User Manual

SMARTer™ PCR cDNA Synthesis Kit User Manual

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I. List of Components

SMARTer PCR cDNA Synthesis Kit

Cat. No. 634925 10 rxns	Cat. No. 634926 20 rxns	
Box 1		
• 10 µl	20 µl	**SMARTer II A Oligonucleotide** (12 µM) 5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3' Rsa I (X = undisclosed base in the proprietary SMARTer oligo sequence)
• 5 µl	5 µl	**Control Mouse Liver Total RNA** (1 µg/µl)
Box 2		
• 10 µl	20 µl	**3′ SMART CDS Primer II A** (12 µM) 5′–AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N–3′ Rsa I (N = A, C, G, or T; N$_{-1}$ = A, G, or C)
• 200 µl	400 µl	**5′ PCR Primer II A** (12 µM)
• 40 µl	80 µl	**5X First-Strand Buffer (RNase-Free)** 250 mM Tris-HCl (pH 8.3) 375 mM KCl 30 mM $MgCl_2$
• 100 µl	200 µl	**dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
• 50 µl	50 µl	**Dithiothreitol** (DTT; 100 mM)
• 10 µl	10 µl	**RNase Inhibitor** (40 U/µl)
• 12 µl	25 µl	**SMARTScribe™ Reverse Transcriptase** (100 U/µl)
• 1 ml	1 ml	**Deionized H_2O**
Box 3		
• 10	20	**CHROMA SPIN™+TE-1000 Columns**

Storage Conditions

- Store Control Mouse Liver Total RNA and SMARTer II A Oligonucleotide at –70°C.
- Store the CHROMA SPIN +TE-1000 Columns at room temperature.
- Store all other reagents at –20°C.

Licensing Information

For important information about the use of SMART technology, please see the Notice to Purchaser at the end of this user manual.

II. Additional Materials Required

The following reagents are required but not supplied:

- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
  We strongly recommend use of the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) for PCR amplification. This kit includes the Advantage 2 Polymerase Mix, which has been specially formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). The Advantage 2 Polymerase Mix is formulated to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).
- **NucleoSpin RNA II Kit** (Cat. Nos. 740955.10, 740955.20, 740955.50 & 740955.250)
  We strongly recommend the use of the NucleoSpin RNA II Kit for RNA purification. We have found that cells or tissues frozen in RA1 buffer are better preserved, resulting in the isolation of higher quality RNA. The RA1 buffer, which contains guanidinium isothiocyanate, protects cellular RNA by inactivating RNases. The higher quality of RNA reduces the number of cycles required to reach the optimal SMARTer cDNA amplification level, resulting in a more representative cDNA pool. Additional Buffer RA1 (Cat. No. 740961) can be purchased separately.
- **NucleoSpin Extract II** (Cat. Nos. 740609.10, 740609.50 & 740609.250)
  We strongly recommend use of NucleoSpin Extract II for PCR product purification.
- **Easy Dilution Solution** (Cat. No. TAK 9160)
  We recommend Takara's Easy Dilution Solution for RNA template dilution, Clontech Cat. No. TAK 9160.
- **TE buffer** (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA)
- **ß-mercaptoethanol** (Sigma Cat. No. M6250)
- **DNA size markers** (1 kb DNA ladder)
- **50X TAE electrophoresis buffer**:

242.0 g	Tris base
57.1 ml	glacial acetic acid
37.2 g	$Na_2EDTA•2H_2O$
x ml	Add H_2O to 1 L

NOTE: If you plan to use the SMARTer method to generate cDNA for use with PCR-Select, please see Appendix A for specific materials required for that application.

III. Introduction & Protocol Overview

The **SMARTer PCR cDNA Synthesis Kit** provides a PCR-based method for producing high-quality cDNA from nanogram quantities of total RNA.The SMARTer PCR cDNA Synthesis Kit is an improved version of our original SMART™ PCR cDNA Synthesis Kit, with a new, SMARTer oligo and **SMARTScribe Reverse Transcriptase** included; it provides higher specificity, lower background and increased yield. This kit allows you to synthesize high-quality cDNA for array probe generation, cDNA subtraction, "Virtual Northern" blots, cDNA sequencing or other applications, from as little as 2 ng of total RNA.The cornerstone of SMARTer cDNA synthesis is SMART (Switching Mechanism At 5′ End of RNA Transcript) technology. SMART technology is especially useful for researchers who have limited starting material, such as RNA derived from laser-capture microscopy samples, cells sorted by flow cytometry, or other extremely small samples.

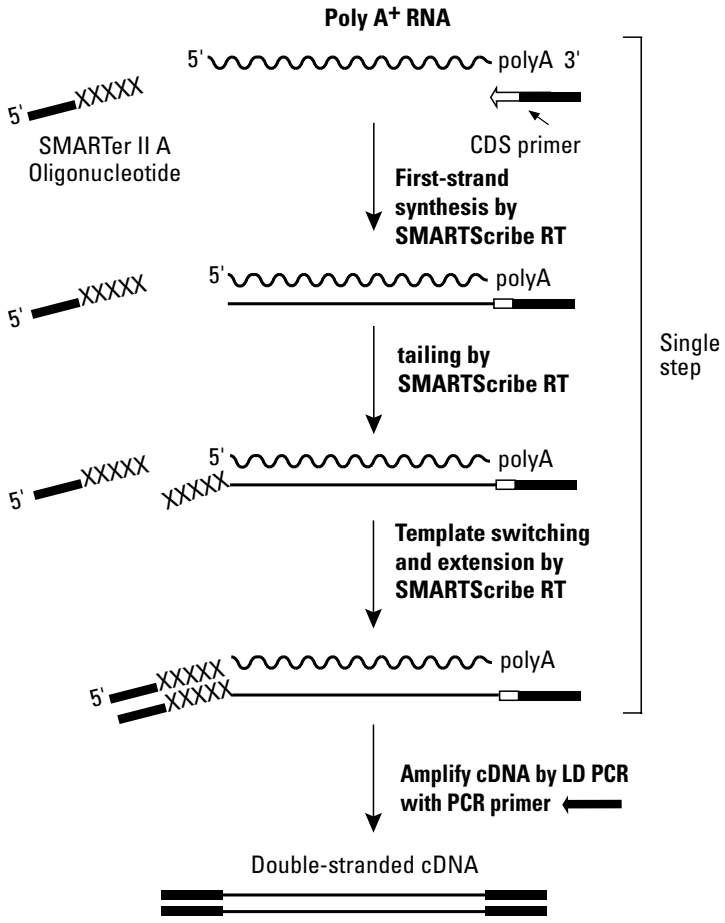


Figure 1. Flowchart of SMARTer cDNA synthesis. The SMARTer II A Oligonucleotide, 3′ SMART CDS Primer II A, and 5′ PCR Primer II A all contain a stretch of identical sequence (see Section I for sequence information).

SMARTer cDNA Synthesis

All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded (ss) DNA in the first-strand reaction. However, because RT cannot always transcribe the entire mRNA sequence, the 5′ ends of genes tend to be underrepresented in cDNA populations. In the absence of RNA degradation, truncated cDNA molecules present in libraries are often due to the tendency of RT to pause before transcription is complete. In contrast, the SMARTer method is able to preferentially enrich for full-length cDNAs.

SMARTer cDNA synthesis starts with nanogram amounts of total RNA. A modified oligo(dT) primer (the 3′ SMART CDS Primer II A) primes the first-strand synthesis reaction (Figure 1). When SMARTScribe RT reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3′ end of the cDNA. The SMARTer Oligonucleotide (patent pending) base-pairs with the non-template

III. Introduction & Protocol Overview, *continued*

nucleotide stretch, creating an extended template. SMARTScribe RT then switches templates and continues replicating to the end of the oligonucleotide (Chenchik *et al.*, 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5′ end of the mRNA, as well as sequences that are complementary to the SMARTer Oligonucleotide. In cases where the RT pauses before the end of the template, the addition of nucleotides is much less efficient than with full-length cDNA-RNA hybrids, thus the overhang needed for base-pairing with the SMARTer Oligonucleotide is absent. The SMARTer anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences, such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly A^- RNA, will not be exponentially amplified. However, truncated RNAs that are present in poor quality RNA starting material will be amplified, and will contaminate the final cDNA library.

Downstream Applications of Synthesized SMARTer cDNA

- **SMARTer cDNA Synthesis for Subtractive Hybridization**

  The **PCR-Select™ cDNA Subtraction Kit** (Cat. No. 637401) provides a powerful method for identifying differentially expressed genes by subtractive hybridization (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996). Subtractive hybridization enables the comparison of two populations of mRNA, and allows researchers to obtain clones of genes that are expressed in one population, but not in the other. 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 this cDNA is used with the PCR-Select Kit, the excess of ribosomal RNA and low concentration of cDNA corresponding to the poly A^+ fraction results in inefficient subtractive hybridization. However, cDNA generated using the SMARTer PCR cDNA Synthesis Kit can be directly used for PCR-Select subtraction—even when total RNA is used as the starting material. See Appendix A for more information on PCR-Select cDNA Subtraction.

- **SMARTer cDNA Synthesis for Virtual Northern Blots and Probes**

  SMARTer cDNA may also be useful for researchers who wish to analyze transcript size and expression patterns by hybridization but lack sufficient poly A^+ or total RNA for Northern blots. This is especially important for researchers who have isolated clones using the PCR-Select Kit and who also need to confirm the differential expression of corresponding mRNAs. "Virtual Northern" blots can be generated using SMARTer cDNA instead of total or poly A^+ RNA (Endege *et al.*, 1999), and can give information similar to that provided by standard Northern blots. See Appendix B for more information on Virtual Northern blots.

- **SMARTer cDNA Synthesis for Array Probes**

  SMART cDNA amplification has been widely used in microarray experiments (Ohtsu *et al.*, 2008; Nygaard *et al.*, 2006; Wilhelm *et al.*, 2006). SMART technology enables researchers to synthesize highly sensitive array probes from minimal starting material (Gonzalez *et al.*, 1999; Livesey *et al.*, 2000). Using the SMARTer method, probes made from small amounts of total RNA produce results that are comparable to those from pure poly A^+ RNA—a clear advantage when only limited amounts of tissues or cells are available.

- **SMARTer cDNA Synthesis for Next-Generation Sequencing**

  SMARTer cDNA synthesis has proven very useful for generating samples for various next-generation sequencing platforms (Cheung *et al.*, 2006; Morin *et al.*, 2008; Andreas *et al.*, 2007). The **SMARTer Ultra Low RNA Kit for Illumina® Sequencing** provides a simple and efficient solution for generating libraries from total RNA that are compatible with Illumina's Genome Analyzer, HiScanSQ™, and HiSeq™ instruments, facilitating transcriptome analysis from as little as 100 pg of input RNA. The integration of Clontech's SMART technology with Illumina sequencing has resulted in the most sensitive sample preparation workflow offered by any next-generation sequencing (NGS) platform. The combination of SMART technology's ability to handle very small quantities of RNA and the Illumina sequencing platform's capacity for single- and paired-end sequencing of millions to billions of long and short reads per

III. Introduction & Protocol Overview, *continued*

run, allows you to annotate coding SNPs, discover transcript isoforms, characterize splice junctions, and determine the relative abundance of transcripts from even the smallest samples.

- **SMARTer cDNA Synthesis for Rapid Amplification of cDNA Ends (RACE)**

  The SMARTer cDNA synthesis method is also optimized for rapid amplification of cDNA ends (RACE; Matz *et al.*, 1999). The **SMARTer RACE cDNA Amplification Kits** (Cat. Nos. 634923 & 634924), allow researchers to synthesize first-strand cDNA, and facilitate both 5′ and 3′ RACE using either poly A$^+$ RNA or total RNA.

Clontech Also Offers SMART cDNA Library Construction Kits

Clontech offers a number of kits that feature SMART technology. The **SMART cDNA Library Construction Kit** (Cat. No. 634901) includes the components for **directional cloning of full-length cDNA**. Please note that cDNA generated using the SMART cDNA Library Construction Kit **cannot be used for PCR-Select cDNA subtraction**. Clontech also offers another SMART library construction kit, the **In-Fusion® SMARTer Directional cDNA Library Construction Kit** (Cat. No. 634929), that allows creation of cDNA libraries in any vector starting from as little as 2 ng of total RNA. There are also several vectors sold separately that can be used with the SMART cDNA Library Construction Kit or the In-Fusion SMARTer Directional cDNA Library Construction Kit. These include the mammalian expression vector **pEXP-Lib** (Cat. No. 635003), and the retroviral expression vector **pRetro-Lib** (Cat. No. 635002).

IV. RNA Preparation & Handling

A. General Precautions

The integrity and purity of your total or poly A$^+$ RNA starting material is an important element in high-quality cDNA synthesis. The following precautions will help you avoid contamination and degradation of your RNA:

- Wear gloves throughout the procedure to protect your RNA samples from degradation by nucleases.
- Use freshly deionized (e.g., MilliQ-grade) H_2O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H_2O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips.
- Ethidium bromide is a carcinogen. Use appropriate precautions when handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manual* by Sambrook & Russell (2001).

B. RNA Isolation

Clontech offers several kits for isolating total or poly A$^+$ RNA from a variety of sources. The **NucleoBond RNA/DNA Kit** contains AX-R tips to isolate total RNA from tissue or cells without using phenol or chloroform. With the **NucleoSpin RNA II Kit**, you can isolate highly pure total RNA from cells, tissues, or cell-free biological fluids without phenol chloroform extractions. The **NucleoTrap mRNA Mini Kit** combines a spin-column filter with oligo(dT)-latex bead technology to isolate high-quality mRNA from total RNA in less than 30 minutes. For more RNA isolation kits, visit the Clontech website at **www.clontech.com**. Many procedures are available for the isolation of poly A$^+$ RNA (Farrell, 1993; Sambrook *et al.*, 1989).

IV. RNA Preparation & Handling, *continued*

C. RNA Purity

The purity of RNA is the key factor for successful cDNA synthesis and SMARTer cDNA Amplification. The presence of residual organics, metal ions, salt or nucleases in your RNA sample could have a large impact on downstream applications by inhibiting enzymatic activity or degrading the RNA. We strongly recommend checking the stability of your RNA to ensure that it is free of contaminants.

To test the stability of your RNA, incubate a small portion of it at 37°C for 2 hours, then compare the sample to a duplicate control stored at –70°C. If the sample incubated at 37°C shows a lower 28S:18S ratio than the control or the RNA shows a significant downward shift on a formaldehyde agarose gel, the RNA may have nuclease contaminants (see Section IV.D. for methods for assessing RNA quality).

Impurities such as salt or organic contaminants can be removed by repeated ethanol precipitation, subsequent washing with 80% ethanol and the complete removal of all remaining ethanol.

If your RNA template is from a plant or some other species with high pigment levels, please pay special attention to polysaccharide/pigment contamination. Polysaccharides/pigments are hard to remove and can't be detected on the agarose gel. These glycoproteins might interfere with primer binding sites of RNA during the first-strand cDNA synthesis leading to reduced cDNA yield.

D. Assessing the Quality of the RNA Template

Methods for Assessing Total RNA Integrity

1. RNA/cDNA Quality Assay:

   The Clontech **RNA/cDNA Quality Assay Kit** (Cat. No. 636841) directly determines the quality of your human and mouse RNA and cDNA samples using reverse transcription (RT) and PCR. Because this assay uses RT-PCR, it provides a direct functional test of your sample for its ability to produce full-length cDNA for your application. You achieve quick results using standard lab equipment, and avoid inconvenient and toxic formaldehyde gels.

2. Formaldehyde agarose gel visualization with Ethidium Bromide (EtBr):

   The integrity of total RNA can be visually assessed by the ratio of 28S:18S RNA on a denaturing formaldehyde agarose gel by staining with EtBr. The theoretical 28S:18S ratio for eukaryotic RNA is approximately 2:1. For mammalian total RNA, you should observe two bright bands at approximately 4.5 and 1.9 kb; these bands represent 28S and 18S ribosomal RNA. The ratio of intensities of these bands should be 1.5–2.5:1. For more information, see Sambrook & Russell (2001).

3. Formaldehyde agarose gel visualization with SYBR® Green or SYBR Gold:

   One drawback of visualizing RNA with Ethidium Bromide is the amount of sample required. Alternative dyes such as SYBR® Green II or SYBR Gold (Invitrogen, CA) allow you to detect as little as 1 or 2 ng of RNA (using SYBR Gold and SYBR Green II, respectively). These dyes are especially useful if you have a limited amount of RNA.

4. Detection with the Agilent 2100 BioAnalyzer (Agilent Technologies, CA):

   This microfluidics-based technology, which provides an alternative to traditional gel-based analysis, requires only 10 ng of RNA per analysis. In addition to assessing RNA quality, this automated system provides a good estimate of RNA concentration.

Methods for Assessing mRNA Integrity

All of the methods mentioned above can be used to assess the quality of your mRNA. However, because mRNA does not contain strong ribosomal bands, the assessment of its quality will be somewhat subjective. Typically, mRNA appears as a smear between 0.5 kb to 6 kb, with an area of higher intensity around 1.5 and 2 kb. This size distribution may be tissue or species-specific. If the average size of your mRNA is lower than 1.5 kb, it could be an indication of degradation.

V. SMARTer cDNA Synthesis

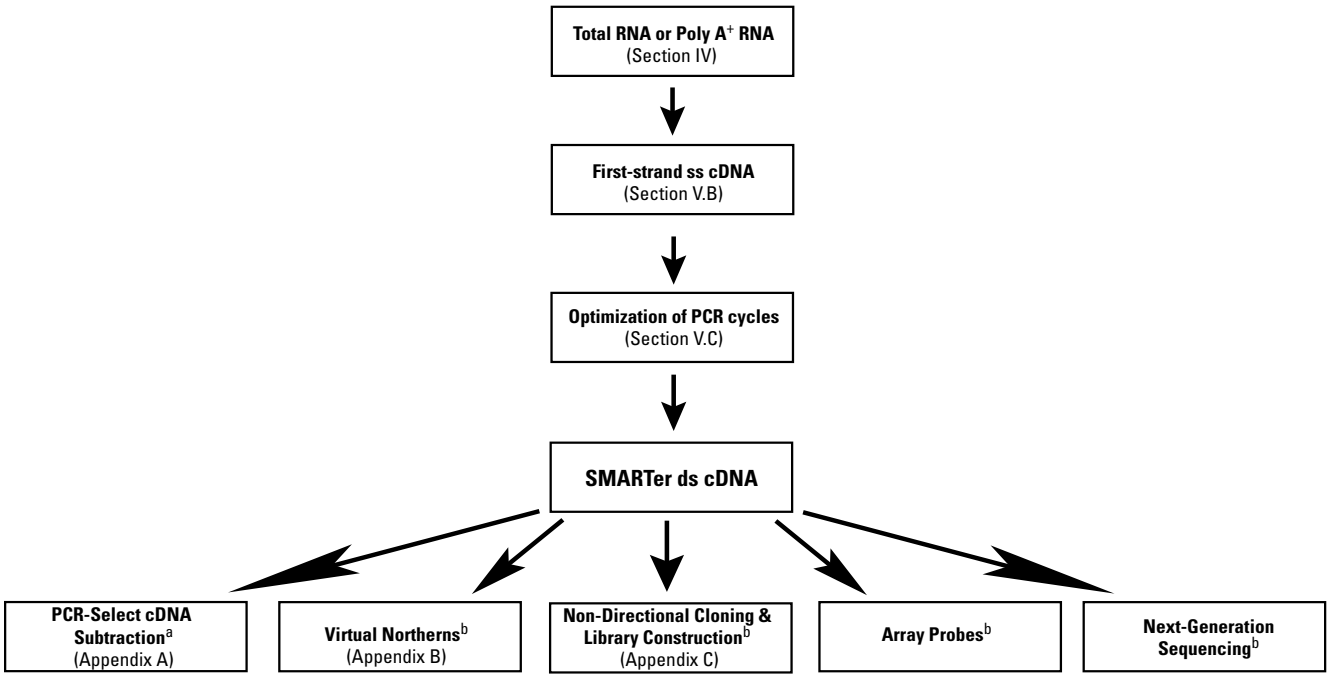
PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Use the following protocol for generating cDNA with Clontech's simple and highly efficient SMARTer technology. This protocol is designed for synthesizing SMARTer cDNA for a variety of applications OTHER THAN LIBRARY CONSTRUCTION.

Attention

IMPORTANT: This protocol is designed for synthesizing SMARTer cDNA for applications ***other than library construction***, such as PCR-Select cDNA Subtraction (See Appendix A) or Virtual Northern Blots (See Appendix B). To synthesize SMART cDNA for library construction, use our **SMART cDNA Library Construction Kit** (Cat. No. 634901) or **In-Fusion SMARTer Directional cDNA Library Construction Kit** (Cat. No. 634933).

If you plan to use cDNA generated by the SMARTer method with our PCR-Select cDNA Subtraction protocol, please refer to the procedure provided in Appendix A before performing first-strand cDNA synthesis. In addition, we recommend reading the User Manual for cDNA Subtraction (PT1117-1). A different RNA control is supplied with Clontech's PCR-Select cDNA Subtraction Kit that should be used to synthesize cDNA according to the PCR-Select User Manual (a non-SMARTer method). In addition, use the control provided in this kit to troubleshoot any problems using the SMARTer protocol. For more information about using these controls, see Appendix A of this User Manual.

If you decide that you want to use your SMARTer cDNA for constructing libraries, please refer to the procedure provided in Appendix C for polishing the ends of SMARTer cDNAs.



[a]Reagents for these procedures are included in the Clontech PCR-Select cDNA Subtraction Kit.

[b]Reagents for these procedures are *not* included in the SMARTer PCR cDNA Synthesis Kit.

Figure 2. Guide to using the SMARTer cDNA synthesis protocol for PCR-Select cDNA Subtraction, Virtual Northerns, Non-Directional Cloning & Library Construction, and other applications.

V. SMARTer cDNA Synthesis, *continued*

A. General Considerations

- We recommend Takara's Easy Dilution Solution (Clontech Cat. No. TAK 9160) for RNA template dilution. Easy Dilution Solution can prevent template from sticking to the tube, and allows correct dilution at low concentration.
- Resuspend pellets and mix reactions by gently pipetting the solution up and down or by tapping the bottom of the tube. Then spin the tube briefly to bring all contents to the bottom.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification protocol and reagents.

B. PROTOCOL: First-Strand cDNA Synthesis

This protocol has been optimized for both total RNA and poly A$^+$ RNA. The minimum amount of starting material for cDNA synthesis is 2 ng of total RNA or 1 ng of poly A$^+$ RNA. However, if your RNA sample is not limiting, we recommend that you start from 1 µg of total RNA or 0.5 µg of poly A$^+$ RNA for cDNA synthesis. Please note that if you are starting from >100 ng of total RNA, you must follow the guidelines in Tables I & II to dilute your first-strand cDNA product before proceeding with cDNA amplification (Section C).

We strongly recommend use of the **Advantage 2 PCR Kit** (Cat. Nos. 639206 & 639207) for PCR amplification (Section C). This kit includes the Advantage 2 Polymerase Mix, which has been specially formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994).

IMPORTANT:

- The success of your experiment depends on the quality of your starting sample of total or poly A$^+$ RNA. For best results we **strongly** recommend that you use the NucleoSpin® RNA II Kit (see Section II for ordering information) to isolate highly pure RNA from cells, tissues or biological fluids (See Section IV.B. RNA Isolation).
- Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants (see Section IV.D. Assessing the Quality of the RNA Template).
- Do not change the size (volume) of any of the reactions. All components have been optimized for the volumes specified.
- The first time you use this kit, you should perform cDNA synthesis with the Control Mouse Liver Total RNA provided in the kit, in parallel with your experimental sample. Performing this control synthesis at least once will verify that all components are working properly and will also help you troubleshoot any problems that may arise.

1. For each sample and Control Mouse Liver Total RNA, combine the following reagents in separate 0.5 ml reaction tubes:

1–3.5 µl	**RNA** (1 ng–1 µg of poly A$^+$ RNA or 2 ng–1 µg total RNA)*
1 µl	**3′ SMART CDS Primer II A** (12 µM)
x µl	**Deionized H_2O**
4.5 µl	**Total Volume**

*For the control synthesis, add 1 µl (1 µg/µl) of Control Mouse Liver Total RNA. PCR-Select users should start with ≥10 ng of total RNA.

V. SMARTer cDNA Synthesis, *continued*

2. Mix contents and spin the tubes briefly in a microcentrifuge.
3. Incubate the tubes at 72°C in a hot-lid thermal cycler for 3 min, then reduce the temperature to 42°C for 2 min.

NOTE: The initial reaction steps (Step 4-6) are critical for first-strand synthesis and should not be delayed after Step 3. You can prepare your master mix (for Step 4) while your tubes are incubating (Step 3) in order to jump start the cDNA synthesis.

4. Prepare a Master Mix for all reaction tubes at room temperature by combining the following reagents in the order shown:

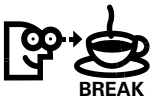
2 µl	**5X First-Strand Buffer**
0.25 µl	**DTT** (100 mM)
1 µl	**dNTP Mix** (10 mM)
1 µl	**SMARTer II A Oligonucleotide** (12 µM)
0.25 µl	**RNase Inhibitor**
1 µl	**SMARTScribe Reverse Transcriptase (100 U)***
5.5 µl	**Total Volume added per reaction**

* Add the reverse transcriptase to the master mix just prior to use. Mix well by pipetting and spin the tube briefly in a microcentrifuge.

5. Aliquot 5.5 µl of the Master Mix into each reaction tube. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
6. Incubate the tubes at 42°C for 1 hour.

NOTE: If you plan to use a downstream application that requires full-length cDNAs, ***extend the incubation time to 90 min***.

7. Terminate the reaction by heating the tubes at 70°C for 10 min.
8. Dilute the first-strand reaction product by adding the appropriate volume of TE buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA):
   - Add 40 µl of TE buffer if you used total RNA as the starting material.
   - Add 190 µl of TE buffer if you used more than 0.2 µg of poly A$^+$ RNA as the starting material.
   - Add 90 µl of TE buffer if you used less than 0.2 µg of poly A$^+$ RNA as the starting material.



9. For PCR-Select cDNA subtraction, proceed with the protocols provided in Appendix A of this User Manual. For all other applications, proceed with Section C. If necessary, cDNA samples can be stored at –20°C (for up to three months) until you are ready to proceed with cDNA amplification (Section C).

V. SMARTer cDNA Synthesis, *continued*

PROTOCOL

C. PROTOCOL: cDNA Amplification by LD PCR

Table I provides guidelines for optimizing your PCR, depending on the amount of total or poly A$^+$ RNA used in the first-strand synthesis. These guidelines were determined using the Control Mouse Liver Total RNA and a hot-lid thermal cycler; optimal parameters may vary with different templates and thermal cyclers. Additional guidelines, based on the amount of starting material, are also provided in Table II.

For each sample and control, set up an extra reaction tube to determine the optimal number of PCR cycles. In our experience, each 100 µl reaction typically yields 1–3 µg of ds cDNA after the PCR and purification steps (Sections C and D). To ensure that you have sufficient cDNA for your application, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

Table I: Guidelines for Setting Up PCR Reactions

Total RNA (ng)	Volume of Diluted ss cDNA[a] for PCR (µl)	Typical Optimal No. of PCR Cycles*
1000	1	18–20
250	4	18–20
50	10	19–21
10	10	21–23
2	10	23–25
Poly A$^+$ RNA (ng)	**Volume of Diluted ss cDNA[a] for PCR (µl)**	**Typical Optimal No. of PCR Cycles***
500	2	15–17
100	4	15–17
20	10	16–18
5	10	18–20
1	10	20–22

[a]From Step V.B.9.

Attention

***IMPORTANT**:
Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles: 15, 18, 21, 24 and 27 cycles.

1. Preheat the PCR thermal cycler to 95°C.
2. For each reaction, aliquot the appropriate volume (see Table I, above) of each diluted first-strand cDNA into a labeled 0.5 ml reaction tube. If necessary, add deionized H_2O to adjust the volume to 10 µl.

V. SMARTer cDNA Synthesis, *continued*

3. Prepare a PCR Master Mix for all reactions, plus one additional reaction. Combine the following reagents in the order shown:

74 µl	**Deionized H_2O**
10 µl	**10X Advantage 2 PCR Buffer**
2 µl	**50X dNTP Mix** (10 mM)
2 µl	**5′ PCR Primer II A** (12 µM)
2 µl	**50X Advantage 2 Polymerase Mix**
90 µl	**Total Volume per reaction**

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 90 µl of the PCR Master Mix into each tube from Step 2.
6. Cap the tube, and place it in the preheated thermal cycler. If you are NOT using a hot-lid thermal cycler, overlay the reaction mixture with two drops of mineral oil.

IMPORTANT:
Typical cycle numbers are provided as a rough guide for those working with extremely small amounts of RNA. We strongly recommend that you perform a range of cycles to determine the optimal number of cycles for your sample and cycling conditions.

Table II: Cycling Guidelines Based on Starting Material

No. of Cells (e.g. HeLa)	Typical Yield of Total RNA (ng)	Typical No. of PCR Cycles
~10	0.15	27
~100	1.5	24
~1,000	15	20
~10,000	150*	18

*We recommend that you do not use the cDNA equivalent of more than 100 ng of reverse transcribed RNA in a single PCR reaction. See Table I for dilution guidelines.

7. Commence thermal cycling using the following program:
   - 95°C 1 min
   - X cycles[a]:

     95°C 15 sec
     65°C 30 sec
     68°C 3 min[b]

     [a]Consult Table I for guidelines. **Subject all tubes to 15 cycles.** Then, divide the PCR reaction mix between the "Experimental" and "Optimization" tubes, using the Optimization tube for each reaction to determine the optimal number of PCR cycles, as described in Step 8. Store the Experimental tubes at 4°C.

     [b]*For applications requiring longer cDNA transcripts, increase to 6 min.*

V. SMARTer cDNA Synthesis, *continued*

8. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30 µl from each tube to a second reaction tube labeled "Optimization". Store the "Experimental" tubes at 4°C. Using the Tester PCR tube, determine the optimal number of PCR cycles (see Figure 3):
   a. Transfer 5 µl from the 15 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   b. Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.
   c. Transfer 5 µl from the 18 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   d. Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.
   e. Transfer 5 µl from the 21 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   f. Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.
   g. Transfer 5 µl from the 24 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   h. Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.
9. Electrophorese each 5 µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Determine the optimal number of cycles required for each experimental and control sample (see Figure 4, Section VI).
10. Retrieve the 15 cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until you reach the optimal number.
11. When the cycling is completed, analyze a 5 µl sample of each PCR product alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 4 to confirm that your reactions were successful.
12. Add 2 µl of 0.5 M EDTA to each tube to terminate the reaction.

After PCR product purification, you now have SMARTer ds cDNA ready-to-use for applications such as the generation of cDNA array probes or Virtual Northern blots.

V. SMARTer cDNA Synthesis, *continued*

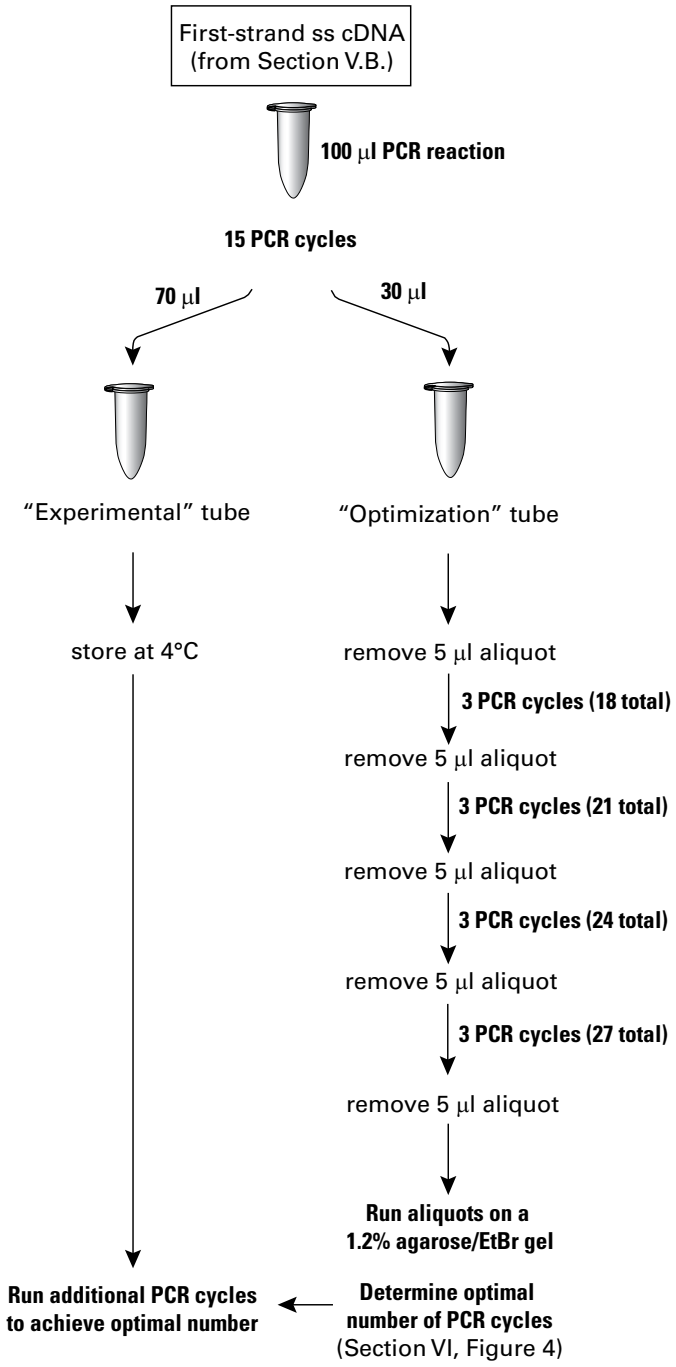


Figure 3. Optimizing PCR parameters for SMARTer cDNA synthesis.

VI. Analysis of cDNA Amplification Results

Figure 4 shows a typical gel profile of ds cDNA synthesized using the Control Mouse Liver Total RNA for SMARTer cDNA synthesis and amplification. In general, cDNA synthesized from mammalian total RNA should appear on a 1.2% agarose/EtBr gel as a moderately strong smear from 0.5 to as high as 5 kb with some distinct bands. The number and position of the bands you obtain will be different for each particular total RNA used. Furthermore, cDNA prepared from some mammalian tissue sources (e.g., human brain, spleen, and thymus) may not display bright bands due to the very high complexity of the RNA.

For the best results, you must optimize the PCR cycling parameters for your experiment, as described in Section V.C. (Figure 3). Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative probe. Undercycling, on the other hand, results in a lower yield of cDNA. The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative: when in doubt, it is better to use fewer cycles than too many.

Figure 4 provides an example of how your analysis should proceed. In this experiment, the PCR reached its plateau after 24 cycles for the 2 ng experiment and 21 cycles for the 50 ng experiment; that is, the yield of PCR products stopped increasing. After 24 and 21 cycles, a smear appeared in the high-molecular-weight region of the gel, indicating that the reactions were overcycled. Therefore, the optimal number of cycles would be 23 for the 2 ng experiment and 20 for the 50 ng experiment.

We have optimized the PCR cycling parameters presented in this User Manual using a hot-lid thermal cycler and the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207). These parameters may vary with different polymerase mixes, templates, and thermal cyclers. We strongly recommend that you optimize the number of PCR cycles with your experimental sample(s) and the Control Mouse Liver Total RNA. Try different numbers of cycles; then, analyze your results by electrophoresing 5 µl of each product on a 1.2% agarose/EtBr gel in 1X TAE buffer.

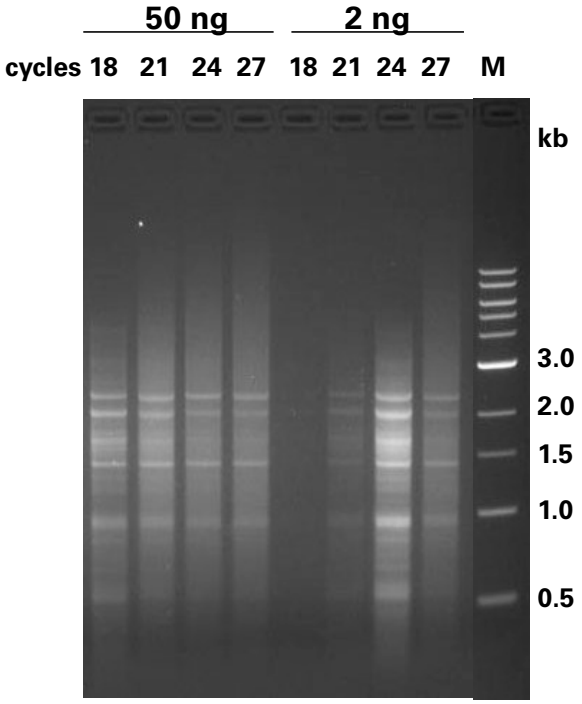


Figure 4. Analysis for optimizing PCR parameters. 2 ng or 50 ng of the Control Mouse Liver Total RNA was subjected to first-strand cDNA synthesis and purification as described in the protocol. 10 µl was used for PCR amplification. A range of PCR cycles were performed (18, 21, 24 and 27). 5 µl of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1X TAE buffer following the indicated number of PCR cycles. The optimal number of cycles determined in this experiment was 23 for the 2 ng reaction, and 20 for the 50 ng reaction. Lane M: 1 kb DNA ladder size markers.

VII. Troubleshooting Guide

Table III: Troubleshooting Guide for First-Strand cDNA Synthesis & SMARTer PCR Amplification

PROBLEM	CAUSE	SOLUTION
Low molecular weight (size distribution < 3 kb, with a majority between 500-200 bp), poor yield, or no PCR product observed for the Control Mouse Liver Total RNA	RNAs may have degraded during storage and/or first-strand synthesis. Poor quality RNA starting material will reduce the ability to obtain full-length cDNAs.	RNA must be stored at –70°C. Your working area, equipment, and solutions must be free of contamination by RNase. For best results, freeze cells/tissue immediately following harvest in Buffer RA1 with an RNase inhibitor, then use the NucleoSpin RNA II Kit to isolate RNA (see Section II. Additional Materials Required, for ordering information).
	You may have made an error during the procedure, such as using a suboptimal incubation temperature or omitting an essential component.	Carefully check the protocol and repeat the first-strand synthesis and PCR with your sample and the control RNA.
	The conditions and parameters for PCR may have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, polymerase mixes, or RNA samples.	Check the protocol and repeat the first-strand synthesis and PCR.
Poor yield or truncated PCR product from your experimental RNA	If your RNA sample was prepared from a nonmammalian species, the apparently truncated PCR product may actually have the normal size distribution for that species. For example, for insects, the normal RNA size distribution may be <2–3 kb.	If you have not already done so, electrophorese a sample of your RNA on a formaldehyde/agarose/EtBr gel to determine its concentration and analyze its quality (see Section IV.D. Assessing the Quality of the RNA Template, for more details).
	The concentration of your experimental RNA is low, but the quality is good.	Repeat the experiment using more RNA and/or more PCR cycles.
	Your experimental RNA has been partially degraded (by contaminating RNases) before or during first-strand synthesis.	Repeat the experiment using a fresh lot or preparation of RNA. Check the stability of your RNA by incubating a small sample in water for 2 hr at 42°C. Then, electrophorese it on a formaldehyde/agarose/EtBr gel alongside an unincubated sample. If the RNA is degraded during incubation, it will not yield good results in the first-strand synthesis. In this case, reisolate the RNA using a different technique, such as our NucleoSpin RNA II Kit (see Section II. Additional Materials Required, for ordering information).
	Your experimental RNA sample contains impurities that inhibit cDNA synthesis.	In some cases, ethanol precipitation of your existing total RNA, followed by washing twice in 80% EtOH, may remove impurities. If this fails, reisolate the RNA using a different technique, such as our NucleoSpin RNA II Kit (see Section II. Additional Materials Required, for ordering information).

VIII. References

For the most recent publications featuring SMART technology, please visit the SMART microsite on the web at **www.clontech.com/smart**

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Appendix A: Protocols for PCR-Select™

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

The following modified SMARTer cDNA synthesis protocol allows you to use your cDNA directly for PCR-Select cDNA Subtraction. Clontech's PCR-Select cDNA Subtraction Kit (Cat. No. 637401) offers an efficient method for selectively amplifying differentially expressed genes—those genes expressed in one mRNA population but reduced or absent in another.

IMPORTANT:
The minimum amount of starting material for PCR-Select cDNA synthesis is **10 ng** of total RNA. However, if your RNA sample is not limiting, we recommend that you start with 20–1,000 ng of total RNA for cDNA synthesis.

A. Additional Materials Required

The following materials are required for PCR-Select but are not supplied:

- **Phenol:chloroform:isoamyl alcohol** (25:24:1)

  Prepare as follows:

  1. Melt phenol.
  2. Equilibrate with an equal volume of sterile 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 (25:24:1) at 4°C away from light for a maximum of two weeks.

- **TE buffer** (10 mM Tris-HCl [pH 7.6], 0.1 mM EDTA)
- **Ethanol**
- **4 M ammonium acetate** (pH 7.0)
- **1X TNE buffer** (10 mM Tris-HCl [pH 8], 10 mM NaCl, 0.1 mM EDTA)
- **NucleoTraPCR Kit** (Cat. No. 740587)
- **Microfiltration columns** (0.45 µm)

B. PROTOCOL: cDNA Amplification by LD PCR

Guidelines for optimizing your PCR, depending on the amount of total RNA used in the first-strand synthesis, are provided in Table I (see Section V). These guidelines were determined using the Control Mouse Liver Total RNA and a hot-lid thermal cycler; optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles: 15, 18, 21, 24 and 27 cycles (Figure 5).

To generate sufficient cDNA for PCR Select subtraction, you should set up three 100 µl PCR reactions, labeled "A", "B", and "C", for each tester and driver sample (Figure 5). In our experience, each PCR reaction will typically yield 1–3 µg of ds cDNA. Subtraction usually requires 2 µg of driver cDNA, so the three combined tubes of SMARTer cDNA should produce sufficient cDNA, taking into account any loss from column chromatography; three tubes will also be ample for the tester. To ensure that you have sufficient cDNA, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

Appendix A: Protocols for PCR-Select™, *continued*

1. Preheat the PCR thermal cycler to 95°C.
2. For each experimental sample, aliquot 30 µl cDNA from Step V.B.9. into a labeled 1.5 ml reaction tube.
3. Prepare enough Master Mix for all PCR reactions and 1 extra reaction to ensure sufficient volume. Combine the following reagents in the order shown:


Recipe

222	µl	**Deionized H_2O**
30	µl	**10X Advantage 2 PCR Buffer**
6	µl	**50X dNTP Mix** (10 mM; in Advantage 2 PCR Kit)
6	µl	**5′ PCR Primer II A** (12 µM)
6	µl	**50X Advantage 2 Polymerase Mix**
270	µl	**Total Volume per reaction**

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 270 µl of the PCR Master Mix into each tube from Step 2. Mix well.
6. Aliquot 100 µl of the resulting PCR reaction mix into three reaction tubes labeled "A", "B", and "C."
7. Cap each tube, and place them in the preheated thermal cycler. If you are NOT using a hot-lid thermal cycler, overlay the reaction mixture with two drops of mineral oil.
8. Commence thermal cycling using the following program:
   - 95°C 1 min
   - X cycles[a]:

     95°C 15 sec
     65°C 30 sec
     68°C 3 min[b]

     [a]Consult Table I for guidelines. **Subject all tubes to 15 cycles.** Then, divide the PCR reaction mix in tube C between the "Experimental" and "Optimization" tubes, using the Optimization tube for each reaction to determine the optimal number of PCR cycles, as described in Step 9 (below). Store Tubes A and B and the Experimental tube at 4°C.

     [b]*For applications requiring full-length cDNA, increase to 6 min.*
9. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30 µl from Tube C to a second reaction tube labeled "Optimization". Store Tubes A and B, and the "Experimental" tube containing the remaining 70 µl of Tube C, at 4°C. Using the Optimization PCR tube, determine the optimal number of PCR cycles (see Figure 5, Appendix A):
   a. Transfer 5 µl from the 15 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   b. Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.
   c. Transfer 5 µl from the 18 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   d. Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.
   e. Transfer 5 µl from the 21 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   f. Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.
   g. Transfer 5 µl from the 24 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   h. Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.

Appendix A: Protocols for PCR-Select™, *continued*

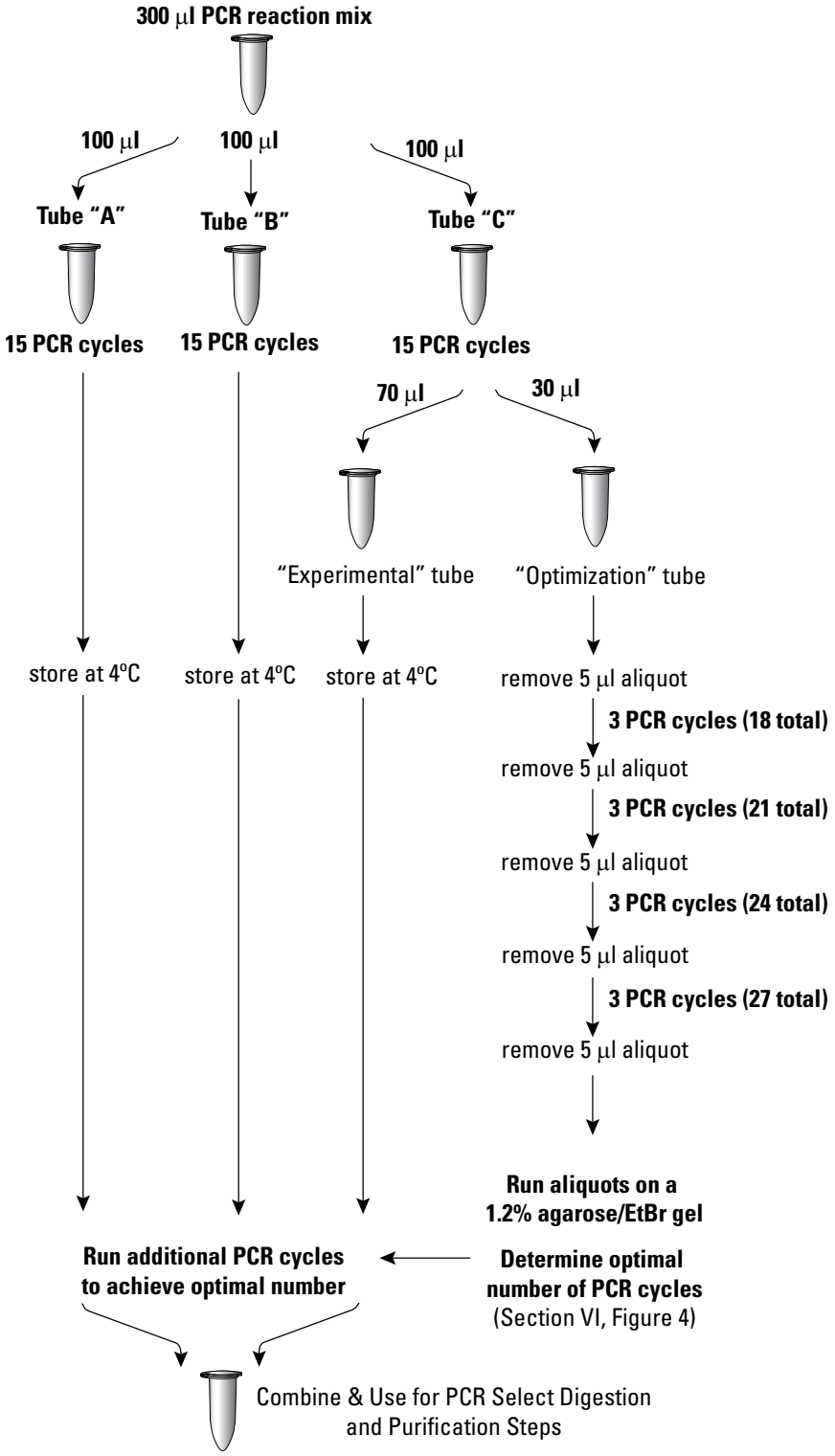

Figure 5. Optimizing PCR parameters for SMARTer cDNA synthesis for use with Clontech PCR-Select.

Appendix A: Protocols for PCR-Select™, *continued*

10. Electrophorese each 5 µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Determine the optimal number of cycles required for each experimental and control sample (see Figure 4, Section VI).
11. Retrieve the 15 cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until you reach the optimal number.
12. When the cycling is completed, analyze a 5 µl sample of each PCR product alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 4 to confirm that your reactions were successful.
13. Add 2 µl of 0.5 M EDTA to each tube to terminate the reaction.

C. PROTOCOL: Column Chromatography

1. For every experimental sample and control, combine the three reaction tubes (A, B, and Experimental) of PCR product into a 1.5 ml microcentrifuge tube. Transfer 7 µl of the raw PCR product to a clean microcentrifuge tube and label this tube "Sample A". Store at –20°C. You will use Sample A for analysis of column chromatography, as described in Section G.
2. To each tube of combined PCR product, add an equal volume of phenol: choloroform:isoamyl alcohol (25:24:1). Vortex thoroughly.
3. Centrifuge the tubes at 14,000 rpm for 10 min to separate the phases.
4. Remove the top (aqueous) layer and place it in a clean 1.5 ml tube.
5. Add 700 µl of n-butanol and vortex the mix thoroughly. Butanol extraction allows you to concentrate your PCR product to a volume of 40–70 µl.

NOTE: Addition of too much n-butanol may remove all the water and precipitate the nucleic acid. If this happens, add water to the tube and vortex until an aqueous phase reappears.

6. Centrifuge the solution at room temperature at 14,000 rpm for 1 min.
7. Remove and discard the upper (n-butanol organic) phase.
8. If you do not end up with a volume of 40–70 µl, repeat steps 6–7 with the same volume of n-butanol.

NOTE: If your volume is <40 µl, add H2O to the aqueous phase to adjust volume to 40–70 µl.

9. Invert a CHROMA SPIN +TE-1000 column several times to completely resuspend the gel matrix.

NOTE:Check for air bubbles in the column matrix. If bubbles are visible, resuspend the matrix in the column buffer by inverting the column again.

10. Remove the top cap from the column, and then remove the bottom cap.
11. Place the column into a 1.5 ml centrifuge tube or a 17 x 100 mm tube.
12. Discard any column buffer that immediately collects in the tube and add 1.5 ml of 1X TNE buffer.
13. Let the buffer drain through the column by gravity flow until you can see the surface of the gel beads in the column matrix.
14. Discard the collected buffer and proceed with purification.
15. Carefully and slowly apply the sample to the center of the gel bed's flat surface. Do not allow any sample to flow along the inner wall of the column.
16. Apply 25 µl of 1X TNE buffer and allow the buffer to completely drain out of the column.

Appendix A: Protocols for PCR-Select™, *continued*

17. Apply 150 µl of 1X TNE buffer and allow the buffer to completely drain out of the column.
18. Transfer column to a clean 1.5 ml microcentrifuge tube.
19. Apply 320 µl of 1X TNE buffer and collect the eluate as your purified ds cDNA fraction. Transfer 10 µl of this fraction to a clean microcentrifuge tube and label this tube "Sample B". Store at –20°C. Use this aliquot for agarose/EtBr gel analysis (Step 21, below).
20. Transfer column to a clean 1.5 ml microcentrifuge tube. Apply 75 µl of 1X TNE buffer and collect the eluate in a clean microcentrifuge tube. Label this tube "Sample C" and store at –20°C. **Save this fraction until after you perform agarose/EtBr gel analysis** (Step 21, below).
21. To confirm that your PCR product is present in the purified ds cDNA fraction, perform the agarose/EtBr gel analysis as described in Appendix A, Section G.2.

D. PROTOCOL: RsaI Digestion

This step generates shorter, blunt-ended ds cDNA fragments, which are necessary for both adaptor ligation and subtraction.

Before proceeding with RsaI digestion, set aside another 10 µl of purified ds cDNA for agarose/EtBr gel analysis to estimate the size range of the ds cDNA products (Step 4, below). Label this tube "Sample D".

1. Add the following reagents to the purified cDNA fraction collected from the CHROMA-SPIN column (Appendix A, Section C.21):

   36 µl **10X RsaI restriction buffer**

   1.5 µl **RsaI** (10 units)

2. Mix well by vortexing and spin briefly in a microcentrifuge.
3. Incubate at 37°C for 3 hr.
4. To confirm that RsaI digestion was successful, electrophorese 10 µl of uncut ds cDNA (Sample D) and 10 µl of RsaI-digested cDNA on a 1.2% agarose/EtBr gel in 1X TAE buffer (see Appendix A, Section G.3 in this User Manual and Section V.B in the PCR-Select User Manual).
5. Add 8 µl of 0.5 M EDTA to terminate the reaction.
6. Transfer 10 µl of the digested cDNA to a clean microcentrifuge tube, label this tube "Sample E", and store at –20°C. You will compare this sample to the PCR product after final purification, as described in Appendix A, Section G.4.

E. PROTOCOL: Purification of Digested cDNA

You may purify your digested cDNA using any silica matrix-based PCR purification system, such as those offered by Clontech. Alternatively, a phenol:chloroform extraction may be performed; however, this may decrease the efficiency of the PCR-Select subtraction. The following purification procedure has been optimized using SMARTer ds cDNA and our NucleoTrapCR Kit (Cat. No. 740587; not included with PCR-Select Kit).

Before you start: Add 95% ethanol to the Wash Buffer NT3 (concentrate) for a final concentration of approximately 85%. The appropriate volume is listed on the Buffer NT3 bottle.

1. Aliquot the RsaI-digested cDNA (Appendix A, Section D.6, above) into two clean,1.5 ml microcentrifuge tubes (approximately 170 µl in each tube).
2. Vortex the NucleoTrap Suspension thoroughly until the beads are completely resuspended.
3. Add 680 µl of Buffer NT2 and 17 µl of NucleoTrap Suspension to each tube of digestion mixture.
4. Incubate the sample at room temperature for 10 min. Mix gently every 2–3 min during the incubation period.
5. Centrifuge the sample at 10,000 x g for 1 min at room temperature. Discard the supernatant.

Appendix A: Protocols for PCR-Select™, *continued*

6. Add 680 µl of **Buffer NT2** to the pellet. Mix gently to resuspend. Centrifuge at 10,000 x g for 1 min at room temperature. Remove the supernatant completely and discard.
7. Add 680 µl of **Buffer NT3** to the pellet. Mix gently to resuspend. Centrifuge the sample at 10,000 x g for 1 min at room temperature. Remove the supernatant completely and discard.
8. Repeat Step 7.
9. Air dry the pellet for 15 min at room temperature (or at 37°C to speed up evaporation).

NOTE:Do not use a speed vac to dry the pellet; speed vacs tend to overdry the beads, which leads to lower recovery rates.

10. Add 50 µl of TE buffer (pH 8.0) to the pellet. Resuspend the pellet by mixing gently. Combine the resuspended pellets into one tube. Mix gently.
11. Elute the DNA by incubating the sample at 50°C for 5 min. Gently mix the suspension 2–3 times during the incubation step.
12. Centrifuge the sample at 10,000 x g for 30 sec at room temperature. Transfer the supernatant, containing the pure DNA, to a clean 1.5 ml microcentrifuge tube.

NOTE:Repeating Steps 10–12 can increase yields approximately 10–15%.

13. Apply the supernatant to a microfiltration column that has been inserted into a 1.5 ml tube. Centrifuge for 5 min and discard the column.
14. Transfer 6 µl of the filtered DNA solution to a clean 1.5 ml microcentrifuge tube containing 14 µl of deionized H_2O. Label this tube "Sample F" and store at –20°C. You will use this sample to analyze the SMARTer cDNA after purification, as described in Appendix A, Section G.4.
15. To precipitate the DNA, add 50 µl of 4 M ammonium acetate and 375 µl of 95% ethanol to the remaining sample from Step 14.
16. Vortex the mix thoroughly and centrifuge the tubes at 14,000 rpm for 20 min at room temperature.
17. Carefully remove and discard the supernatant.
18. Overlay the pellet with 500 µl of 80% ethanol.
19. Centrifuge the tube at 14,000 rpm for 10 min. Carefully remove the supernatant and discard.
20. Air dry the pellets for 5–10 min.
21. Dissolve the pellet in 6.7 µl of 1X TNE buffer.
22. Transfer 1.2 µl to a clean 1.5 ml microcentrifuge tube containing 11 µl of deionized H_2O, label this tube "Sample G," and store the remaining sample at –20°C. Use 10 µl of the diluted DNA to assess the yield of DNA by UV spectrophotometry. For each reaction, we usually obtain 1–3 µg of SMARTer cDNA after purification. For two tubes, you should obtain a total of 2–6 µg of cDNA. If your yield is lower than this, perform the agarose/EtBr gel analysis described in Appendix A, Section G.4.
23. If your DNA concentration is >300 ng/µl, dilute your cDNA to a final concentration of 300 ng/µl in 1X TNE buffer, and follow the adaptor ligation step in accordance with the PCR-Select cDNA subtraction protocol.
24. Your digested ds cDNA is now ready for adaptor ligation, as described in Section IV.F of the User Manual for our PCR-Select cDNA Subtraction Kit (Cat. No. 637401). Be sure to read Section F for important cDNA subtraction control procedures.

Appendix A: Protocols for PCR-Select™, *continued*

F. Controls for PCR-Select cDNA Subtraction

We strongly recommend that you perform the following control subtractions. Please refer to Section IV of the PCR-Select User Manual.

1. Control subtraction using the **human skeletal muscle poly A$^+$ RNA (included in the PCR-Select Kit):**

   Use the conventional method (as described in the PCR-Select User Manual) to synthesize ds cDNA from the control human skeletal muscle poly A$^+$ RNA provided in the PCR-Select Kit. Then, set up a "mock" subtraction: use a portion of the human skeletal muscle cDNA as driver, and mix another portion with a small amount of the control HaeIII-digested ϕX174 DNA from the PCR-Select Kit for tester. This control subtraction, which is described in detail in the PCR-Select User Manual, is the best way to confirm that the multistep subtraction procedure works in your hands.

2. Control subtraction using the **mouse liver total RNA (included in the SMARTer kits):**

   Use the SMARTer kit to amplify the control mouse liver total RNA; then, perform a mock subtraction as described for control #1: use a portion of the mouse liver cDNA as driver, and mix another portion with a small amount of the control HaeIII-digested ϕX174 DNA from the PCR-Select Kit for tester. If control #1 works, but control #2 does not, you may assume that the SMARTer cDNA amplification and/or purification failed. In this case, try reducing the number of PCR cycles for the cDNA amplification and troubleshoot your purification protocol (Appendix A, Section E).

G. Analysis of Results of SMARTer PCR cDNA Synthesis for PCR-Select cDNA Subtraction

Figure 4 shows a typical gel profile of ds cDNA synthesized using the Control Mouse Liver Total RNA and the SMARTer protocol outlined in Section V. In general, cDNA synthesized from mammalian total RNA should appear on a 1.2% agarose/EtBr gel as a moderately strong smear from 0.5–5 kb with some distinct bands. The number and position of the bands you obtain will be different for each particular total RNA used. Furthermore, cDNA prepared from some mammalian tissue sources (e.g., human brain, spleen, and thymus) may not display bright bands due to the very high complexity of the RNA. For nonmammalian species, the size distribution may be smaller (see Section H for more details).

1. **Determining the Optimal Number of PCR Cycles (Section B):**

   For best results, you must optimize the PCR cycling parameters for your experiment, as described in Section B (Figure 5). Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA is a very poor template for cDNA subtraction. Undercycling, on the other hand, results in a lower yield of your PCR product. The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative: when in doubt, it is better to use fewer cycles than too many.

   We have optimized the PCR cycling parameters presented in this User Manual using an authorized hot-lid thermal cycler and the Advantage 2 PCR Kit (Cat. No. 639207). These parameters may vary with different polymerase mixes, templates, and thermal cyclers. We strongly recommend that you optimize the number of PCR cycles with your experimental sample(s) and the control mouse liver total RNA. Try different numbers of cycles; then, analyze your results by electrophoresing 5 µl of each product on a 1.2% agarose/EtBr gel in 1X TAE buffer.

   Figure 4 provides an example of how your analysis should proceed. In this experiment, the PCR reached its plateau after 21 and 24 cycles; that is, the yield of PCR products stopped increasing. After 21 and 24 cycles, a smear appeared in the high molecular weight region of the gel, indicating that the reaction was overcycled. Because the plateau was reached after 24 cycles for the 2 ng reaction and after 21 cycles for the 50 ng reaction, the optimal number of cycles determined in this experiment would be 23 for the 2 ng reaction, and 20 for the 50 ng reaction.

Appendix A: Protocols for PCR-Select™, *continued*

2. **Column Chromatography (Section C):**

   To analyze the ds cDNA after column chromatography, electrophorese 3 µl of the unpurified PCR product (Sample A, from Step C.1) alongside 10 µl of the PCR product purified by column chromatography (Sample B, from Section C) and 10 µl of the second fraction (Sample C, from Section C) on a 1.2% agarose/EtBr gel. Compare the intensities of Sample A and Sample B, and estimate the percentage of PCR product that remains after column chromatography. The yield of cDNA after column chromatography is typically 50%. If your yield is <30%, check to see if it is present in the second fraction, Sample C. If this second fraction has a higher yield of cDNA than the first, combine the fractions and proceed with Section D. Otherwise if the cDNA is not present in Sample C, repeat the PCR and column chromatography steps.

3. **RsaI Digestion (Section D):**

   To confirm that RsaI digestion was successful, electrophorese 10 µl of uncut ds cDNA (Sample D, from Appendix A, Section D) alongside 10 µl of RsaI-digested cDNA (from Step D.4) on a 1.2% agarose/EtBr gel. Compare the profiles of both samples. Before RsaI digestion, ds cDNA should appear as a smear from 0.5–10 kb with bright bands corresponding to abundant mRNAs. (For some RNA samples from nonmammalian species, the size distribution may be only 0.5–3 kb.) After RsaI digestion, the smear should range from 0.1–2 kb. This result will be similar to that shown in the PCR-Select Kit User Manual.

4. **Purification of Digested cDNA (Section E):**

   To analyze the yield of purified SMARTer cDNA, electrophorese 10 µl of RsaI-digested cDNA before purification (Sample E, from Section D) alongside 10 µl of purified diluted cDNA before ethanol precipitation (Sample F, Section E) and 1.8 µl of purified diluted cDNA after ethanol precipitation (Sample G, from Section E) on a 1.5% agarose/EtBr gel. Compare the intensities of the samples and estimate what percentage of RsaI-digested PCR product remains after purification and ethanol precipitation. The yield of cDNA after purification using the NucleoTrapCR Kit and ethanol precipitation is typically 70 percent. If your yield is <30 percent, troubleshoot your purification protocol or consult the troubleshooting guide of the User Manual for that particular purification kit.

Appendix A: Protocols for PCR-Select™, *continued*

H. Troubleshooting

For troubleshooting the actual PCR-Select subtraction procedure, please refer to the PCR-Select User Manual PT1117-1. Here, we provide a troubleshooting guide for preparing SMARTer cDNA for substraction (described in Appendix A, Sections B–E) in Table IV.

Table IV: Troubleshooting Guide for Preparing SMARTer cDNA for Subtraction

PROBLEM	CAUSE	SOLUTION
Low yield of cDNA after column chromatography (Appendix A, Section C)	You may have applied the wrong volume of buffer to the CHROMA-Spin column, or collected the wrong volume of buffer from the column.	Carefully check the protocol and repeat column chromatography.
	Your column may have leaked during shipping.	If your column contains less than 750 µl of matrix, discard it and use another column.
Failure of RsaI Digestion (Appendix A, Section D)	If the size distribution of your sample and/or control cDNA is not reduced after RsaI digestion, your TNE buffer mix may be suboptimal.	Check the recipe for TNE buffer. If you used the correct recipe for TNE buffer, perform phenol:chloroform extraction and ethanol precipitation; then, repeat the RsaI digestion.
Low yield of cDNA after purification of digested cDNA (Appendix A, Section E)	Loss of cDNA during purification.	Troubleshoot your purification procedure.
	Loss of cDNA during ethanol precipitation	Check the volumes of the ammonium acetate and ethanol. Repeat purification and ethanol precipitation.
	Your PCR did not reach the plateau (i.e., the reaction was undercycled).	Perform more PCR cycles. Optimize the number of cycles as described in Appendix A, Section B.

Appendix B: Virtual Northern Blots

After cloning your subtracted cDNA fragments, you should confirm that they represent differentially expressed genes. Typically, this is accomplished by hybridization to Northern blots of the same RNA samples used as driver and tester for subtraction. If, however, you have limited sample material, you may wish to use Virtual Northern blots for analysis. By using the same SMARTer PCR-amplified tester and driver cDNA used for subtraction, you can obtain information that is similar to that provided by standard Northern analysis. Even if a cDNA does not give a single band when hybridized to a Virtual Northern blot, you can still detect whether or not it is differentially expressed. Multiple bands on a Virtual Northern blot may result from different causes. The cDNA may belong to a multi-gene family, or may contain a nucleotide repeat. Alternatively, a truncated copy of the gene may be present. To distinguish between these possibilities, analysis should also include other methods, such as genomic DNA sequencing or RACE.

To prepare a Virtual Northern blot, electrophorese your SMARTer PCR-amplified cDNA (before purification) on an agarose/EtBr gel and use a Southern transfer onto a nylon membrane (see Sambrook &Russell, 2001). At Clontech, we use the Turboblotter equipment and protocol from Schleicher & Schuel. Figure 6 shows how Virtual Northern blots can be used to confirm differential expression of subtracted cDNAs.

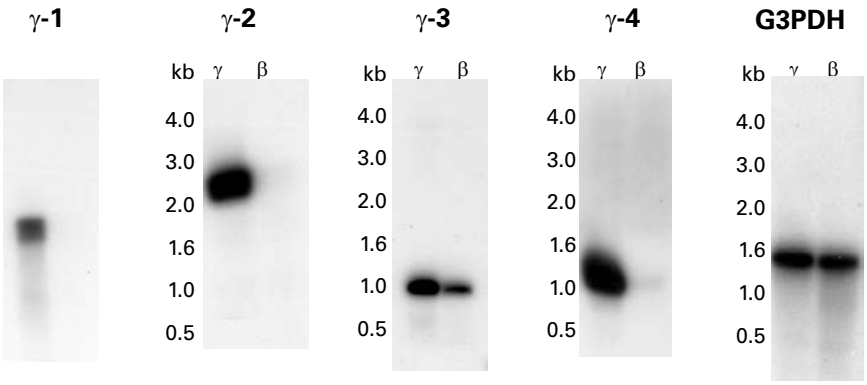

Figure 6. Virtual Northern blot analysis of cDNA fragments expressed in cells producing γ-globin. PCR-Select cDNA subtraction was performed to isolate cDNAs that were preferentially expressed in cells producing γ-globin. 1 µg of total RNA from cells producing γ-globin was used as tester; 1 µg of total RNA from cells producing β-globin was used as driver. Tester and driver cDNAs were synthesized using the SMART PCR cDNA Synthesis Kit and were subjected to PCR-Select subtraction. 84 subtracted cDNA clones were arrayed on a nylon membrane for differential screening. 13 of these subtracted cDNAs showed differential signals and were therefore candidates for further analysis by Virtual Northern blots. Differential expression of all 13 clones was confirmed; four examples are shown in this figure. Virtual Northern blots were prepared using the same SMART PCR-amplified cDNA that was used for subtraction. Each lane contains 0.5 µg of SMART cDNA. Subtracted cDNA fragments (γ-1, γ-2, γ-3, and γ-4) were labeled with [32P]-dCTP and hybridized to the Virtual Northern blots. Hybridization with G3PDH serves as a control for loading. Lane γ: Cells producing γ-globin. Lane β: Cells producing β-globin.

Appendix C: Protocol for Non-Directional Cloning of SMARTer cDNA

We recommend the following procedure for polishing the ends of SMARTer cDNAs for constructing libraries.

A. Additional Materials Required

The following materials are required for ds cDNA polishing but are not supplied:

- **Proteinase K** (20 µg/µl; Roche Applied Science Cat. No. 03 115 887)
- **T4 DNA Polymerase** (New England BioLabs Cat. No. M0203S)

B. PROTOCOL: ds cDNA Polishing

1. Combine 50 µl (2–5 µg) of the amplified ds cDNA with 2 µl of Proteinase K (20 µg/µl) in a sterile 0.5 ml microcentrifuge tube. Store the remainder of the PCR mixture at –20°C.

NOTE: Proteinase K treatment is necessary to inactivate the DNA polymerase activity before proceeding with the ligation steps.

2. Mix contents and spin the tube briefly.
3. Incubate at 45°C for 20 min. Spin the tube briefly.
4. Heat the tube at 90°C for 8–10 min to inactivate the Proteinase K.
5. Chill the tube in ice water for 2 min.
6. Add 3 µl (15 units) of T4 DNA Polymerase.
7. Incubate the tube at 16°C for 30 min.
8. Heat the tube at 72°C for 10 min.
9. Add 27.5 µl of 4 M ammonium acetate.
10. Add ~210 µl of room temperature 95% ethanol.
11. Mix thoroughly by inverting the tube.
12. Spin the tube immediately at 14,000 rpm for 20 min at room temperature.

NOTE: Do not chill the tube at –20°C or on ice before centrifuging. Chilling the sample will **result in coprecipitation of impurities.**

13. Carefully remove the supernatant.
14. Wash pellet with 80% ethanol.
15. Air dry the pellet (~10 min) to evaporate residual ethanol.
16. Add deionized H_2O to resuspend the pellet. The amount added will depend on your cDNA library construction protocol.

NOTE: This preparation of blunt-ended cDNA may now be ligated to any adaptor you choose. Consult your protocol for cDNA library construction.

Notes

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This document has been reviewed and approved by the Clontech Quality Assurance Department.