Super SMART™ PCR cDNA Synthesis Kit User Manual

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I. Introduction

The Super SMART™ PCR cDNA Synthesis Kit provides a novel, PCR-based method for producing high-quality cDNA from nanogram quantities of total RNA. This kit allows you to synthesize high-quality cDNA for array probe generation, cDNA subtraction, "Virtual Northern" blots, or other applications, from as little as 2 ng of total RNA. 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.

Super SMART™ —a modified SMART protocol

To develop the Super SMART method, we modified the traditional SMART protocol by increasing the reaction volumes and performing an additional column purification step (Table I). With this improved protocol, it is possible to use only 2 ng of total RNA compared to the 50 ng required for use in the standard SMART protocol—a 25-fold reduction in sample size. The Super SMART 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 SMART PCR amplification. These modifications produce yields of ds cDNA ranging from 1–2 µg.

SMART™ cDNA synthesis technology

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. This is often the case for long mRNAs, especially if the first-strand synthesis is primed only with oligo(dT) primers, or if the mRNA has a persistent secondary structure. 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 SMART method is able to preferentially enrich for full-length cDNAs.

SMART 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 RT reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART™ Oligonucleotide (patent pending), which has an oligo(G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. 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 SMART Oligonucleotide. In cases where RT pauses before the end of the template, the addition of deoxycytidine nucleotides is much less efficient than with full-length cDNA-RNA hybrids,

I. Introduction *continued*

TABLE I. COMPARISON OF SMART™ PROTOCOLS*

Standard SMART™	Super SMART™
50–1,000 ng total RNA **volume ≥ 3.5 µl**	**2–1,000 ng total RNA** **volume ≥ 50 µl**
SMART first-strand cDNA synthesis **Volume = 10 µl** **1.5 hours**	SMART first-strand cDNA synthesis **Volume = 106 µl** **1.5 hours**
Dilute 1:5 with TE **Volume = 50 µl**	**Purify with NucleoSpin®** **column**
Use 10 µl cDNA for SMART PCR amplification • 100 µl reaction • cycle optimization and scale-up	**Use 79 µl cDNA** for SMART PCR amplification • 100 µl reaction • cycle optimization and scale up
Purify PCR products with NucleoSpin®	Purify PCR products with NucleoSpin®
Yields 1 to 2 µg ds DNA	Yields 1 to 2 µg ds DNA

* Differences between protocols appear in **bold**.

thus the overhang needed for base-pairing with the SMART Oligonucleotide is absent. The SMART 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.

Super SMART™ cDNA synthesis for Array Probes

When used in conjunction with the Super SMART™ PCR cDNA Synthesis Kit, the Atlas SMART cDNA Probe Amplification Kit allows researchers 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^+ RNA—a clear advantage when only limited amounts of tissues or cells are available. In fact, the Atlas SMART cDNA Probe Am-

I. Introduction *continued*

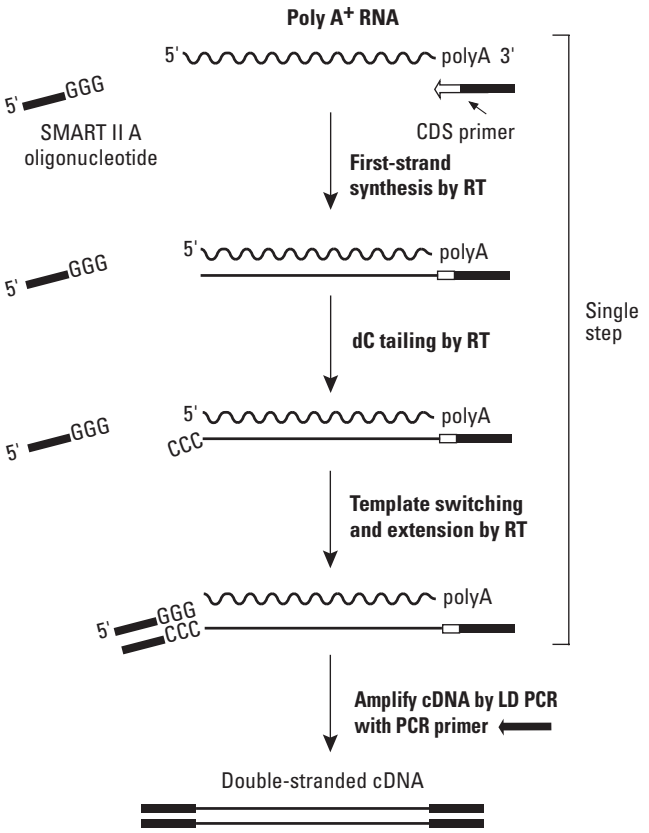


Figure 1. Flow chart of SMART™ technology. The SMART II A Oligonucleotide, 3' SMART CDS Primer II A, and 5' PCR Primer II A all contain a stretch of identical sequence (see Section II for complete sequence information).

plification Kit allows you to generate array probes starting with RNA from as little as 100 cells or 0.01 mg tissue.

Creator™ System Compatibility

The design of the SMART II A oligonucleotide ensures that cDNAs made with this kit are compatible with the Creator™ Cloning and Expression System. The Creator System unites our wide array of expression products,

I. Introduction *continued*

providing access to many strategies for gene function analysis. SMART cDNAs can be cloned into one of our Creator donor vectors, via our Creator Cloning Kits. Once your gene is directionally cloned into a donor vector, it is easily shuttled to a range of expression acceptor vectors, ready for functional analysis.

Super SMART cDNA synthesis for PCR-Select subtraction

The Clontech 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^+ 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 Super SMART PCR cDNA Synthesis Kit can be directly used for PCR-Select subtraction—even when total RNA is used as the starting material.

Virtual Northern blots and probes

SMART 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 SMART cDNA instead of total or poly A^+ RNA (Endege *et al.*, 1999), and can give information similar to that provided by standard Northern blots. For more information on Virtual Northern blots, please see the Appendix.

Synthesize SMART™ cDNA for a wide variety of applications

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 (Cat. No. 634901) **cannot** be used for PCR-Select cDNA subtraction. In the SMART library construction protocol, each PCR-amplified cDNA molecule has an extra SMART sequence on each end which decreases the efficiency of subtraction of cDNA amplified. The SMART II A Oligonucleotide and 3' SMART CDS Primer II A provided in the Super SMART PCR cDNA Synthesis Kit each have an *Rsa* I site to facilitate removal of these identical sequences from the PCR-amplified cDNA molecules.

I. Introduction *continued*

The SMART cDNA synthesis method is also optimized for rapid amplification of cDNA ends (RACE; Matz *et al.,* 1999). The SMART™ RACE Kit (Cat. No. 634914), allows you to perform both 5' and 3' RACE using either poly A^+ RNA or total RNA.

Advantage® 2 PCR Kit

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 comprised of TITANIUM™ *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and a minor amount of a proofreading polymerase. This combination allows you to efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).

II. List of Components

Store NucleoSpin® Extract II Kit at room temperature.
Store Control RNA and SMART II A Oligo at –70°C.
Store all other reagents at –20°C.

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

Box 1:

- 55 µl **SMART II A Oligonucleotide** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
  RsaI
- 55 µl **3' SMART CDS Primer II A** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N-3'
  RsaI
  (N = A, C, G, or T; N$_{-1}$ = A, G, or C)
- 160 µl **5X First-Strand Buffer**
  250 mM Tris-HCl (pH 8.3)
  375 mM KCl
  30 mM $MgCl_2$
- 100 µl **5' PCR Primer II A** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGT-3'
- 80 µl **dNTP Mix** (10 mM of each dNTP)
- 20 µl **Dithiothreitol** (DTT; 100 mM)
- 5 µl **Control Human Placenta Total RNA** (1 µg/µl)
- 1 ml **Deionized H_2O**

Box 2:

- 1 **NucleoSpin® Extract II Kit**
  - NucleoSpin® Extract II Columns
  - NucleoSpin Collecting Tubes (2 ml)
  - Buffer NT
  - Buffer NT3 (Add 95% ethanol before use as specified on the label.)
  - Buffer NE

III. Additional Materials Required

The following reagents are required but not supplied:

- **MMLV Reverse Transcriptase** (Please see Addendum PT3980-4 for details on the choice of RT enzyme.)
- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
- **NucleoSpin® RNA II Kit** (Cat. No. 635990)

  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 SMART cDNA amplification level, resulting in a more representative cDNA pool. Additional Buffer RA1 (Cat. No. 636037) can be purchased separately.
- β-**mercaptoethanol** (Sigma Cat. No. M6250)
- **RNase Inhibitor** (20 U/µl)

  We recommend Ambion's SUPERase•In RNase inhibitor (Cat. No. 2696)
- **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	$Na_2EDTA•2H_2O$

  Add H_2O to 1 L.

Note: If you plan to use the Super SMART 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 Super SMART PCR cDNA Synthesis Kit** and must be purchased separately.

IV. General Considerations

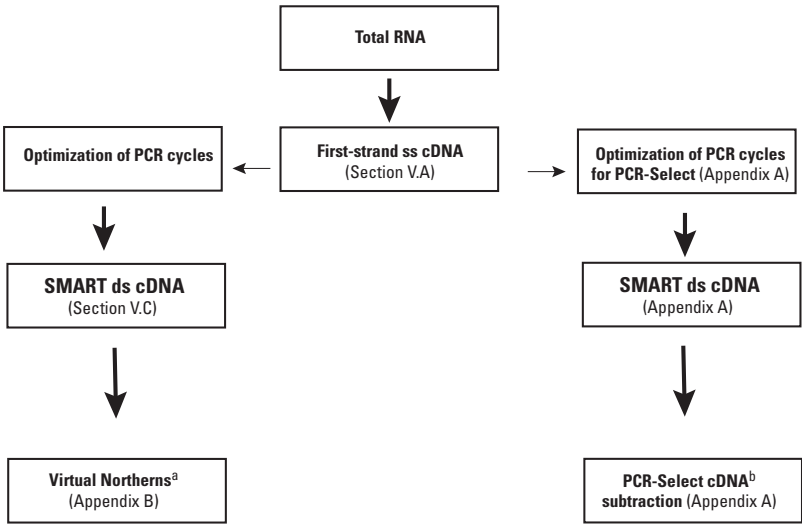
PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

- Whatever your application may be, the success of your experiment depends on the quality of your starting sample of total RNA. For best results we **strongly** recommend that you use the NucleoSpin® II Kit (Cat. No. 635990). Alternatively, you may wish to use one of our premade Premium Total RNAs (visit our web site at **www.clontech.com**).
- Before you begin first-strand synthesis, we strongly recommend that you check the integrity of your RNA by electrophoresing a sample on a formaldehyde/agarose/EtBr gel, if you have sufficient RNA. 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).
- Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.
- The first time you use this kit, you should perform cDNA synthesis with the control human placenta total RNA provided in the kit, in parallel with your experimental sample. Performing this control at least once will verify that all components are working properly, including the reverse transcriptase activity, and will also help you troubleshoot any problems that may arise.
- The cycling parameters in this protocol have been optimized using both hot-lid and non-hot-lid thermal cyclers. Optimal parameters may vary with different thermal cyclers and templates.
- To resuspend pellets and mix reactions, gently pipet them up and down and centrifuge the tube briefly to deposit contents at the bottom.
- Add enzymes to reaction mixtures last, and thoroughly incorporate the enzyme by gently pipetting the reaction mixture up and down.
- Do not increase the amount of enzyme added or concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized.

V. Super SMART™ cDNA Synthesis Protocol

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

If you plan to use cDNA generated by the Super SMART 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. 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-SMART method). In addition, use the control provided in this kit to troubleshoot any problems using the SMART protocol. For more information about using these controls, see Appendix A of this User Manual.



[a]Reagents for these procedures are *not* included in the Super SMART PCR cDNA Synthesis Kit.

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

Figure 2. Guide to using the protocol for SMART™ cDNA synthesis for Virtual Northerns, PCR-Select cDNA subtraction and other applications.

V. Super SMART™ cDNA Synthesis Protocol *continued*

This protocol has been optimized for total RNA. The minimum amount of starting material for standard cDNA synthesis is 2 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 dilute your first strand cDNA product before proceeding with cDNA amplification (Section C).

A. First-Strand cDNA Synthesis

1. For each sample and Control Human Placenta RNA, combine the following reagents in a sterile 0.5 ml reaction tube:

1–50 µl	RNA sample* (2–1,000 ng of total RNA)
7 µl	3' SMART CDS Primer II A (12 µM)
7 µl	SMART II A Oligonucleotide (12 µM)
x µl	Deionized H_2O
64 µl	Total volume

*For the control synthesis, add 10 ng of control human placenta total RNA. PCR-Select users should start with ≥10 ng of total RNA.

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 65°C in a hot-lid thermal cycler for 2 min, then reduce the temperature to 42°C.
4. Add the following to each reaction tube:

20 µl	5X First-Strand Buffer
2 µl	DTT (100 mM)
10 µl	50X dNTP (10 mM)
5 µl	RNase Inhibitor (20 U/ µl)
5 µl	MMLV Reverse Transcriptase*
42 µl	Total added per reaction

*Please see Addendum PT3980-4 for details on the choice of RT enzyme.

5. Gently pipet up and down to mix, then spin the tubes briefly in a microcentrifuge.
6. Incubate the tubes at 42°C for 30 min* in a hot-lid thermal cycler.

   *If you plan to use a downstream application that requires full-length cDNAs, ***extend the incubation time to 90 min***.
7. Add 2 µl of 0.5 M EDTA to stop the reaction. If necessary, cDNA can be stored at -20°C until you are ready to proceed with column chromatography (Section B).

V. Super SMART™ cDNA Synthesis Protocol *continued*

B. Column Chromatography

To purify the SMART 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 212 µl of Buffer NT to each cDNA synthesis reaction; mix well by pipetting.
2. Place a NucleoSpin® Extract II Column into a 2 ml collection tube. Pipet the sample into the column. Centrifuge at 14,000 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. Place the column back into the collection tube. Centrifuge at 14,000 rpm for 2 min to remove any residual Wash Buffer NT3.
5. Transfer the NucleoSpin Columns into a fresh 1.5 ml microcentrifuge tube. Add 50 µl of sterile Milli-Q H_2O to the column. Allow the column to stand for 2 min with the caps open. .
6. Close the tube and centrifuge at 14,000 rpm for 1 min to elute the sample.
7. Repeat elution with 35 µl of sterile Milli-Q H_2O in the same 1.5 ml microcentrifuge tube. The total recovered elution volume should be 80–85 µl per sample. If necessary, add sterile Milli-Q H_2O to bring the total volume up to 80 µl.
8. 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 below. Samples can also be stored at –20°C for up to three months.

C. cDNA Amplification by LD PCR

Table II 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 human placenta total RNA, and both hot-lid and non-hot-lid thermal cyclers; optimal parameters may vary with different templates and thermal cyclers. Additional guidelines, based on the amount of starting material, is also provided in Table III.

In our experience, each 100 µl reaction typically yields 1–2 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 SMART cDNA by UV spectrophotometry.

V. Super SMART™ cDNA Synthesis Protocol *continued*

TABLE II: GUIDELINES FOR SETTING UP PCR

Total RNA (ng)	Volume of ss cDNA[a] (µl)	Volume of H_2O (µl)	Typical optimal No. of PCR cycles[b]
~2	80 µl	–	24–28
~5	80 µl	–	21–24
~25	80 µl	–	17–20
~50	40 µl	40 µl	17–20
~100	25 µl	55 µl	17–20
~250	10 µl	70 µl	17–20
~500	5 µl	75 µl	17–20
~1,000	2.5 µl	77.5 µl	17–20

[a] From Step V.B.8.

[b] 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 II, above) of each first-strand cDNA into a labeled 0.5 ml reaction tube. If necessary, add deionized H_2O to adjust the volume to 80 µl.
3. Prepare a Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

per rxn	
4 µl	Deionized H_2O
10 µl	10X Advantage 2 PCR Buffer
2 µl	50X dNTP (10 mM)
2 µl	5' PCR Primer II A (12 µM)
2 µl	50X Advantage 2 Polymerase Mix
20 µl	Total volume

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 21 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 necessary, overlay the reaction mixture with two drops of mineral oil.

V. Super SMART™ cDNA Synthesis Protocol *continued*

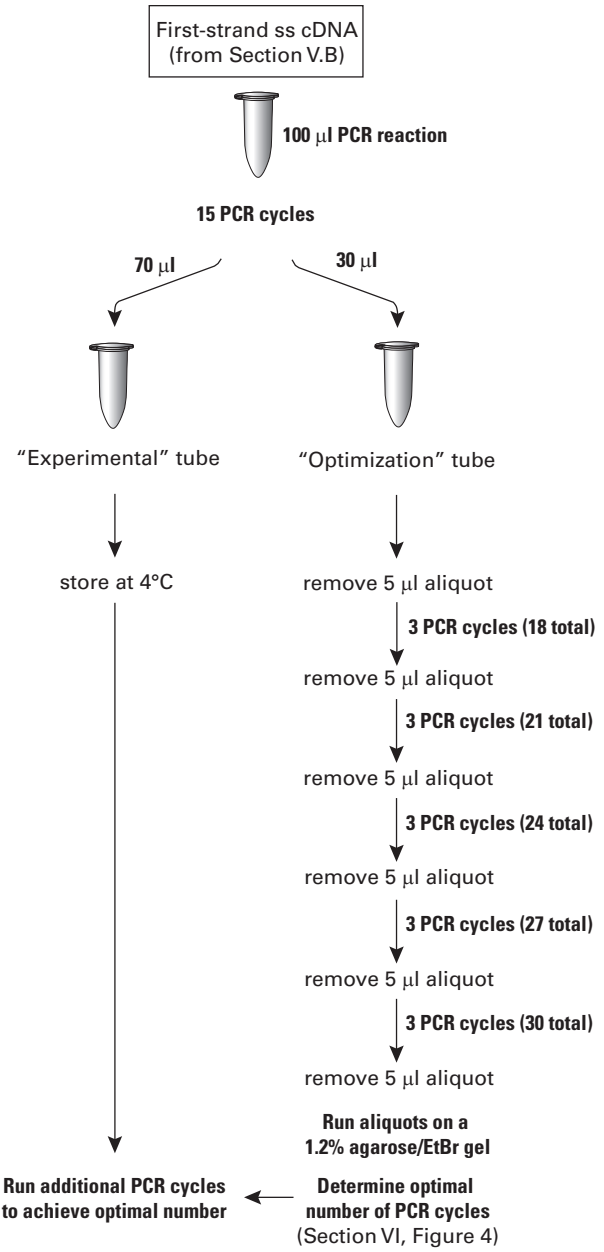


Figure 3. Optimizing PCR parameters for SMART™ cDNA synthesis.

V. Super SMART™ cDNA Synthesis Protocol *continued*

TABLE III: CYCLING GUIDELINES BASED ON STARTING MATERIAL

No. of cells (e.g. HeLa)	Typical yield Total RNA (ng)	Typical No. of PCR cycles*
~10	0.15	27
~100	1.5	24
~1,000	15	18
~10,000	150†	15

*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.

†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 II for dilution guidelines.

7. Commence thermal cycling using the following:

Non-hot-lid thermal cycler:		Hot-lid thermal cyclers:	
• 95°C	1 min	•95°C	1 min
• x cycles[a]:		•x cycles[a]:	
95°C	15 sec	95°C	5 sec
65°C	30 sec	65°C	5 sec
68°C	3 min[b]	68°C	3 min[b]

[a] Consult Table II 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 (below). Store the Experimental tubes at 4°C.

[b] *For applications requiring full-length cDNA, increase to 6 min.*

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 4):
   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.

V. Super SMART™ cDNA Synthesis Protocol *continued*

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.

i. Transfer 5 µl 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 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 1XTAE 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 1XTAE buffer. Compare your results to Figure 4 to confirm that your reactions were successful.

D. Spin Column Purification of PCR Products

Note: For the following steps, use the columns and reagents supplied in the NucleoSpin® Extract II Kit (provided). Before use, be sure to add 95% ethanol directly to Buffer NT3 as specified on the bottle.

1. Add 300 µl NT Buffer to each 70-µl PCR reaction. Mix well by pipetting.
2. Place a NucleoSpin® column into a 2 ml Collection Tube, and pipet the sample onto the filter. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough.
3. Insert the NucleoSpin® column into a fresh 2-ml Collection Tube. Add 500 µl Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard flowthrough.
4. Repeat Step 5 twice.
5. Discard flowthrough and spin again at 14,000 rpm for 1 min to remove final traces of ethanol to dry filter.

V. Super SMART™ cDNA Synthesis Protocol *continued*

6. Transfer the NucleoSpin® column to a clean 1.5 ml microcentrifuge tube. Pipette 50 µl Buffer NE directly onto the filter, being careful not to touch the filter surface with the pipette tip. Allow 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 column.
8. Determine the yield of each PCR product by measuring the A_{260}. For each reaction, we usually obtain 1–2 µg of SMART cDNA after purification.
9. If no product is detected, perform elution (Steps 8 and 9) a second time, using a fresh 1.5 ml microcentrifuge tube.

You now have SMART 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 Human Placenta Total RNA for SMART cDNA synthesis and amplification. As indicated by the arrow, you should observe a distinct band at 900 bp. 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 6 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 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.

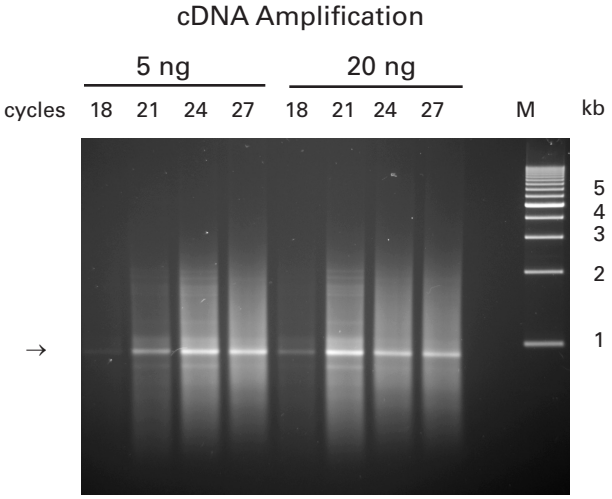


Figure 4. Analysis for optimizing PCR parameters. 5 ng or 20 ng of the control human placental total RNA was subjected to the first strand cDNA synthesis and purification as described in the protocol. 80 µl was used for PCR amplification. A range of PCR cycles were performed (18, 21, 24, and 27). 5 ul of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1XTAE buffer following the indicated number of PCR cycles. The optimal number of cycles determined in this experiment was 23 for the 5 ng reaction, and 20 for the 20 ng reaction. Lane M: 1 kb DNA ladder size markers. The arrow indicates the strong band at 900 bp typically seen for human placenta total RNA.

VI. Analysis of cDNA Amplification Results *continued*

Figure 4 provides an example of how your analysis should proceed. In this experiment, the PCR reached its plateau after 24 cycles for the 5 ng experiment and 21 cycles for the 20 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 5 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 Human Placenta 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.

VII. Troubleshooting Guide

First-Strand cDNA Synthesis and SMART™ PCR Amplification

1. Low molecular weight (size distribution < 3 kb, with a majority between 500-200 bp), poor yield, or no PCR product observed for the control placenta total RNA
   a. 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 Related Products for ordering information).
   b. 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.
   c. 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.
2. Poor yield or truncated PCR product from your experimental RNA

   If the reaction with the control human placenta total RNA was successful, but your experiment failed, your experimental RNA sample may be too dilute or degraded, or may contain impurities that inhibit first-strand synthesis. 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 for more details).
   a. The concentration of your experimental RNA is low, but the quality is good.

      Repeat the experiment using more RNA and/or more PCR cycles.

VII. Troubleshooting Guide

b. 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 IX for ordering information).

c. 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 IX for ordering information).

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IX. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

Appendix A: Protocols 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. With slight modifications to the standard protocol, Super SMART cDNA can be used directly for PCR-Select cDNA subtraction.

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 reagents are required for PCR-Select but are not supplied:

- **CHROMA SPIN™-1000 DEPC-H_2O 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 to the remaining layer. Mix thoroughly. Remove and discard the top layer.
  6. Store the bottom layer of phenol:chloroform:isoamyl alcohol (24:1) at 4°C away from light for a maximum of two weeks.

  - **TE buffer** (10 mM Tris [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)
- **NucleoTrap® Purification Kit** (Cat. No. 636020)

  NucleoTrap Suspension

  80 ml Buffer NT2

  16 ml Buffer NT3

Appendix A: Protocols for PCR-Select *continued*

B. 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 II (see Section V). These guidelines were determined using the control human placenta total RNA and both hot-lid and non-hot-lid thermal cyclers; 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 SMART 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 SMART 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.B.8 into a labeled 1.5-ml reaction tube.
3. Prepare a Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

per rxn	
172 µl	Deionized H_2O
30 µl	10X Advantage 2 PCR Buffer
6 µl	50X dNTP (10 mM)
6 µl	5' PCR Primer II A (12 µM)
6 µl	50X Advantage 2 Polymerase Mix
220 µl	Total volume

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 necessary, overlay the reaction mixture with two drops of mineral oil.

Appendix A: Protocols for PCR-Select *continued*

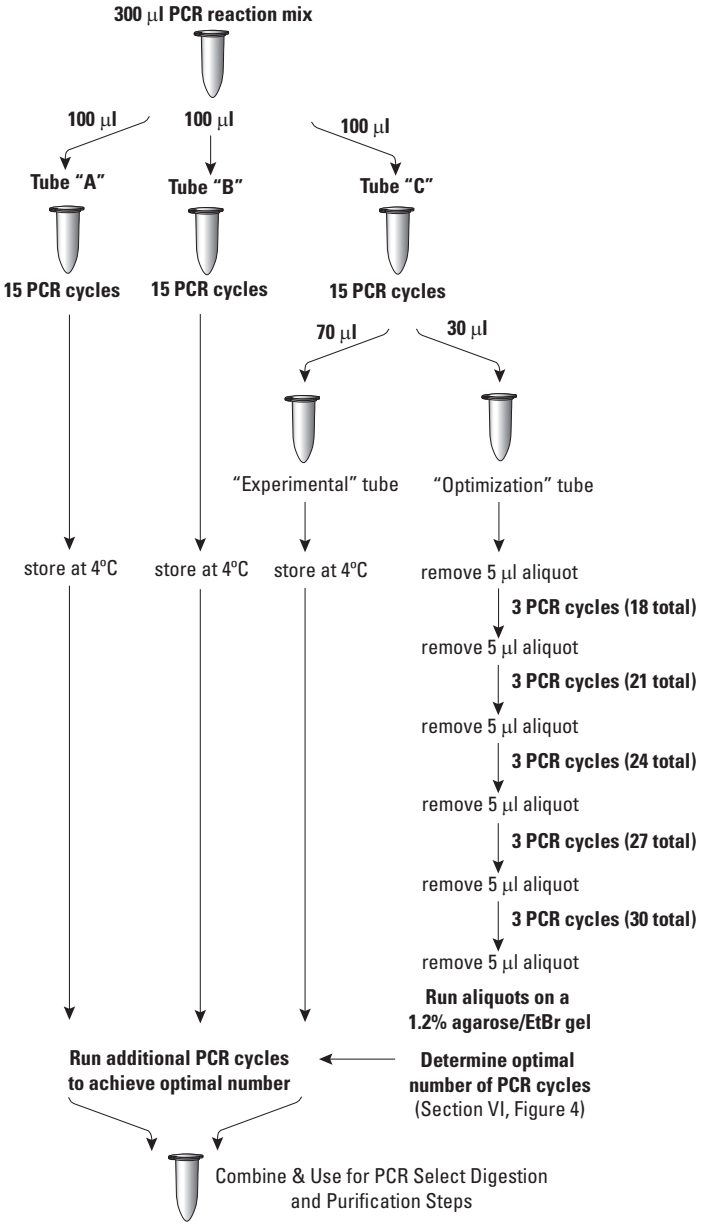

Figure 5. Optimizing PCR parameters for SMART™ cDNA synthesis for use with Clontech PCR-Select™.

Appendix A: Protocols for PCR-Select *continued*

8. Commence thermal cycling using the following:

Non-hot-lid thermal cycler:		Hot-lid thermal cycler:	
• 95°C	1 min	• 95°C	1 min
• x cycles[a]:		• x cycles[a]:	
95°C	15 sec	95°C	5 sec
65°C	30 sec	65°C	5 sec
68°C	3 min[b]	68°C	3 min[b]

[a] Consult Table II 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 8 (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 4):
   a. Transfer 5 µl from the 15 cycle PCR 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 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.
   i. Transfer 5 µl from the 27 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

Appendix A: Protocols for PCR-Select *continued*

j. Run three additional cycles (for a total of 30) with the remaining 5 µl of PCR mixture.

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 1XTAE 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 1XTAE 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. 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 H_2O to the aqueous phase to adjust volume to 40–70 µl.

Appendix A: Protocols for PCR-Select *continued*

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 µl of 1X TNE buffer and allow the buffer to completely drain out of the column.
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. 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 Section G.2.

D. RsaI Digestion

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

Appendix A: Protocols for PCR-Select *continued*

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 (Step C.21):

10X RsaI restriction buffer	36 µl
RsaI (10 units)	1.5 µl

2. Mix 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 1XTAE 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 Section G.4.

E. Purification of Digested cDNA

You may purify your digested cDNA using any silica matrix-based PCR purification system, such as those offered by Clontech (see Section IX). 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 SMART ds cDNA and our NucleoTrap® PCR Kit (Cat. No. 636020; not included with PCR-Select Kit).

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

1. Aliquot the RsaI-digested cDNA (Step 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.

Appendix A: Protocols for PCR-Select *continued*

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 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 SMART cDNA after purification, as described in 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.

Appendix A: Protocols for PCR-Select *continued*

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 SMART 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 Section G.4.
23. If your DNA concentration is >300 ng/µl, dilute your cDNA to a final concentration of 300 ng/µl in 1XTNE 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. 636020). 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$^+$ 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 **human placenta total RNA** (included in the SMART kit)

   Use the SMART kit to amplify the control human placenta total RNA; then, perform a mock subtraction as described for control #1: use a portion of the human placenta 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 SMART 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 (Section E).

Appendix A: Protocols for PCR-Select *continued*

G. Analysis of Results for PCR-Select Subtraction

Figure 4 shows a typical gel profile of ds cDNA synthesized using the control human placenta RNA and the SMART protocol outlined in Section VII. As indicated by the arrow, you should observe a strong, distinct band at 900 bp. In general, cDNA synthesized from mammalian total RNA should appear on a 1.2% agarose/EtBr gel as a moderately strong smear from 0.5–6 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 non-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 human placenta 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 18 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 18 cycles, the optimal number of cycles for this experiment would be 17.

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 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 SMART 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 NucleoTrap PCR 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. Here, we provide a troubleshooting guide for preparing SMART cDNA for subtraction (described in Appendix A, Sections B–E).

1. Low yield of cDNA after column chromatography (Section C)

   Possible reasons for low yield include the following:

   a. 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.

   b. Your column may have leaked during shipping. If your column contains less than 750 µl of matrix, discard it and use another column.

2. Failure of RsaI digestion (Section D)

   If the size distribution of your sample and/or control cDNA is not reduced after RsaI digestion, 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.

3. Low yield of cDNA after purification of digested cDNA (Section E)

   Possible reasons for low yield include the following:

   a. Loss of cDNA during purification. Troubleshoot your purification procedure.

   b. Loss of cDNA during ethanol precipitation. Check the volumes of the ammonium acetate and ethanol. Repeat purification and ethanol precipitation.

   c. 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 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 SMART 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 SMART 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.

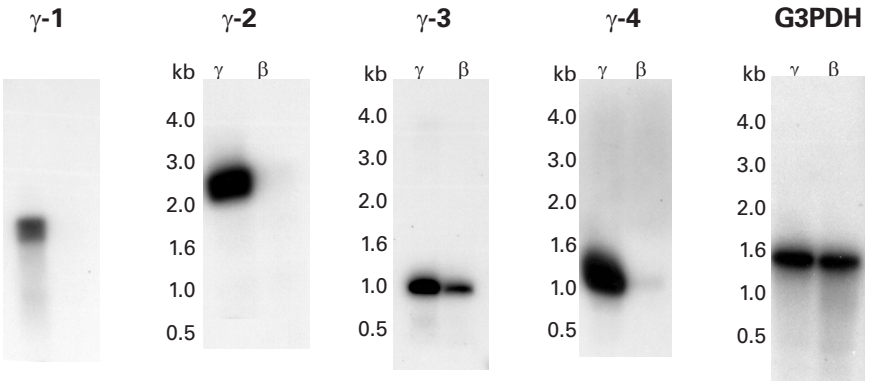

Figure 6. Virtual Northern blot analysis of cDNA fragments expressed in cells producing γ-globin. PCR-Select cDNA subtraction was performed to isolate cDNAs that were preferentially expressed in cells producing γ-globin. 1 µg of total RNA from cells producing γ-globin was used as tester; 1 µg of total 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 [^{32}P]-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.

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