

Clontech Laboratories, Inc.

# SMARTer® Pico PCR cDNA Synthesis Kit User Manual

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## I. Introduction & Protocol Overview

### A. Summary

The **SMARTer Pico PCR cDNA Synthesis Kit** provides a PCR-based method for producing high-quality cDNA from picogram quantities of total RNA. The SMARTer Pico PCR cDNA Synthesis Kit is an improved version of our original Super SMART® PCR cDNA Synthesis Kit, with a new, SMARTer oligo and **SMARTScribe Reverse Transcriptase** included; it provides higher specificity, lower background and increased yield. The 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 1 ng of total RNA at extremely low concentration (or from a very diluted sample). The cornerstone of SMARTer Pico cDNA synthesis is SMART (Switching Mechanism At 5’ End of the RNA Template) 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.

### B. SMARTer Pico—a Modified SMARTer Protocol

To develop the SMARTer Pico method, we modified the SMARTer protocol by increasing the reaction volumes and performing an additional column purification step (Table 1). With this modified protocol, it is possible to use only 1 ng of total RNA in a 50 µl reaction. Therefore, a sample concentration as low as 20 pg/µl can be used. The SMARTer Pico protocol also includes a purification step after first-strand synthesis that makes it possible to use the entire volume of purified single-stranded cDNA for a single SMARTer Pico PCR amplification. These modifications produce yields of ds cDNA ranging from 1–2 µg.

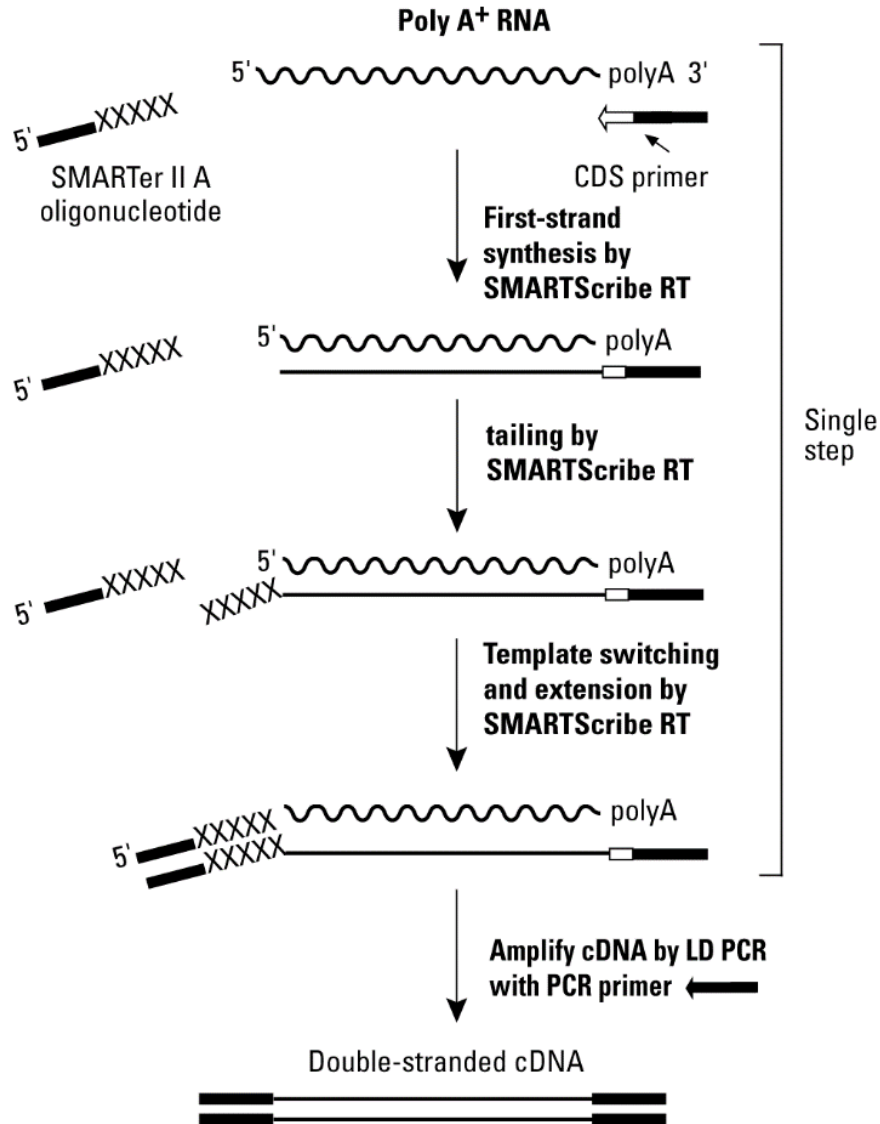
Table 1. Comparison of SMARTer Protocols\*

SMARTer	SMARTer Pico
<b>2–1000 ng total RNA</b>	<b>1–1000 ng total RNA</b>
<b>RT reaction volume up to 3.5 µl</b>	<b>RT reaction volume up to 50 µl</b>
<b>total RNA template concentration as low as 0.6 ng/µl</b>	<b>total RNA template concentration as low as 20 pg/µl</b>
<b>SMARTer first-strand cDNA synthesis</b>	<b>SMARTer Pico first-strand cDNA synthesis</b>
<b>Volume = 10 µl</b>	<b>Volume = 106 µl</b>
<b>Dilute 1:5 with TE Buffer</b>	<b>Purify with NucleoSpin Column</b>
<b>Volume = 50 µl</b>	<b>Column Elution Volume = 80 µl</b>
Use <b>10 µl cDNA</b> for SMARTer PCR amplification	Use <b>80 µl cDNA</b> for SMARTer Pico PCR amplification
<ul style="list-style-type: none"> <li>• 100 µl reaction</li> <li>• cycle optimization and scale-up</li> </ul>	<ul style="list-style-type: none"> <li>• 100 µl reaction</li> <li>• cycle optimization and scale-up</li> </ul>
Purify PCR products with NucleoSpin	Purify PCR products with NucleoSpin
Yields 1–2 µg ds cDNA	Yields 1–2 µg ds cDNA

\*Differences between protocols appear in bold.

### C. 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.



**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 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 Reverse Transcriptase 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 base-pairs with the non-template 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 non-template 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.

## D. Downstream Applications of Synthesized SMARTer cDNA

- **SMARTer Pico cDNA Synthesis for PCR-Select Subtraction**

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). When total RNA is used for cDNA synthesis by conventional methods, ribosomal RNA is transcribed along with the poly A<sup>+</sup> 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<sup>+</sup> fraction results in inefficient subtractive hybridization. However, cDNA generated using the SMARTer Pico 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 Pico 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<sup>+</sup> 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<sup>+</sup> 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 Pico 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). With the SMARTer Pico PCR cDNA Synthesis Kit researchers are able to synthesize highly sensitive array probes from minimal starting material (Gonzalez et al., 1999; Livesey et al., 2000). Using this method, probes made from small amounts of total RNA produce results that are comparable to those from pure poly A<sup>+</sup> RNA—a clear advantage when only limited amounts of tissues or cells are available.

- **Synthesize SMARTer cDNA 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 5'/3' Kits** (Cat. Nos. 634858 & 634859), allow researchers to synthesize first-strand cDNA, and facilitate both 5' and 3' RACE using either poly A<sup>+</sup> RNA or total RNA.

## E. 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 an alternative SMART library construction kit, the **In-Fusion® SMARTer Directional cDNA Library Construction Kit** (Cat. No. 634933), 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).

## II. List of Components

The SMARTer Pico PCR cDNA Synthesis Kit (Cat. No. 634928) includes Cat. No. 634927 (not sold separately) and 2 x Cat. No. 740609.10.

**SMARTer Pico PCR cDNA Synthesis Kit Contents** (Cat. No. 634927; Box 1 & 2)

### Box 1

- 2 x 35 µl **SMARTer II A Oligonucleotide** (12 µM)  
5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3'  
Rsa I  
(X = undisclosed base in the proprietary SMARTer oligo sequence)
- 5 µl **Control Mouse Liver Total RNA** (1 µg/µl)

### Box 2

- 70 µl **3' SMART CDS Primer II A** (12 µM)  
5'–AAGCAGTGGTATCAACGCAGAGTACT<sub>(30)</sub>N<sub>-1</sub>N–3'  
Rsa I  
(N = A, G, C, or T; N<sub>-1</sub> = A, G, or C)
- 200 µl **5' PCR Primer II A** (12 µM)
- 250 µl **5X First-Strand Buffer (RNase-Free)**
  - 250 mM Tris (pH 8.3)
  - 375 mM KCl
  - 30 mM MgCl<sub>2</sub>
- 250 µl **dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
- 50 µl **Dithiothreitol (DTT)** (100 mM)
- 55 µl **RNase Inhibitor (40 U/µl)**
- 55 µl **SMARTscribe™ Reverse Transcriptase** (100 U/µl)
- 2 x 1 ml **Deionized H<sub>2</sub>O**

## NucleoSpin Gel and PCR Clean-Up (2 x Cat. No. 740609.10; Box 3 & 4)

### Box 3 & 4

- 2 x 10 **NucleoSpin Gel and PCR Clean-Up Columns**
- 2 x 10 **Collection Tubes** (2 ml)
- 2 x 10 ml **Binding Buffer NT**
- 2 x 6 ml **Wash Buffer NT3 Concentrate** (add 95% ethanol before use as specified on the label)
- 2 x 5 ml **Elution Buffer NE**

### Storage Conditions

- Store Control Mouse Liver Total RNA and SMARTer II A Oligonucleotide at  $-70^{\circ}\text{C}$ .
- Store NucleoSpin Gel and PCR Clean-Up Kit at room temperature.
- Store all other reagents at  $-20^{\circ}\text{C}$ .

## III. 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 Kit** (Cat. Nos. 740955.50 & 740955.250)  
We strongly recommend the use of the NucleoSpin RNA 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.
- **EASY Dilution (for Real Time PCR)** (Cat. No. 9160)  
We recommend our EASY Dilution (for Real Time PCR) solution (Cat. No. 9160) for RNA template dilution.
- **$\beta$ -mercaptoethanol** (Sigma Cat. No. M6250)
- **50X TAE electrophoresis buffer:**
  - 242.0 g Tris base
  - 57.1 ml glacial acetic acid
  - 37.2 g  $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$
  - X ml Add  $\text{H}_2\text{O}$  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. Please note that CHROMA SPIN™ Columns are not supplied with the SMARTer Pico PCR cDNA Synthesis Kit and must be purchased separately.



## IV. RNA Preparation & Handling

### A. General Precautions

The integrity and purity of your total or poly A<sup>+</sup> 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<sub>2</sub>O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H<sub>2</sub>O. 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 Green & Sambrook (2012).

### B. RNA Isolation

Clontech offers several kits for isolating total or poly A<sup>+</sup> 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 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](http://www.clontech.com). Many procedures are available for the isolation of poly A<sup>+</sup> RNA (Farrell, 1993; Green & Sambrook, 2012).

### C. RNA Purity

The purity of RNA is the key factor for successful cDNA synthesis and SMARTer Pico 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.

**IMPORTANT: 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 cannot 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. 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 Green & Sambrook (2012).

#### 2. 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.

#### 3. 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 above methods can be used to assess mRNA quality. However, since mRNA does not contain strong ribosomal bands, this assessment will be somewhat subjective. Typically, mRNA appears as a smear between 0.5 kb –6 kb, with an area of higher intensity around 1.5 and 2 kb. This size distribution may be tissue or species-specific. An average mRNA size lower than 1.5 kb could indicate degradation.

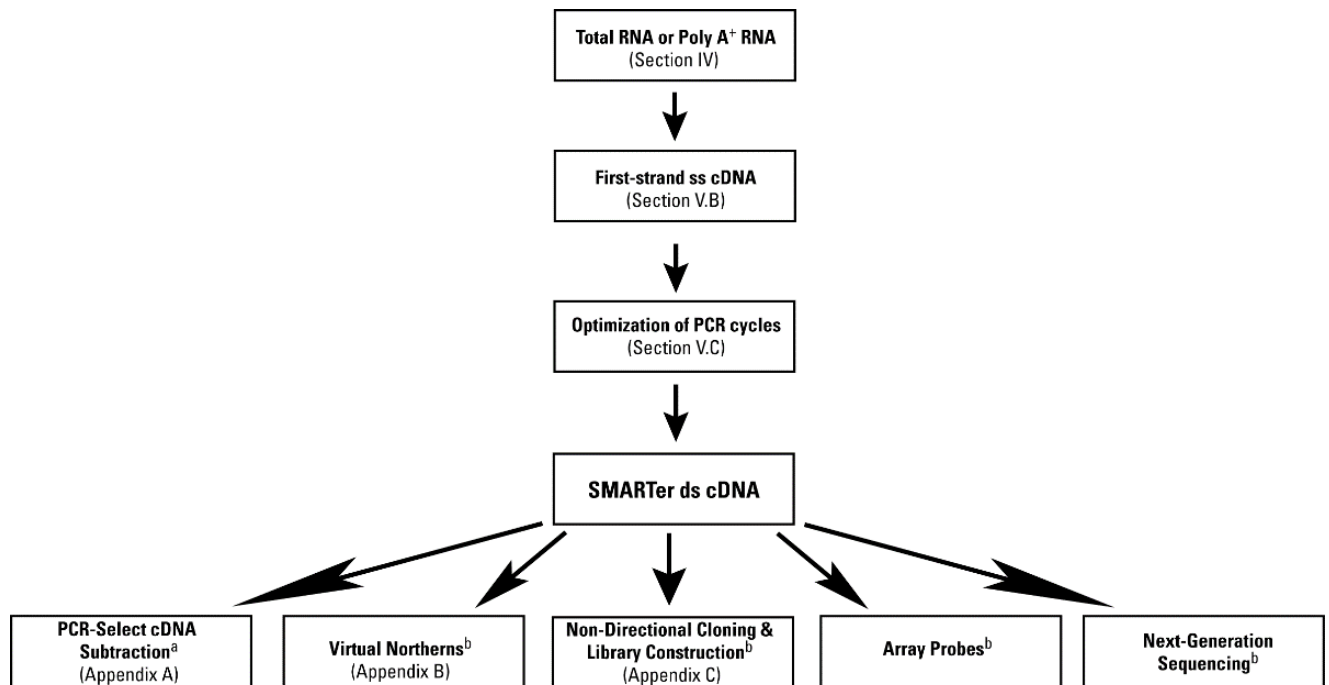
## V. SMARTer Pico cDNA Synthesis

**PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.** Use the following protocol for generating cDNA from very low amounts of RNA with Clontech’s simple and highly efficient SMART technology. This protocol is designed for synthesizing SMARTer cDNA for applications **OTHER THAN LIBRARY CONSTRUCTION**.

**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 SMARTer 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 Pico 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 Pico 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.



<sup>a</sup>Reagents for these procedures are included in the Clontech PCR-Select cDNA Subtraction Kit.

<sup>b</sup>Reagents for these procedures are *not* included in the SMARTer Pico PCR cDNA Synthesis Kit.

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

## A. General Considerations

- We recommend our **EASY Dilution (for Real Time PCR)** solution (Cat. No. 9160) for RNA template dilution. It prevents 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 total RNA. The minimum amount of starting material for cDNA synthesis is 1 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. Please note that if you are starting from >100 ng of total RNA, you must follow the guidelines in Table 2 to dilute your first-strand cDNA product before proceeding with cDNA amplification (Section D).

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).

### IMPORTANT:

- The success of your experiment depends on the quality of your starting sample of RNA. For best results we strongly recommend that you use the NucleoSpin RNA 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.

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

1–50 µl	RNA (1–1,000 ng of total RNA)*
7 µl	3' SMART CDS Primer II A (12 µM)
x µl	Deionized H <sub>2</sub> O
<hr/>	
57 µl	<b>Total Volume</b>

\*For the control synthesis, add 10 ng of Control Mouse Liver Total RNA. PCR-Select users should start with >10 ng of total RNA.

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 cool the tubes to 42°C.

**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:

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

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

5. Aliquot 49 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 long transcripts, **extend the incubation time to 90 min.**

7. Terminate the reaction by heating the tubes at 70°C for 10 min.
8. If necessary, cDNA samples can be stored at -20°C (for up to three months) until you are ready to proceed with spin-column purification (Section C).

### C. Protocol: Column cDNA Purification using NucleoSpin Gel and PCR Clean-Up

To purify the SMARTer cDNA from unincorporated nucleotides and small (<0.1 kb) cDNA fragments, follow this procedure for each reaction tube. Before use, be sure to add 95%–100% ethanol directly to Wash Buffer NT3 as specified on the bottle label.

1. Add 350 µl of Buffer NT to each cDNA synthesis reaction; mix well by pipetting.
2. Place a NucleoSpin Gel and PCR Clean-Up Column into a 2 ml collection tube. Pipette the sample into the column. Centrifuge at 8,000 rpm for 1 min. Discard the flowthrough.
3. Return the column to the collection tube. Add 600 µl of Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
4. Return the column to the collection tube. Add 250 µl of Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
5. Place the column back into the collection tube. Centrifuge at 14,000 rpm for 2 min to remove any residual Wash Buffer NT3.
6. Transfer the NucleoSpin Columns into a fresh 1.5 ml microcentrifuge tube. Add 50 µl of sterile Milli-Q H<sub>2</sub>O to the column. Allow the column to stand for 2 min with the caps open.
7. Close the tube and centrifuge at 14,000 rpm for 1 min to elute the sample.

8. Repeat elution with 35  $\mu$ l of sterile Milli-Q H<sub>2</sub>O in the same 1.5 ml microcentrifuge tube. The recovered elution volume should be 80–85  $\mu$ l per sample. If necessary, add sterile Milli-Q H<sub>2</sub>O to bring the total volume up to 80  $\mu$ l.
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 D. Samples can be stored at –20°C (for up to three months) until you are ready to proceed with cDNA amplification by LD PCR (Section D).

## D. Protocol: cDNA Amplification by LD PCR

Table 2 provides guidelines for optimizing your PCR, depending on the amount of total 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 3.

In our experience, each 100  $\mu$ l reaction typically yields 1–2  $\mu$ g of ds cDNA after the PCR and purification steps (Sections D and E). To ensure that you have sufficient cDNA for your application, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

**Table 2. Guidelines for Setting Up PCR Reactions**

Total RNA (ng)	Volume of Diluted ss cDNA <sup>a</sup> for PCR ( $\mu$ l)	Volume of H <sub>2</sub> O ( $\mu$ l)	Typical Optimal No. of PCR Cycles*
1000	2.5	77.5	18–20
250	10	70	18–20
100	25	55	18–20
50	40	40	18–20
20	80	none	19–21
5	80	none	21–23
1	80	none	24–27

<sup>a</sup> From Step V.C.9.

**\*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, 27 and 30 cycles.

1. Preheat the PCR thermal cycler to 95°C.
2. For each reaction, aliquot the appropriate volume (see Table 2, above) of each diluted first-strand cDNA into a labeled 0.5-ml reaction tube. If necessary, add deionized H<sub>2</sub>O to adjust the volume to 80  $\mu$ l.

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

4 $\mu$ l	Deionized H <sub>2</sub> O
10 $\mu$ l	10X Advantage 2 PCR Buffer
2 $\mu$ l	50X dNTP Mix (10 mM)
2 $\mu$ l	5' PCR Primer II A (12 $\mu$ M)
2 $\mu$ l	50X Advantage 2 Polymerase Mix
20 $\mu$ l	Total volume per reaction

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 20  $\mu$ l 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 3. 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 2 for dilution guidelines.

7. Commence thermal cycling using the following program:

- 95°C for 1 min
- X cycles<sup>a</sup>:
  - 95°C for 15 sec
  - 65°C for 30 sec
  - 68°C for 3 min<sup>b</sup>

<sup>a</sup> Consult Tables II & III 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.

<sup>b</sup> For applications requiring longer cDNA transcripts, increase to 6 min.

8. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30  $\mu$ l 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  $\mu$ l 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  $\mu$ l of PCR mixture.
  - c. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - e. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - g. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - i. Transfer 5  $\mu$ l from the 27-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
  - j. Run three additional cycles (for a total of 30) with the remaining 5  $\mu$ l of PCR mixture.
9. Electrophorese each 5  $\mu$ l aliquot of the PCR reaction alongside 0.1  $\mu$ g 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  $\mu$ l sample of each PCR product alongside 0.1  $\mu$ g 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  $\mu$ l of 0.5 M EDTA to each tube to terminate the reaction.



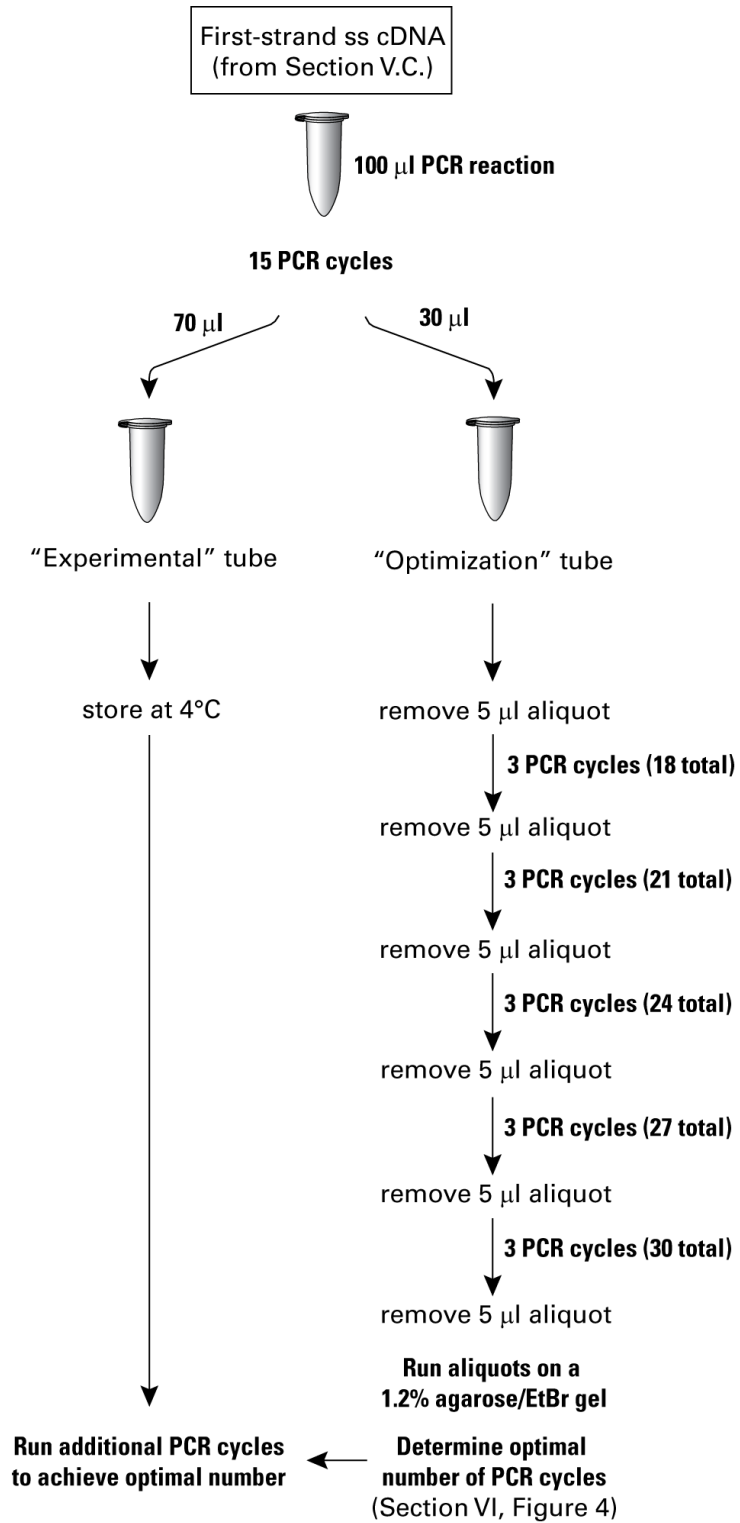


Figure 3. Optimizing PCR parameters for SMARTer Pico cDNA synthesis.

## E. Protocol: Column Purification of PCR Products using NucleoSpin Gel and PCR Clean-Up

1. Add 300  $\mu$ l Binding NT Buffer to each 70  $\mu$ l PCR reaction. Mix well by pipetting.
2. Place a NucleoSpin column into a 2 ml Collection Tube, and pipette the sample onto the filter. Centrifuge at 8,000 rpm for 1 min. Discard the Collection Tube and flowthrough.
3. Insert the NucleoSpin column into a fresh 2 ml Collection Tube. Add 600  $\mu$ l Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
4. Return the column to the Collection Tube. Add 250  $\mu$ l Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
5. Discard the flowthrough and spin again at 14,000 rpm for 1 min to remove the final traces of ethanol to dry the filter.
6. Transfer the NucleoSpin column to a clean 1.5 ml microcentrifuge tube. Pipette 50  $\mu$ l Elution Buffer NE directly onto the filter, being careful not to touch the surface of the filter with the tip of the pipette. Allow the filter to soak for 2 min with the lid open.
7. Close the tube and centrifuge at 14,000 rpm for 1 min to elute PCR product. Save the column.
8. Determine the yield of each PCR product by measuring the  $A_{260}$ . For each reaction, we usually obtain 1–2  $\mu$ g of SMARTer cDNA after purification.
9. If no product is detected, perform elution (Steps 6 and 7) a second time, using a fresh 1.5 ml microcentrifuge tube.

You now have SMARTer ds cDNA ready-to-use for applications such as the generation of cDNA array probes or Virtual Northern blots.

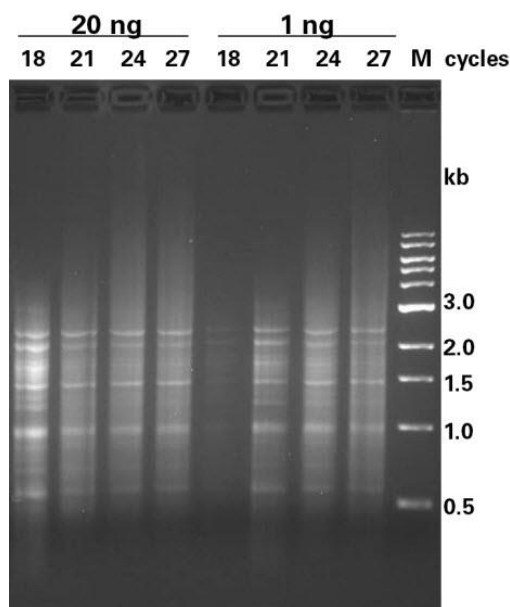
## VI. Analysis of cDNA Amplification Results.

Figure 4 shows a typical gel profile of ds cDNA synthesized using the Control Mouse Liver Total RNA for SMARTer Pico 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 4 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.D. (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 26 cycles for the 1 ng experiment and 21 cycles for the 20 ng experiment; that is, the yield of PCR products stopped increasing. After 26 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 25 for the 1 ng experiment and 20 for the 20 ng experiment.

We have optimized the PCR cycling parameters presented in this User Manual using both hot-lid and non-hot-lid thermal cyclers 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. Analysis for optimizing PCR parameters.** 1 ng or 20 ng of the control mouse liver total RNA was subjected to first-strand cDNA synthesis and purification as described in the protocol. 80 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 25 for the 1 ng reaction, and 20 for the 20 ng reaction. Lane M: 1 kb DNA ladder size marker.

## VII. Troubleshooting Guide

Table 4. Troubleshooting Guide for First-Strand cDNA Synthesis & SMARTer Pico PCR Amplification

Problem	Possible Explanation	Solution
<b>Low molecular weight (size distribution &lt; 3 kb, with a majority between 500-200 bp), poor yield, or no PCR product observed for the control mouse liver total RNA</b>	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 $-70^{\circ}\text{C}$ . 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 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.
<b>Poor yield or truncated PCR product from your experimental RNA</b>	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 $42^{\circ}\text{C}$ . 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 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 Kit (see Section II. Additional Materials Required, for ordering information).

## VIII. References

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## Appendix A. Protocols for PCR-Select

### **PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.**

The following modified SMARTer Pico cDNA synthesis protocol allows you to use your cDNA directly for PCR-Select cDNA Subtraction. The **Clontech 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:

- **CHROMA SPIN-1000+DEPC-H<sub>2</sub>O Columns** (Cat. No. 636093)
- **Microfiltration columns** (0.45 µm)
- **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], 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)

**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 2 (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, 27, and 30 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 1–2 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.

1. Preheat the PCR thermal cycler to 95°C.
2. For each experimental sample, aliquot 80 µl cDNA from Step V.C.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:

172 µl	Deionized H <sub>2</sub> O
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
<hr style="border: 0.5px solid black;"/>	
220 µl	Total volume per reaction

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 220 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 for 1 min
  - X cycles<sup>a</sup>:
    - 95°C for 15 sec
    - 65°C for 30 sec
    - 68°C for 3 min<sup>b</sup>

<sup>a</sup> Consult Table 2 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.

<sup>b</sup> For applications requiring full-length cDNA, increase to 6 min.

9. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30  $\mu$ l from Tube C to a second reaction tube labeled "Optimization". Store Tubes A and B, and the "Experimental" tube containing the remaining 70  $\mu$ l 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  $\mu$ l 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  $\mu$ l of PCR mixture.
  - c. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - e. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - g. Transfer 5  $\mu$ l 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  $\mu$ l of PCR mixture.
  - i. Transfer 5  $\mu$ l from the 27-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
  - j. Run three additional cycles (for a total of 30) with the remaining 5  $\mu$ l of PCR mixture.
10. Electrophorese each 5  $\mu$ l aliquot of the PCR reaction alongside 0.1  $\mu$ g 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  $\mu$ l sample of each PCR product alongside 0.1  $\mu$ g 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  $\mu$ l of 0.5 M EDTA to each tube to terminate the reaction.



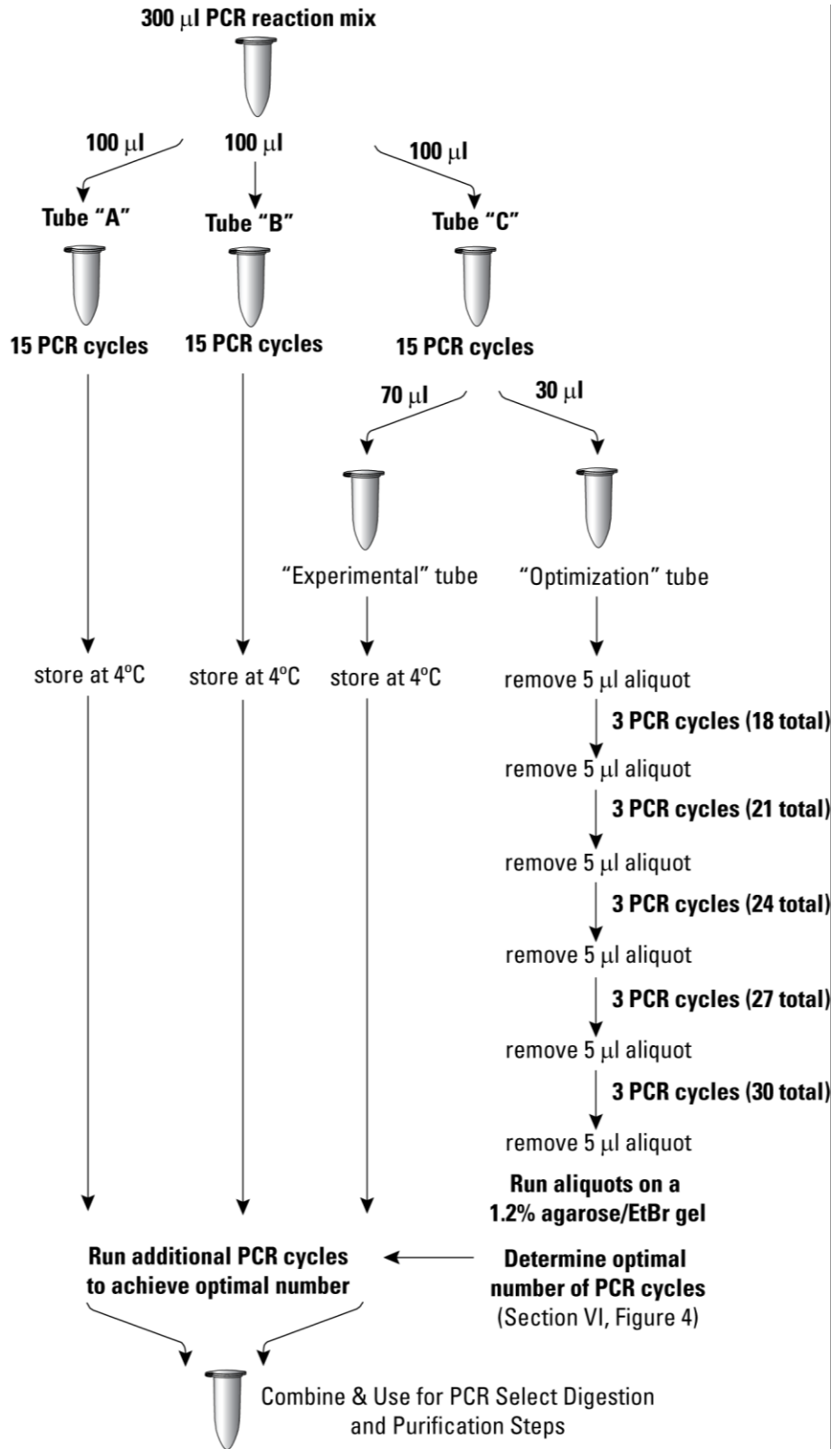


Figure 5. Optimizing PCR parameters for SMARTer Pico cDNA synthesis for use with Clontech PCR-Select.

### 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  $\mu$ l of the raw PCR product to a clean microcentrifuge tube and label this tube "Sample A". Store at  $-20^{\circ}\text{C}$ . 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: chloroform: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  $\mu$ l of n-butanol and vortex the mix thoroughly. Butanol extraction allows you to concentrate your PCR product to a volume of 40–70  $\mu$ l.

**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  $\mu$ l, repeat steps 6–7 with the same volume of n-butanol.

**NOTE:** If your volume is  $<40$   $\mu$ l, add  $\text{H}_2\text{O}$  to the aqueous phase to adjust volume to 40–70  $\mu$ l.

9. Invert a CHROMA SPIN-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. The top of the column matrix should be at the 0.75 ml mark on the wall of the column. If your column contains much less matrix, discard it and use another column.
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  $\mu$ l of 1X TNE buffer and allow the buffer to completely drain out of the column.
17. Apply 150  $\mu$ l 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  $\mu$ l of 1X TNE buffer and collect the eluate as your purified ds cDNA fraction. Transfer 10  $\mu$ l of this fraction to a clean microcentrifuge tube and label this tube "Sample B". Store at  $-20^{\circ}\text{C}$ . Use this aliquot for agarose/EtBr gel analysis (Step 21, below).
20. Transfer column to a clean 1.5 ml microcentrifuge tube. Apply 75  $\mu$ l of 1X TNE buffer and collect the eluate in a clean microcentrifuge tube. Label this tube "Sample C" and store at  $-20^{\circ}\text{C}$ . 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 PT1117-1).
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.
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. Centrifuge the pellet again at 10,000 x g for 1 min at room temperature. 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 7.6) 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<sub>2</sub>O. 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<sub>2</sub>O, 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, below for important cDNA subtraction control procedures.

## 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<sup>+</sup> 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<sup>+</sup> 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  $\phi$ X174 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 Pico kit)**:

Use the SMARTer Pico 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  $\phi$ X174 DNA from the PCR-Select Kit for tester. If control #1 works, but control #2 does not, you may assume that the SMARTer Pico 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 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 Pico 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 0.5–4 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 a 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 total RNA. Try different numbers of cycles; then, analyze your results by electrophoresing 5  $\mu$ l 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 26 cycles; that is, the yield of PCR products stopped increasing. After 21 and 26 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 26 cycles for the 1 ng reaction and after 21 cycles for the 20 ng reaction, the optimal number of cycles determined in this experiment would be 25 for the 1 ng reaction, and 20 for the 20 ng reaction.

### **Column Chromatography (Section C):**

To analyze the ds cDNA after column chromatography, electrophorese 3  $\mu$ l of the unpurified PCR product (Sample A, from Step C.1) alongside 10  $\mu$ l of the PCR product purified by column chromatography (Sample B, from Section C) and 10  $\mu$ l 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.

### **2. RsaI Digestion (Section D):**

To confirm that RsaI digestion was successful, electrophorese 10  $\mu$ l of uncut ds cDNA (Sample D, from Appendix A, Section D) alongside 10  $\mu$ l 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.

### **3. Purification of Digested cDNA (Section E):**

To analyze the yield of purified SMARTer cDNA, electrophorese 10  $\mu$ l of RsaI-digested cDNA before purification (Sample E, from Section D) alongside 10  $\mu$ l of purified diluted cDNA before ethanol precipitation (Sample F, Section E) and 1.8  $\mu$ l 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.

## 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 subtraction (described in Appendix A, Sections B–E) in Table 5.

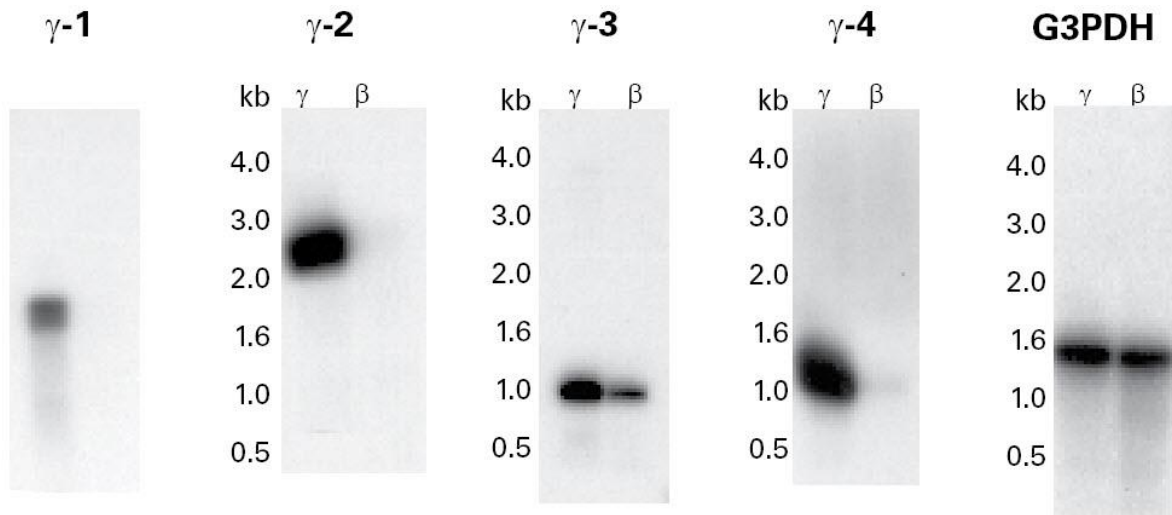
**Table 5. Troubleshooting Guide for Preparing SMARTer cDNA for Subtraction**

<b>Problem</b>	<b>Possible Explanation</b>	<b>Solution</b>
<b>Low yield of cDNA after column chromatography (Appendix A, Section C)</b>	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.
<b>Failure of RsaI Digestion (Appendix A, Section D)</b>	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.
<b>Low yield of cDNA after purification of digested cDNA (Appendix A, Section E)</b>	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 Pico 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 Pico PCR-amplified cDNA (before purification) on an agarose/EtBr gel and use a Southern transfer onto a nylon membrane (see Green & Sambrook, 2012). 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  $\gamma$ -globin.** PCR-Select cDNA subtraction was performed to isolate cDNAs that were preferentially expressed in cells producing  $\gamma$ -globin. 1  $\mu$ g of total RNA from cells producing  $\gamma$ -globin was used as tester; 1  $\mu$ g of total RNA from cells producing  $\beta$ -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  $\mu$ g of SMART cDNA. Subtracted cDNA fragments ( $\gamma$ -1,  $\gamma$ -2,  $\gamma$ -3, and  $\gamma$ -4) were labeled with [ $^{32}$ P]-dCTP and hybridized to the Virtual Northern blots. Hybridization with G3PDH serves as a control for loading. Lane  $\gamma$ : Cells producing  $\gamma$ -globin. Lane  $\beta$ : Cells producing  $\beta$ -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<sub>2</sub>O 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.

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