PCR-Select™ cDNA Subtraction Kit User Manual

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I. Introduction

Subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA: we refer to the cDNA that contains specific (differentially expressed) transcripts as **tester**, and the reference cDNA as **driver**. Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA.

Although traditional subtractive hybridization methods have been successful in some cases, they require several rounds of hybridization and are not well suited for the identification of rare messages (Duguid & Dinauer, 1990; Hara et al., 1991; Hedrick et al., 1984; Sargent & Dawid, 1983; and Davis et al., 1984). The PCR-Select™ cDNA Subtraction Kit is based on a unique method of selective amplification of differentially expressed sequences, which overcomes the technical limitations of traditional subtraction methods (Diatchenko et al., 1996; Gurskaya et al., 1996). Figure 1 presents a brief overview of the PCR-Select procedure. The entire procedure requires only 0.5–2 µg of poly A$^+$ RNA, takes 3–4 days, and does not require physical separation of single-stranded (ss) and double-stranded (ds) molecules (Duguid & Dinauer, 1990; Sargent & Dawid, 1983; and Hedrick et al., 1984). Furthermore, **suppression PCR** (U.S. Patent No. 5,565,340; described in Appendix A) prevents undesirable amplification during enrichment of target molecules.

Molecular basis of PCR-Select cDNA subtraction

Figure 2 details the molecular events that occur during PCR-Select cDNA subtraction. First, cDNA is synthesized from 0.5–2 µg of poly A$^+$ RNA generated from the two types of tissues or cells being compared. The tester and driver cDNAs are digested with Rsa I, a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA is then subdivided into two portions, and each is ligated with a different cDNA adaptor. The ends of the adaptor do not contain a phosphate group, so only one strand of each adaptor attaches to the 5' ends of the cDNA. The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in. (See Appendix B for detailed sequences of the primers and adaptors).

Two hybridizations are then performed. In the first, an excess of driver is added to each sample of tester. The samples are then heat denatured and allowed to anneal, generating the type **a**, **b**, **c**, and **d** molecules in each sample (Figure 2). The concentration of high- and low-abundance sequences is equalized among the type **a** molecules because reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization (see Nucleic Acid Hybridization, ed. by James & Higgins). At the same time,

I. Introduction continued

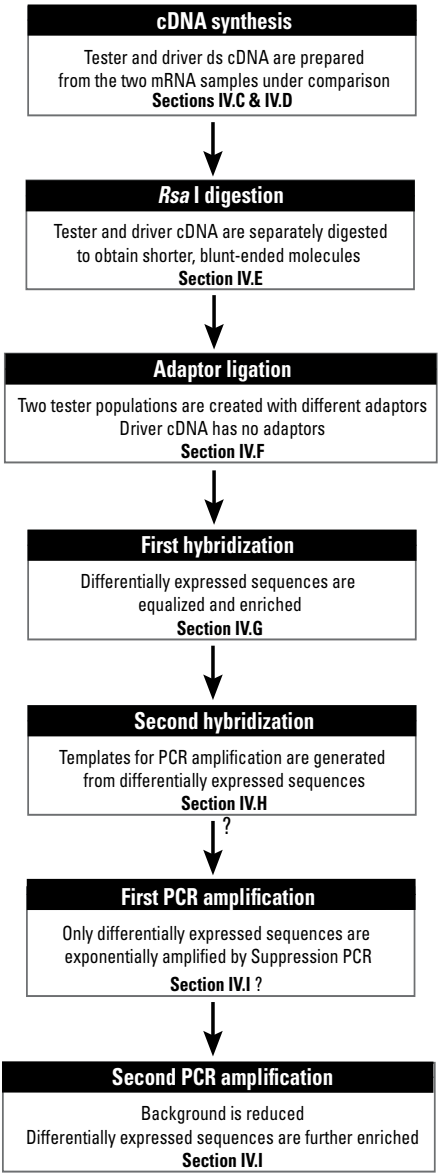
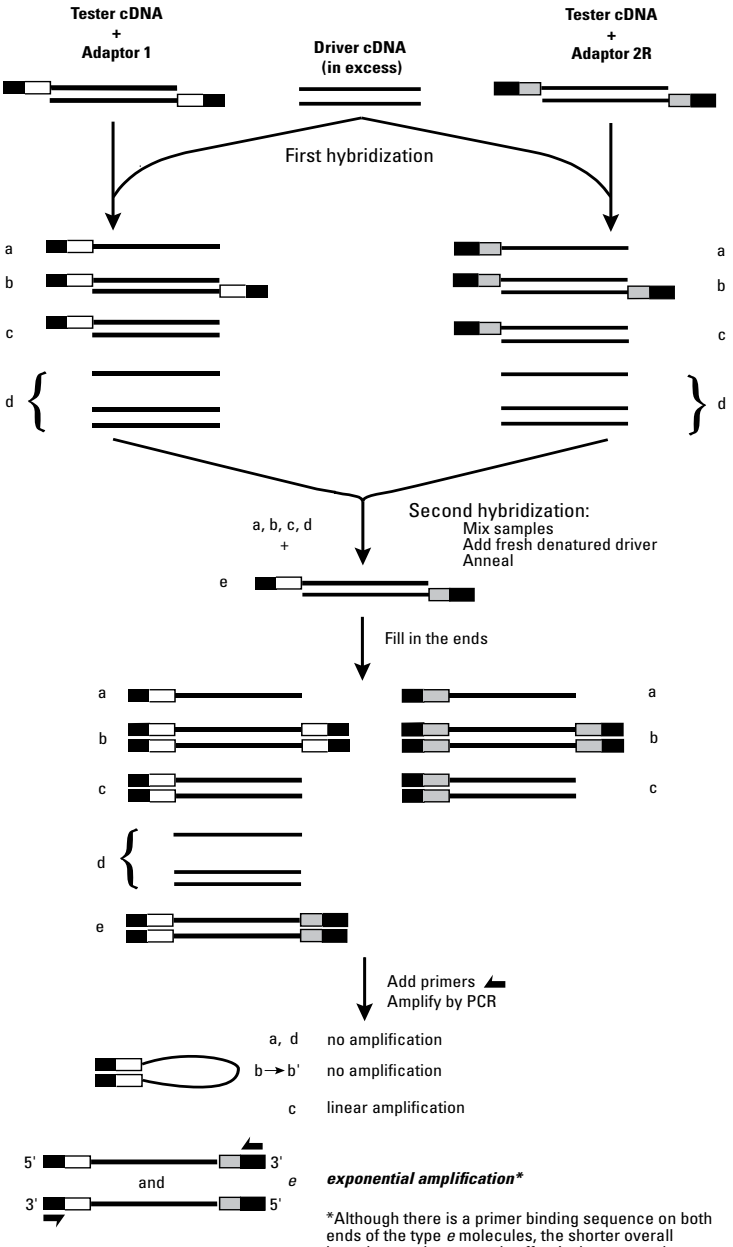


Figure 1. Overview of the PCR-Select procedure. The cDNA in which specific transcripts are to be found is referred to as **tester** and the reference cDNA is referred to as **driver**. If you have used the Super SMART PCR cDNA Synthesis Kit for cDNA synthesis you should begin with the Adaptor Ligation Step (Section IV.F) shown above.



*Although there is a primer binding sequence on both ends of the type *e* molecules, the shorter overall homology at the two ends effectively negates the suppression PCR effect—except for very short molecules. See Appendix A for more details on suppression PCR.

I. Introduction continued

type **a** molecules are significantly enriched for differentially expressed sequences while cDNAs that are not differentially expressed form type **c** molecules with the driver.

During the second hybridization, the two primary hybridization samples are mixed together without denaturing. Now, only the remaining equalized and subtracted ss tester cDNAs can reassociate and form new type **e** hybrids. These new hybrids are ds tester molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA is added (again, without denaturing the subtraction mix) to further enrich fraction **e** for differentially expressed sequences. After filling in the ends by DNA polymerase, the type **e** molecules—the differentially expressed tester sequences—have different annealing sites for the nested primers on their 5' and 3' ends.

The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences. During this PCR, type **a** and **d** molecules are missing primer annealing sites, and thus cannot be amplified. Due to the suppression PCR effect, most type **b** molecules form a pan-like structure that prevents their exponential amplification (see Appendix A for more details.) Type **c** molecules have only one primer annealing site and amplify linearly. **Only type e molecules**—the equalized, differentially expressed sequences with two different adaptors—**amplify exponentially**.

Next, a secondary PCR amplification is performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences. The cDNAs can then be directly inserted into a T/A cloning vector. Alternatively, site-specific cloning can be performed using the Not I (also Sma I, Xma I) site on Adaptor I and the Eag I site on Adaptor 2R. Blunt-end cloning requires use of the Rsa I site at the adaptor/cDNA junction. This cloning allows identification of differentially expressed RNAs by sequence and/or hybridization analysis. Additionally, the PCR mixture can be used as a hybridization probe to screen DNA libraries.

PCR-Select differential screening

After the subtracted cDNA library has been obtained, it is important to confirm that individual clones indeed represent differentially expressed genes. This is typically accomplished by probing Northern blots with randomly-selected, subtracted clones. However, this method can be time-consuming and inefficient, especially when two closely related RNA populations are being

Figure 2 (facing page). Schematic diagram of PCR-Select cDNA subtraction. Type **e** molecules are formed only if the sequence is upregulated in the tester cDNA. Solid lines represent the Rsa I-digested tester or driver cDNA. Solid boxes represent the outer part of the Adaptor 1 and 2R longer strands and corresponding PCR primer 1 sequence. Clear boxes represent the inner part of Adaptor 1 and the corresponding Nested PCR primer 1 sequence. Shaded boxes represent the inner part of Adaptor 2R and the corresponding Nested PCR primer 2R sequence.

I. Introduction continued

compared—a situation in which the subtracted library is likely to contain a significant number of false positives. Differential screening of the subtracted library before undertaking Northern blot analysis helps to eliminate false positives and saves time and effort. Such screening of subtracted cDNA libraries can be performed using the Clontech™ PCR-Select Differential Screening Kit (Cat. No. 637403). Dot blot arrays of clones from the subtracted library can be hybridized with labeled probes from either tester or driver populations and those that are recognized by the tester probe and not by the driver probe are confirmed to be differentially expressed. For more details, please see the Clontech™ PCR-Select Differential Screening Kit User Manual (PT3138-1).

To maximize the sensitivity of the Clontech PCR-Select Differential Screening Kit, two subtractions should be performed: the original intended subtraction (forward subtraction), and a reverse subtraction in which tester serves as the driver and the driver as tester. For additional information about differential screening, see Section VI.

Using the Super SMART™ PCR cDNA Synthesis Kit

If your starting material is limited, the Super SMART PCR cDNA Synthesis Kit (Cat. No. 635000) can be used to preamplify total RNA samples for use with the PCR-Select cDNA Subtraction Kit (Matz et al., 1999 and Chenchik et al., 1998). When total RNA is used for cDNA synthesis by conventional methods, ribosomal RNA is transcribed along with the poly A^+ fraction, even if synthesis is oligo(dT)-primed. If cDNA generated in this manner is used with the PCR-Select cDNA Subtraction Kit, the excess of ribosomal RNA coupled with a low concentration of cDNA (corresponding to the poly A^+ fraction) can cause inefficient subtractive hybridization. cDNA generated using the Super SMART PCR cDNA Synthesis Kit can be directly used for PCR-Select subtraction—even if total RNA is used as starting material.

To use experimental cDNA generated with the Super SMART Kit, you should begin with the Adaptor Ligation Step (Section IV.F). However, it is highly recommended that you perform the PCR-Select control cDNA synthesis and subtraction described in Section IV.C. Please note that cDNA generated using the SMART PCR cDNA Library Construction Kit (Cat. No. 634901) is **not compatible** with the PCR-Select cDNA Subtraction Kit.

Once differentially expressed cDNAs have been identified with the PCR-Select method, the SMART™ RACE cDNA Amplification Kit (Cat. No. 634914) and Marathon-Ready™ cDNAs (many) provide excellent tools for rapidly cloning the corresponding full-length cDNAs (Chenchik et al., 1996). For more information, see Related Products (Section VIII).

II. List of Components

Store RNA at –70°C. Store 4X Hybridization buffer at room temperature. Store all other reagents at –20°C.

This kit includes enough reagents for seven cDNA syntheses. For best results, use 2 µg of poly A^+ RNA per reaction; differentially expressed cDNAs for rare transcripts may be lost during subtraction if less poly A^+ RNA is used. The seven cDNA syntheses are equivalent to six complete subtraction experiments and one control, assuming the cDNA from each synthesis is used for tester and driver in separate experiments (for identifying up- and down-regulated cDNAs in a particular system). Enough PCR reagents are provided for 50 primary and 100 secondary PCRs. Refer to Appendix B for detailed adaptor and primer sequences.

First-strand synthesis

- 7 µl AMV Reverse Transcriptase (20 units/µl)
- 10 µl cDNA Synthesis Primer (10 µM)
- 200 µl 5X First-Strand Buffer
  - 250 mM Tris-HCl (pH 8.5)
  - 40 mM $MgCl_2$
  - 150 mM KCl
  - 5 mM Dithiothreitol

Second-strand synthesis

- 28 µl 20X Second-Strand Enzyme Cocktail
  - DNA polymerase I, 6 units/µl
  - RNase H, 0.25 units/µl
  - E. coli DNA ligase, 1.2 units/µl
- 200 µl 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 BSA
- 14 µl T4 DNA Polymerase (3 units/µl)

Endonuclease digestion

- 300 µl 10X Rsa I Restriction Buffer
  - 100 mM Bis Tris Propane-HCl (pH 7.0)
  - 100 mM $MgCl_2$
  - 1 mM DTT
- 12 µl Rsa I (10 units/µl)

II. List of Components continued

Adaptor ligation

- 21 µl T4 DNA Ligase (400 units/µl; contains 3 mM ATP)
- 200 µl 5X DNA Ligation Buffer
  - 250 mM Tris-HCl (pH 7.8)
  - 50 mM $MgCl_2$
  - 10 mM DTT
  - 0.25 mg/ml BSA
- 30 µl Adaptor 1 (10 µM)
- 30 µl Adaptor 2R (10 µM)

Hybridization

- 200 µl 4X Hybridization Buffer
- 1.4 ml Dilution buffer (pH 8.3)
  - 20 mM HEPES (pH 6.6)
  - 20 mM NaCl
  - 0.2 mM EDTA (pH 8.0)

PCR amplification

- 50 µl PCR Primer 1 (10 µM)
- 100 µl Nested PCR primer 1 (10 µM)
- 100 µl Nested PCR primer 2R (10 µM)
- 10 µl PCR Control Subtracted cDNA

Control reagents

- 5 µl Control Poly A^+ RNA (1 µg/µl; from human skeletal muscle)
- 5 µl Control DNA (3 ng/µl)
  (Hae III-digested bacteriophage ϕX174 DNA)
- 50 µl G3PDH 5' Primer (10 µM)*
- 50 µl G3PDH 3' Primer (10 µM)*

*These primers will amplify human, mouse, and rat species G3PDH genes.

General reagents

- 20 µl dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
- 100 µl 20X EDTA/Glycogen Mix (0.2 M EDTA; 1 mg/ml glycogen)
- 400 µl NH_4OAc (4 M)
- 1 ml sterile H_2O

III. Additional Materials Required

The following reagents are required but not supplied.

- **Hae III digest of bacteriophage fX174**
  We recommend New England Biolabs DNA size markers (Cat. Nos. N3026S & N3026L)
- **0.5-ml PCR reaction tubes** We recommend GeneAmp 0.5-ml reaction tubes (Cat. No. N801-0737 or N801-0180).
- **80% Ethanol & 96% Ethanol**
- **Phenol:chloroform:isoamyl alcohol (25:24:1)**
- **Chloroform:isoamyl alcohol (24:1)**
- **50X PCR enzyme mix**
  We recommend our Advantage™ cDNA Polymerase Mix (Cat. No. 639105; also provided in Advantage cDNA PCR Kits [Cat. Nos. 639101 & 639102]).The protocol contained in this User Manual was optimized using this mix, which was developed for long and accurate PCR amplification of cDNA (Barnes, 1994; Cheng et al., 1994). The 50X mix contains KlenTaq-1 DNA Polymerase (an exo-minus, N-terminal deletion ofTaq DNA polymerase), a proofreading polymerase, and TaqStart™ Antibody for automatic hot start (Kellogg et al., 1994). Alternatively,Taq DNA polymerase alone can be used, but 3–5 additional thermal cycles are needed in both the primary and secondary PCR steps. Note that these additional cycles may increase background, lowering the percentage of differentially expressed clones in your subtracted library.

  Note: If you do not use Advantage cDNA Polymerase Mix, we highly recommend using TaqStart Antibody (Cat. Nos. 639250 & 639251), manual hot start, or hot start with wax beads to reduce levels of nonspecific DNA synthesis.

- **10X PCR buffer**
  Use the 10X reaction buffer supplied with your DNA polymerase or mix (included with the Advantage cDNA Polymerase Mix [Cat. No. 639105] and the Advantage cDNA PCR Kits [Cat. Nos. 639101 & 639102]).
- **dNTP Mix for PCR** (10 mM each dATP, dCTP, dGTP, dTTP)
- **50X TAE electrophoresis buffer**

  242 g Tris base
  57.1 ml Glacial acetic acid
  37.2 g $Na_2EDTA•2H_2O$

  Add H_2O to 1 L. For 1X TAE buffer, dilute 50X stock solution 1:49 with H_2O.

IV. PCR-Select cDNA Subtraction Protocols

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

- Wear gloves to protect RNA and cDNA samples from degradation by nucleases.
- The cycling parameters in this protocol have been optimized on the Gene Amp DNA Thermal Cycler 480 and GeneAmp Systems 2400/9600. Optimal parameters may vary with different thermal cyclers, polymerase mixes, and templates.
- If you use the PCR-Select Differential Screening Kit (Cat. No. 637403) to differentially screen your samples before performing Northern blot analysis, you will need to perform two subtractions: your original intended subtraction (forward subtraction), and a reverse subtraction, in which tester serves as driver and driver as tester. For more information about differential screening, see Section VI and the PCR-Select Differential Screening Kit User Manual (PT3831-1).
- A hot start **MUST** be used to reduce nonspecific DNA synthesis during the setup of the PCR. We recommend using either TaqStart Antibody (Kellogg et al., 1994) or manual hot start (D'Aquila et al., 1991). This protocol was optimized using our TaqStart™ Antibody (individually available as Cat. Nos. 639250 & 639251; also included in our Advantage™ cDNA Polymerase Mix [Cat. No. 639105]).
- To resuspend pellets and mix reactions, gently pipet them up and down and centrifuge the tube briefly to deposit contents at the bottom.
- Mix phenol:chloroform extractions by vortexing.
- Add enzymes to reaction mixtures last and thoroughly mix by gently pipetting the reaction mixture up and down.
- Do not increase the amount of enzyme or concentration of DNA in the reactions as these have been carefully optimized.
- Although not required, we recommend that you add [α-^{32}P]dCTP to the first-strand synthesis reaction to help quantify cDNA yield, determine efficiency of DNA precipitation, and troubleshoot cDNA synthesis.

B. RNA Preparation and Handling

1. General precautions

   Intact, pure poly A$^+$ RNA is essential for the synthesis of high-quality cDNA. To avoid RNA contamination and degradation, as well as minimize the presence of RNases, use the following precautions: wear gloves to prevent RNase contamination from your hands and

IV. PCR-Select cDNA Subtraction Protocols continued

use aerosol-free pipette tips for dispensing small volumes (or sterile disposable pipettes for larger volumes).

2. RNA isolation

   See Farrell et al., 1993 or Sambrook et al., 2001 for procedures. Whenever possible, the total RNA samples being compared should be purified side-by-side using the same reagents and protocol. This practice reduces the likelihood of false positives.

3. RNA analysis

   After total and poly A$^+$ RNA isolation, examine RNA integrity by electrophoresing samples on a denaturing, formaldehyde 1% agarose/EtBr gel. Intact total mammalian RNA typically exhibits two bright bands—corresponding to ribosomal 28S and 18S RNA—at ~4.5 and 1.9 kb, respectively, with a ratio of intensities of ~1.5–2.5:1. Mammalian poly A$^+$ RNA appears as a smear from 0.5–12 kb with weak ribosomal RNA bands present. The size distribution may be smaller (0.5–3 kb) for nonmammalian species.

   If your experimental RNA appears on an agarose gel smaller than expected (e.g., no larger than 1–2 kb), as a smear <1–2 kb, and/or the ratio of intensity of 28S to 18S observed is <1:1, your RNA may have been impure or degraded. We suggest you test all RNA isolation reagents for the presence of RNase or other impurities. If contamination is found, you must prepare RNA again using fresh reagents if necessary. If problems persist, you may need to identify another source of tissue or cells, such as our Premium Poly A$^+$ RNAs (see Related Products, Section VIII). Poor quality and/or degraded RNA produces high background in the subtraction procedure and should not be used as starting material.

C. First-Strand cDNA Synthesis

Perform this procedure with each experimental tester and driver poly A$^+$ RNA, and with the Control Poly A$^+$ RNA (from human skeletal muscle) provided with the kit. The skeletal muscle cDNA made in this section serves as control driver cDNA in later steps. In Section F, mock tester cDNA is generated by adding a small amount of the Control DNA (Hae III-digested ϕX174) to an aliquot of the skeletal muscle ds cDNA. You should then perform a complete control subtraction with these skeletal

muscle tester and driver cDNAs in parallel with your experimental subtraction. The control subtraction allows you to estimate both the yield and size distribution of synthesized ds cDNA.

1. For each tester, driver, and the Control Poly A$^+$ RNA (from human skeletal muscle), combine the following components in a sterile 0.5-ml microcentrifuge tube. (Do not use a polystyrene tube).

	per rxn
poly A$^+$ RNA (2 µg)	2–4 µl*
cDNA Synthesis Primer (10 µM)	1 µl

* For the control synthesis, add 2 µl of the skeletal muscle control poly A$^+$ RNA.

If needed, add sterile H_2O to a final volume of 5 µl. Mix contents and spin briefly in a microcentrifuge.

2. Incubate at 70°C for 2 min in a thermal cycler.
3. Cool on ice for 2 min and briefly centrifuge.
4. Add the following to each reaction:

	per rxn
5X First-Strand Buffer	2 µl
dNTP Mix (10 mM each)	1 µl
sterile H_2O*	1 µl
AMV Reverse Transcriptase (20 units/µl)	1 µl

* To monitor the progress of the cDNA synthesis, dilute 1 µl of [α^{32}P]dCTP (10 mCi/ml, 3,000 Ci/mmol) with 9 µl of H_2O, and substitute 1 µl of this diluted label for the H_2O component above.

5. Gently vortex and briefly centrifuge the tubes.
6. Incubate the tubes at 42°C for 1.5 hr in an air incubator.
   Note: Do not use a water bath or thermal cycler. Evaporation can reduce the reaction mixture volume, and therefore, reaction efficiency.
7. Place on ice to terminate first-strand cDNA synthesis and immediately proceed to Section D.

D. Second-Strand cDNA Synthesis

Perform the following procedure with each first-strand tester, driver, and the control skeletal muscle cDNA.

1. Add the following components to the first-strand synthesis reaction tubes (containing 10 µl):

	per rxn
sterile H_2O	48.4 µl
5X Second-Strand Buffer	16.0 µl
dNTP Mix (10 mM)	1.6 µl
20X Second-Strand Enzyme Cocktail	4.0 µl

2. Mix contents and briefly spin. The final volume should be 80 µl.
3. Incubate at 16°C for 2 hr in water bath or thermal cycler.
4. Add 2 µl (6 u) of T4 DNA Polymerase. Mix contents well.

IV. PCR-Select cDNA Subtraction Protocols continued

5. Incubate at 16°C for 30 min in a water bath or thermal cycler.
6. Add 4 µl of 20X EDTA/Glycogen Mix to terminate second-strand synthesis.
7. Add 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
8. Vortex thoroughly, and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
9. Carefully collect the top aqueous layer and place in a fresh 0.5-ml microcentrifuge tube. Discard the inter- and lower phases and dispose of them appropriately.
10. Add 100 µl of chloroform:isoamyl alcohol (24:1).
11. Repeat steps 8 and 9.
12. Add 40 µl of 4 M NH_4OAc and 300 µl of 95% ethanol.

    Note: Proceed immediately with precipitation. **Do not** store tubes at -20°C. Prolonged exposure to this temperature can precipitate undesired salts.
13. Vortex thoroughly and centrifuge at 14,000 rpm for 20 min at room temperature.
14. Carefully collect the supernatant. [If you labelled with [α-^{32}P]dCTP, check for the pellet using a Geiger counter.]
15. Overlay the pellet with 500 µl of 80% ethanol.
16. Centrifuge at 14,000 rpm for 10 min.
17. Remove the supernatant. [If you labelled with [α-^{32}P]dCTP, check for the pellet using a Geiger counter.]
18. Air dry the pellet for about 10 min to evaporate residual ethanol.
19. Dissolve precipitate in 50 µl of sterile H_2O.
20. Transfer 6 µl to a fresh microcentrifuge tube. Store this sample at –20°C until after Rsa I digestion (for agarose gel electrophoresis) to estimate yield and size range of ds cDNA products synthesized (see Section V.A)

E. Rsa I Digestion

Perform the following procedure with each experimental ds tester and driver cDNA, as well as with the control skeletal muscle cDNA. This step generates shorter, blunt-ended ds cDNA fragments which are optimal for subtraction and required for adaptor ligation in Section F.

1. Add the following reagents:

	per rxn
ds cDNA	43.5 µl
10X Rsa I Restriction Buffer	5.0 µl
Rsa I (10 units/µl)	1.5 µl

2. Mix by vortexing and briefly centrifuge.
3. Incubate at 37°C for 1.5 hr.

IV. PCR-Select cDNA Subtraction Protocols continued

4. Set aside 5 µl of the digest mixture to analyze the efficiency of Rsa I digestion as described in Section V.B.
5. Add 2.5 µl of 20X EDTA/Glycogen Mix to terminate the reaction.
6. Add 50 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
7. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
8. Carefully collect the top aqueous layer and place in a fresh 0.5-ml tube.
9. Add 50 µl of chloroform:isoamyl alcohol (24:1).
10. Repeat steps 7 and 8.
11. Add 25 µl of 4 M NH_4OAc and 187.5 µl of 95% ethanol.

    Note: Proceed immediately with precipitation. **Do not** store tubes at -20°C. Prolonged exposure to this temperature can precipitate undesired salts.
12. Repeat step 7.
13. Remove the supernatant.
14. Gently overlay the pellets with 200 µl of 80% ethanol.
15. Centrifuge at 14,000 rpm for 5 min.
16. Carefully remove the supernatant. [If you labelled with [α-^{32}P] dCTP, check for the pellet using a Geiger counter.]
17. Air dry the pellets for 5–10 min.
18. Dissolve the pellet in 5.5 µl of H_2O and store at –20°C.
    These 5.5-µl samples of Rsa I digested cDNA will serve as your **experimental driver cDNA** and your **control skeletal muscle driver cDNA**. In the next section, these samples will be ligated with adaptors to create your tester cDNAs for forward, control, and reverse (if applicable) subtractions.
19. Check your Rsa I-digested cDNA from Step IV.E.4 using agarose/EtBr gel electrophoresis, as described in Section V.B. Then, proceed to Section IV.F to finish preparing your experimental and control skeletal muscle **tester cDNAs**.

F. Adaptor Ligation

Figure 3 shows the experimental flowchart for preparing adaptor-ligated tester cDNA. If you plan to perform differential screening of the subtracted library (discussed in detail in Section VI), you must perform subtractions in both directions for each tester/driver cDNA pair. The forward subtraction experiment shown in Figure 3A is designed to enrich for differentially expressed sequences present in poly A^+ RNA sample 1 (cDNA 1, tester) but not poly A^+ RNA sample 2 (cDNA 2, driver). Figure 3B shows the reverse subtraction, in which cDNA 2 serves as tester and cDNA 1 serves as driver. The result is two subtracted cDNA populations: the forward-subtracted cDNA contains

IV. PCR-Select cDNA Subtraction Protocols continued

Figure 3. Preparing adaptor-ligated tester cDNAs for hybridization and PCR. Each tester cDNA (i.e., each different experimental cDNA and your control skeletal muscle tester cDNA) must be ligated to the appropriate adaptors as shown above. **Panel A.** The forward subtraction is your intended experiment. **Panel B.** A second subtraction in reverse (i.e., tester as driver, driver as tester is required for differential screening of the subtracted cDNA library (Section VI). **Panel C.** Control subtraction with skeletal muscle cDNA.

IV. PCR-Select cDNA Subtraction Protocols continued

sequences that are specific to sample 1, and the reverse-subtracted cDNA contains sequences that are specific to sample 2. Even if you are only interested in sequences specific to sample 1, the reverse-subtracted cDNA can be used for differential screening (Section VI).

To perform subtractions in both directions, you will need to prepare tester cDNA corresponding to each of your poly A^+ RNA samples. You should also perform a control subtraction (Panel C). In Step 2 (below), you will prepare tester cDNA for this control subtraction by mixing the control skeletal muscle cDNA with ϕX174/Hae III DNA.

As illustrated in Figure 3, three separate adaptor ligations must be performed for each experimental tester cDNA and the control skeletal muscle tester cDNA. Each cDNA is aliquotted into two separate tubes: one aliquot is ligated with Adaptor 1 (Tester 1-1, 2-1, and 3-1), and the second is ligated with Adaptor 2R (Tester 1-2, 2-2, and 3-2). After the ligation reactions are set up, portions of each tester tube are combined so that the cDNA is ligated with both adaptors (unsubtracted tester control 1-c, 2-c, and 3-c). Each unsubtracted tester control cDNA serves as a positive control for ligation, and later serves as a negative control for subtraction.

Note: Through the rest of the procedure, it is helpful to label tubes using the nomenclature described in this User Manual. Labeling tubes of intermediate products with the appropriate step number in which they were created may prove helpful as well. Referring to Figure 3 will help you keep track of the multiple samples.

Adaptors will not be ligated to the driver cDNA.

1. Dilute 1 µl of each Rsa I-digested **experimental** cDNA (Step IV.E.19) with 5 µl of sterile H_2O.
   If you have used the Super SMART PCR cDNA Synthesis Kit to prepare your cDNA, use the purified, Rsa I-digested cDNAs from the Super SMART procedure for this dilution.
2. **Prepare your control skeletal muscle tester cDNA**:
   a. Dilute the ϕX174/Hae III Control DNA with sterile H_2O to a final concentration of 150 ng/ml.
   b. Mix 1 µl of control skeletal muscle cDNA (Step IV.E.19) with 5 µl of the diluted ϕX174/Hae III Control DNA (150 ng/ml).

   This is your **control skeletal muscle tester cDNA**. It contains 0.2% Hae III-digested ϕX174 DNA; each fragment corresponds to about 0.02% of the total cDNA. After subtraction of the skeletal muscle tester cDNA against the skeletal muscle driver cDNA, the primary bands produced in the final PCR should correspond to these control fragments.

IV. PCR-Select cDNA Subtraction Protocols continued

If you have used the Super SMART PCR cDNA Synthesis Kit to prepare your cDNA, you should repeat Step 2 above using the human placenta cDNA from the Super SMART procedure (as described in the Super SMART cDNA Synthesis Kit User Manual PT3656-1). For the rest of the PCR-Select protocol, you should analyze the control human placenta cDNA in parallel with the control skeletal muscle cDNA.

Prepare your adaptor-ligated tester cDNA:

3. Prepare a ligation Master Mix by combining the following reagents in a 0.5-ml microcentrifuge tube. To ensure that you have sufficient Master Mix, prepare enough for all ligations plus one additional reaction.

	per rxn
sterile H_2O	3 µl
5X Ligation Buffer	2 µl
T4 DNA Ligase (400 units/µl)	1 µl

Note: The ATP required for ligation is a component of the T4 DNA Ligase mix (3 mM initial, 300 µM final).

4. For each experimental tester cDNA and for the control skeletal muscle tester cDNA, combine the reagents in Table I in the order shown in 0.5 ml microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.

TABLE I: SETTING UP THE LIGATION REACTIONS
(REPEAT FOR EACH EXPERIMENTAL TESTER cDNA & THE CONTROL SKELETAL MUSCLE TESTER cDNA)

	Tube Number	
Component	**1 Tester 1-1***	**2 Tester 1-2***
Diluted tester cDNA	2 µl	2 µl
Adaptor 1 (10 µM)	2 µl	–
Adaptor 2R (10 µM)	–	2 µl
Master Mix	6 µl	6 µl
Final volume	10 µl	10 µl

* Use the same setup for Tester 2-1 and 2-2, 3-1 and 3-2.

5. In a fresh microcentrifuge tube, mix 2 µl of Tester 1-1 and 2 µl of Tester 1-2. After ligation is complete, this will be your unsubtracted tester control 1-c (see Figure 3). Do the same for each additional experimental tester cDNA and the control skeletal muscle tester

cDNA. After ligation, approximately 1/3 of the cDNA molecules in each unsubtracted tester control tube will bear two different adaptors.

6. Centrifuge briefly, and incubate at 16°C overnight.
7. Add 1 µl of EDTA/Glycogen Mix to stop ligation reaction.
8. Heat samples at 72°C for 5 min to inactivate the ligase.
9. Briefly centrifuge the tubes. Your experimental and control skeletal muscle Adaptor-Ligated Tester cDNAs and unsubtracted tester controls are now complete.
10. Remove 1 µl from each unsubtracted tester control (1-c, 2-c, 3-c) and dilute into 1 ml of H_2O. These samples will be used for PCR (Section IV.I).
11. Store samples at –20°C.

Perform the ligation efficiency analysis described in Section V.C. prior to proceeding with the hybridizations on Section IV.G.

G. First Hybridization

Perform the ligation efficiency analysis in Section V.C. **before proceeding with the hybridizations described below**. If your ligation was not efficient, repeat the ligation step before proceeding any further.

In the following procedure, an excess of driver cDNA is added to each tester cDNA, samples are heat denatured, and allowed to anneal. The remaining ss cDNAs (available for the second hybridization) are dramatically enriched for differentially expressed sequences because non-target cDNAs present in the tester and driver cDNA form hybrids.

Important: Before you begin the hybridization, **allow the 4X Hybridization buffer to warm up to room temperature for at least 15–20 min**. Verify that there is no visible pellet or precipitate before using the buffer. If necessary, heat the buffer at 37°C for ~10 min to dissolve any precipitate.

1. For each of the experimental and skeletal muscle subtractions, combine the reagents in Table II in 0.5-ml tubes in the order shown.

IV. PCR-Select cDNA Subtraction Protocols continued

TABLE II: SETTING UP THE FIRST HYBRIDIZATION
(REPEAT FOR EACH EXPERIMENTAL TESTER cDNA & THE CONTROL SKELETAL MUSCLE cDNA)

	Hybridization Sample 1	Hybridization Sample 2
Component	**Tester 1-1***	**Tester 1-2***
Rsa I-digested Driver cDNA (IV.E.18)	1.5 µl	1.5 µl
Adaptor 1-ligated Tester 1-1* (IV.F.9)	1.5 µl	—
Adaptor 2R-ligated Tester 1-2 (IV.F.9)	—	1.5 µl
4X Hybridization Buffer	1.0 µl	1.0 µl
Final volume	4.0 µl	4.0 µl

* Use the same setup for Tester 2-1 and 2-2, 3-1 and 3-2.

2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples at 98°C for 1.5 min in a thermal cycler.
4. Incubate samples at 68°C for 8 hr*.

   *Samples may hybridize for 6–12 hrs. **Do not let the incubation exceed 12 hours.**

H. Second Hybridization

The two samples from the first hybridization are mixed together, and fresh denatured driver DNA is added to further enrich for differentially expressed sequences. New hybrid molecules are formed which consist of differentially expressed cDNAs with different adaptors on each end.

Important: Do not denature the primary hybridization samples at this stage. Also, do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver.

Repeat the following steps for each experimental tester cDNA and for the control skeletal muscle cDNA.

1. Add the following reagents into a sterile tube:

	per rxn
Driver cDNA (Step IV.E.21)	1 µl
4X Hybridization Buffer	1 µl
sterile H_2O	2 µl

2. Place 1 µl of this mixture in a 0.5-ml microcentrifuge tube and overlay it with 1 drop of mineral oil.
3. Incubate at 98°C for 1.5 min in a thermal cycler.
4. Remove the tube of freshly denatured driver from the thermal cycler. Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (prepared in Section IV.G; see Table II). This ensures that the two hybridization samples mix together only in the presence of freshly denatured driver.

IV. PCR-Select cDNA Subtraction Protocols continued

a. Set a micropipettor at 15 µl.
b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
c. Carefully draw the entire sample partially into the pipette tip. Do not be concerned if a small amount of mineral oil is transferred with the sample.
d. Remove the pipette tip from the tube, and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket.
f. Transfer the entire mixture to the tube containing hybridization sample 1.
g. Mix by pipetting up and down.

5. Briefly centrifuge if necessary.
6. Incubate reaction at 68°C overnight.
7. Add 200 µl of dilution buffer and mix by pipetting.
8. Heat at 68°C for 7 min in a thermal cycler .
9. Store at –20°C.

I. PCR Amplification

Differentially expressed cDNAs are selectively amplified during the two reactions described in this section. Prior to thermal cycling, the missing strands of the adaptors are filled in by a brief incubation at 75°C (Step IV.I.6); this creates the binding site for PCR Primer 1 (see Figure 2). In the first amplification, only ds cDNAs with different adaptor sequences on each end are exponentially amplified. In the second amplification, nested PCR is used to further reduce background and enrich for differentially expressed sequences.

A minimum of seven PCR reactions are recommended as described in Figure 3: (1) forward-subtracted experimental cDNA, (2) unsubtracted tester control (1-c), (3) reverse-subtracted experimental cDNA, (4) unsubtracted tester control for the reverse subtraction (2-c), (5) subtracted control skeletal muscle cDNA, (6) unsubtracted tester control for the control subtraction (3-c), and (7) PCR control-subtracted cDNA. The PCR control subtracted cDNA provides a positive PCR control and contains a successfully subtracted mixture of Hae III-digested ϕX174 DNA. We recommend that you also perform a standard PCR control (i.e., the positive control template in the Advantage cDNA PCR Kit) to verify that your enzyme is performing efficiently.

IV. PCR-Select cDNA Subtraction Protocols continued

Notes:

- All cycling parameters were optimized on a GeneAmp DNA Thermal Cycler 480 and GeneAmp PCR Systems 2400/9600. Cycling parameters for other thermal cycler machines may require optimization.
- If you do not use Advantage cDNA Polymerase Mix, you can use Taq DNA polymerase alone; however, 3–5 more cycles will be needed in the primary and secondary PCR steps. You must also use a hot start (for more information, see Section IV.A). Our Taq Start Antibody (Cat. Nos. 639250 & 639251; also included in the cDNA Polymerase Advantage Mix [Cat. No. 639105]) works best. Alternatively, you can perform hot start as follows: (1) Prepare the primary PCR Master Mix without Taq Polymerase. (2) Mix PCR samples and heat the reaction mix to 75°C for 1 min. (3) Quickly add the necessary amount of Taq polymerase. (4) Incubate the reaction at 75°C for 5 min. (5) Perform PCR as described in step 8 below.

1. Prepare the PCR templates:
   a. Aliquot 1 µl of each diluted cDNA (i.e., each subtracted sample from Step IV.H.9 and the corresponding diluted unsubtracted tester control from Step IV.F.10) into an appropriately labeled tube.
   b. Aliquot 1 µl of the PCR control subtracted cDNA into an appropriately labeled tube.
2. Prepare a Master Mix for all of the primary PCR tubes plus one additional tube. For each reaction planned, combine the reagents in Table III in the order shown:

TABLE III: PREPARATION OF THE PRIMARY PCR MASTER MIX

Reagent	Per Rxn	7-rxn Mix*
sterile H_2O	19.5 µl	156.0 µl
10X PCR reaction buffer	2.5 µl	20.0 µl
dNTP Mix (10 mM)	0.5 µl	4.0 µl
PCR Primer 1 (10 µM)	1.0 µl	8.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	4.0 µl
Total volume	24.0 µl	192.0 µl

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

3. Mix well by vortexing, and briefly centrifuge the tube.
4. Aliquot 24 µl of Master Mix into each of the reaction tubes prepared in step 1.
5. Overlay with 50 µl of mineral oil.
6. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)

IV. PCR-Select cDNA Subtraction Protocols continued

Note: This step "fills in" the missing strand of the adaptors (see Figure 2), thus creating binding sites for the PCR primers.

7. Immediately commence thermal cycling:

GeneAmp® DNA Thermal Cycler 480:	GeneAmp® PCR Systems 2400 or 9600:
27 cycles:	• 94°C 25 sec
• 94°C 30 sec	27 cycles:
• 66°C 30 sec	• 94°C 10 sec
• 72°C 1.5 min	• 66°C 30 sec
	• 72°C 1.5 min

8. Analyze 8 µl from each tube on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section V.D for expected results.) Alternatively, you can set these 8 µl aliquots aside and run them on the same gel used to analyze the secondary PCR products (step 16).
9. Dilute 3 µl of each primary PCR mixture in 27 µl of H_2O. [If applicable: this diluted primary PCR product is used in the PCR-Select Differential Screening Kit procedure.]
10. Aliquot 1 µl of each diluted primary PCR product mixture from Step 9 into an appropriately labeled tube.
11. Prepare Master Mix for the secondary PCR reactions plus one additional reaction by combining the reagents in Table IV in the order shown:

TABLE IV: PREPARATION OF THE SECONDARY PCR MASTER MIX

Component	Per Rxn	7-rxn Mix*
sterile H_2O	18.5 µl	148.0 µl
10X PCR reaction buffer	2.5 µl	20.0 µl
Nested PCR primer 1 (10 µM)	1.0 µl	8.0 µl
Nested PCR primer 2R (10 µM)	1.0 µl	8.0 µl
dNTP Mix (10 mM)	0.5 µl	4.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	4.0 µl
Total volume	24.0 µl	192.0 µl

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

IV. PCR-Select cDNA Subtraction Protocols continued

12. Mix well by vortexing, and briefly centrifuge.
13. Aliquot 24 µl of Master Mix into each reaction from step 10.
14. Overlay with 1 drop of mineral oil.
15. Immediately commence thermal cycling:

GeneAmp® DNA Thermal Cycler 480:	GeneAmp® PCR Systems 2400 or 9600:
10–12 cycles:	10–12 cycles:
• 94°C 30 sec	• 94°C 10 sec
• 68°C 30 sec	• 68°C 30 sec
• 72°C 1.5 min	• 72°C 1.5 min

16. Analyze 8 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section V.D for expected results.)
17. Store reaction products at –20°C.

The PCR mixture is now enriched for differentially expressed cDNAs. In addition, differentially expressed transcripts that varied in abundance in the original mRNA sample should now be present in roughly equal proportions. Refer to Sections V.D and V.E. Figure 6 shows the results of a successful control subtraction experiment with cDNA made from the skeletal muscle poly A^+ RNA. We strongly recommend that you perform a subtraction efficiency test as shown in Figures 7 and 8.

The uncloned subtracted mixture is an ideal hybridization probe for screening libraries of genomic DNA, full-length cDNA, YAC, BAC, or cosmid clones (Diatchenko et al., 1996). For all other applications, you should clone the products to make a subtracted cDNA library. The cDNAs can be directly inserted into a T/A cloning vector. Alternatively, site-specific cloning can be performed using the Not I (also Sma I, Xma I) site on Adaptor 1 and the Eag I site on Adaptor 2R. Blunt-end cloning requires use of the Rsa I site at the adaptor/cDNA junction.

For further analysis of your subtracted library, several options are available:

- Differential screening
  Our PCR-Select Differential Screening Kit (Cat. No. 637403) contains the necessary reagents for differential screening, along with controls. For more information about differential screening, see Section VI and the PCR-Select Differential Screening Kit User Manual (PT3138-1).
- Northern analysis
  You may confirm the expression pattern of individual clones

using Northern blot analysis. In our experience, the percentage of clones in the subtracted library that corresponds to differentially expressed mRNAs varies considerably, from as high as 95% (Diatchenko et al., 1996), to a mid-range of 40–60% (Gurskaya et al., 1996; von Stain et al., 1997), and as low as 5% (Diatchenko et al., 1998). We recommend that you randomly pick 10–20 clones from the subtracted library for use as probes on Northern blots. If fewer than two clones are confirmed as differentially expressed genes, you should perform the differential screening procedure described above to eliminate false positives.

- Virtual Northerns
  If you lack sufficient poly A^+ RNA for standard Northern blot analysis, you can create "Virtual" Northern blots, which yield similar information (Endege et al., 1999). To make a Virtual Northern blot, use the Super SMART PCR cDNA Synthesis Kit (Cat. No. 635000) to generate SMART cDNA from your total or poly A^+ RNA sample. Then, electrophorese the SMART cDNA on an agarose/EtBr gel, denature it in situ, and transfer it onto a nylon membrane. For more information on Super SMART cDNA synthesis technology and Virtual Northern blots, please see Chenchik et al., 1998 and visit our web site at **www.clontech.com**

V. Analysis of Results & Troubleshooting Guide

A. Analysis of ds cDNA Synthesis Products

1. General recommendations
   a. To monitor the progress and yield of cDNA synthesis, perform first- and second-strand cDNA synthesis with your experimental sample and the control skeletal muscle poly A$^+$ RNA provided. We recommend that you monitor the cDNA synthesis and purification by including [α-^{32}P]dCTP in the first-strand reaction mixture.
   b. Use high-quality poly A$^+$ RNA. The yield of ds cDNA depends on the RNA quality. 2 µg of the Control poly A$^+$ RNA from skeletal muscle will typically produce about 2 µg of ds cDNA. Similar amounts (1–2 µg) are typically obtained from high-quality experimental poly A$^+$ RNAs. You should analyze the efficiency of cDNA synthesis and Rsa I digestion by agarose gel as shown in Figure 4 (next page).
2. Troubleshooting of ds cDNA synthesis
   a. If agarose gel analysis indicates that the yield of your experimental ds cDNA is low in comparison with the ds cDNA produced from the skeletal muscle poly A$^+$ RNA, but the size distribution is similar, you may still use your cDNA. However, it is highly likely that you may have lost some low-abundance, differentially-expressed sequences. Alternatively, you may repeat the synthesis using a higher concentration of poly A$^+$ RNA for first-strand cDNA synthesis.

V. Analysis of Results & Troubleshooting Guide contin-

b. If your experimental ds cDNA appears on an agarose gel as a smear <1–2 kb, the RNA may have been impure or degraded. Electrophorese the RNA used as starting material on a denaturing, formaldehyde 1% agarose/EtBr gel. Intact total mammalian RNA typically exhibits two bright bands—corresponding to ribosomal 28S and 18S RNA—at ~4.5 and 1.9 kb, respectively, with a ratio of intensities of ~1.5–2.5:1. Mammalian poly A^+ RNA appears as a smear from 0.5–12 kb with weak ribosomal RNA bands present. The size distribution may be smaller (0.5–3 kb) for nonmammalian species.

   If your RNA appears on an agarose gel smaller than expected (e.g., no larger than 1–2 kb), as a smear <1–2 kb, and/or the ratio of intensity of 28S to 18S observed is <1:1, these indicate that your RNA may have been of poor quality. We suggest you test all RNA isolation reagents for the presence of RNase or other impurities. If contamination is found, you must prepare RNA again using fresh reagents if necessary. If problems persist, you may need to identify another source of tissue or cells, such as our Premium Poly A^+ RNAs (see Related Products, Section VIII). Poor quality and/or degraded RNA produces high background in the subtraction procedure and should not be used as starting material.

c. The optimal concentration of poly A^+ RNA for first-strand cDNA synthesis is 50–200 µg/ml. If you use a lower concentration of RNA, the size distribution of cDNA products synthesized may be reduced.

B. Analysis of Rsa I Digestion

Electrophorese 2.5 µl of undigested, ds cDNA (from Section IV.D) and 5 µl of Rsa I-digested cDNA (from Section IV.E) on a 1% agarose/EtBr gel in 1XTAE buffer side-by-side. cDNA derived from poly A^+ RNA appears as a smear from 0.5–10 kb. Bright bands correspond to abundant mRNAs or rRNAs. (Size distribution may be only 0.5–3 kb for some RNA samples from nonmammalian species.) After Rsa I digestion, the average cDNA size is smaller (0.1–2 kb compared to 0.5–10 kb). Typical results are shown in Figure 4. If the size distribution of your sample and/or control cDNA is not reduced after Rsa I digestion, repeat the phenol/chloroform extraction, ethanol precipitation, and digestion steps.

[Optional] To determine if a sample is completely digested, remove a small sample of DNA at 60 and 80 min. Compare the samples on an agarose gel. If the DNA size distribution for both samples is identical, digestion has progressed to completion.

V. Analysis of Results & Troubleshooting Guide contin-

C. Analysis of Ligation

We recommend that you perform the following PCR experiment to verify that at least 25% of the cDNAs have adaptors on both ends. This experiment is designed to amplify fragments that span the adaptor/cDNA junctions of Testers 1-1 and 1-2. (See Section IV.F.) You should also perform this analysis on your adaptor-ligated control skeletal muscle cDNA (3-1 and 3-2) and, if doing the reverse subtraction, with your second experimental tester cDNA (2-1 and 2-2). The G3PDH primers in this control experiment will work for human, mouse, and rat genes. For other species, you will need to design suitable primers.

Note: We recommend that you also include a standard PCR control (such as the positive control template in the Advantage cDNA PCR Kit [Cat. Nos. 639101 & 639102]) to verify that your enzyme is performing efficiently.

1. Dilute 1 µl of each ligated cDNA from Sec. IV.F (e.g., the Testers 1-1 and 1-2) into 200 µl of H_2O.
2. Combine the reagents in Table V in four separate tubes:

TABLE V: SETTING UP THE LIGATION ANALYSIS
(REPEAT FOR EACH EXPERIMENTAL TESTER cDNA & THE CONTROL SKELETAL MUSCLE cDNA)

	Tube (µl)			
Component	**1**	**2**	**3**	**4**
Tester 1-1* (ligated to Adaptor 1)	1	1	–	–
Tester 1-2* (ligated to Adaptor 2R)	–	–	1	1
G3PDH 3' Primer (10 µM)	1	1	1	1
G3PDH 5' Primer (10 µM)	–	1	–	1
PCR Primer 1 (10 µM)	1	–	1	–
Total volume	3	3	3	3

* Use the same set-up for Tester 2-1 and 2-2, 3-1 and 3-2.

3. Prepare a Master Mix for all of the reaction tubes plus one additional tube. For each reaction planned, combine the reagents in Table VI in the order shown:

TABLE VI: PREPARATION OF THE LIGATION ANALYSIS PCR MASTER MIX

Component	**Per Rxn**	**4-rxn Mix**
sterile H_2O	18.5 µl	92.5 µl
10X PCR reaction buffer	2.5 µl	12.5 µl
dNTP Mix (10 mM)	0.5 µl	2.5 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	2.5 µl
Total volume	22.0 µl	110.0 µl

V. Analysis of Results & Troubleshooting Guide contin-

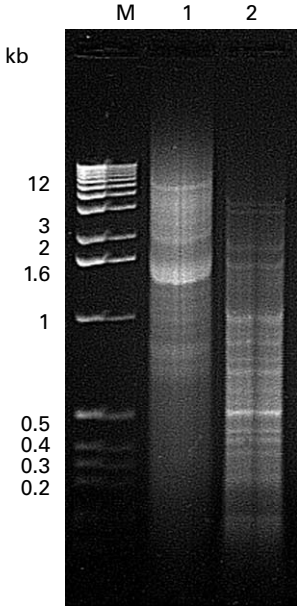


Figure 4. Positive control skeletal muscle ds cDNA before (Lane 1) and after (Lane 2) Rsa I digestion. cDNA was synthesized as described in the protocol using the human skeletal muscle control poly A^+ RNA included in the kit. Lane M: DNA size markers.

4. Mix well by vortexing and briefly centrifuging.
5. Aliquot 22 µl of Master Mix into each of the reactions from step 2.
6. Mix well by vortexing and briefly centrifuging.
7. Overlay with 50 µl of mineral oil.
8. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)

   Note: This step "fills in" the missing strand of the adaptors (see Figure 2), thus creating binding sites for the PCR primers.
9. Immediately commence thermal cycling:

GeneAmp® DNA Thermal Cycler 480:	GeneAmp® PCR Systems 2400 or 9600:
20 cycles:	• 94°C 30 sec
• 94°C 30 sec	20 cycles:
• 65°C 30 sec	• 94°C 10 sec
• 68°C 2.5 min	• 65°C 30 sec
	• 68°C 2.5 min

V. Analysis of Results & Troubleshooting Guide contin-

10. Analyze 5 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TAE buffer.

Typical results are shown in Figure 5. If you cannot detect a product after 20 cycles, perform 5 additional cycles, and analyze by gel electrophoresis. Additional PCR cycles may be necessary as G3PDH expression varies among tissues—its abundance in skeletal muscle is relatively high. As shown in Figure 5, the PCR product using one gene-specific primer (G3PDH 3' Primer) and PCR Primer 1 should be about the same intensity as the PCR product amplified using two gene-specific primers (G3PDH 3' and 5' Primers). If the band intensity for these PCR products differs by more than 4-fold, your ligation was less than 25% complete and will significantly reduce subtraction efficiency.

If you are working with mouse or rat cDNA, the PCR product amplified using the G3PDH 3' Primer and PCR Primer 1 will be ~1.2 kb instead of 0.75 kb for human cDNA (rat and mouse G3PDH cDNAs lack an Rsa I restriction site). However, if you are working with human cDNA (which does contain the Rsa I site), and you observe this 1.2-kb band along with a band of the expected size, your cDNA is not fully digested. If there is a significant amount of this undigested product, you should repeat the Rsa I digestion.

If the above analysis shows poor ligation efficiency, it is likely that either your cDNA was contaminated by undesired salt during a precipitation step, or second-strand synthesis was inefficient. Therefore, we recommend that you repeat the PCR-Select procedure starting with First-Strand cDNA Synthesis (Section IV.C).

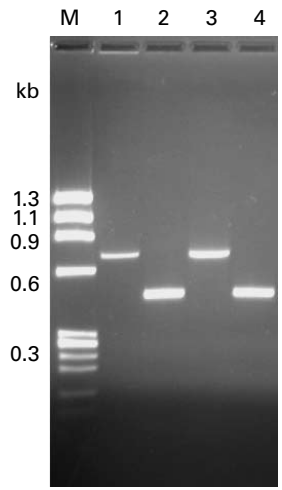


Figure 5. Typical results of ligation efficiency analysis. The results shown here are for human samples; if you are working with mouse or rat samples, the PCR product amplified using the G3PDH 3' Primer and PCR Primer 1 (Lane 3) will be ~1.2 kb instead of 0.75 kb. Lane 1: PCR products using Tester 1-1 (Adaptor 1-ligated) as the template and the G3PDH 3' Primer and PCR Primer 1. Lane 2: PCR products using Tester 1-1 (Adaptor 1-ligated) as the template, and the G3PDH 3' and 5' Primers. Lane 3: PCR products using Tester 1-2 (Adaptor 2R-ligated) as the template, and the G3PDH 3' Primer and PCR Primer 1. Lane 4: PCR products using Tester 1-2 (Adaptor 2R-ligated) as the template, and the G3PDH 3' and 5' Primers. 2% agarose/EtBr gel. Lane M: ϕX174 DNA/Hae III digest size markers.

V. Analysis of Results & Troubleshooting Guide contin-

Alternatively, if you have an insufficient quantity of RNA to resynthesize cDNA, you can reprecipitate the remaining aliquots of each Rsa I-digested experimental and control cDNA (you should have 4.5 µl remaining from Step IV.E.21). Add 2.5 µl of 4 M NH_4OAc and 20 µl of 95% ethanol to each cDNA sample and follow the procedure of Section IV.E from Steps 14–21 before repeating the adaptor ligation procedure. We **do not** recommend reprecipitation as a primary troubleshooting solution for adaptor ligation failure because the recovery of cDNA may be inefficient, resulting in a low subtraction efficiency.

D. Analysis of PCR Products

1. Agarose/EtBr gel electrophoresis of primary PCR

   Perform your primary PCR side-by-side with the PCR control subtracted cDNA. With the PCR control subtracted cDNA, the major bands appearing after 27 cycles should correspond to the ϕX174/Hae III fragments. This result should look similar to the skeletal muscle subtraction you performed; however, the correct ϕX174/Hae III bands may appear only after secondary PCR. The experimental primary PCR subtraction products usually appear as a smear from 0.2–2 kb, with or without some distinct bands.

   a. If you cannot see any products after 27 cycles, use 3 additional cycles, and analyze by gel electrophoresis.

   b. If you cannot detect PCR products in the subtracted or unsubtracted (unsubtracted tester control 1-c) samples nor PCR control subtracted mixture, you must verify that your polymerase is working. If the problem is not with your polymerase mix, try optimizing the PCR cycling parameters in Step IV.I.7 by decreasing the annealing and extension temperature in **small** increments—each degree lower can dramatically increase the background. A starting point is to reduce the annealing temperature from 66°C to 64°C and the extension temperature from 72°C to 71°C.

   c. If you can detect PCR products in the unsubtracted (unsubtracted tester control 1-c) samples, but not in the subtracted sample, perform additional cycles of secondary PCR.

2. Agarose/EtBr gel analysis of secondary PCR

   The patterns of secondary PCR products from the PCR Control Subtracted cDNA and from your skeletal muscle subtraction

V. Analysis of Results & Troubleshooting Guide contin-

should resemble Figure 6. A few additional bands may appear. The experimental subtracted samples usually appear as smears with or without a number of distinct bands.

a. If you do not observe ϕX174/Hae III bands in the PCR control subtracted mixture, you must optimize the PCR conditions.

b. If you can clearly see ϕX174/Hae III bands in your unsubtracted skeletal muscle control, it is likely that either the yield of your cDNA synthesis is very low, or you lost a majority of your skeletal muscle cDNA during phenol:chloroform extraction or ethanol precipitation.

c. If you obtain the expected ϕX174/Hae III bands in the PCR control subtracted mixture, but not in your skeletal muscle control subtraction (e.g., smear or random size bands), it is likely that your subtraction was not optimal. One of the most likely explanations is poor ligation efficiency. For troubleshooting, see Section V.C.

d. If the ligation efficiency is high, verify your protocol and repeat the subtraction. Problems during hybridization are uncommon.

e. If you cannot see a product after 12 cycles, use 3 additional cycles, and analyze by gel electrophoresis. Add cycles sparingly as too many cycles can increase background.

f. In a successful subtraction, the banding pattern of your unsubtracted cDNA ligated with both adaptors (unsubtracted tester control 1-c) should be different from the banding pattern of your experimental subtracted DNA samples (Figure 6).

E. PCR Analysis of Subtraction Efficiency

Either PCR (this Section) or hybridization analysis (Section V.F) can be used to estimate the efficiency of subtraction by comparing the abundance of known cDNAs before and after subtraction. Ideally this is done with both a non-differentially expressed gene (e.g., a housekeeping gene), and with a gene known to be differentially expressed between the two RNA sources being compared. PCR provides a quicker test than hybridization analysis.

The test described below uses the G3PDH primers provided with the kit to confirm the reduced relative abundance of G3PDH following the PCR-Select procedure. Note that these G3PDH primers can only be used for human, mouse, and rat genes. For other species, you will need to design suitable primers.

Not all housekeeping gene transcripts are subtracted evenly. Although G3PDH is subtracted very efficiently in most tissues and cell lines, there are some exceptions, including skeletal muscle and heart. For this reason, we do not recommend the use of G3PDH abundance

V. Analysis of Results & Troubleshooting Guide contin-

to analyze subtraction in the skeletal muscle control. Most other housekeeping genes are subtracted very efficiently from skeletal muscle and can therefore be used. In general, if the abundance

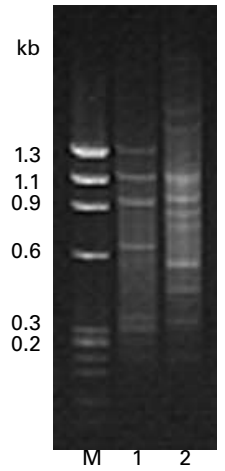


Figure 6. Typical results of control skeletal muscle subtraction analysis. The secondary PCR product of the subtracted skeletal muscle sample contains mostly DNA fragments corresponding to the ϕX174/Hae III digest. The adaptor sequences on both ends of DNA fragments cause the mobility shift of these PCR products in comparison with original, digested ϕX174 DNA. Lane M: ϕX174 DNA/Hae III digest size markers. Lane 1: Secondary PCR products of subtracted skeletal muscle tester cDNA with 0.2% ϕX174/Hae III-digested DNA. Lane 2: Secondary PCR products of unsubtracted skeletal muscle tester cDNA ligated with both Adaptors 1 and 2R (generated in Section IV.F) and containing 0.2% ϕX174/Hae III-digested DNA. Samples are electrophoresed on a 2% agarose/EtBr gel.

of G3PDH did not decrease significantly after subtraction, you should check the abundance of other housekeeping genes (such as α-tubulin). However, in the control skeletal muscle subtraction experiment, the agarose gel banding pattern of the ϕX174/Hae III digest (Figure 6, Lane 2) has already indicated whether or not subtraction was successful.

In certain instances a particular housekeeping gene is present at different levels in tester and driver poly A^+ RNA. If the concentration of G3PDH message is even 2-fold higher in the tester sample, G3PDH will not be efficiently subtracted out. If you perform subtraction in both directions (see Section IV.F) and have unsubtracted tester control for both the subtraction and the reverse subtraction, the PCR analysis of subtraction efficiency will indicate if there is any difference in G3PDH abundance in the two cDNA samples being compared. If this is the case, choose another housekeeping gene as a control for subtraction efficiency.

Clontech offers a number of RT-PCR Control Amplimer Sets for housekeeping genes that can be used as positive controls for your studies. These include human, mouse, and rat Control Amplimer Sets for ß-actin and G3PDH (see Related Products).

1. Dilute the subtracted and unsubtracted (unsubtracted tester control 1-c and 2-c) secondary PCR products 10-fold in H_2O. The

V. Analysis of Results & Troubleshooting Guide contin-

concentration of subtracted and unsubtracted product should be roughly equal.

2. Combine the following reagents in 0.5-ml microcentrifuge tubes in the order shown:

	Rxn 1	Rxn 2
Diluted subtracted cDNA (2° PCR product)	1.0 µl	–
Diluted Unsubtracted tester control 1-c (2° PCR product)	–	1.0 µl
G3PDH 3' Primer (10 µM)	1.2 µl	1.2 µl
G3PDH 5' Primer (10 µM)	1.2 µl	1.2 µl
sterile H_2O	22.4 µl	22.4 µl
10X PCR reaction buffer	3.0 µl	3.0 µl
dNTP Mix (10 mM)	0.6 µl	0.6 µl
50X Advantage cDNA Polymerase Mix	0.6 µl	0.6 µl
Total volume	30.0 µl	30.0 µl

3. Mix by vortexing and briefly centrifuging.
4. Overlay with one drop of mineral oil.
5. Use the following thermal cycling program:

   18 cycles:

   - 94°C 30 sec
   - 60°C 30 sec
   - 68°C 2 min

6. Remove 5 µl from each reaction and place it in a clean tube. Put the rest of the reaction back into the thermal cycler for 5 additional cycles.
7. Repeat step 6 twice (i.e., remove 5 µl after 28 and 33 cycles).
8. Examine the 5 µl samples (i.e., the aliquots that were removed from each reaction after 18, 23, 28, and 33 cycles) on a 2.0% agarose/EtBr gel.

Figure 7 shows an example of G3PDH reduction in a successfully subtracted mixture. For the unsubtracted cDNA, you should see a G3PDH product after 18–23 cycles, depending on the abundance of G3PDH in the particular cDNA. For reference, in skeletal muscle and heart poly A^+ RNA, G3PDH is extremely abundant. With your subtracted samples, you should detect a product at ~5–15 cycles later.

If you do not observe a decrease in G3PDH abundance in the subtracted sample, repeat the PCR amplification (Section IV.I). Perform two independent primary and secondary PCRs and check G3PDH abundance in both. During the first hybridization step of the subtraction procedure, equalization of the ss tester molecules takes place. As a result, a very low concentration of cDNA molecules with different adaptors on each

V. Analysis of Results & Troubleshooting Guide contin-

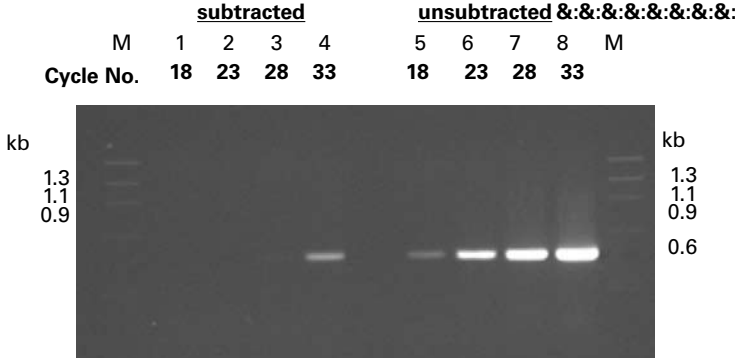


Figure 7. Reduction of G3PDH abundance by PCR-Select subtraction. Tester cDNA was prepared from human testis poly A$^+$ RNA. Driver cDNA was prepared from a mixture of poly A$^+$ RNA samples prepared from 10 different human tissues. PCR was performed on the subtracted (Lanes 1–4) or unsubtracted (Lanes 5–8) secondary PCR product with the G3PDH 5' and 3' primers included in the kit. Lanes 1 & 5: 18 cycles; Lanes 2 & 6: 23 cycles; Lanes 3 & 7: 28 cycles; Lanes 4 & 8: 33 cycles. Lane M: marker.

end forms during the second hybridization (see Introduction & Figure 2); only about 10,000 of these target molecules are present in 1 µl of diluted subtracted cDNA. If one such molecule representing G3PDH mRNA happens to be present, G3PDH abundance will not appear to decrease. If you see reduction of G3PDH abundance in the independent PCR-amplified subtracted cDNA, it indicates that this random event did occur.

As a positive control for the enrichment of differentially expressed genes, repeat the procedure above using PCR primers for a gene known to be expressed in the tester RNA, but not in the driver RNA. This cDNA should become enriched during subtraction. The difference in the number of cycles required for equal amplification of the corresponding PCR product in subtracted and unsubtracted samples indicates the efficiency of your subtraction. 5 cycles corresponds roughly to a 20-fold cDNA enrichment. Because of the equalization that occurs during subtraction, the level of enrichment will depend on the initial abundance of each differentially expressed gene, as well as the difference in abundance of each gene in tester and driver. Differentially expressed genes that are present in low abundance in the tester cDNA will be enriched more than differentially expressed genes that are present in high abundance.

Notes:

- Do not use PCR primers that amplify a cDNA fragment that contains an Rsa I restriction site between the PCR priming sites.
- Certain sequences may not rehybridize or be amplified, while other cDNA fragments from the same cDNAs are dramatically enriched (Hubank & Schatz, 1994; Wang & Brown, 1991). Hybridization analysis may provide more information about some cDNA fragments that appear to be lost.

F. Hybridization Analysis of Subtraction Efficiency

Dot or Southern blot analysis of subtracted and unsubtracted (unsubtracted tester control 1-c) secondary PCR products (with different genes as probes) is helpful in evaluating the success of your subtraction. As shown in Figure 8, the abundance of housekeeping cDNAs drops after subtraction, while the abundance of known, up-regulated cDNAs rises.

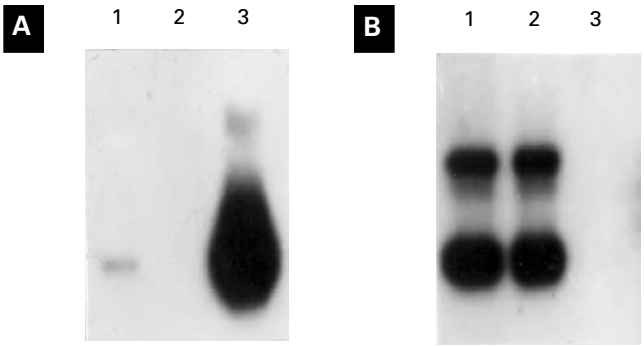


Figure 8. Enrichment of a differentially expressed gene and reduction of an abundant housekeeping gene in Jurkat cells. Tester cDNA was prepared from human Jurkat cells that were incubated with 2 µg/ml phytohemagglutinin (PHA) and 2 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72 hr. Driver cDNA was prepared from untreated cells.15 µl of the secondary PCR products generated from unsubtracted tester cDNA, unsubtracted driver cDNA, and subtracted cDNA were electrophoresed on a 1.5% agarose gel (0.3 µg per lane), transferred to nylon filters, and hybridized with either an IL-2R probe (Panel A) or a G3PDH probe (Panel B). Lane 1: unsubtracted tester cDNA. Lane 2: unsubtracted driver cDNA. Lane 3: subtracted cDNA.(Gurskaya et al., 1996)

V. Analysis of Results & Troubleshooting Guide *continued*

Although Southern blot analysis is a sensitive indicator of subtraction efficiency, you may occasionally observe background bands of unpredicted sizes. Two criteria should be applied to distinguish background from "true bands" (i.e., bands that truly represent a subtracted gene).

First, the intensity of true bands should increase proportionally to the number of PCR cycles used during subtraction. Occasionally, very intense bands will appear only in a fraction that was subjected to a high number of cycles (i.e., > 30 primary cycles of amplification). Such bands should be considered background. In addition, a band sometimes may appear in one fraction, but not in others. These bands may be due to contamination, or result from a random event that occurred during that particular PCR because of the very low concentration of target molecules present after subtractive hybridization.

The second criterion for true bands is that they should be amplified by both sets of primers (primary and nested). Since the products resulting from PCR with the nested primers are slightly smaller than those produced by the flanking primers, true bands undergo a slight downward shift in molecular weight after secondary PCR amplification.

Figure 9 shows an example of the type of background that may be observed on Southern blots. For this experiment, PCR-Select cDNA subtraction was performed with poly A^+ RNA from stimulated and unstimulated T-cell hybridomas (i.e., with and without treatment with an antibody for the T-cell receptor). The efficiency of subtraction was assessed by Southern blot hybridization to a probe for G3PDH and α-tubulin. (These data were kindly provided by Wong et al. at Rockefeller University. For details see the article in the July 1996 issue of Clontechniques.)

In the Southern blot probed with G3PDH (Panel A), band A is considered to be a true band because it meets both criteria: its intensity increased in proportion to the number of PCR cycles, and its molecular weight shifted slightly downward after secondary PCR. In contrast, the other bands can be attributed to background because they do not meet the criteria. The three bands (B) in the subtracted fraction probably represent PCR products that were highly enriched in the subtracted library and cross-hybridized with the G3PDH probe simply because of their abundance. The probe also cross-hybridized with the molecular weight marker (C) which contains no G3PDH DNA but is also present in excess. These bands may also result from partial homology of the PCR primers to gene-specific sequences.

In contrast, the Southern blot probed with α-tubulin (Panel B) displays very little background; all of these bands are most likely genuine results. As evident from both blots, exceeding 30 primary PCR cycles and 14 secondary cycles dramatically increases background.

V. Analysis of Results & Troubleshooting Guide *continued*

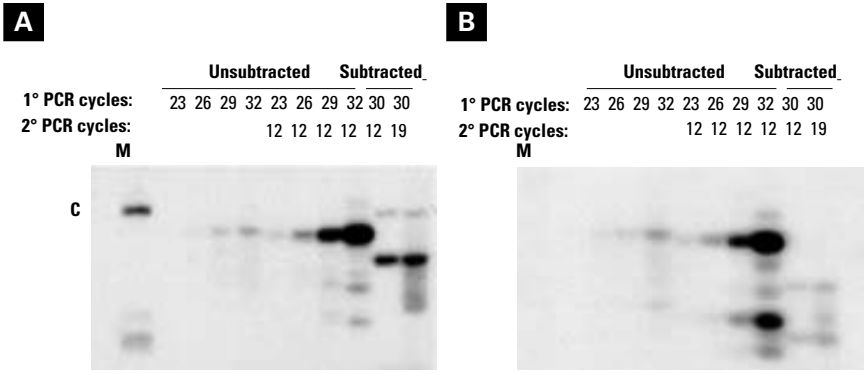


Figure 9. Southern blot analysis of PCR-Select subtraction. Tester cDNA was prepared fromT-cell hybridomas (KMls-8.3.5.1) that were treated with anti-T-cell-receptor antibody. Driver cDNA was prepared from untreated cells. PCR products were run on a 2.0% agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes for housekeeping genes. The number of cycles used for 1° and 2° PCR amplification is indicated. M = DNA size markers. **Panel A.** Southern blot hybridized with a G3PDH probe. This blot is an example of high background with a significant amount of bands corresponding to background. **A** designates "true bands"; while **B** and **C** are bands resulting from two different types of background. **Panel B.** Southern blot hybridized with an α-tubulin probe. Unlike the blot in Panel A, this blot exhibits clean bands with much lower background.

VI. Differential Screening of the Subtracted Library

In most cases, the PCR-Select cDNA Subtraction Kit method greatly enriches for differentially expressed genes; nevertheless, the subtracted sample will still contain some cDNAs that correspond to mRNAs common to both the tester and driver samples. Although this background may depend somewhat on the quality of RNA purification and performance of the particular subtraction, it chiefly arises when very few mRNA species are differentially expressed in tester and driver. In general, a limited set of differentially expressed mRNAs and low quantitative difference in expression produces higher background—even if you obtain sufficient enrichment of differentially expressed cDNAs. With high background, picking random clones from the subtracted library for Northern blot analysis is extremely time-consuming and inefficient. We highly recommend that you perform differential screening before embarking on Northern blot analysis.

There are two approaches for differentially screening the subtracted library. The first is to hybridize the subtracted library with ^{32}P-labeled probes synthesized as first-strand cDNA from tester and driver (Hedrick et al., 1984; Sakaguchi et al., 1986). Clones corresponding to differentially expressed mRNAs will hybridize only with the tester probe, and not with the driver probe. Although this approach is widely used, it has one major disadvantage: only cDNA molecules corresponding to highly abundant mRNAs (i.e., mRNAs which constitute more than about 0.2% of the total cDNA in the probe) will produce detectable hybridization signals (Wang & Brown, 1991). Clones corresponding to low-abundance differentially expressed mRNAs will not be detected by this screening procedure.

The second approach bypasses the problem of losing low-abundance sequences. In this method, the subtracted library is hybridized with forward- and reverse-subtracted cDNA probes (Lukyanov et al., 1996; Wang & Brown, 1991). To make the reverse-subtracted probe, subtractive hybridization is performed with the original tester cDNA as a driver and the driver cDNA as a tester. Clones representing mRNAs that are truly differentially expressed will hybridize only with the forward-subtracted probe; clones that hybridize with the reverse-subtracted probe may be considered background. This approach requires one additional step: before they can be used as probes, the forward- and reverse-subtracted probes must undergo restriction enzyme digestion to remove the adaptor sequences. Despite their small size, these adaptors cause a very high background when the subtracted probes are hybridized to the subtracted cDNA library.

Our PCR-Select Differential Screening Kit (Cat. No. 637403) contains everything needed to generate subtracted and unsubtracted cDNA probes to screen your subtracted cDNA library. This ensures a good ratio of signal to background in your Northern analyses. For more details, please see the PCR-Select Differential Screening Kit User Manual (PT3831-1) and our website at **www.clontech.com**.

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Specific References for PCR-Select Technology

Suppression subtractive hybridization—the technology that all of our PCR-Select kits are built upon—was originally described by Diatchenko et al. (1996). The following selected articles—found within the above references list—contain published experimental data obtained using PCR-Select™ technology.

Cao et al. (2004)
Jeong et al. (2004)
Knaup et al. (2004)
Lian et al. (2004)
Norton et al. (2004)
Pan et al. (2004)

For a more comprehensive listing of PCR-Select citations, please visit our web site at:

http://www.clontech.com/clontech/citations/index.shtml

Appendix A: Suppression PCR

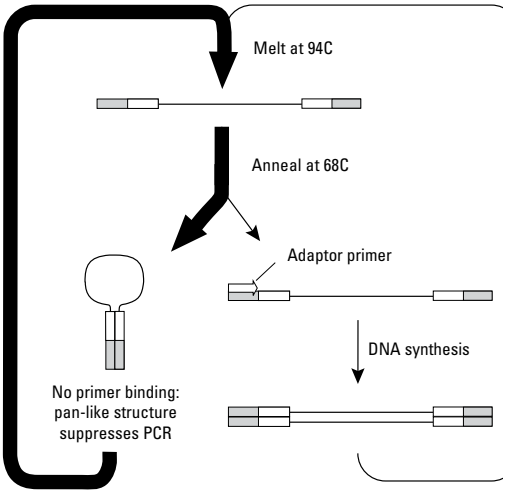
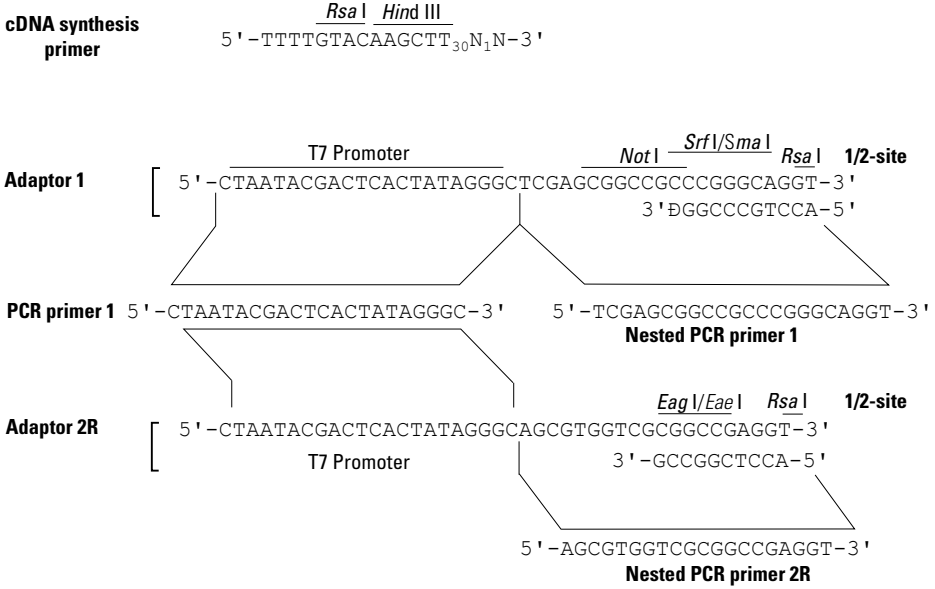


Figure 10. Suppression PCR.

The PCR-Select cDNA adaptors are engineered to prevent undesirable amplification during PCR by means of a method called **suppression PCR** (U.S. Patent No. 5,565,340; Siebert et al., 1995). Suppression occurs when complementary sequences are present on each end of a single-stranded cDNA. During each primer annealing step, the hybridization kinetics strongly favor (over annealing of the shorter primers) the formation of a pan-like secondary structure that prevents primer annealing. Occasionally when a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-like structure.Thus during PCR, nonspecific amplification is efficiently suppressed, and specific amplification of cDNA molecules with different adaptors at both ends can proceed normally.

The 5' ends of Adaptors 1 and 2R have an identical stretch of 22 nt. Primary PCR therefore requires only one primer for amplification, eliminating the problem of primer dimerization (Lukyanov et al., 1995). Furthermore, the identical sequences on the 3' and 5' ends of the differentially expressed molecules introduces a slight suppression PCR effect. Since these identical sequences are the same length as PCR Primer 1, the suppression effect becomes significant only for very short cDNAs (under 200 nt), because the formation of pan structures for shorter molecules is more efficient (Lukyanov et al., 1995). Thus, longer molecules are preferentially enriched, balancing the inherent tendency of the subtraction procedure to favor short cDNA fragments. These shorter fragments are more efficiently hybridized, amplified, and cloned than longer fragments.

Appendix B: Adaptor and Primer Sequences



Control Primers:

G3PDH 5' Primer	5'-ACCACAGTCCATGCCATCAC-3'
G3PDH 3' Primer	5'-TCCACCACCCTGTTGCTGTA-3'

Figure 11. Sequences of the PCR-Select cDNA synthesis primer, adaptors, PCR primers, and Control Primers. When Adaptors 1 and 2R are ligated to Rsa I-digested cDNA, the Rsa I site is restored.

Notes

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