL TRI reagent) by centrifugation at 12,000g for 8 min at 4°C, and the RNA pellet was washed twice with 75% ethanol and then centrifuged at 7500g for 5 min at 4°C.

3.2. Removal of DNA Contamination

Because chromosomal DNA contamination of the RNA samples can lead to amplification of DNA, thereby complicating the pattern of displayed bands, residual DNA was removed via a MessageClean kit. Briefly, 100 µg total cellular RNA were incubated with 10 U of RNase-free DNase I in 1X Reaction

Buffer for 40 min at 37°C. The DNase was subsequently inactivated by adding an equal volume of phenol:chloroform (3:1) to the samples, vortexing briefly, incubating on ice for 10 min, and briefly spinning at low speed (*see Note 2*). The purified RNA was subsequently recovered from the aqueous phase by ethanol precipitation. The integrity of the resultant RNA was checked before and after DNase I treatment by electrophoresis of 1–3 µg of each sample in a 7% formaldehyde/agarose gel. RNA samples were stored at concentrations >1 mg/mL at –80°C prior to DD.

3.3. Reverse Transcription

Three reverse transcriptions of each RNA sample were performed in 0.5-mL microfuge tubes (GenHunter) each containing one of the three different anchored oligo(dT) primers (GenHunter) in a 20 µL reaction mixture containing: distilled H₂O, 9.4 µL; 5X RT buffer, (4.0 µL); dNTP (250 µM), 1.6 µL; total RNA (DNA-free), 2.0 µL (0.1 µg/µL freshly diluted); AAGCT₁₁M (2 µM), 2.0 µL (M is G, A, or C). The thermocycler was programmed as follows: 5 min at 65°C, 60 min at 37°C, 5 min at 75°C, hold at 4°C. After the first 10 min at 37°C 1 µL MMLV Reverse Transcriptase was added to each tube and mixed quickly and thoroughly by fingertipping. The samples were returned to the thermocycler and incubation at 37°C was continued. At the conclusion of the reverse transcription reaction, the tubes were centrifuged briefly to collect condensation and placed on ice for PCR (or stored at –80°C for later use).

3.4. Differential Display

3.4.1. Polymerase Chain Reaction

For DD we utilized RNAimage Kits (GenHunter) according to the company's directions. Briefly, PCR reactions were set up at room temperature as follows (20 µL final volume for each primer set combination): distilled H₂O, 10 µL; 10X PCR buffer, 2.0 µL; dNTP (25 µM), 1.6 µL; arbitrary 13mer primers (2 µM), 2 µL; AAGCT₁₁M (2 µM), 2.0 µL; RT mixture (from **step 1**), 2.0 µL; [α^{32} P]dATP, 2000 ci/mmol, 0.2 µL; AmpliTaq (5 U/µL), 0.2 µL. Core mixes were made to avoid pipetting errors (*see Note 3*). Samples were mixed well by pipetting up and down. The PCR reaction cycles were as follows: 30 s at 94°C, 2 min at 42°C, 30 s at 72°C for 40 cycles followed by 5 min at 72°C and hold at 4°C.

3.4.2. Visualization of PCR Products

PCR products were visualized via electrophoresis in a 6% denaturing polyacrylamide gel (Sequagel-6) in TE buffer (*see Note 4*). The gel was allowed to polymerize >2 h and was prerun for 30 min. 3.5 µL of each sample were mixed with 2 µL loading dye and incubated at 80°C for 2 min immediately before

loading. For optimum resolution of bands, the wells were flushed with buffer (four to six at a time) during loading. Electrophoresis was conducted for approx 3.5 h at 60 W constant power (not to exceed 1700 V) until the xylene dye (the slower dye) reached the bottom. The power was turned off and the gel was transferred to Whatman 3MM paper and covered with plastic wrap. It was dried for 2 h at 80°C under vacuum. The dried gel (and subsequent autoradiogram) were oriented with radioactive ink prior to exposing the gel to X-ray film overnight (approx 16 h).

3.4.3. Extraction, Reamplification, and Subcloning of Differentially Expressed Bands

Two bands that appeared to be differentially expressed in depressed patients vs normal controls were excised by cutting through the film with a clean razor blade. The gel slices/3MM paper were soaked in 100 μ L H₂O for 10 min in Eppendorf tubes and then boiled for 15 min. The tubes were centrifuged for 2 min to pellet the gel and paper debris and to collect condensation. The supernatant was then transferred to fresh tubes and 10 μ L 3 M sodium acetate, 5 μ L glycogen (10 mg/mL), and 450 μ L of 100% ethanol were added. The mixture was incubated for 30 min at -80°C and the DNA was pelleted by centrifugation for 10 min at 4°C. The DNA pellets were rinsed with 200 μ L ice-cold 85% ethanol and then dissolved in 10 μ L H₂O. 4 μ L were used for reamplification and the remainder was stored at -20°C.

Reamplification of the cDNAs of interest was performed using the original conditions (primer sets and PCR program) with two exceptions: (1) The dNTP concentration was 20 μ M (using 250 μ M dNTP stock) instead of 2–4 μ M; and (2) no radioisotope was added. We used a 40 μ L reaction mix (GenHunter) prepared as follows: distilled H₂O, 20.4 μ L; 10X PCR buffer, 4.0 μ L; dNTP (250 μ M) 3.2 μ L; HAP₆₄ (2 μ M), 4.0 μ L; HT₁₁C (2 μ M), 4.0 μ L; cDNA template (from previous step), 4.0 μ L; AmpliTaq (5 U/ μ L), 0.4 μ L.

Following PCR amplification, 10–15 μ L of the PCR products were electrophoresed in a 1.5% agarose gel to verify that the sizes of the reamplified products were consistent with their sizes on the denaturing polyacrylamide gel. The amplified cDNAs were then subcloned via the PCR-TRAP cloning system according to the company's instructions (for later use in preparation of probes, etc.) and sequenced in the Vanderbilt University DNA Sequencing Core facility.

3.5. Results and Discussion

Comparison of the sequences of the two apparently differentially expressed cDNAs (**Fig. 1**) revealed that they were identical except for a single 4-base tandem repeat (TGAT) in the 3'prime-noncoding region of the cDNA (**Fig. 2**).

According to our DD analysis, the two randomly selected normal control subjects had three tandemly repeated TGAT sequences, whereas the randomly selected two melancholic depressed patients had four at the same locus. However, the analysis by DD of a larger sample of fibroblasts from control subjects ($n = 7$) and patients with a DSM-IV diagnosis of major depression ($n = 8$) revealed that this polymorphism detected in the 3'-noncoding region of this gene (α -E-catenin) is not specific for patients with major depression. The observation reported demonstrates, however, that in addition to detecting the presence or absence of gene transcripts, DD can also detect finite changes in mRNA sequences, including deletions or insertions of nucleotide repeats that are often linked to genetic diseases, e.g., Huntington disease, Fragile X syndrome, and Myotonic dystrophy (for a review, *see* **ref. 5**).

4. Notes

1. RNA remains exclusively in the aqueous phase; DNA and proteins remain in the interphase and organic phase.
2. Phenol/chloroform is the only completely reliable method to remove DNase before reverse transcription.
3. Making core mixes is always a good idea wherever possible to improve reproducibility and increase the chance of finding real differences.
4. Our sequencing apparatus for DD is from GenHunter, but any standard large sequencing gel apparatus will do.

Acknowledgments

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Silencing in Yeast

Identification of Clr4 Targets

Sergey V. Ivanov and Alla V. Ivanova

Summary

Efficient handling of multiple reactions is a crucial prerequisite for productive RNA differential display (DD) analysis. To identify transcriptional targets of the histone H3 Lys9-specific methyltransferase Clr4, we applied a multifomat modification of DD to compare between *clr4*⁺ and *clr4*⁻ transcriptomes of *Schizosaccharomyces pombe*. As a result, 14 differentially expressed bands were identified among 720 polymerase chain reaction (PCR) studied. The content of these bands was then analyzed by cloning, sequencing, and Northern analysis. In the final stage of verification, four Clr4 targets were isolated based on their expression in six Clr4 chromo and SET domain mutant strains. The step-by-step description of the multifomat DD provided below includes RNA purification, cDNA synthesis, 96-well PCR, electrophoretic separation of PCR products, isolation of DNA fragments from differentially expressed bands, and verification of candidate genes by Northern analysis.

Key Words: RNA differential display; *clr4*; silencing; yeast; histone-specific methyltransferase; transcriptional regulator.

1. Introduction

The *clr4* gene is essential for silencing of centromeres (**1**) and the mating-type loci in *Schizosaccharomyces pombe* (**2,3**) and encodes a histone methyltransferase specific for H3 Lys 9 (**4,5**). The Clr4 protein consists of two evolutionary conserved functional domains: the SET domain responsible for methyltransferase activity in vitro (**4**) and the chromodomain required, most likely, for targeting Clr4 to the *mat2/3* region and centromeres (**5**). In vivo and in vitro experiments suggest that Clr4, similar to other SET- and

chromodomain-containing proteins, such as SU(VAR) 3-9, the Polycomb-group, and the trithorax-group, is involved in regulation of gene expression via higher-order chromatin assembly. To provide further insight into Clr4 activity as a transcriptional regulator, we used SET- and chromodomain mutants of Clr4 and developed a multiformat assay for RNA differential display (DD) on yeast. In this article, we describe the technique in detail and report on four Clr4 gene targets.

2. Materials

1. DNase, amplification grade (Invitrogen; Carlsbad, CA).
2. RNase block (Stratagene, La Jolla, CA) or RNasin (Promega).
3. RNAeasy Mini Kit (Qiagen, Valencia, CA).
4. RLT buffer (Qiagen).
5. RPE buffer (Qiagen).
6. SuperScript reverse transcriptase (Invitrogen).
7. 96-well polymerase chain reaction (PCR) microplates.
8. 36 × 43 cm vertical gel box, glass plates, and gel dryer.
9. 0.4 mm 72-well shark tooth comb and gel spacers.
10. Single-channel automatic pipettors.
11. 12-channel automatic pipet (Matrix Technologies, Hudson, NH).
12. 8-channel gel-loading syringe (Hamilton, Reno, NV).
13. PGEM-T cloning kit (Promega, Madison, WI).
14. YPD medium: 1% yeast extract, 2% peptone, 2% dextrose.
15. AE buffer: 50 mM Na acetate, pH 5.3, 10 mM ethylenediaminetetraacetic acid (EDTA).
16. 3 M Na acetate, pH 5.3 (Quality Biologicals, Gaithersburg, MD).
17. Phenol (Invitrogen) saturated with AE buffer.
18. Diethyl pyrocarbonate (DEPC)-treated water (Quality Biologicals).
19. RT pre-mix (for 20 reactions): 168 μ L of 125 mM Tris-HCl, pH 8.3, 187.5 mM KCl, 7.5 mM MgCl₂, 25 mM dithiothreitol (DTT), 1.25 mM dNTP, and 4200 U of SuperScript.
20. PCR pre-mix (for one 96-well plate): 1 mL of 12.5 mM Tris-HCl, pH 8.3, 62.5 mM KCl, 3.75 mM MgCl₂, 0.3 U Taq DNA polymerase, 12.5 μ M each dNTP, and 0.06 μ Ci of α -[³³P]-dATP.
21. 10X RNA gel buffer (Quality Biologicals).
22. RNA gel loading solution (Quality Biologicals).
23. Nylon membrane Hybond-XL (Amersham Biosciences, Piscataway, NJ).
24. Oligo-dT-"anchored" downstream primers for RT and PCR: HT₁₁A, HT₁₁C, or HT₁₁G where H stands for *Hind*III restriction site.
25. Upstream primers for PCR:

XGTCACAC;	XCATAGCC
XAGTGAGC;	XCTTGATG
XCTATGGC;	XCCAGTAC
XCTGTGTC;	XCGCATTG
XACGGACG;	XCTCCGTC
XATGCACG;	XTAAAGGG
XAGCAGCT;	XCATGGTC
XTGAGCGT;	XTGGCTCC
XGCGCAAC;	XTTCGCAG
XGAAGCGT;	XCTAAGCG
XTGGCATG;	XCTGACAC
XGCGAGGT;	XCTAACCG
XGTCTAAA;	XATTGGTC
XCTATTTC;	XACCAATC
XTGAATTC;	XCAATCGC
XAAATCGA;	XGTCATAG
XTTATTTCG;	XCTGACTG
XGTTATAG;	XATACAGG
XAGTTATC;	XAACGAGG
XCAAGTTT;	XCAAGTCC
X, <i>Xba</i> I site.	

3. Methods

The procedure described in **Subheadings 3.1.–3.7.** is a modification of the original DD protocol developed by Liang and Pardee (6). It includes: (1) isolation of yeast total RNA; (2) synthesis of cDNA on yeast total RNA; (3) multi-format PCR on cDNA; (4) analysis of PCR profiles in denaturing acrylamide gel; and (5) cloning and Northern analysis of differentially expressed fragments.

3.1. RNA Isolation

We adopted a heating/freezing technique for yeast RNA isolation from (7), using ordinary precautions to avoid ribonuclease contamination. For DD, three independent RNA isolations were used for each yeast strain to overcome the problem of false-positive bands.

1. Grow yeast cells in 10 mL of YPD media and harvest the cells by centrifugation.
2. Resuspend the cells in 400 mL of AE buffer and transfer them in a 1.5-mL Eppendorf tube.
3. Add 40 μ L of 10% SDS and vortex.
4. Add 400 μ L of phenol equilibrated with AE buffer, vortex, and incubate at 65°C for 4 min.
5. Rapidly chill the mixture in a dry ice/ethanol bath until phenol crystals appear and then centrifuge for 2 min at maximum speed in a microcentrifuge to separate aqueous and phenol phases.
6. Transfer the upper (aqueous) phase to a fresh Eppendorf tube and extract with phenol/chloroform at room temperature.
7. Transfer the extracted phase to a new tube and bring it to approx 0.3 *M* Na acetate, pH 5.3, by adding 40 μ L of 3 *M* Na acetate, pH 5.3.
8. Precipitate RNA with 2.5 vol (approx 1 mL) of absolute ethanol, centrifuge for 10 min at maximum speed, wash with 80% ethanol, dry briefly at room temperature, and resuspend in 20 μ L DEPC water. Assess RNA concentration by spectrophotometry.
9. Store the RNA solution at -70°C.

3.2. DNase Treatment

A simple and reliable DNase treatment procedure combined with RNeasy Mini Kit (Qiagen) purification protocol described below is an efficient solution for the problem of DNA contamination in total RNA samples (*see Note 1*).

1. Incubate 2–5 μ g of RNA with 2.5 U of amplification grade DNase I and 10 U of RNA block (or RNasin) in 12.5 μ L of DNase buffer supplied by the manufacturer at 37°C for 1 h.
2. Purify RNA samples with RNeasy kit as suggested by the manufacturer:
 - a. Add 10 μ L of β -mercaptoethanol per 1 mL of RLT buffer available from the kit.
 - b. Adjust RNA sample to 100 μ L with DEPC water and add 350 μ L of RLT buffer.
 - c. Add 250 μ L absolute ethanol to the diluted RNA, mix thoroughly by pipetting.
 - d. Apply the mixture (700 μ L) to an RNeasy mini column placed in a 2-mL collection tube.
 - e. Close the tube gently and centrifuge for 15 s at 3000–5000*g*.
 - f. Discard the flow-through and the collection tube.
 - g. Transfer the column into a new 2-mL collection tube and pipet 500 μ L of RPE buffer onto the column.
 - h. Close the tube gently, centrifuge as in **step e**, and discard the flow-through.
 - i. Add another 500 μ L of RPE to the column and centrifuge at 3000–5000*g* for 2 min to dry the RNeasy silica-gel membrane.
 - j. To elute, transfer the column to a new 1.5-mL collection tube and pipet 50 μ L DEPC water directly onto the RNeasy silica-gel membrane.
 - k. Close the tube gently and centrifuge at 3000–5000*g* for 1 min.
 - l. Store purified RNA samples at -70°C.

3.3. cDNA Synthesis

For correct comparison of RT-PCR products, concentration and quality of the purified RNA sample must be assessed.

1. Estimate RNA concentration by spectrophotometry and analyze aliquots (0.5–1 µg) in a regular 1% agarose gel (*see Note 2*). Cool agarose solution down to approx 60°C and add 10 µL of ethidium bromide (EtBr) solution (10 mg/mL) per 100 mL agarose. Pour gel and perform brief electrophoresis (bromophenol at approx 1/2 of the gel length) in 1X TAE buffer, using a DNA ladder as a reference, and photograph the gel. Continue separation and make additional pictures if necessary. Good-quality RNA samples show prominent 18S and 28S RNA bands and a smear representing heterogeneous poly(A)⁺ RNA.
2. Perform RT reactions in 0.2-mL tubes for easy handling and storage of the product. Mix 1 µg of each purified RNA sample with 10 pkmol of each of oligo-dT-“anchored” primer (*see Subheading 2.*) and DEPC water in a total volume of 12 µL, incubate at 70°C for 5 min, and rapidly chill on wet ice. For three independent RNA isolations from two yeast strains, we used six separate RT reactions with each oligo-dT-anchored primer, 18 reactions in total.
3. Add 8 µL of RT pre-mix (*see Subheading 2.*) to each tube, mix by pipetting, and incubate at 42°C for 1 h.
4. Incubate the reaction mixture at 95°C for 10 min and use for PCR immediately or store the RT product at –20°C.

3.4. Multiple PCR on cDNA Templates

Successful DD analysis relies on reproducibility of PCR conditions. To standardize PCR parameters and separate false positives from “real” differentially expressed bands, we employed a multiformat assay (*see Note 3*). In a single 96-well plate, we were able to screen two triplicated RNA samples using one oligo-dT anchored and 16 upstream primers ($2 \times 3 \times 16 = 96$).

1. Prepare a fresh 96-well plate and add 1 µL of each of six cDNA samples synthesized with the same anchored primer to each well using a single-channel automatic pipettor. Follow the order shown in **Fig. 1**. Make sure that aliquots are dispensed on the bottom of the well (*see Note 4*).
2. Add 1 µL containing 25 pmol of each of 16 downstream primer (*see Subheading 2.*) to each set of 6 wells (**Fig. 1**) using automatic pipettor. Dispense aliquots on the side of the well near the bottom.
3. Add 400 pmol of the anchored primer chosen in **step 1** to 1 mL PCR premix (*see Subheading 2.*), and mix by pipetting. Dispense 8 µL of the premix in each of the 96 wells using a single-channel or a 12-channel automated pipettor. Avoid cross-contamination by dispensing on the well side opposite to the previously used. Discard the leftover according to radiation safety regulations. Spin the plate briefly if necessary.
4. Overlay each sample with 10 mL mineral oil using 12-channel pipettor or use a thermal cycler with a heated lid.

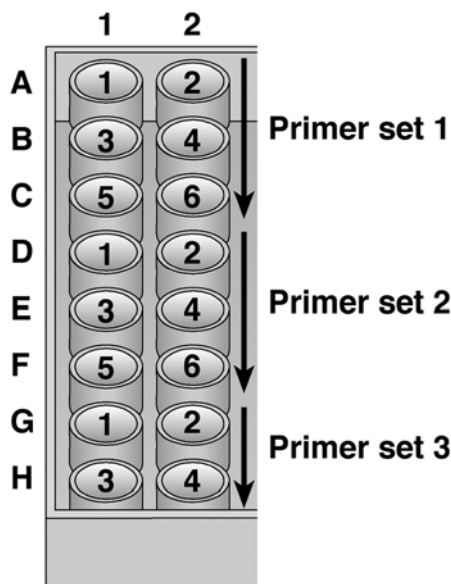


Fig. 1. Arrangement of samples in a 96-well plate for loading on a denaturing gel with an 8-channel syringe. Each set of six PCR reactions (samples 1–3 represent RNA no. 1, samples 4–6, RNA no. 2) is loaded as indicated. For simplicity, only first two columns are shown.

- For PCR, use the following cycling program: initial incubation at 94°C for 3 min, then 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s (40 cycles). Extend the last cycle by a 5-min incubation at 72°C. Store the plate at –20°C.

3.5. Denaturing PAAG Electrophoresis

- Prepare one or more 5% denaturing PAAG using 0.4 mm spacers and 72-well shark tooth comb (8). It takes three gels to screen two 96-well plates.
- Using 12-channel pipet, add 10 µL of formamide loading dye to each well; mix by pipetting and incubate in the PCR heating block at 94°C for 3 min.
- Eight-channel Hamilton syringe loads every other well of a 72-well shark tooth comb. First, load the column A1-H1 on odd lanes (1, 3, 5...15). Then load the column A2-H2 on even lanes (2, 4, 6...16) (Fig. 2). Continue with the rest of the plate, using the same loading strategy (see Note 5).
- Run denaturing electrophoresis until xylene cyanol reaches approx 4/5 of the gel.

3.6. Recovery and Reamplification of Differentially Expressed Bands

- Dry gels on Whatman 3MM paper in a gel dryer, label with radioactive ink, and make exposure using X-ray film.
- Match the gel with its picture, staple them together with gel on top, and cut out one of each reproducible differentially expressed bands with a razor blade using

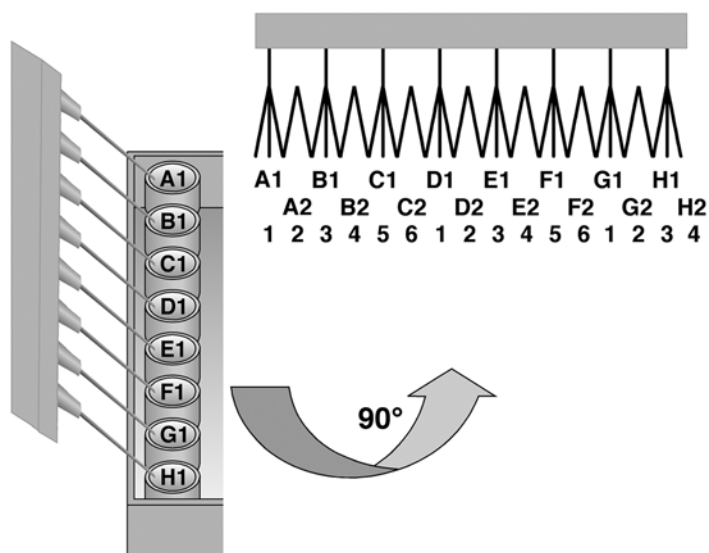


Fig. 2. Loading gel from a 96-well plate with an 8-channel Hamilton syringe and a shark-tooth comb. First, load column 1 (A1-H1) on odd lanes, then load column 2 (A2-H2) on even lanes.

a light box and a marker to label the band on the film if necessary. Make another exposure (**Fig. 3**) to make sure that bands were targeted precisely. Dried gels can be stored at room temperature and used again for bands excision, if required.

3. Extract DNA by incubation gel piece in 100 μ L of water at 90°C for 15 min.
4. Reamplify 2 μ L of extracted differentially expressed material using the same PCR conditions and primers and analyze the product in a regular 5% PAAG.
5. Clone 0.5 μ L of PCR product in PGEM-T vector (*see Note 6*), select white colonies and analyze them for inserts, using *HindIII/XbaI* restriction digestion.
4. Sequence two to four clones representing each band to determine heterogeneity within the band and check for possible redundancy or cloning artefacts (*see Note 7*).

3.7. Verification of Differential Expression by Northern Analysis

In the first stage of verification, we screened all clones by Northern analysis with the same *clr4*⁺ and *clr4*⁻ RNA samples that were used for DD (data not shown). In the second stage we used total RNA samples isolated from *clr4* mutants to further verify candidate genes and find out what protein domains are involved in the gene expression regulation (**Fig. 4; [9]**). Generally, total RNA is acceptable for Northern analysis except for the transcripts that do not co-migrate with 28S or 18S rRNA bands. Poly(A)⁺ RNA samples, however, make Northern

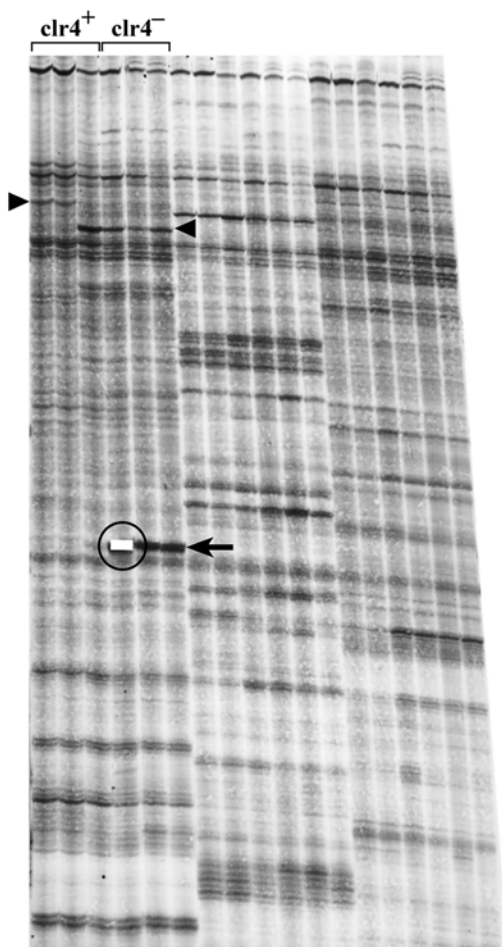


Fig. 3. *S. pombe* *clr4*⁺ and *clr4*⁻ reverse transcriptase polymerase chain reaction (RT-PCR) products separated in 5% denaturing PAAG. Each set of six lanes represents a separate primer pair. All reactions were performed in triplicates. Inconsistent bands (false positives) are shown with arrowheads. A differentially expressed band reproduced in all three lanes is shown with arrow. The piece of gel, which was cut out and used for DNA recovery, is circled.

analysis much more sensitive and straightforward. The procedure described below is a routine RNA gel electrophoresis protocol that has been used for a long time (8).

1. Mix 1.2 g of agarose with 110 mL water and melt in a microwave.
2. Cool the mixture down to approx 50°C, add 15 mL 10X RNA gel buffer and 25

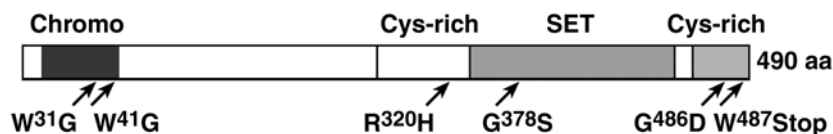


Fig. 4. Mutant Clr4 proteins used in Northern analysis.

- mL 37% formaldehyde. Mix well, cast gel, and let the gel mature for 2–4 h.
3. For loading, use 4.5 μ L of DEPC water containing 10 μ g of total RNA or 2–4 μ g of poly-A(+) RNA. Also, prepare 4.5 μ L DEPC water with 2–4 μ g of an RNA ladder. Add 10 μ L of formamide, 3.5 μ L 37% formaldehyde, and 1 μ L 10X RNA gel buffer to each sample, mix, and incubate samples at 65°C for 5 min. Add 2 μ L of RNA gel loading solution to each sample, load on the gel, and separate in 1X RNA gel buffer until bromophenol blue reaches approx 1/2 to 3/4 of gel length.
4. Soak the gel in distilled water on a rocking platform for 1 h to let formaldehyde diffuse out of the gel.
5. Transfer separated RNA samples on nylon membrane in 20X SSC (8).
6. After transfer, irradiate the membrane using Stratagene transilluminator and stain it for 2–3 min in 0.04% methylene blue solution in 0.5 M sodium acetate, pH 5.2. Rinse the membrane briefly in distilled water several times. The picture will develop during first wash and background will be minimal after five to six washes. Put the wet membrane between two sheets of thick plastic (i.e., plastic folder cover), scan the picture, and store the digital image. Because methylene blue will be washed out during hybridization, label RNA ladder bands with a pencil.
7. Hybridize the membrane with the insert of interest labeled with ^{32} P to verify whether the cloned fragment is differentially expressed in your model.

3.8. Conclusions

Out of 720 PCR reactions analyzed, 14 bands were detected that showed up- and downregulation by Clr4. Northern analysis confirmed four Clr4 targets: (1) the *cdl1*(clr4-dependent locus 1) gene (C10D6 in Z98951) that encodes a hypothetical 421 a.a. protein product (NP_588366) specific for *S. pombe*; (2) the *cdl2* fragment that was not found in the Genbank; (3) the *cdl3* gene (represented in the cosmid Z69727 by pos.23914–24495) that encodes a putative gluconate kinase (NP_593694); and (4) the *ubi4* gene (AF095794) that encodes 8 ubiquitins fused in tandem (9,10).

The *cdl1* gene is located at the end of the 31-kb centromeric clone (c10D6, Sanger Centre). This gene is downregulated by Clr4 (Fig. 5). The fact that R³²⁰H and W31G mutants show high levels of *cdl1* expression indicates that both SET and chromodomains are involved in the suppression. Clr4 had previously been shown to cause strong repression of certain sites within the centromere but to have little or no effect on two sites just outside *cen1* (11). Our

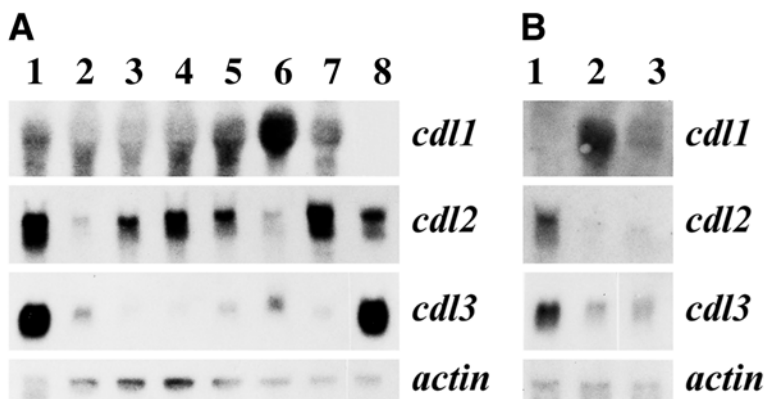


Fig. 5. Northern blot analysis showing that regulation of *cdl* genes depends on *clr4* function. (A) Analysis of *clr4*-(SET domain mutations) and *swi6* -strains: 1, *clr4* + (strain SP837); 2, *clr4* - ::*ura4* + (AP43); 3, *clr4* - G 378 S (PG906); 4, *clr4* - W 487 * (PG910); 5, *clr4* - G 486 D (PG912); 6, *clr4* - R 320 H (PG916); 7, *swi6* - (SP926); 8, *clr4* + copy integrated into the AP43 genome. Hybridization with *actin* was used as a loading control. (B) Chromo-domain mutants: 1, *clr4* + (PG9); 2, *clr4* - W 31 G (AP126); 3, *clr4* - W 41 G (AP130). (Reproduced with permission from ref. 9.)

data now suggest that the repressive effect of Clr4 spreads to nearby sequences, although this effect is allele-specific.

Genes *cdl2* and *cdl3* were both upregulated by the wild-type *clr4* gene in an allele-specific manner (Fig. 5) supporting pleiotropic effects of *clr4* suggested by previous experimentation (9).

The *ubi4* gene was found to be upregulated by wild-type Clr4 (not shown). Okazaki et al. (12) demonstrated that the *ubi4* gene plays an important role during *S. pombe* meiosis. Because upregulation of polyubiquitin synthesis may be a part of histone ubiquitination process, it would be interesting to see if *ubi4* regulation by Clr4 is related to the activity of Rhp6, a ubiquitin-conjugating enzyme implicated in silencing (13).

Overall, the 96-well application of DD and the set of primers we described, proved to be efficient and reliable not only on the yeast model, but also on other objects, such as human cell lines (14) and rat kidney explants (15).

4. Notes

1. DNA carryover in RNA samples represents one of the major sources of false-positive bands in DD gels. Fortunately, this problem is easy to deal with. After DNase digestion, RNA samples can be tested by "cold" PCR without reverse transcription: because regular PCR is DNA-specific, no product will be observed

with DNA-free RNA samples. However, the described procedures of RNA isolation and DNase treatment are already optimized to bring DNA carryover to an undetectable level.

2. Conventional agarose gel is a fast and convenient, although not common, tool to characterize RNA quality and to roughly estimate its concentration. Agarose mini-gels help in saving RNA and shortening electrophoresis to 15–30 min.
3. Variations in PCR parameters are widely acknowledged as another source of false positives. To overcome this problem, we isolated RNA in triplicates, used pre-mixes to minimize pipetting errors where possible, and adopted 96-well format with programmable pipettors to carry out multiple PCR reactions in one block. Altogether, we performed 720 PCR reactions that represented 120 different primer pairs (9). Before using radioactive assay, consistency and efficiency of multiple PCR may be assessed in a pilot “cold” experiment using the same PCR premix (*see Subheading 2.*) but with dNTP concentration increased to 0.1 mM. In this case, electrophoresis in a small, regular 5% PAAG with subsequent EtBr staining can be used to visualize the PCR products.
4. The order shown in **Fig. 1** is designed for loading the 96-well plate on a PAAG gel with a 8-channel syringe. To make this task easier, a grid that shows the loading sequence can be printed out and used as a reference.
5. You may be able to use less than 96 samples per plate and still take advantage of 8-channel syringe if remaining plate wells are loaded with water to avoid air bubbles.
6. We found that no purification step was necessary for cloning the PCR product and performed ligation reaction with an aliquot of PCR reaction.
7. Strain separation during denaturing gel electrophoresis may lead to duplicated bands representing the same DNA fragment. Cloning and sequencing helps not only in recognizing such duplicates, but also in separating heterogeneous products extracted from the same gel piece and assigning priorities to genes-candidates based on BLAST search and *in silico* functional analysis.

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Identification of mRNA Bound to RNA Binding Proteins by Differential Display

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Summary

A large number of RNA binding proteins have recently been identified that influence various human genetic disorders. However, the specific function of many of these proteins and what role they may play in a particular disease remains unclear. Identification of the substrate mRNA bound by an RNA binding protein will provide insights into the function of that protein and how its aberrant expression could lead to a disease phenotype. We have developed a technique termed SNAAP, for isolation of specific nucleic acids associated with proteins, to identify natural mRNA substrates for an RNA binding protein. The technique couples affinity purification of specific mRNAs bound by an RNA binding protein, with the identification of that mRNA using differential display (DD). Methods are described herein for the isolation and identification of endogenous mRNAs bound by any RNA binding protein, as well as methodology to validate the specificity of the binding. The availability of technologies to isolate the cognate substrate mRNAs potentially bound and regulated by an RNA binding protein involved in genetic disorders will greatly expedite our etiological understanding of the disorder and provide modalities for intervention.

Key Words: Differential display; mDAZL; RNA binding protein; RNA isolation; SNAAP.

1. Introduction

RNA binding proteins are critical determinants of the posttranscriptional pathway of gene expression and in many instances their misregulation directly correlates to a disease state in humans, including azoospermia (lack of sperm production), fragile X mental retardation, and myotonic dystrophy (*1–6*). Despite the identification of specific RNA binding proteins as the causative

agents in a given disease, the functional significance of these proteins in the disease is generally unclear. Identification of the substrate mRNAs bound by and regulated by the specific RNA binding proteins will provide insights into the function of the protein and potential avenues for therapeutic intervention. One step in elucidating an RNA binding protein's function is to identify the specific substrate RNAs for the particular protein. It is important to note that all RNA binding proteins possess some level of nonspecific binding affinity to RNA. Consequently, the identification of unknown substrate RNAs for a particular RNA binding protein requires that the methods employed in the identification of substrate RNA ensure specificity. The use of cellular extract as the source of RNA substrates permits the inclusion of cellular proteins that appear to be effective in providing natural competition to select mRNAs (7). Additionally, the mRNAs present in the cellular extract are within the context of their endogenous ribonucleoprotein structures, which potentially may promote binding with high affinity to the protein of interest. In this chapter, a technique termed SNAAP (isolation of specific nucleic acids associated with proteins), which enables the isolation and identification of endogenous RNA substrates is described. We utilize the mouse DAZL (mDAZL) RNA binding protein as a representative example and detail the methods employed in the preparation of cellular extracts, expression and purification of recombinant fusion protein, the isolation of specific substrate ribonucleoproteins that associate with the recombinant protein, and finally, confirmation of specificity of binding. The experimental procedures described are easily adaptable to any RNA binding protein and any source of cellular RNA.

2. Materials

1. Dounce homogenizer.
2. Sonicator.
3. Ultracentrifuge.
4. Swinging bucket rotor.
5. Beckman polypropylene tubes.
6. Bio-Rad protein assay reagent.
7. Bovine serum albumin (BSA).
7. Glycerol.
8. *Escherichia coli* BL21 competent cells.
9. Lurea Broth (LB).
10. Ampicillin sodium salt.
11. Isopropyl- β -D-thiogalactoside (IPTG).
12. pGEX-6P-1 vector.
13. Glutathione resin beads (Amersham).
14. Triton X-100 (TX).

15. Micrococcal nuclease.
16. CaCl_2 .
17. 5 mM EGTA (ethylene glycol-bis [β -aminoethyl ether]-*N, N, N', N'*-tetraacetic acid).
18. Nutator.
19. Heparin.
20. Phenol:chloroform (1:1).
21. Chloroform.
22. Glycogen (20 mg/mL).
23. Ethanol.
24. GenHunter RNAimage® mRNA Differential Display Kit.
25. Polymerase chain reaction (PCR) machine.
26. MMLV reverse transcriptase.
27. AmpliTaq (Perkin-Elmer).
28. α -[^{35}S]dATP AmpliTaq (Perkin-Elmer).
29. Dithiothreitol (DTT).
30. SP6 or T7 polymerase (Promega).
31. 5X Transcription Buffer (Promega).
32. Diethyl pyrocarbonate (DEPC)-treated dH_2O .
31. Rnasin RNase Inhibitor (Promega).
32. RQ1 DNase.
33. [^{32}P]-UTP.
34. rATP, rCTP, rGTP, and UTP.
35. RNase T1.
36. RNase A.
37. tRNA(40 mg/mL).
38. pGEM-T vector (Promega).
39. Oligonucleotide (**Subheading 3.6.2.**).
40. [γ ^{32}P]dATP.
41. T4 Polynucleotide Kinase.
42. 10X T4 kinase buffer.
43. Prime RNase Inhibitor (Eppendorf).
44. Potter-Elvehjem tissue homogenizer (optional).
45. UV light source (optional).

Buffers

46. 1X PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , and 8.1 mM Na_2HPO_4 .
47. Lysis buffer: 20 mM HEPES-HCl, pH 7.6, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol (DDT), 2 $\mu\text{g/mL}$ leupeptin, and 0.5% (v/v) aprotinin.
48. Buffer A: 10 mM Tris-HCl, pH 7.5, 1 mM KOAc, 1.5 mM MgOAc, 2 mM DTT, 2 $\mu\text{g/mL}$ leupeptin, and 0.5% (v/v) aprotinin.
49. Buffer B: 10 mM Tris-HCl, pH 7.5, 1 mM KOAc, 1.5 mM MgOAc, 2 mM DTT, 30% sucrose (w/v), 2 mg/mL leupeptin and 0.5% (v/v) aprotinin.
50. RNA Binding Buffer (RBB buffer): 10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl_2 , 150 mM KCl, 0.5 mM DTT, 2 $\mu\text{g/mL}$ leupeptin, 0.5% (v/v) aprotinin.

51. 1X TBE: 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA.
52. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
53. TES: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS).
54. DNA/RNA loading dye: 80% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol.
55. Protein Transfer Buffer: 50 mM Tris-HCl pH 8.3, 14.5 mM glycine, 20% methanol.
56. RNase Buffer: 10 mM Tris-HCl, pH 7.5, 400 U/mL micrococcal nuclease, 1 mM CaCl_2 , 1% (v/v) aprotinin, 2 mg/mL leupeptin/pepstatin, 0.1 mg/mL RNase A.
57. 2X SB (SDS-PAGE loading dye): 0.175 M Tris-HCl, pH 6.8, 40% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.25% bromophenol blue.

3. Methods

The methods describe in **Subheadings 3.1.–3.6.** outline: (1) preparation of extract from tissue and tissue culture cells; (2) expression and purification of recombinant protein from *E. coli*; (3) affinity isolation of bound mRNA by an RNA binding protein; (4) Identification of bound mRNA by differential display; (5) assays for confirmation of specificity of binding; and (6) identification of binding sites within the identified mRNA (**Fig. 1**).

3.1. Preparation of Extract

The steps described in **Subheadings 3.1.1.** and **3.1.2.** outline the procedure for the preparation of extract from tissues (mouse testis) or cell lines. Tissues and cell lines for the preparation of extract should be selected based on the expression of the particular protein of interest. Total extracts prepared from mouse testis were utilized for the identification of mouse DAZL (mDAZL) substrates. Expression of mDAZL in testis and the requirement for the DAZL protein in spermatogenesis has been demonstrated (8). In addition to total extracts, additional fractionation of extracts by ultracentrifugation may be preferred. For example, depending on the known localization of the protein of interest, cytoplasmic extracts devoid of nuclei may be preferred over total extract.

3.1.1. Isolation of Total Cellular Extract From Mouse Testis (or Other Tissue)

1. Pre-chill buffers at 4°C and perform all steps on ice to minimize degradation.
2. Wash testis twice in 1X PBS and slice organ into small pieces with a clean razor (see **Note 1**).
3. Add 1 mL Lysis Buffer per 5 mg of tissue.
4. Sonicate the lysed cells with three 10-s bursts at setting three while keeping samples on ice at all times.
5. Centrifuge sonicate (15,000g; 15 min) at 4°C to pellet insoluble matter.
6. Collect supernatant and supplement with glycerol to 5% (v/v).

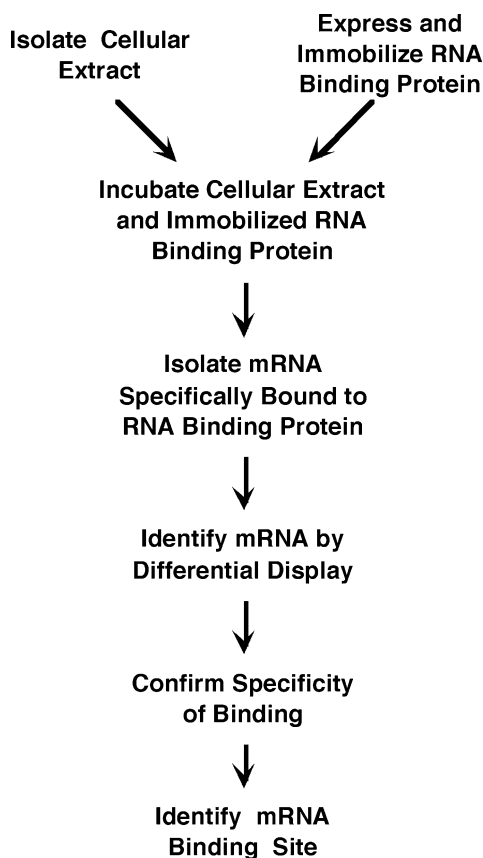


Fig. 1. An overview of the SNAAP approach is shown outlining the steps involved in the identification and confirmation of endogenous mRNAs bound by an RNA binding protein.

7. Determine protein concentration with Bio-Rad Protein Assay Reagent using BSA standards. Resulting extract is approx 5 mg/mL.
8. Store aliquots at -70°C .

3.1.2. Isolation of S130 From Tissue Culture Cell Suspension

1. Pre-chill buffers at 4°C and perform all steps on ice to minimize degradation.
2. Collect tissue culture cells by centrifugation (300g; 3 min).
3. Wash cells twice with 1X PBS (*see Note 2*).

4. Resuspend cells in 1.5 mL of Buffer A per 10^8 cells.
5. Lyse cells with 25 strokes of a type B pestle in a Dounce homogenizer.
6. Pellet Nuclei (4000g; 20 min) at 4°C.
7. Layer 7 mL of supernatant over 2 mL of Buffer B in Beckman polypropylene tubes.
8. Ultracentrifuge sample (130,000g; 2 h) in pre-chilled swinging bucket rotor.
9. Gently aspirate off the thin layer of lipid that collects on the surface and discard.
10. Collect supernatant without disturbing the supernatant–sucrose interface.
11. Supplement with glycerol to 5% (v/v) and determine protein concentration.
12. Store in aliquots at -70°C (see **Note 3**).

3.2. Expression and Immobilization of Recombinant Protein

The isolation of mRNAs that are specifically associated with the RNA binding protein of interest requires that the protein of interest first be purified and immobilized onto an affinity matrix. Once bound, extract can be incubated with the protein and co-purifying mRNAs can then be separated from other RNA and proteins present in the cellular extract. The experiments detailed in the following sections make use of the bacterially expressed, GST-tagged mDAZL, which is immobilized onto glutathione agarose resin (see **Note 4**).

3.2.1. Preparation of Bacterial Extract Containing Recombinant Protein

1. Construct pGEX-6P recombinant plasmid for expression of GST-mDAZL fusion protein (see **Note 5**).
2. Transform plasmid into *E. coli* BL21 cells and plate onto LB media containing Ampicillin (LB + AMP) (50 $\mu\text{g/mL}$) at 37°C overnight.
3. Incubate a single colony of *E. coli* cells into 5 mL LB + AMP media for 12–15 h at 37°C with vigorous shaking.
4. Dilute culture 1:100 into a 500 mL culture of LB + AMP and grow at 37°C with vigorous shaking until the OD_{600} reaches 0.5.
5. Induce expression of the recombinant protein with 0.1–1 mM IPTG for 4 h at 30°C .
6. Centrifuge cells (7700g; 10 min) at 4°C .
7. Wash cell pellet with 1X PBS, then re-pellet by centrifugation as in **step 6**.
8. Resuspend cells in 5 mL of 1X PBS.
9. Lyse cells by sonicating three times for 30 s at setting three while keeping samples on ice.
10. Centrifuge sonicate (31,000g; 10 min) to pellet cellular debris.
11. Retain supernatant and transfer to fresh container.
12. Determine protein concentration with Bio-Rad protein Assay Reagent.

3.2.2. Micrococcal Nuclease Treatment of Bacterial Extract Containing Recombinant Protein (see **Note 6**)

1. Determine volume of GST-mDAZL and GST-control protein extracts that contain 20 μg fusion protein (see **Note 7**).

2. Add CaCl_2 to 1 mM.
3. Add 200 U/mL micrococcal nuclease to extract.
4. Incubate at 30°C for 15 min.
5. Stop reaction with 5 mM EGTA.
6. Centrifuge cell extract (13,000g; 5 min) at 4°C to pellet any precipitate.

3.2.3. Preparation of Glutathione Sepharose Resin

1. Transfer 80 μL of glutathione beads into an Eppendorf tube.
2. Centrifuge (800g; 10 s) and remove residual ethanol.
3. Wash in 500 μL of RBB/0.5% Triton X-100 (RBB/0.5% TX) by inverting gently and tapping.
4. Centrifuge as in **step 2** and remove supernatant and repeat.
5. Resuspend the beads in RBB buffer (80 μL) by pipetting and divide equally into two tubes.

3.2.4. Immobilization of Recombinant Protein

1. Combine micrococcal nuclease treated GST fusion protein bacterial extracts with 40 μL of pre-washed glutathione resin beads in a final volume of 1 mL of RBB/0.5% TX and incubate for 30 min to 1 h at 4°C.
2. Centrifuge (800g; 30 s) and aspirate supernatant.
3. Wash beads four times with 1 mL of RBB/0.5% TX to remove any unbound protein.
4. Wash beads twice with 1 mL of RBB to remove Triton X-100 before allowing protein and RNA complex formation.
5. Resuspend each sample in 350 μL of RBB.

3.2.5. Preclearing of Cellular Extract With Glutathione Resin

1. Add 1% β -mercaptoethanol to testis (cellular) extract.
2. Add 1 mL Prime RNase Inhibitor ACE per 30 μL of extract to 300 μg of total testis extract to prevent RNA degradation.
3. Incubate on ice for 15 min.
4. Transfer treated extract to 20 μL of washed glutathione beads.
5. Increase volume to 350 μL with RBB and allow binding to occur for 10 min at 4°C while mixing on a nutator to remove mRNA and protein that bind the resin nonspecifically.
6. Pellet beads with a low-speed spin (800g; 3 min) and utilize supernatant in next steps.

3.3. Co-Purification and Isolation of mRNA Bound to an RNA Binding Protein

In order to isolate bound mRNA, cellular extract is incubated with the bound fusion protein and washed extensively. Elution of the RNAs that associate with

the protein can be identified by DD and determined to be specific for the protein of interest. It may be necessary to try increased stringency washes to reduce nonspecifically binding RNA background.

3.3.1. Binding of RNA to Fusion Proteins on Resin

1. Add 300 µg of precleared testis total extract to each glutathione resin containing the washed, immobilized recombinant protein.
2. Incubate for 1 h at 4°C on a nutator.
3. Spin briefly (800g; 3 min) and aspirate supernatant.
4. Add 500 µL of RBB/0.25% TX and resuspend samples.
5. Add 1 mg/mL Heparin to each sample to minimize nonspecific RNA–protein interactions.
6. Incubate for 10 min at 4°C on a nutator
7. Wash beads four times with 700 mL of RBB/0.25% TX.
8. Remove all excess supernatant with gel loading tip.

3.3.2. Isolation of Bound RNAs.

1. Add 200 µL of TES to resin, vortex, and boil for 3 min to elute RNA from beads.
2. Centrifuge sample (15,000g; 1 min) and extract two times with 200 µL phenol/chloroform (1:1).
3. Transfer Supernatant to new Eppendorf tube and chloroform extract twice.
4. Ethanol precipitate RNA for 15 min at –20°C with 20 µg of glycogen as a carrier.
5. Centrifuge (15,000g; 10 min) at room temperature.
6. Wash with 70% ethanol and centrifuge (15,000g; 5 min) at room temperature.
7. Resuspend in 10 µL of DEPC-treated H₂O.
8. Heat to 65°C for 5 min and vortex and chill on ice. RNA is ready to utilize for DD.

3.4. Identification of mRNA by Differential Display

The DD is carried out under the recommendations of the manufacturer and using reagents supplied by GenHunter Corporation RNAimage® Kit and involves the following steps: reverse transcription (RT), PCR amplification, and separation by denaturing polyacrylamide gel, and reamplification of cDNA.

*3.4.1. Reverse Transcription (see **Note 8**)*

1. Prepare three different RT reactions for each RNA sample. Each reaction should contain one of the three different anchored 3' primers (provided by manufacturer) that contain a stretch of 11 T nucleotides followed by an A, C, or G that anneals to the poly(A) tail of each mRNA.
2. In a total volume of 20 µL mix on ice:
 - 3 µL of RNA
 - 4 µL of 5X MMLV reverse transcriptase buffer
 - 1.6 µL of 250 µM dNTPs

- 2 μL of a 2 μM 3' primers provided in GenHunter kit
- 9.4 μL sterile dH_2O
- 3. Denature at 65°C for 5 min and anneal at 37°C for 10 min.
- 4. Add 200 U of MMLV reverse transcriptase, mix, and incubate the reaction for 60 min at 37°C to generate cDNAs.
- 5. Inactivate the MMLV reverse transcriptase by incubation at 75°C for 5 min.

3.4.2. PCR Amplification

- 1. Mix reactions in a total volume of 20 μL that contains:
 - 2 μL of RT product (from **Subheading 3.4.1.**)
 - 2 μL of 10X PCR buffer
 - 1.6 μL of dNTPs (25 μM)
 - 2 μL of the same 3' primer used for the RT (2 μM)
 - 2 μL of distinct 5' primers provided in GenHunter kit. (*see Note 9*).
 - 1 μL of $\alpha[^{35}\text{S}]\text{dATP}$ (1250 Ci/mmol)
 - 0.2 μL of AmpliTaq
 - 9.2 μL sterile dH_2O
- 2. Amplify PCR products with 30–40 cycles in which the DNA is denatured at 94°C for 30 s annealed at 40°C for 2 min extended at 72°C for 30 s, and follow with a final extension for 5 min at 72°C (*see Note 10*).

3.4.3. Separation by Denaturing Polyacrylamide Gel

- 1. Prepare a 6% polyacrylamide/7 M urea denaturing gel. Polymerize 2 h.
- 2. Pre-run gel for 30 min at 60 W constant power.
- 3. Flush wells of urea with syringe immediately prior to loading samples.
- 4. Mix 3.3 μL of PCR amplified sample and 3 μL DNA Loading dye. Incubate at 80°C for 2 min and immediately load onto gel.
- 5. Run gel at 60 W constant power (do not exceed 1700 V) until xylene dye (slower migrating dye) reaches the bottom of the gel (approx 3.5 h).
- 6. Blot gel onto 3 M paper, cover with plastic wrap, and dry under vacuum on a gel dryer at 80°C for 1 h and then visualize by autoradiography (overnight to 72-h exposure).

3.4.4. Reamplification of cDNA Probe

- 1. Identify specific bands on the film present in GST-DAZL lanes but not present in GST-RBD and mark with a clean pencil.
- 2. Remove band from film with a razor blade.
- 3. Overlay film onto dried gel and excise RNA that corresponds to band with a clean razor.
- 4. Elute DNA from dried gel slice and 3M paper by soaking gel in 100 μL H_2O for 10 min in Eppendorf tube.
- 5. Boil the tube for 15 min. Use parafilm to seal cap tightly.
- 6. Spin for 2 min to pellet gel and 3M paper.
- 7. Transfer supernatant to new tube.

8. Precipitate cDNA with 1/10 vol of 3 M sodium acetate, 2.5 μ L of glycogen (20 mg/mL) and 2.5 vol 100% ethanol at -70°C for a minimum of 10 min.
9. Spin sample for 10 min at 4°C to pellet DNA. Aspirate supernatant.
10. Rinse pellet with 200 μ L of 70% ethanol and spin 5 min. Aspirate supernatant.
11. Resuspend DNA in 10 μ L of sterile dH_2O .
12. Reamplify DNA with the same 3' and 5' primers and PCR conditions used in the differential display.

Set up a 40 μ L reaction:

- 4 μ L of cDNA template from **step 11**
 - 4 μ L of 10X PCR buffer
 - 3.2 μ L of dNTPs (250 μ M)
 - 4 μ L of the same 3' primer used for the RT (2 μ M)
 - 4 μ L of the distinct, 5' primer provided in GenHunter kit
 - 0.4 μ L of AmpliTaq
 - 20.4 μ L sterile dH_2O
13. Amplify cDNA with 30–40 cycles in which the DNA is denatured at 94°C for 30 s, annealed at 40°C for 2 min, extended at 72°C for 30 s.
Follow with a final extension for 5 min at 72°C .
 14. Electrophorese 30 μ L of PCR samples on a 1.5% agarose gel containing ethidium bromide (EtBr).
 15. Verify that size of reamplified PCR product is consistent with size on DNA sequencing gel.
 16. Extract reamplified cDNA fragment from agarose gel utilizing gel elution kit.
 17. Clone cDNA fragment into pGEM-T vector.
 18. Sequence the cDNA and search NCBI GenBank database to obtain the full-length cDNA sequence.

3.5. Confirmation of Specificity of Binding

Once the cDNA of a specific RNA has been cloned, it is critical to confirm the RNA is bound specifically by the RNA binding protein. Typically the co-purification and isolation and subsequent identification of mRNA bound to the RNA-Binding Protein is repeated in duplicate and only RNA that are reproducibly co-purified are further analyzed. Three commonly utilized methods for analyzing RNA–protein interactions to confirm specificity of binding are detailed in this section. Outlined in **Subheading 3.5.1.** is the method for preparing the radiolabeled RNA substrate utilized in the co-purification assay, the electrophoretic mobility shift assay (EMSA), and UV-crosslinking assay. These assays can be completed with recombinant protein and RNA to demonstrate direct binding. If direct binding cannot be detected, the reactions can be supplemented with cellular extract. Additional cellular proteins may be required for binding of the RNA by the protein of interest.

3.5.1. ³²P- Labeled RNA Generation

1. Linearize the plasmid containing the full-length cDNA insert identified by DD-PCR with restriction enzymes.
2. Set up 25 mL reaction:
 - 2 μ L 0.5 μ g/ μ L linearized DNA template
 - 5 μ L 5X transcription buffer
 - 5 μ L rNTPs (2.5 mM rATP, rCTP, rGTP, and 0.25 mM UTP)
 - 2.5 μ L 100 mM DTT
 - 1.0 μ L RNasin Rnase inhibitor
 - 2.5 μ L 1mg/mL BSA
 - 2.0 μ L [α -³²P]UTP (approx 50 mCi of 400 Ci/nmol)
 - 1.0 μ L SP6 or T7 Polymerase
 - 9 μ L DEPC-treated dH₂O
3. Mix and incubate reaction at 37°C for 1 h.
4. Add 1 μ L RQ1 DNase (RNase-Free) and incubate at 37°C for 15 min to degrade template DNA.
5. Pass sample through G-50 column to remove unincorporated nucleotides.
6. Precipitate RNA with 1/10 vol 3 M sodium acetate, 20 μ g of glycogen, and 2.5 vol 100% ethanol at -70°C for a minimum of 10 min.
7. Centrifuge sample (15,000g; 10 min) at 4°C to pellet RNA. Aspirate supernatant.
8. Rinse pellet with 200 μ L of 70% ethanol and centrifuge (15,000g; 5 min). Aspirate supernatant.
9. Resuspend RNA in 10 μ L of DEPC-treated dH₂O.

3.5.2. Co-Purification Assay

1. Incubate 4 μ g GST-tagged recombinant protein with 20 μ L of glutathione Sepharose beads for 15 min at 4°C in RBB/0.5% TX.
2. Wash beads four times with 1 mL of RBB/0.5% TX.
3. Wash twice with 1 mL of RBB.
4. Incubate in vitro transcribed ³²P-labeled RNA (prepared in **Subheading 3.5.1.**) with the beads from **step 3** in RBB/0.25% TX for 1 h at 4°C.
5. Wash four times with 1 mL RBB/0.25% TX (*see Note 11*).
6. Elute bound RNA from beads with 200 μ L of TES and boil for 3 min.
7. Phenol/chloroform extract supernatant twice and chloroform extracted twice.
8. Ethanol precipitate the RNA for 10 min at -70°C with 2.5 vol 100% EtOH, 1/10 volume 3 M sodium acetate, and 20 μ g glycogen.
9. Centrifuge RNA (15,000g; 10 min) at 4°C.
10. Gently wash pellet with 70% ethanol and spin 5 min. Aspirate all ethanol from pellet.
11. Resuspend entire pellet in RNA loading dye and heat 2 min at 95°C
12. Resolve RNA on a 5% polyacrylamide/7 M urea gel.
13. Blot gel onto 3M paper, cover with plastic wrap, and dry under vacuum on a gel dryer at 80°C for 1 h, and then visualize by autoradiography (overnight).

3.5.3. Electrophoretic Mobility Shift Assay (EMSA)

1. If extract is to be used, add 1 μL Prime RNase Inhibitor per 150 μg extract and incubate on ice for 15 min. Use 50–100 μg extract per reaction.
2. Incubate 2–6 μg of purified GST-mDAZL (with or without extract) with 0.5 ng of in vitro transcribed uniformly ^{32}P -labeled RNA (approx 10,000 cpm) in RBB for 20 min at 25°C in a total volume of 20 μL .
3. Degrade unbound RNA with 1 U RNase T1 and 10 ng RNase A for 10 min at 25°C (see **Note 12**).
4. Add heparin to a final concentration of 2 mg/mL and incubate 10 min at 25°C (see **Note 13**).
5. Resolve RNase-resistant complexes on a 5% polyacrylamide gel (60:1 acrylamide:bis) in 0.5X TBE buffer at 8 V/cm.
6. Blot gel onto 3M paper, cover with plastic wrap, and dry under vacuum on a gel dryer at 80°C for 1 h and then visualize by autoradiography (overnight).
7. Confirm that the detected binding is specific by repeating EMSA in the presence of competitor RNA. For competition assays, prepare reactions in which 1:1, 1:10, and 1:100 ratio of the labeled RNA to either self or unrelated cold competitor RNA (such as vector polylinker transcribed sequence) is added at the beginning of the reaction. The ability of the cold RNA of interest to compete complex formation, while unrelated RNA is unable to at the equivalent concentration, would indicate specificity of RNA binding (see **Note 14**).

3.5.4. UV-Crosslinking Assay

1. Carry out all reactions in an Eppendorf cap.
2. If extract is to be used, treat extract with 40 mg/mL tRNA, 1 μL Prime RNase Inhibitor per 150 μg extract, and 1% β -mercaptoethanol. Use 50 μg extract per reaction.
3. Incubate 0.5–1 μg recombinant protein with 5 ng of in vitro transcribed uniformly ^{32}P -labeled (approx 100,000 cpm) RNA substrate in RBB for 20 min at 25°C in a total volume of 20 μL . If cold competitor RNA is to be used, add at same time as labeled RNA (see, **Subheading 3.5.2., step 5** for competition assay).
4. Transfer caps to a tray containing ice and UV crosslink with 15W germicidal lamp for 7–10 min. Extract should be 5 cm from the UV light source.
5. After crosslinking, transfer content of cap to an Eppendorf tube.
6. Add 20 μL RNase Buffer and incubate at 37°C for 1 h.
7. Heat the extract at 75°C for 5 min.
8. Add an equal volume of 2XSB and resolve on an SDS-PAGE gel.
9. Blot gel onto 3M paper, cover with plastic wrap, and dry under vacuum on a gel dryer at 80°C for 1 h, and then visualize by autoradiography (overnight).

3.6. Identification of mRNA Binding Site

Once an mRNA is identified and confirmed as a substrate, the binding site within the RNA can be determined. The identification of a specific region of

the RNA that is involved in binding may allow for the identification of specific consensus sequence recognized by the RNA binding protein. Consensus sequences can then be used to identify additional substrates by computational analysis to be tested. Analysis of mRNA substrates that bind mDAZL yielded 21 specific mRNAs that all contained the mDAZL binding site (9). These mRNA included two proteins critical for sperm production and fertility, the testis-specific TPX-1 mRNA involved in germ cell-Sertoli cell adhesion (10,11) and the testis-enriched Trf2 protein transcription factor (12,13).

3.6.1. Determining the Region of RNA Required for Protein Binding

1. Initially focus on the 5'UTR, the coding region, and the 3'UTR to begin narrowing down the region of RNA that may contain the protein of interest binding site. If the coding region is too large, two or more probes may need to be prepared.
2. Design primers to amplify the DNA corresponding to these individual regions. Introduce a T7 promoter at the 5' end for preparation of radiolabeled RNA substrate. Set up standard PCR reactions to generate templates. Run PCR on agarose gel to confirm PCR products are expected size.
3. Prepare RNA substrates as described in **Subheading 3.5.1**.
4. Analyze the ability of recombinant protein to bind to RNA fragments using co-purification assay, EMSA, or UV-crosslink assay, as detailed in **Subheading 3.5**. (see **Note 15**).

3.6.2. Further Delineation of the RNA Binding Site

The binding site within a fragment of the RNA can be further delineated with the use of radiolabeled oligonucleotides.

1. Order deoxynucleotides that span the region of RNA binding site (i.e., the 3'UTR).
2. End label the oligonucleotide in a 10 μ L reaction:
 - 1 μ L Oligonucleotide
 - 1 μ L [$g^{32}P$] dATP
 - 1 μ L 10X kinase buffer
 - 0.5 μ L T4 polynucleotide kinase
 - 6.5 μ L H₂O
3. Incubate at 37°C for 30 min.
4. Increase the volume of the reaction to 50 μ L.
5. Remove unincorporated nucleotides with a G-25 spin column.
6. Analyze the ability of recombinant protein to bind to RNA fragments using co-purification assay, EMSA, or UV-crosslink assay, as detailed in **Subheading 3.5**.
7. Further refinement of the binding site can be attained by competing binding to labeled RNA oligonucleotide substrate with cold RNA oligonucleotides that contain 5–10 continuous nucleotide substitutions that span the fragment (**Fig. 3B**). (see **Note 16**).

4. Notes

1. For larger tissues, after tissues are sliced into small pieces with razor blade, disrupt tissue slices with a Portter-Elvehjem tissue homogenizer as necessary and proceed to sonication step.
2. The cell pellet can be stored at -70°C . Freezing the cells at this step aids in obtaining a more efficient lysis in the subsequent step.
3. Prepare cytoplasmic S100 in an analogous manner reducing the ultracentrifugation spin step to 100,000g. Nuclei pellets or ribosomal containing pellets from the fractionation may also be used as extract source. Gently wash pellets with Buffer A three times and fully resuspend. Supplement with 10% glycerol (v/v) and store in aliquots at -70°C .
4. In addition to GST-tagged proteins, any tag may be used to purify and immobilize the recombinant protein. Myc-tagged recombinant protein has been successfully employed by the authors. The use of the hexahistidine tag (6XHis) tag is not recommended owing to its highly charged nature that may influence RNA binding. In addition to the use of recombinant proteins, endogenous proteins of interest may also be used. The availability of a direct monoclonal antibody (MAb) specific for the endogenous protein is required. Immobilization can be carried out utilizing protein A Sepharose beads that bind to the antibody.
5. The preparation and use of a GST-tagged control RNA binding protein in subsequent steps is an important control for specificity. GST alone is not considered to be a sufficient control.
6. The removal of bacterial nucleic acids that may be bound to the recombinant protein is an important step in the preparation of the protein for binding to the cellular extract. Micrococcal nuclease requires CaCl_2 for activity and is then rendered inactive upon the addition of EGTA, which chelates the calcium.
7. To determine the amount of GST-protein that is expressed in the *E. coli* extract, binding experiments (as detailed in **Subheading 3.2.3.**) can be scaled down and completed and the bound protein can be run on an SDS-PAGE gel and stained with Coomassie Blue stain. This will allow for a close approximation in the amount of *E. coli* extract required for equivalent amounts (20 μg) of test and control proteins to be immobilized.
8. It is critical for RNA to be free of chromosomal DNA contamination. Total RNA is recommended by the manufacturer over poly(A) RNA for better results.
9. A combination of four 5'primers can be used per reaction because the pool of RNAs have already been greatly reduced by the co-purification. This reduces the number of PCR reactions and allows the screening of a larger number of potential sequences in a shorter amount of time.
10. The number of cycles needs to be determined empirically depending on the efficiency of the co-purification and the abundance of the isolated mRNA.
11. Control RNAs should be used in a parallel experiment in order to confirm that the detected binding is specific. To increase specificity of interaction, increase the salt concentration in the RBB up to 500 mM and/or include 1 mg/mL heparin in

- the washes. The addition of up to 350 mM urea can also greatly reduce nonspecific interactions.
12. The RNases degrade regions of the RNA not complexed with protein, thus allowing for the clearer identification of RNA–protein complexes.
 13. The addition of Heparin will minimize nonspecific RNA–protein interactions. It may be necessary to try several concentrations to obtain the best conditions ranging from 1–10 mg/mL Heparin.
 14. For cold (unlabeled) RNA transcription reactions, follow protocol in **Subheading 3.5.1.** with the following modifications: increase the UTP concentration in the rNTP mix from 0.25 mM to 2.5 mM and omit labeled ^{32} P-UTP. Quantitation of the RNA may be carried out by UV spectrophotometer. It is recommended the unlabeled RNA is run on a 2% agarose gel (preferably alongside the labeled RNA) to ensure the RNA is intact and quantitation is accurate.
 15. We recommend using the co-purification assay in the initial analysis to enable a more rapid initial identification of the binding region within a large RNA fragment. Subsequent refinements could employ the EMSA or UV-crosslinking approaches
 16. Many RNA binding proteins will also bind single-stranded DNA oligonucleotides in a sequence-specific manner. Therefore, DNA oligonucleotides, which are cheaper and easier to obtain, can initially be tested prior to using RNA oligonucleotides.

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