

# CLONTECH

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Discovery

## Delta<sup>®</sup> Differential Display Kit User Manual

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Storage Conditions: Positive control RNAs –70°C  
All other components –20°C

FOR RESEARCH USE ONLY

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## I. Introduction

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The Delta® Differential Display Kit (formerly named the Delta RNA Fingerprinting Kit) enables researchers to identify RNAs that are expressed in one RNA population but missing in another (Diachenko *et al.*, 1996). The identification of such “differentially expressed” RNAs is a powerful tool for studying differentiation, the cell cycle, carcinogenesis, inductive events, and other biological phenomena that involve changes in gene expression.

Delta Differential Display is a highly reproducible method for detecting differentially expressed RNAs. In our hands, more than 95% of Delta results are reproducible, and more than 85% of the differentially expressed bands can be confirmed by Northern blot analysis. The method requires only 2 µg of total RNA per sample, and the primers in the kit give you more than 150 different combinations. The protocol requires only a single cDNA synthesis for each different RNA sample, in contrast to the multiple cDNA synthesis reactions required for similar methods. The longer primers in the Delta Kit allow higher stringency PCR, which, in turn, leads to much greater reproducibility of the resulting displays. The use of enzyme mixes suitable for long-distance PCR enables the Delta system to resolve differentially expressed bands of up to 2 kb and results in a higher fidelity of PCR products to the original RNA templates.

### Protocol overview

The Delta Kit is based on improvements to the methods described by McClelland *et al.* (1993). The protocol consists of two stages: cDNA synthesis and differential display PCR (Figure 1). First-strand cDNA is synthesized using each of the RNAs of interest as a template and oligo(dT) as a primer. In contrast to the 9–12 syntheses required for each different RNA sample in other differential display protocols (Liang & Pardee, 1992; Liang *et al.*, 1993), the Delta protocol requires only a single cDNA synthesis reaction for each RNA sample. (The multiple cDNA syntheses in other methods use different oligo(dT)<sub>11</sub> NM primers to subdivide the pool of total RNA. In Delta Differential Display, this subdivision occurs during the PCR.) The single cDNA synthesis saves considerable time, and, since reverse transcriptase is an expensive reagent, it reduces costs as well. We also find the subsequent PCR to be more reproducible.

Another time- and cost-saving feature of the Delta Kit is that each cDNA synthesis reaction uses only 2 µg of total RNA. In our experience, the Delta Kit gives similar results with total and poly A<sup>+</sup> RNA, so there is no need to purify poly A<sup>+</sup> RNA. Whenever possible, RNA samples should be prepared side-by-side using the same reagents and purification protocol.

In differential display PCR, sequences are amplified—in the presence of [ $\alpha$ -<sup>32</sup>P]dATP—based on chance homology to arbitrary P primers. The Delta PCR cycling program differs substantially from conventional PCR cycling programs. Three initial cycles are performed at low stringency (i.e., low annealing temperature) to allow the primers to anneal and initiate DNA synthesis. Because of the low stringency, each P primer will bind sites on many cDNAs with imperfect

# I. Introduction *continued*

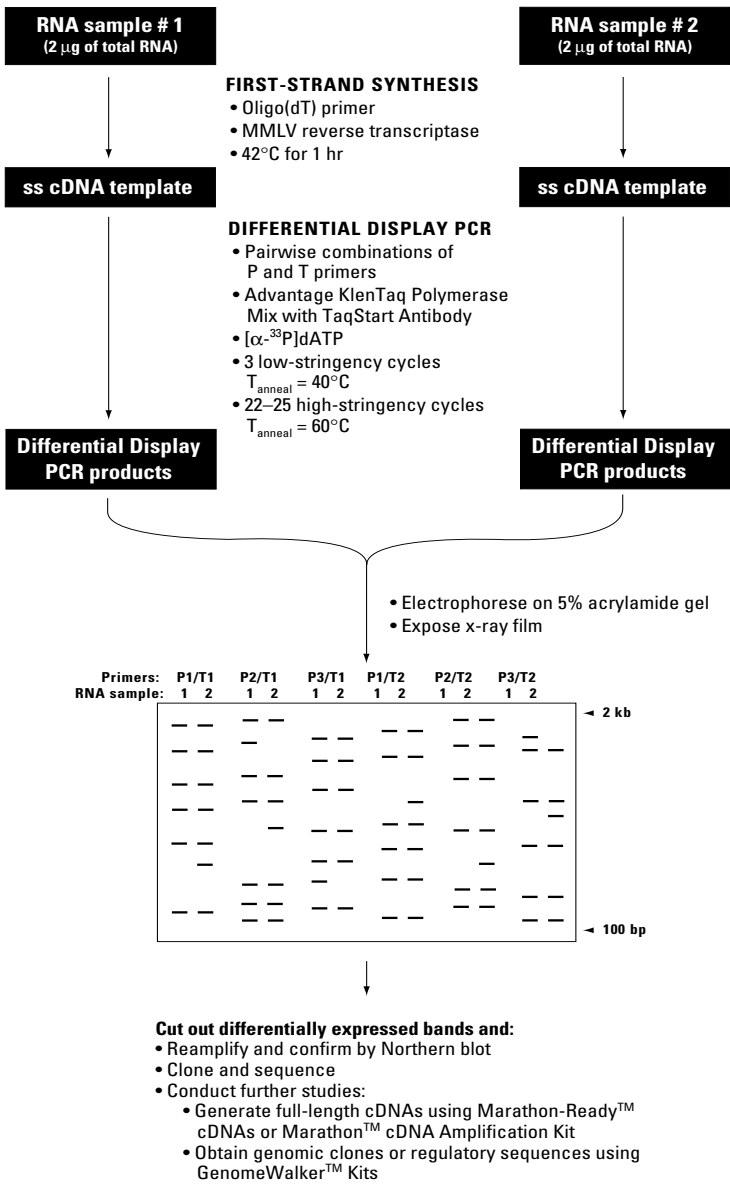
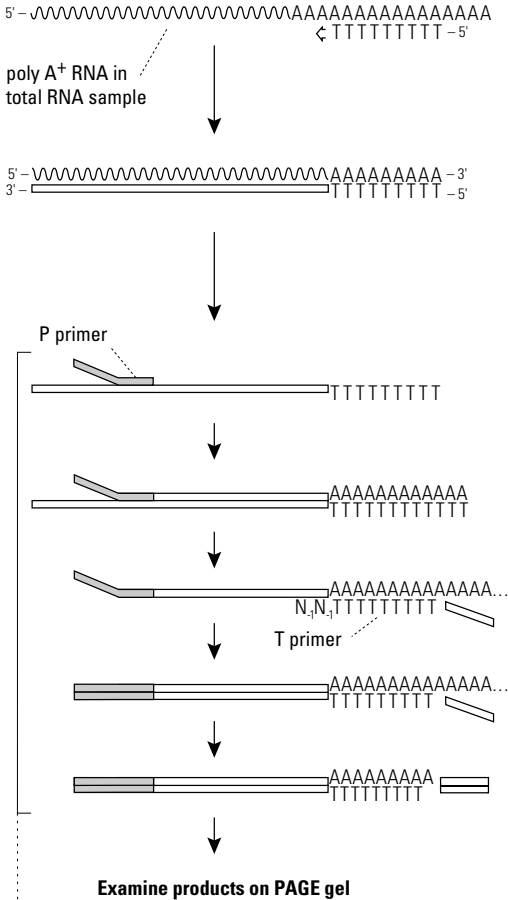


Figure 1. Overview of the Delta Differential Display protocol. In the actual protocol, each differential display PCR is performed using two different dilutions of each cDNA sample.

## I. Introduction *continued*



### FIRST-STRAND SYNTHESIS

- 2 µg of each total RNA sample
- Add reverse transcriptase and oligo(dT)
- 42°C for 1 hr
- Prepare A and B dilutions

### DIFFERENTIAL DISPLAY PCR

- Add one P and one T primer to the A and B dilutions of each cDNA
  - Add dNTPs and [ $\alpha$ -<sup>32</sup>P]dATP
  - Add long-distance PCR enzyme mix with TaqStart Antibody (e.g., Advantage KlenTaq Polymerase Mix)
- **Three low-stringency cycles**  
 $T_{\text{anneal}} = 40^{\circ}\text{C}$ , so the P primer can bind (imprecisely) to various cDNA strands
- **22–25 high-stringency cycles**  
 Primer "tails" allow shift to  $T_{\text{anneal}} = 60^{\circ}\text{C}$ , leading to higher yields of specific products, lower backgrounds, & high reproducibility

For clarity, only one set of annealing/extension events is shown. During the low-stringency cycles, many other cDNA strands are primed with the same P primer, producing the mix of PCR products that are amplified in the later, high-stringency cycles to produce the differential display for each RNA sample.

**Figure 2. Detailed flow chart of the cDNA synthesis and PCR in the Delta Differential Display protocol.** This figure shows differential display PCR using one P primer and one T primer. Although these are the most commonly performed reactions, Delta Differential Display can also be performed using P primers alone or pairs of P primers. Similarly, Delta Differential Display can be performed using T primers alone or pairs of T primers. See Section IV.E for a discussion of choosing primers for your Delta experiments.

## I. Introduction *continued*

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and/or incomplete matches. The products of these early cycles are then amplified (using the P primer and the downstream T primer) during 22–25 high-stringency PCR cycles. When examined by polyacrylamide electrophoresis and autoradiography, these reactions produce characteristic banding patterns, or “displays,” of the starting RNA.

A sample display using the positive control reagents can be seen in Figure 3 in Section V (Expected Results and Troubleshooting Guide). Each different pair of primers will produce a different display and may therefore identify a different set of differentially expressed RNAs. Once differentially expressed bands have been identified, they can be eluted from the gel and reamplified to make probes for Northern blot confirmation of differential expression, as discussed in Section VI (Additional Procedures). Confirmed positives can then be cloned and further characterized.

### The Delta Differential Display primers

The design of the Delta primers is critical to the success and proven reproducibility of the Delta Differential Display Kit. The design features that distinguish the Delta primers from the primers used in previously described methods and in other commercial kits are discussed in detail in Appendix A.

With 10 arbitrary “P” primers and 9 oligo(dT) or “T” primers, the kit has 90 possible combinations of upstream and downstream primers. Furthermore, each P primer can be used alone or in pairwise combinations with other P primers. Similarly, the T primers can be used alone or in combination with other T primers. See Section IV.E for a discussion on choosing primers.

### Long-distance PCR with the Advantage® cDNA PCR Kit

Delta Differential Display uses the conditions of long-distance (LD) PCR (Barnes, 1994; Cheng *et al.*, 1994), in which a combination of thermophilic DNA polymerases produces much larger PCR products than are possible with conventional PCR. Yields are also increased relative to conventional PCR. Furthermore, the presence of a minor amount of a DNA polymerase with 3'→5' exonuclease (i.e., “proofreading”) activity makes LD PCR more accurate than conventional PCR with a single DNA polymerase (Barnes, 1994; Frey & Suppmann, 1995; Nelson *et al.*, 1995).

We recommend CLONTECH's Advantage KlenTaq Polymerase Mix (#8417-1), which was specifically developed for LD PCR using cDNA templates and is the enzyme mix used to optimize the protocols in this booklet. This 50X mix contains KlenTaq-1 DNA Polymerase (an exo-minus, N-terminal deletion of *Taq* DNA polymerase) as the primary polymerase, a minor amount of a secondary polymerase which provides 3'→5' proofreading activity, and TaqStart™ Antibody to provide a convenient and automatic form of “hot-start” PCR (Kellogg *et al.*, 1994). Advantage KlenTaq Polymerase Mix is also available in the Advantage cDNA PCR Kit (#K1905-1, -y).

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## I. Introduction *continued*

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There are several advantages to using LD PCR for the identification of differentially expressed RNAs. First, in combination with the longer primers used in this kit, LD PCR allows us to reduce the total number of PCR cycles from 40 to 25. This means that abundant products are not overcycled and differentially expressed bands are easier to detect. Secondly, the larger range of PCR products (up to 2 kb) increases your chances of finding differentially expressed bands with each primer combination. Finally, the higher fidelity of LD PCR means that bands identified by Delta Differential Display and subsequently cloned will have greater fidelity to the original mRNA sequence.

### **CLONTECH PCR-Select™ cDNA Subtraction Kit**

Another method for comparing RNA populations is CLONTECH's PCR-Select cDNA Subtraction Kit (#K1804-1), a unique PCR-based method for powerful, reproducible enrichment of differentially expressed genes. The PCR-Select method allows you to selectively and simultaneously amplify all sequences that are differentially expressed in a given tissue or cell type, and also enriches for rare mRNAs. CLONTECH's suppression PCR technology (U.S. Patent #5,565,340) prevents amplification of sequences that are not differentially expressed.

## II. List of Components

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Store total RNA at  $-70^{\circ}\text{C}$ . Store all other reagents at  $-20^{\circ}\text{C}$ .

The following reagents are sufficient for 12 cDNA syntheses and 900 PCRs.

### For optional DNase I treatment of total RNA

- 12  $\mu\text{l}$  **DNase I** (RNase-free, 1 unit/ $\mu\text{l}$ )

### Reagents for first-strand cDNA synthesis

- 30  $\mu\text{l}$  **Oligo(dT) primer** (1  $\mu\text{M}$ )
- 12  $\mu\text{l}$  **MMLV reverse transcriptase** (200 units/ $\mu\text{l}$ )
- 500  $\mu\text{l}$  **5X First-strand buffer**:
  - 250 mM Tris (pH 8.3)
  - 30 mM  $\text{MgCl}_2$
  - 375 mM KCl

### Control reagents

- 5  $\mu\text{l}$  **Positive control RNA 1** (1 mg/ml of total RNA from adult liver)
- 5  $\mu\text{l}$  **Positive control RNA 2** (1 mg/ml of total RNA from fetal liver)

### PCR primers for Delta Differential Display

- 10 x 90  $\mu\text{l}$  **Arbitrary primers** (20  $\mu\text{M}$ ):
  - P1: 5'-ATTAACCCTCACTAAATGCTGGGGA-3'
  - P2: 5'-ATTAACCCTCACTAAATCGGTCATAG-3'
  - P3: 5'-ATTAACCCTCACTAAATGCTGGTGG-3'
  - P4: 5'-ATTAACCCTCACTAAATGCTGGTAG-3'
  - P5: 5'-ATTAACCCTCACTAAAGATCTGACTG-3'
  - P6: 5'-ATTAACCCTCACTAAATGCTGGGTG-3'
  - P7: 5'-ATTAACCCTCACTAAATGCTGTATG-3'
  - P8: 5'-ATTAACCCTCACTAAATGGAGCTGG-3'
  - P9: 5'-ATTAACCCTCACTAAATGTGGCAGG-3'
  - P10: 5'-ATTAACCCTCACTAAAGCACCGTCC-3'
- 9 x 90  $\mu\text{l}$  **Oligo(dT) primers** (20  $\mu\text{M}$ ):
  - T1: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
  - T2: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
  - T3: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
  - T4: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
  - T5: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
  - T6: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
  - T7: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
  - T8: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
  - T9: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

### General reagents

- 200  $\mu\text{l}$  **dNTPs** (5 mM each dATP, dCTP, dGTP, dTTP)
- 2 x 4 ml **Sterile  $\text{H}_2\text{O}$**



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### III. Additional Materials Required

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The following reagents are required but not supplied:

#### For optional DNase I treatment of total RNA

- 0.5 M **Tris-HCl** (pH 7.5)
- 0.5 M **MgCl<sub>2</sub>**
- 3 M **NaOAc**
- 0.2 M **EDTA**
- **Phenol:chloroform:isoamyl alcohol (25:24:1)**

Prepare as follows:

1. Melt phenol.
2. Equilibrate with an equal volume of sterile TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA).
3. Incubate the mixture at room temperature for 2–3 hr.
4. Remove and discard the top layer.
5. Add an equal volume of chloroform:isoamyl alcohol (24:1) to the remaining layer. Mix thoroughly. Remove and discard the top layer.
6. Store the bottom layer of phenol:chloroform:isoamyl alcohol at 4°C away from light for a maximum of 2 weeks.

- **Chloroform:isoamyl alcohol (24:1)**

#### For Delta Differential Display

- **50X polymerase mix**

You will need a *Taq*-based 50X polymerase mix suitable for LD PCR. The Delta protocol has been optimized with CLONTECH's Advantage KlenTaq Polymerase Mix (#8417-1), which contains KlenTaq-1 DNA Polymerase (an exo-minus, N-terminal deletion of *Taq* DNA polymerase) as the primary polymerase, a minor amount of a 3'→5' proofreading polymerase, and TaqStart Antibody for hot start (Kellogg *et al.*, 1994). Advantage KlenTaq Polymerase Mix is available in the Advantage cDNA PCR Kit (#K1905-1, -y).

If you choose not to use Advantage KlenTaq Polymerase Mix, Table I gives instructions for mixing TaqStart Antibody with other commercially available LD PCR-licensed enzyme mixes. In our experience, Delta Differential Display reactions are somewhat less efficient with these other enzyme mixes. In general, we recommend that the primary enzyme in the mix be *Taq* DNA polymerase (or a *Taq* derivative) instead of *Tth* DNA polymerase, which results in higher backgrounds and more smearing.

*Taq* DNA polymerase can be used by itself; however, more cycles will be needed. Furthermore, the size range of the products will be much smaller, so you will obtain fewer differentially expressed bands per display and miss the larger bands that would be identified using the LD PCR mixes. Also, LD PCR has a significantly lower error rate than PCR with *Taq* DNA polymerase alone (Barnes, 1994), so the sequence of your PCR products will be more

### III. Additional Materials Required *continued*

faithful to the original mRNA. This is a particularly important consideration for designing primers for subsequent experiments, such as Marathon™ cDNA amplification or sequencing.

Using TaqStart Antibody in the 50X polymerase mix greatly reduces nonspecific synthesis in Delta Differential Display PCR. (TaqStart Antibody has already been added to CLONTECH's Advantage KlenTaq Polymerase Mix.) TaqStart is an effective method for hot-start PCR that is simpler and more convenient than wax-based or manual methods. The TaqStart Antibody binds to and inactivates *Taq* DNA polymerase (native or truncated) and thus eliminates DNA synthesis from nonspecifically bound primers while reactions are being assembled. PCR amplification proceeds efficiently after an initial incubation at 94°C which irreversibly inactivates the TaqStart Antibody. TaqStart Antibody is available from CLONTECH (#5400-1, -2).

- **10X PCR reaction buffer**

(Included in the Advantage cDNA PCR Kit)

Use the 10X reaction buffer supplied with your source of native or truncated *Taq* DNA polymerase in all reactions that call for 10X PCR buffer. (Of the three buffers supplied with the Expand™ Long Template PCR System, we obtained the best results with buffer #1.)

If you are using a buffer system in which the Mg<sup>2+</sup> is added separately, we recommend using a final [Mg<sup>2+</sup>] of 1.5 mM.

- **[ $\alpha$ -<sup>33</sup>P]dATP** (1000-3000 Ci/mmmole; 3.3  $\mu$ M)

[ $\alpha$ -<sup>33</sup>P]dATP produces much sharper bands than [ $\alpha$ -<sup>32</sup>P]dATP and thus increases the resolution of your differential displays (and therefore the number of bands that you can distinguish). [ $\alpha$ -<sup>35</sup>S]dATP can also be used; however, longer exposure times will be required. Also, volatilization of [ $\alpha$ -<sup>35</sup>S]dATP upon heating may lead to low-level contamination of thermal cyclers (Trentmann *et al.*, 1995).

- **0.5-ml PCR tubes**

We recommend Perkin-Elmer GeneAmp® 0.5-ml reaction tubes (Cat. #N801-0737 or N801-0180).

- **X-ray film**

We recommend Kodak BioMax MR film.

- **Gel electrophoresis apparatus**

For best results, use a temperature-controlled (“thermostatic”) gel electrophoresis apparatus, such as the Macrophor™ System from Pharmacia Biotech AB or the genomylxLR™ DNA Sequencer from Genomylx Corporation.

- **Gel loading buffer:**

- 95.0% Formamide
- 0.2% Bromophenol blue
- 0.2% Xylene cyanol

### III. Additional Materials Required *continued*

TABLE I: PREPARATION OF 50X POLYMERASE MIX

| Supplier/kit<br>(Cat. #)  | DNA polymerases<br>(conc. supplied)           | Concentration<br>in PCR      | Proportions for<br>50X enzyme mix <sup>b</sup>                                 |
|---|---|------------------------------|--|
| CLONTECH's Advantage<br>cDNA PCR Kit (#K1905-1, -y) <sup>a</sup>      | Premade enzyme<br>mix (50X)                   | See Advantage<br>User Manual | Ready-to-use<br>(contains TaqStart Antibody)                                   |
| Takara LA PCR Kit (#RR011)<br>merase                                  | ExTaq (5 units/μl)                            | 50 units/ml                  | Mix 10 μl of ExTaq DNA Poly-<br>with 10 μl of TaqStart Antibody <sup>b</sup>   |
| Boehringer Mannheim<br>Expand Long Template<br>PCR System (#1681 834) | Taq (3.5 units/μl) &<br>Pwo (conc. not given) | 50 units/ml<br>???           | Mix 14.3 μl of <i>Taq/Pwo</i><br>with 5.7 μl of TaqStart Antibody <sup>b</sup> |

<sup>a</sup> Best results in Delta Differential Display

<sup>b</sup> 50X enzyme mixes made with TaqStart Antibody can be stored at -20°C. (TaqStart Antibody is provided at 1.1 mg/ml in 50% glycerol. Do not dilute the antibody prior to adding it to the 50X mix.)

To prepare 50X enzyme mixes without TaqStart Antibody, simply replace the specified volume of TaqStart Antibody with the 1X PCR reaction buffer supplied with the DNA polymerase. Because of the lower glycerol content, 50X enzyme mixes made with 1X PCR reaction buffer should be stored at 4°C.

## IV. Protocol for Delta Differential Display

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PLEASE READ ENTIRE PROTOCOL BEFORE STARTING

### A. General Considerations

1. The cycling parameters throughout this protocol have been optimized using a Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp PCR Systems 2400/9600, Advantage KlenTaq Polymerase Mix, and ss cDNA templates prepared from the RNA controls provided in the Delta Kit. The optimal cycling parameters may vary with different thermal cyclers.
2. We recommend that you use some form of hot start for Delta Differential Display PCR. The following protocol has been optimized using TaqStart Antibody (Kellogg *et al.*, 1994) in the 50X polymerase mix. Hot-start PCR can also be performed using wax beads (D'Aquila *et al.*, 1991) or manually (Chou *et al.*, 1992).
3. Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.
4. When mixing reactions, gently pipet the solution up and down or tap the bottom of the tube and spin briefly to collect the contents at the bottom of the tube.
5. Set up all reactions on ice, unless otherwise indicated.
6. Add enzymes to reaction mixtures last. Make sure that the enzyme is thoroughly mixed with the reaction mixture by pipeting the mixture up and down.
7. Use the recommended amounts of enzyme. These amounts have been carefully optimized for the Delta protocol and reagents.

### B. Preparation and Handling of Total RNA

#### 1. General precautions

The integrity and purity of the total RNA used as starting material is an important element of high-quality cDNA synthesis and the subsequent PCR. The following precautions will help you avoid contamination and degradation of your RNA.

- a. Wear gloves to avoid RNase contamination from your hands.
- b. Treat H<sub>2</sub>O used in steps that involve RNA with diethyl pyrocarbonate (DEPC).

**Note:** Do not use DEPC to treat solutions containing Tris, nucleotides, or other amines.

- c. Rinse all glassware with 0.5 N NaOH, followed by DEPC-treated H<sub>2</sub>O. Then bake the glassware at 160–180°C for 4–9 hr.
- d. Use 70% ethanol or isopropanol to wipe all pipettes before use with RNA.

## IV. Protocol for Delta Differential Display *continued*

### 2. RNA isolation

Whenever possible, total RNA samples being compared in a Delta experiment should be purified side-by-side using the same reagents and protocol. Two RNA samples purified from the same source but by a different procedure can give different banding patterns in differential display. Thus, more false positives may arise if the RNAs being compared were prepared in different ways.

See Farrell (1993) or Sambrook *et al.* (1989) for reviews of procedures for isolating total RNA.

### 3. RNA analysis

After isolating total RNA, we recommend that you examine the RNA by electrophoresis of a sample on a denaturing formaldehyde/agarose/EtBr gel. On denaturing gels, total RNA typically gives bright 28S and 18S ribosomal RNA bands at approximately 4.5 and 1.9 kb, respectively, with a ratio of intensities of about 1.5–2.5:1. If this ratio is less than 1:1, we suggest you prepare fresh RNA after checking your RNA purification reagents for RNase and other impurities. If problems persist, you may have to find another source of tissues/cells.

### C. (Optional) DNase I Treatment of Total RNA

Unless total RNA samples are seriously contaminated with genomic DNA, DNase I treatment is not necessary. If analysis of total RNA on a denaturing formaldehyde/agarose/EtBr gel reveals visible genomic DNA contamination (indicated by EtBr-stained material stuck in the wells), then you should treat your samples with DNase I before continuing with the first-strand cDNA synthesis. Likewise, if the total RNA control display patterns are identical to your experiments, you should treat your RNA with DNase I.

1. For each total RNA sample, prepare an equal volume of DNase I mixture. Combine the following components in a sterile 0.5-ml microfuge tube:

| <u>per 25-<math>\mu</math>l RNA sample*</u> | <u>components</u>                    |
|---|--------------------------------------|
| 1 $\mu$ l                                   | 0.5 M Tris-HCl (pH 7.5)              |
| 1 $\mu$ l                                   | 0.5 M MgCl <sub>2</sub>              |
| 22 $\mu$ l                                  | Sterile H <sub>2</sub> O             |
| 1 $\mu$ l                                   | RNase-free DNase I (1 unit/ $\mu$ l) |
| <hr/>                                       |                                      |
| 25 $\mu$ l total                            |                                      |

\* We generally recommend that you use 25  $\mu$ l of each RNA sample at a concentration of 0.1–1 mg/ml. For multiple RNA samples, scale up the amounts of the components of the DNase I mixture proportionately. To avoid pipeting errors, do not make less than 25  $\mu$ l of the DNase I mixture.

## IV. Protocol for Delta Differential Display *continued*

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2. In appropriately labeled, sterile 0.5-ml microfuge tubes, add an equal volume of the DNase I mixture to each RNA sample. (Do not use polystyrene tubes, which may melt during phenol:chloroform extraction.)
3. Incubate the tubes at 37°C for 30 min.
4. To each tube, add 2.5 µl of 0.2 M EDTA and 2 µl of 3 M NaOAc.
5. If you started with less than 25 µl of RNA, adjust the reaction mixture volume to 50 µl by adding a solution of 10 mM Tris (pH 7.5) and 50 mM NaOAc.  
**Note:** The concentration of total RNA at this point should be at least 20 µg/ml. Decreasing the RNA concentration below this optimum level may result in loss of RNA during phenol:chloroform extraction and precipitation.
6. To each tube, add a volume of phenol:chloroform:isoamyl alcohol (25:24:1) equal to the reaction volume.
7. Vortex the tubes thoroughly.
8. Centrifuge the tubes at 14,000 rpm for 10 min to separate phases.
9. For each sample, transfer the top aqueous layer to a clean 0.5-ml tube.
10. To each tube, add a volume of chloroform:isoamyl alcohol (24:1) equal to the volume of the aqueous layer.
11. Vortex the tubes thoroughly.
12. Centrifuge the tubes at 14,000 rpm for 10 min to separate phases.
13. For each sample, transfer the top aqueous layer to a clean 0.5-ml tube.
14. To each tube, add 1/10 volume of 3 M NaOAc and 2.5 volumes (of the total resulting volume) of 95% ethanol.
15. Vortex the tubes thoroughly.
16. Centrifuge the tubes for 20 min at 14,000 rpm at room temperature.
17. Carefully remove the supernatants.
18. Gently overlay the pellets with 200 µl of 80% ethanol.
19. Centrifuge at 14,000 rpm for 5 min.
20. Carefully remove the supernatants.
21. Air dry the pellets for 5–10 min.
22. Dissolve each pellet in 1.5 µl of H<sub>2</sub>O for each 2 µg of starting RNA. If possible, measure the final concentration of each RNA sample. Store samples at –20°C.

## IV. Protocol for Delta Differential Display *continued*

### D. First-Strand cDNA Synthesis

The 10- $\mu$ l reaction described below is designed to convert 2  $\mu$ g of total RNA into first-strand cDNA. This kit contains enough components for 12 separate cDNA syntheses. In most applications, you will perform two or more separate cDNA syntheses in parallel—one for each tissue, stage, or cell-culture condition that you wish to compare.

The following protocol is written for total RNA. Poly A<sup>+</sup> RNA can also be used, but in our experience poly A<sup>+</sup> RNA gives no more differentially expressed bands than total RNA. If you use poly A<sup>+</sup> RNA, you will need fewer cycles for PCR (Section IV.E).

In addition to your experimental sample, we recommend that you perform two positive control cDNA syntheses with the human adult liver and fetal liver total RNA included in the kit (positive control RNAs 1 & 2, respectively). This will provide templates for positive controls and thus verify that the system performs in your hands.

1. For each RNA sample (including positive controls), label a sterile 0.5-ml microcentrifuge tube with a number followed by "A".

**Note:** This will distinguish each sample from the "B" dilution prepared in Steps 16–18. If you are comparing three experimental RNA samples, you might label your experimental tubes 1A, 2A, and 3A, and your positive controls PC1A and PC2A.)

2. Combine the following in the appropriately labeled tubes:

2  $\mu$ g Total RNA sample\*  
1  $\mu$ l cDNA synthesis primer (1  $\mu$ M)

\* The volume depends on the concentration of each RNA sample. At CLONTECH, our total RNA samples are typically 0.5–1.0 mg/ml. For the positive control RNAs (1 mg/ml), add 2  $\mu$ l.

3. Add sterile H<sub>2</sub>O to a final volume of 5  $\mu$ l.
4. Mix contents and spin the tubes briefly in a microcentrifuge.
5. Incubate the tubes at 70°C for 3 min. (Temperatures >70°C may lead to degradation of your RNA.)
6. Cool the tubes on ice for 2 min.
7. Spin the tubes briefly in a microcentrifuge.
8. Prepare enough Master Mix for all of your cDNA synthesis reactions plus one additional tube:

| <u>per</u> | <u>5</u>    | <u>Components</u>                               |
|------------|-------------|---|
| <u>rxn</u> | <u>rxns</u> |   |
| 2 $\mu$ l  | 10 $\mu$ l  | 5X First-strand buffer                          |
| 2 $\mu$ l  | 10 $\mu$ l  | dNTP mix (5 mM)                                 |
| 1 $\mu$ l  | 5 $\mu$ l   | MMLV reverse transcriptase (200 units/ $\mu$ l) |

9. Add 5  $\mu$ l of the Master Mix to each reaction tube.

## IV. Protocol for Delta Differential Display *continued*

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10. Mix the contents of the tubes by gently pipeting.
11. Spin the tubes briefly in a microcentrifuge.
12. Incubate the tube at 42°C for 1 hr in an air incubator.  
**Note:** Using a water bath or thermal cycler for this incubation may reduce the volume of the reaction mixture (due to evaporation) and therefore reduce the efficiency of first-strand synthesis.
13. Terminate the reactions by incubating at 75°C for 10 min.
14. Place the tubes on ice.
15. Spin the tubes briefly in a microcentrifuge.
16. For each RNA sample (including positive controls), label a sterile 0.5-ml microcentrifuge tube with a number followed by “B” (i.e. tubes should be labeled 1B, 2B, PC1B, PC2B....). This is for the “B” dilution of each cDNA sample.
17. Transfer 2 µl of each reaction to the appropriate “B” tube.
18. Add 78 µl of sterile H<sub>2</sub>O to each “B” tube.
19. Add 72 µl of sterile H<sub>2</sub>O to the tube containing the remaining 8 µl of each ss cDNA. This is the “A” dilution of each cDNA sample.
20. Store all cDNA dilutions at –20°C until ready for use.



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## IV. Protocol for Delta Differential Display *continued*

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### E. Design Considerations for Delta Experiments

#### 1. Choice of primers and number of primer combinations

The Delta Differential Display Kit contains 10 arbitrary P primers and 9 oligo(dT) T primers. This gives you 90 possible two-way primer combinations. The arbitrary P primers can also be used alone (i.e., without a downstream primer) or in pair-wise combinations with other P primers (McClelland *et al.*, 1990). This gives you 55 additional primer combinations. Although there is some overlap, some bands will be detected with the P primer alone that would have been missed in pair-wise combinations with T primers. The T primers can also be used alone or in combination with each other. (Presumably, the oligo(dT) portion of the primer anneals to internal runs of As during the initial low-stringency cycles.) Note that combinations of P and T primers (or T primers alone) will generally produce PCR products derived from the 3' end of the mRNA, while P primers alone (or combinations of P primers) will favor internal regions of the mRNA.

Reactions using a P/T primer pair typically generate 40–80 bands. When comparing two different RNA samples, the number of differentially expressed RNAs will vary depending on the relatedness of the two sources. For example, 4% of the mRNAs differ between SV40-transformed and untransformed mouse cells (Scott *et al.*, 1983). Similarly, 2% of the transcripts differ between T- and B- lymphocytes (Hedrick *et al.*, 1984), while over 100 genes are activated during T-cell differentiation following antigenic stimulation (Ulman *et al.*, 1990).

In our experiments comparing human adult and fetal liver total RNA (i.e., the positive control RNAs in the Delta Kit), we typically obtained 1–2 differentially expressed bands for each combination of P and T primers. When these bands were used as probes on Northern blots, more than 85% were confirmed as unique, differentially expressed mRNAs, each having a different size. In contrast, in studies comparing stimulated and unstimulated mouse macrophages (Raw cell lines) or stimulated and unstimulated T-cells (Jurkat cell line), we typically obtained 1 differentially expressed band for every 2–3 combinations of P and T primers.

#### 2. Use of controls

##### a. Negative controls

##### i. H<sub>2</sub>O control

For each pair of primers you use, it is important to perform an H<sub>2</sub>O control using 1 µl of H<sub>2</sub>O as the “template.” Although there is no template in this reaction, it nevertheless produces a light banding pattern in some cases. In other cases, there will be no signal after the specified number of cycles. Whatever bands are present in the H<sub>2</sub>O control should take at least 5 additional cycles to reach the same intensity as the bands in real differential displays. If present, the light H<sub>2</sub>O banding pattern is an

## IV. Protocol for Delta Differential Display *continued*

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inherent background in all differential display reactions performed with that primer pair.

This is an important control for the quality of your cDNA synthesis. If your cDNA synthesis reaction does not work well, then your experimental differential display PCR will be of similar intensity to the H<sub>2</sub>O control. If you get no signal in your H<sub>2</sub>O controls and experimental samples, you may have a problem with your DNA polymerase. Once you are familiar with the H<sub>2</sub>O control for a primer pair, it is not necessary to repeat the control with every new experiment.

ii. Total RNA control

Perform a total RNA control the first time you use each freshly made cDNA template for differential display PCR. For this control, use 1 µl of a 1/100 dilution of the RNA that served as a template for cDNA synthesis. Initially, this control only needs to be done with one primer pair. The resulting differential display pattern should be compared to the H<sub>2</sub>O control performed with the same set of primers. If there was genomic DNA contamination in your RNA, you will see a significant number of bands in the RNA control that are not present in the H<sub>2</sub>O control. Such contamination can lead to many “false positives” when a contaminated sample is being compared to a non-contaminated sample. If your total RNA control differs from your H<sub>2</sub>O control, you can still use the cDNA as a template for PCR, but you should repeat the total RNA control for each different primer pair. Alternatively, you may wish to repeat your cDNA synthesis after either making fresh RNA or treating your RNA with RNase-free DNase. Once you have determined that a given RNA was not contaminated with genomic DNA, it is not necessary to repeat the total RNA control in later experiments.

b. Positive control

For a positive control, perform differential display PCR with the positive control cDNAs (made in Section C from the positive control RNAs) and the P10 and T8 primers. This should produce two good displays with a strong differentially expressed band of ~380 bp in the fetal lane (PC2; see Figure 3 in Section V). To prevent running out of the P10 and T8 primers, you may wish to use one of your own successful differential display reactions as the positive control in your later experiments.

3. Use both the A and B dilutions of each cDNA sample as templates for differential display PCR

In some cases a band will show up in one dilution, but will be absent from the other dilution; however, real differentially expressed bands always appear in both dilutions. Thus, using both dilutions as templates provides a positive control for true differentially expressed bands.

## IV. Protocol for Delta Differential Display *continued*

### F. Differential Display PCR

Delta Differential Display experiments tend to get very large—the more primer combinations you try, the more differentially expressed bands you may identify. (See Table III in Appendix B for examples of how the size of Delta experiments varies with the number of primer combinations and RNA samples.) **We recommend that you keep your initial experiments small.** For your first experiment, try using two T primers and three P primers for a total of six PCRs with each template. With two RNA samples and positive controls, this requires a total of 39 PCRs, and the results can be examined on 1–2 large gels.

- 24 Experimental displays (no. of RNA samples x 2 dilutions of each cDNA sample x no. of primer pairs)
- 6 H<sub>2</sub>O controls
- 2 Total RNA controls
- 7 Complete positive control experiment  
(4 positive controls, 2 total RNA controls, 1 H<sub>2</sub>O control)

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- 39 Total PCRs

A good organizational plan and labeling system is very helpful. Table II gives an example of how Delta experiments are often set up at CLONTECH.

**TABLE II: SAMPLE ORGANIZATIONAL AND LABELING CHART FOR SETTING UP DELTA DIFFERENTIAL DISPLAY PCR**

| Tube Label            | cDNA Sample | Primers | Tube Label   | cDNA Sample      | Primers  |
|-----------------------|-------------|---------|--|------------------|----------|
| Experimental displays |             |         | Water controls for each primer pair                                    |                  |          |
| 1                     | 1A          | P1 & T9 | H1   | H <sub>2</sub> O | P1 & T9  |
| 2                     | 1B          | P1 & T9 | H2   | H <sub>2</sub> O | P1 & T8  |
| 3                     | 2A          | P1 & T9 | H3   | H <sub>2</sub> O | P2 & T9  |
| 4                     | 2B          | P1 & T9 | and so on ....   |                  |          |
| 5                     | 1A          | P1 & T8 | Total RNA controls for each RNA<br>(Use any of the above primer pairs) |                  |          |
| 6                     | 1B          | P1 & T8 | R1   | RNA 1            | P1 & T9  |
| 7                     | 2A          | P1 & T8 | R2   | RNA 2            | P1 & T9  |
| 8                     | 2B          | P1 & T8 | and so on ....   |                  |          |
| 9                     | 1A          | P2 & T9 | Complete positive control (PC) experiment                              |                  |          |
| 10                    | 1B          | P2 & T9 | PC1A   | PC1A             | P10 & T8 |
| 11                    | 2A          | P2 & T9 | PC1B   | PC1B             | P10 & T8 |
| 12                    | 2B          | P2 & T9 | PC2A   | PC2A             | P10 & T8 |
| and so on ....        |             |         | PC2B   | PC2B             | P10 & T8 |
|                       |             |         | HPC  | H <sub>2</sub> O | P10 & T8 |
|                       |             |         | RPC1   | PC RNA 1         | P10 & T8 |
|                       |             |         | RPC2   | PC RNA 2         | P10 & T8 |

## IV. Protocol for Delta Differential Display *continued*

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The Delta Differential Display protocol has been optimized with CLONTECH's Advantage KlenTaq Polymerase Mix, which includes TaqStart Antibody for automatic hot-start PCR. If you choose not to use Advantage KlenTaq Polymerase, Table I (Section III) provides instructions for mixing TaqStart Antibody with other commercially available LD PCR-licensed enzyme mixes. We recommend using some form of hot-start PCR (i.e., TaqStart Antibody, wax beads, or manual hot start) to minimize background and false positives in your differential display reactions.

**Note on Steps 1–3:** If you are performing PCR with a single P or T primer, use 2  $\mu$ l of that primer. If you are performing PCR with 2 P primers or 2 T primers, use 1  $\mu$ l of each primer.

1. For each different experimental display (i.e., each pair of cDNAs and each combination of primers), combine the following reagents in an 0.5-ml PCR tube.
  - 1  $\mu$ l Experimental cDNA sample
  - 1  $\mu$ l P primer
  - 1  $\mu$ l T primer
2. [Recommended] For each pair of primers, prepare an H<sub>2</sub>O control by combining the following reagents in an 0.5-ml PCR tube:
  - 1  $\mu$ l Sterile H<sub>2</sub>O
  - 1  $\mu$ l P primer
  - 1  $\mu$ l T primer
3. [Recommended] For each different template, prepare a total RNA control by combining the following reagents in an 0.5-ml PCR tube:
  - 1  $\mu$ l Total RNA (1/100 dilution of RNA used as cDNA template)
  - 1  $\mu$ l P primer
  - 1  $\mu$ l T primer
4. [Recommended] For the positive control experiment, combine the following reagents in an 0.5-ml PCR tube (include H<sub>2</sub>O and RNA controls):
  - 1  $\mu$ l Positive control cDNA sample
  - 1  $\mu$ l P10 primer
  - 1  $\mu$ l T8 primer

## IV. Protocol for Delta Differential Display *continued*

5. Prepare enough Master Mix for all of the PCRs plus one additional tube.

| Component   | per rxn | 25 rxns | 100 rxns |
|---|---------|---------|----------|
| 10X KlenTaq PCR reaction buffer   | 2 µl    | 50 µl   | 200 µl   |
| Sterile H <sub>2</sub> O  | 14.2 µl | 355 µl  | 1420 µl  |
| dNTP mix (5 mM each;<br>final concentration 50 µM)                                    | 0.2 µl  | 5 µl    | 20 µl    |
| [α- <sup>33</sup> P]dATP* (1000–3000 Ci/mmmole;<br>3.3 µM; final concentration 50 nM) | 0.2 µl  | 5 µl    | 20 µl    |
| Advantage KlenTaq Polymerase<br>Mix (50X)   | 0.4 µl  | 10 µl   | 40 µl    |
| Final volume  | 17 µl   | 425 µl  | 1700 µl  |

Mix well by vortexing and briefly spin the tube in a microcentrifuge.

\* [α-<sup>33</sup>P]dATP produces sharper bands than [α-<sup>32</sup>P]dATP and thus increases the resolution of differential displays (and the number of bands that can be distinguished). If you use fresh label, you should obtain good overnight exposures. With older label (or for faster exposures), you can increase the amount of label to 0.3–0.5 µl and add proportionately less H<sub>2</sub>O. [α-<sup>35</sup>S]dATP is not recommended because it volatilizes upon heating, leading to contamination of equipment (Trentmann *et al.*, 1995).

6. Aliquot 17 µl of PCR Master Mix into each reaction tube. This gives a final volume of 20 µl.
7. Add 1 drop of mineral oil on top of PCR mixture and tightly cap the tubes.
8. Begin thermal cycling. (Use a cycler designated for radioactive use.)

Perkin-Elmer DNA  
Thermal Cyclers 480:

- 1 cycle:
  - 94°C 5 min
  - 40°C 5 min
  - 68°C 5 min
- 2 cycles:
  - 94°C 2 min
  - 40°C 5 min
  - 68°C 5 min
- 22–25 cycles\*:
  - 94°C 1 min
  - 60°C 1 min
  - 68°C 2 min

- 68°C for an additional 7 min.

Perkin-Elmer GeneAmp  
PCR Systems 2400/9600:

- 1 cycle:
  - 94°C 5 min
  - 40°C 5 min
  - 68°C 5 min
- 2 cycles:
  - 94°C 30 sec
  - 40°C 30 sec
  - 68°C 5 min
- 23 cycles:
  - 94°C 20 sec
  - 60°C 30 sec
  - 68°C 2 min

- 68°C for an additional 7 min.

\* For poly A<sup>+</sup> RNA, start with 15 cycles.

9. Store the differential display reactions at –20°C until you are ready to examine them on a gel.

## IV. Protocol for Delta Differential Display *continued*

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### G. Electrophoresis and Autoradiography

Delta Differential Display products can be up to 2 kb. For maximum resolution, we recommend using some form of temperature-controlled (“thermostatic”) electrophoresis system such as the MacroPhor System from Pharmacia Biotech AB or the genomyxLR DNA Sequencer from Genomyx Corporation. Nonthermostatic electrophoresis systems do not resolve differential display bands as well, resulting in increased band overlap.

1. Pour a denaturing 5% polyacrylamide/8 M urea gel in 0.5X TBE buffer. The acrylamide/bis-acrylamide stock should be 19:1. At CLONTECH, we use 0.2-mm spacers for maximum resolution. 0.2-mm thick gels are very fragile. For large experiments, you will need more than one gel.
2. Prerun the gel at 33 mA (constant current) for at least 20 min.\*
3. For each reaction, combine 5 µl of the PCR mixture with 5 µl of loading buffer in a clean 0.5-ml tube.
4. If desired, prepare a molecular weight marker.
5. Denature all your samples by incubating at 94°C for 2 min.
6. Place tubes on ice immediately.
7. Rinse wells of the gel prior to loading.
8. Load 2 µl samples with a Hamilton syringe or equivalent device.  
**Note:** Load each H<sub>2</sub>O control adjacent to the corresponding set of displays. Load each RNA control adjacent to the H<sub>2</sub>O control for the same primer combination.
9. Electrophorese at 70W (constant power) for 2.75 hr or until the xylene cyanol dye has migrated through the entire gel.\*
10. Dismantle the electrophoresis apparatus. Allow the glass plates to cool down to room temperature before attempting to separate the glass from the gel. After removing the top notched plate, transfer the gel to Whatman paper. Use caution: 0.2-mm gels are very fragile. Finally, place plastic wrap carefully over the gel.
11. Dry the gel under vacuum at 75°C for 35–40 min.
12. Expose x-ray film to the gel at –70°C overnight with an intensifying screen. We recommend Kodak BioMax MR film.
13. **Save the dried gel** so that you can cut out and reamplify the differentially expressed bands.

\* These current/power settings apply only when using 5% polyacrylamide/8 M urea gels/0.5X TBE that are 0.2 mm thick.

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## V. Expected Results and Troubleshooting Guide

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### A. Expected results

The positive control PCRs using primers P10 and T8 to compare the cDNA derived from the positive control RNAs from adult and fetal liver should give two very similar displays, except that a single, strong band of ~380 bp should be present in the fetal liver display (positive control RNA 2) but absent from the adult liver display (positive control RNA 1) (Figure 3). The exact pattern of the positive control displays may vary with different enzymes, different thermal cyclers, and other experimental variables, but the 380-bp fetal band should always be present in the fetal liver sample (PC2).

Your experimental displays will vary depending on the source (and quality) of the RNA samples and the particular combination of primers. In general, displays will consist of patterns of multiple bands ranging from 100–2,000 nt. Typically, several dozen distinct bands will be evident in each lane. Depending on the relatedness of the RNA samples, the number of differentially expressed bands can vary from one or more per display to one per 3–4 displays (or more for closely related RNA samples.) (See the discussion Design Considerations for Delta Experiments in Section IV.E.)

Ideally, total RNA controls should give similar banding patterns to the H<sub>2</sub>O controls using the same primers. The pattern in these controls (if any) may be detectable as a weak background in actual displays.

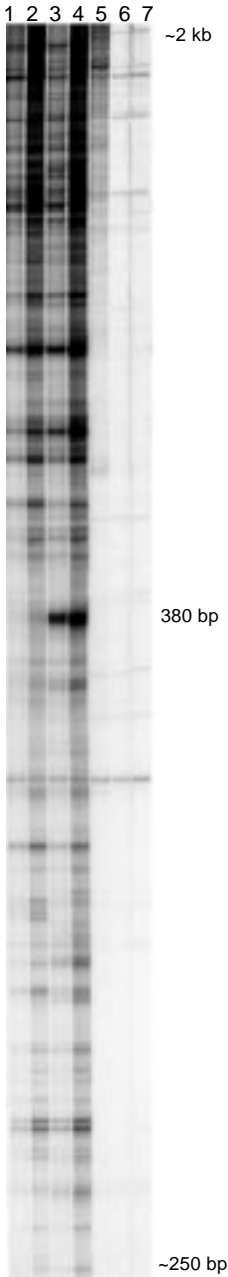
### B. Troubleshooting guide

#### 1. No bands in either the controls or in your experimental samples

If you do not see any bands in either the positive controls or in your experimental samples, perform 5 additional cycles with the remaining 15 µl of your PCRs.

- a. If no products are detectable after the additional cycles, you may have a problem with your DNA polymerase. Determine that your *Taq* DNA polymerase is good by performing a conventional PCR with primers and templates that produce a known product in your hands. If necessary, repeat the PCR using fresh 50X polymerase mix.
- b. If, after five additional cycles, bands appear and the intensity and pattern in all displays (including the positive controls) is similar to the patterns in the corresponding total RNA controls, your cDNA synthesis probably failed. Repeat the cDNA synthesis using fresh RNA.

## V. Expected Results and Troubleshooting Guide *cont'd*



**Figure 3. A typical positive control experiment using the P10 & T8 primers to compare the positive control RNAs** (total RNA from human adult and fetal liver). First-strand cDNA synthesis (using 2 µg of total RNA) and PCR were performed as described in this User Manual.

Lane    PCR template

- 1    Dilution B of cDNA made from positive control RNA 1
- 2    Dilution A of cDNA made from positive control RNA 1
- 3    Dilution B of cDNA made from positive control RNA 2
- 4    Dilution A of cDNA made from positive control RNA 2
- 5    1/100 dilution of positive control RNA 1
- 6    1/100 dilution of positive control RNA 2
- 7    H<sub>2</sub>O (Water control for the primer pair)

The exact pattern of the positive control display may vary depending on the particular enzyme mix you use and the lot of RNA. However, the strong differentially expressed band at ~380 bp should always be detectable in cDNA made from positive control RNA 2.



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## V. Expected Results and Troubleshooting Guide *cont'd*

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2. Positive control works, but experimental displays look identical to total RNA

If the positive controls show the expected patterns, but your experimental samples have the same banding pattern as the total RNA, your initial total RNA may be contaminated with genomic DNA. Treat your RNA samples with DNase I before performing first-strand cDNA synthesis (see Section IV.C.)

3. Positive control works, but experimental displays, total RNA, and H<sub>2</sub>O controls look identical
  - a. If the positive controls show the expected patterns, but your experimental samples have the same banding pattern as the total RNA and H<sub>2</sub>O negative controls, your initial total RNA may have been of poor quality. If you have more RNA, you can check the quality by examining several µg on a denaturing formaldehyde/1% agarose/EtBr gel. If the relative intensity of the 28S RNA band to the 18S RNA band is less than 1:1, you may need to make fresh RNA.
  - b. Alternatively, if you performed the optional DNase I treatment of your RNA samples, you may have lost your RNA during purification. Repeat DNase I treatment on a new aliquot of RNA.
4. Control displays, experimental displays, total RNA, and H<sub>2</sub>O controls look identical

If you treated your RNA samples with DNase I, you may have lost your RNA during purification. Repeat DNase I treatment.
5. Smearing in all lanes

If you observe smears in all your lanes, you have problems with either your PAGE gels or with your PCR. See Sambrook *et al.* (1989) for a general discussion of troubleshooting PAGE gels. If you suspect you have problems with the PCR, try using a fresh batch of 50X polymerase mix or reducing the MgCl<sub>2</sub> concentration in the PCR buffer.

6. Smearing only in experimental samples

If the positive controls show the expected patterns, but you observe smears in your experimental lanes, your experimental RNAs have impurities which inhibit the reverse transcription and/or PCRs. Repeat the phenol:chloroform extraction and ethanol precipitation and repeat the Delta procedure.

7. Ladders

In rare (and apparently random) instances, we have observed a ladder of fairly even bands instead of a display (i.e., an irregular pattern of bands of varying intensities). If this occurs, repeat the PCR.

## VI. Additional Procedures

Once a differentially expressed band has been identified, the next step is to elute the band from the PAGE gel and reamplify it for use as a probe in Northern analysis. If you have many differentially expressed bands, focus your initial efforts on well-isolated bands that are *completely absent* from the other RNA sample. Bands that overlap or are very close to non-differentially expressed bands may be difficult to isolate and clone. Bands that are strongly expressed in one RNA sample and weakly expressed in other samples may not correspond to true differentially expressed mRNAs.

### A. Purification of DNA Fragments from Dried Polyacrylamide Gels

1. Align the autoradiograph on top of the dried gel (on Whatman paper). Use tape or some other method to keep the autoradiograph firmly and precisely aligned on top of the dried gel during the following steps.
2. Using a sharp pin or needle, mark the differentially expressed bands by poking holes through the film and the gel beneath.
3. Using a clean, sharp razor or scalpel, excise the differentially expressed band(s) from the gel using the pin marks as guides. Place each fragment of dried gel in a separate 0.5-ml tube. *Use a clean blade for each band.* Even small amounts of carryover contamination can cause problems during reamplification and subsequent analysis.
4. Add 40  $\mu\text{l}$  of Tricine-EDTA buffer (10 mM Tricine [pH 9.5], 0.2 mM EDTA) to each tube. (TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] or sterile  $\text{H}_2\text{O}$  can also be used.)
5. Overlay with 2–3 drops of mineral oil.
6. Heat at 100°C for 5 min.
7. Carefully remove the Whatman paper. Leave as much liquid as possible in the tube. Discard the Whatman paper in the appropriate radioactive waste container.
8. Store eluted bands at  $-20^\circ\text{C}$ .

### B. Reamplification of the Band of Interest

Reamplify each differentially expressed band using the primer(s) used in the original PCR.

1. For each band, combine the following reagents in an 0.5-ml PCR tube.

|                    |                              |
|--------------------|------------------------------|
| 7 $\mu\text{l}$    | Eluted DNA                   |
| 5 $\mu\text{l}$    | 10X PCR buffer               |
| 0.5 $\mu\text{l}$  | 5 mM dNTP                    |
| 2.5 $\mu\text{l}$  | P primer (20 $\mu\text{M}$ ) |
| 2.5 $\mu\text{l}$  | T primer (20 $\mu\text{M}$ ) |
| 1 $\mu\text{l}$    | 50X polymerase mixture       |
| 31.5 $\mu\text{l}$ | Sterile $\text{H}_2\text{O}$ |
| 50 $\mu\text{l}$   | Total                        |

**Note:** If you performed PCR with a single P or T primer, use 5  $\mu\text{l}$  of that primer.

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## VI. Additional Procedures *continued*

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2. Overlay each reaction with 1–2 drops of mineral oil and place caps firmly on tubes.
3. Commence cycling in a Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp PCR Systems 2400/9600:
  - 20 cycles:
    - 94°C 1 min
    - 60°C 1 min
    - 68°C 2 min
4. Analyze 10 µl of your product on a 2% agarose/EtBr gel. The reamplified product should be the same size as the original band.
5. Store the remaining material at –20°C.
6. [Optional] Purify the PCR products from the primers and unincorporated nucleotides using a CHROMA SPIN™-100 Column (#K1302-1, -2) from CLONTECH or other comparable method.
7. Ethanol precipitate the reamplified PCR product and resuspend in 10 µl of sterile H<sub>2</sub>O.

The reamplified product can now be used to make a probe for Northern analysis (see next section) and then cloned into a T/A-cloning vector for additional analysis. The AdvanTage™ PCR Cloning Kit (#K1901-1) provides all the reagents needed for T/A cloning.

### C. Northern Blot Analysis

The most direct way to verify differential expression of bands identified by Delta Differential Display is to use the reamplified bands as probes on Northern blots of poly A<sup>+</sup> RNA from the original RNA sources. Examples of Northern blot confirmation of differentially expressed bands can be seen in the article that appeared in the April 1995 issue of *CLONTECHniques* (pp. 5–7).

#### 1. Labeling probes

Each reamplified, differentially expressed band can be used as a template for random-primer labeling (Sambrook *et al.*, 1989). If you have followed the steps in Section B for reamplifying, purifying, precipitating, and resuspending your PCR products, use 5 µl as a template for making the random-primed probe.

#### 2. Make Northern blots with poly A<sup>+</sup> RNA

Use poly A<sup>+</sup> RNA for Northern blots whenever possible. Many differentially expressed bands identified by Delta Differential Display will correspond to mRNAs of average or low abundance. Such mRNAs may not be detectable on a Northern blot of total RNA.

## VI. Additional Procedures *continued*

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### 3. Exposure times may vary

The exposure time required for Northern blots varies dramatically from band to band. This is because Delta Differential Display can detect both abundant and relatively rare RNAs. For example, in our experiments with adult and fetal liver (i.e., the positive control RNAs), the exposure needed for Northern blot confirmation of differentially expressed bands ranged from 10 min to 7 days.

The products of differential display PCR are complex mixtures containing many different sequences. In some cases, differentially expressed bands will overlap with bands that are present in both RNA samples (albeit at much lower levels). In these cases, the reamplified band will often detect multiple bands when used as a probe on a Northern blot. The differentially expressed band may or may not be detectable on a Northern blot under these conditions. For example, if the differentially expressed band corresponds to a rare mRNA, the signal may “disappear” in a high background of signal generated from the more abundant mRNAs. This does not necessarily mean that your original band is false; however, isolating the true, differentially expressed RNA from the background of non-differentially expressed RNAs may be difficult, since it requires cloning different PCR products of the same size and determining which of the clones correspond to a differentially expressed cDNA (Bauer *et al.*, 1993). Consequently, if you have other differentially expressed bands that are easily confirmed by Northern analysis, your time may be better spent characterizing them.

One way to reduce band overlap is to use temperature-controlled gel electrophoresis systems (Section IV.G), such as the Macrophor System from Pharmacia Biotech AB or the genomyxLR DNA Sequencer from Genomyx Corporation. These thermostatic systems increase the resolution of differential display, thereby reducing band overlap.

## D. Additional Experiments

### 1. Notes on cloning

Reamplified bands are most readily cloned by T/A cloning; we recommend the AdvanTAge PCR Cloning Kit (#K1901-1). As discussed above, reamplified bands often contain multiple cDNA species; though minor, these other cDNAs will also be cloned into your vector. We therefore recommend that you pick a clone and test it by making a probe and reprobng a Northern blot. If you do not detect the same differentially expressed band that you detected with your reamplified cDNA, the particular clone you picked is probably a minor cDNA band that is not differentially expressed. The correct clone is usually present in your transformation. Simply pick another colony and check it in the same fashion.

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## VI. Additional Procedures *continued*

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### 2. Marathon cDNA amplification

Once some sequence information has been obtained from differentially expressed cDNAs, many researchers will want to clone the corresponding full-length cDNAs as rapidly as possible. The Marathon cDNA Amplification Kit (#K1802-1) and Marathon-Ready™ cDNAs (many) from CLONTECH are ideally suited to this task.

Marathon cDNA amplification is an advanced method for rapid amplification of cDNA ends, or RACE. Unlike conventional RACE, Marathon amplification enables you to clone full-length cDNAs without screening libraries. Like the Delta Differential Display method, Marathon RACE reactions use LD PCR enzyme mixes to allow amplification of much larger cDNAs (up to 9 kb) than can be amplified by conventional RACE. Furthermore, many different cDNAs can be amplified from the products of a single Marathon cDNA synthesis reaction. Because only 23–28 nt of gene-specific sequence information are needed, the Marathon method is ideally suited to situations where a researcher rapidly identifies many different cDNAs that she or he then wants to clone—as is often the case with Delta Differential Display.

By using Marathon-Ready cDNAs, researchers can save time while ensuring the success of three critical aspects of the Marathon method—purification of high-quality poly A<sup>+</sup> RNA, synthesis of full-length cDNA, and efficient, complete adapter ligation. Marathon-Ready cDNAs are available from a wide variety of tissues and organisms.

At CLONTECH, we have used the Marathon Kit to obtain full-length cDNAs for numerous genes identified by Delta Differential Display.

### 3. GenomeWalker™ Kits

Besides cloning the full-length cDNA, many researchers will also want to clone the corresponding locus in genomic DNA, particularly the upstream region containing the promoter and other *cis*-regulatory elements. Conventional methods involve screening genomic libraries with cDNA probes—a very time-consuming task. The GenomeWalker technique is a novel, PCR-based method that allows researchers to walk upstream (or downstream) in uncloned genomic DNA from known sequences (Siebert *et al.*, 1995a, 1995b).

Human, Mouse, and Rat GenomeWalker Kits (#K1803-1, #K1805-1, #K1806-1, respectively) include five PCR-ready pools of uncloned genomic DNA. The Universal GenomeWalker Kit (#K1807-1) supplies the reagents needed to construct these genomic DNA pools, enabling you to walk in the genome of any species. The use of LD PCR, together with nested gene-specific primer and nested adaptor primers, allows steps of up to 6 kb in uncloned genomic DNA adjacent to known sequences—such as the first exon of a differentially expressed cDNA identified using the Delta Kit.

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## VIII. Related Products

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For the latest and most complete listing of all CLONTECH products, please go to **[www.clontech.com](http://www.clontech.com)**.

- Advantage® KlenTaq Polymerase Mix 8417-1
- Advantage® cDNA PCR Kit K1905-1
- TaqStart™ Antibody 5400-1, -2
- CLONTECH PCR-Select™ cDNA Subtraction Kit K1804-1
- Marathon™ cDNA Amplification Kit K1802-1
- Marathon-Ready™ cDNAs many
- CHROMA-SPIN™ Columns many
- GenomeWalker™ DNA Walking Kits many
- Multiple Tissue Northern (MTN®) Blots many
- cDNA Libraries many

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## Appendix A: Design of the Delta Primers

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(The sequences of the P and T primers are given in Section II, List of Components.)

The most important feature of the Delta primers is that they are longer than the primers used in previously described methods. Each P primer is 25 nt long, while each T primer is 29 nt long. The longer primer means that, following three initial low-stringency PCR cycles at  $T_{\text{anneal}} = 40^{\circ}\text{C}$ , the annealing temperature can be increased to  $60^{\circ}\text{C}$  for the final 22–25 cycles. The resulting increase in stringency is critical to the proven repro-ducibility and relatively low backgrounds of Delta Differential Display. In addition, the optimal primer length for *Taq* DNA polymerase is greater than 11 nt (D'yachenko *et al.*, 1994), so the Delta primers work more efficiently in PCR than the shorter primers used in most other similar protocols.

### P primer design

Several factors were considered in choosing the actual sequence of the P primers. First, the primers are incapable of forming significant, stable secondary structure. Secondly, the 3' end of each primer is not significantly complementary to any other sequence in the primer. Finally, we placed several additional restrictions on the nine bases at the 3' ends of the P primers. (These positions are the most significant for priming events.) These positions favor common sequence motifs found in the coding region of eukaryotic (especially mammalian) mRNAs; however, the Delta Kit also yields good results with nonmammalian species, including plants. The probability of two primer binding sites occurring within one mRNA molecule is less than 3%. We also carefully avoided sequences that might allow “slippage” during DNA synthesis or other artifacts (Chenchik *et al.*, 1992).

To ensure optimal performance of the Delta Kit, we synthesized a large pool of primers that met the above criteria. We then performed many Delta experiments to select the best primers for the kit.

### T primer design

The T primers in the Delta Kit have the general structure 5'-anchor-(dT)<sub>9</sub>N<sub>-1</sub>N<sub>-1</sub>, where N<sub>-1</sub> = A, G, or C. The 5' anchor adds the extra length needed for the high-stringency cycling described above. We have also reduced the oligo(dT) portion to 9 T residues to increase the influence of the N<sub>-1</sub> bases; the protocol described by Liang & Pardee (1992) uses oligo(dT)<sub>11</sub>. Also, the Delta Kit does not include primers with T in the terminal position (i.e., 5'-anchor-(dT)<sub>9</sub>N<sub>-1</sub>T), since these primers produced high backgrounds in our experiments. This result is probably due to the higher priming specificity of *Taq* DNA polymerase relative to reverse transcriptase (D'yachenko *et al.*, 1993), since primers with a 3'-terminal T are used to prime cDNA syntheses in other differential display procedures (Liang & Pardee, 1992; Liang *et al.*, 1993).



## Appendix B: Size of Delta Differential Display Experiments

**TABLE III: SIZE OF DELTA DIFFERENTIAL DISPLAY EXPERIMENTS**

|   |    |    |    |    |     |     |     |     | Your<br>Experiment |
|---|----|----|----|----|-----|-----|-----|-----|--------------------|
| Number of primer combinations   | 1  | 6  | 6  | 12 | 20  | 20  | 45  | 90  |                    |
| Number of RNA samples:  | 2  | 2  | 3  | 2  | 2   | 3   | 2   | 2   |                    |
| Experimental displays (no. of RNA<br>samples x 2 dilutions of each cDNA<br>x no. of primer pairs)         | 4  | 24 | 36 | 48 | 80  | 120 | 180 | 360 |                    |
| H <sub>2</sub> O controls   | 1  | 6  | 6  | 12 | 20  | 20  | 45  | 90  |                    |
| Total RNA controls  | 2  | 2  | 3  | 2  | 2   | 3   | 2   | 2   |                    |
| Positive control experiment<br>(4 positive controls, 1 H <sub>2</sub> O control,<br>2 total RNA controls) | 7  | 7  | 7  | 7  | 7   | 7   | 7   | 7   | 7                  |
| Total PCRs:   | 14 | 39 | 52 | 69 | 109 | 150 | 234 | 459 |                    |

# Notes

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## Notes

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