SMART™ PCR cDNA Synthesis Kit User Manual

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I. Introduction

The SMART™ PCR cDNA Synthesis Kit provides a novel, PCR-based method for producing high-quality cDNA from nanograms of total or poly A^+ RNA. SMART technology is especially useful for researchers who have limited starting material, such as total RNA from a small sample.

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) cDNA in the first-strand reaction. However, because RT cannot always transcribe the entire mRNA sequence, the 5' ends of genes tend to be under-represented 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 RT pausing before transcription is complete. Regardless, the SMART method is able to preferentially enrich for full-length cDNAs.

SMART cDNA synthesis starts with either total or poly A^+ 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™ II A Oligonucleotide, 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, thus preventing base-pairing with the SMART Oligonucleotide. The SMART anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. Therefore, cDNA without these sequences due to prematurely terminated cDNAs caused by incomplete RT activity, 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, which will contaminate the final cDNA library.

Synthesize SMART™ cDNA for a wide variety of applications

The first kit to feature SMART technology is the SMART cDNA Library Construction Kit (Cat. No. 634901). This kit includes the components for directional cloning of full-length cDNA. To expand the range of applications, the SMART PCR cDNA Synthesis Kit (Cat. No. 634902; Figure 2) was introduced shortly after. This kit allows you to synthesize high-quality cDNA for library construction using your own vector and ligation reagents. Other applications include Clontech PCR-Select™

I. Introduction *continued*

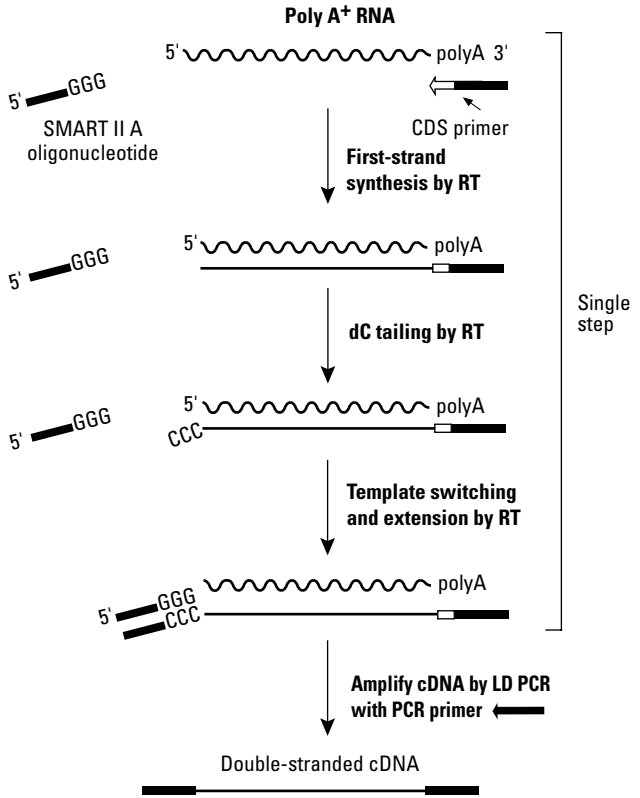


Figure 1. Flow chart of SMART™ technology. The SMART II A Oligonucleotide, 3' SMART CDS Primer II A, and 5' PCR Primer II A all contain a stretch of identical sequence (see Section II for complete sequence information).

cDNA Subtraction (Cat. No. 637401), "Virtual" Northern blots, and probe generation. Please note that the SMART II™ A Oligonucleotide is specially engineered for use with the PCR-Select method. cDNA generated using the SMART cDNA Library Construction Kit **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 amplified cDNA.

I. Introduction *continued*

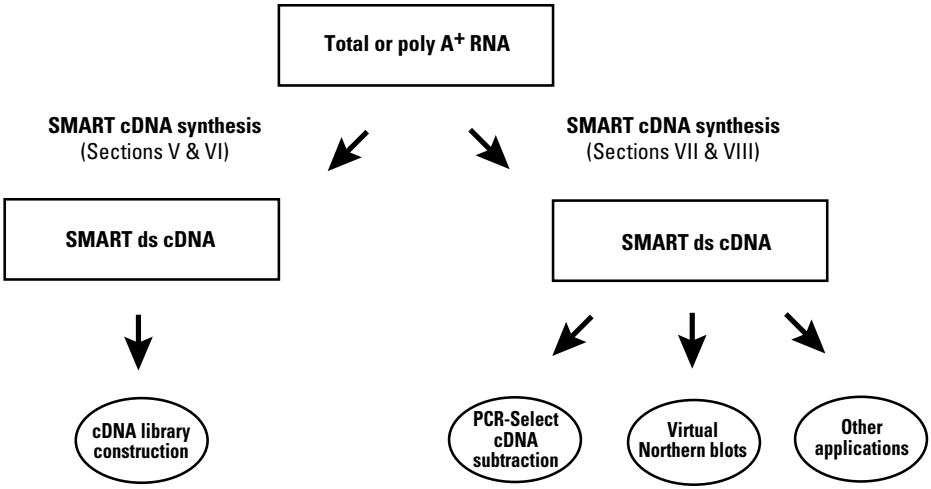


Figure 2. Guide to SMART™ cDNA synthesis protocols. Be sure to follow the appropriate protocol for your application.

The SMART II A Oligonucleotide and 3' SMART CDS Primer II A provided in the SMART PCR cDNA Synthesis Kit each have an *Rsa* I site to facilitate removal of these identical sequences from the PCR-amplified cDNA molecules.

This User Manual includes two protocols for cDNA synthesis. These protocols have been designed to strike a balance between maintaining gene representation and reducing nonspecific background amplification. In the first protocol (Sections V and VI), undiluted first-strand ss cDNA is subjected to the fewest possible number of PCR cycles. This protocol is ideal for cDNA library construction, where high representation is most important (Zhu *et al.*, 2001). In the second protocol (Sections VII and VIII), the first-strand ss cDNA template is diluted and more PCR cycles are performed. This greatly reduces nonspecific amplification, which is crucial for PCR-Select cDNA subtraction and other non-library applications. Be sure to choose the appropriate protocol for your application.

The SMART cDNA synthesis method is now optimized for rapid amplification of cDNA ends (RACE; Matz *et al.,* 1999). The SMART™ RACE cDNA Amplification Kit (Cat. No. 634914) integrates our Marathon® cDNA Amplification Kit (Chenchik *et al.,* 1995; 1996) with our SMART cDNA synthesis technology and allows you to perform both 5' and 3' RACE using either poly A^+ or total RNA. Clontech has rigorously tested our new SMART RACE Kit to verify that it performs even better than the Marathon Kit (January 1999 *Clontechniques*).

I. Introduction *continued*

SMART™ cDNA synthesis for cDNA subtraction

The Clontech PCR-Select cDNA Subtraction Kit (Cat. No. 637401) provides a powerful method for identifying differentially expressed genes (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 SMART PCR cDNA Synthesis Kit can be directly used for PCR-Select subtraction—even if total RNA was used as starting material.

Virtual Northern blots and probes

The SMART PCR cDNA Synthesis Kit 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 Clontech 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.

Other applications for SMART cDNA include preparing probes for hybridization to high-density cDNA or genomic DNA arrays (Pietu *et al.*, 1996) or for the cDNA selection-based positional cloning method (Morgan *et al.*, 1992). Please see these references for more information about these applications.

Advantage® 2 PCR Kit and PowerScript™ Reverse Transcriptase

We strongly recommend the use of the Advantage 2 PCR Kits (Cat. Nos. 639206 & 639207) for PCR amplification. These kits include 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 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).

Each SMART kit also includes PowerScript™ Reverse Transcriptase, a point mutant of Moloney murine leukemia virus (MMLV) reverse transcriptase (RT). PowerScript RT lacks RNase H activity, but retains wild-type polymerase activity, so it can synthesize longer cDNA fragments than wild-type MMLV RT. Our rigorous purification method also ensures that each PowerScript preparation is not contaminated with RNase and DNase.

II. List of Components

Store CHROMA SPIN and Microfiltration columns at room temperature.
Store 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 read the Notice to Purchaser at the end of this User Manual.

Box 1:

- 7 µl **SMART II™ A Oligonucleotide** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
  Rsa I
- 7 µl **3' SMART™ CDS Primer II A** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGTAC$T_{(30)}$V N-3'
  (N = A, C, G, or T; V = A, G, or C) *Rsa* I
- 7 µl **PowerScript™ Reverse Transcriptase**
- 200 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'
- 70 µl **dNTP Mix** (10 mM of each dNTP)
- 200 µl **Dithiothreitol** (DTT; 20 mM)
- 5 µl **Control Human Placental Total RNA** (1 µg/µl)
- 1 ml **Deionized H_2O**

Box 2:

- 7 **CHROMA SPIN™ 1000 Columns**
- 7 **Microfiltration Columns** (0.45 µm)

III. Additional Materials Required

The following reagents are required but not supplied:

First-strand cDNA synthesis and SMART™ PCR cDNA amplification

- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
- [Optional] **Mineral oil** (Sigma Cat. No. M3516**)**
- **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)
- **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.

ds cDNA polishing for library construction

- **Proteinase K** (20 µg/µl; Roche Applied Science Cat. No. 0161519)
- **T4 DNA Polymerase** (New England Biolabs Cat. No. M0203S)

Purification for Clontech PCR-Select™ cDNA Subtraction

- **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

IV. General Considerations

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

- This kit is designed for the construction of high-quality SMART cDNA for a variety of applications. This User Manual provides two protocols for cDNA synthesis: one for cDNA library construction (Sections V and VI), and one for other applications, including Clontech PCR-Select cDNA Subtraction (Sections VII and VIII). **Be sure to follow the appropriate protocol for your application (see Figure 2).**

- Proper template switching, which is essential to the SMART technology, requires the use of an MMLV RNase H^- point mutant (*not* deletion mutant) reverse transcriptase such as PowerScript Reverse Transcriptase (included with each SMART Kit).

- The protocols have been optimized for both total and poly A^+ RNA. The minimum amount of starting material for cDNA synthesis is 50 ng of total RNA or 25 ng of poly A^+ RNA. However, if your RNA sample is not limiting, we recommend that you start from 1 µg of total RNA or 0.5 µg of poly A^+ RNA for cDNA synthesis.

- Whatever your application may be, the success of your experiment depends on the quality of your starting sample of total or poly A^+ RNA. There are several procedures available for RNA isolation (Chomczynski & Sacchi, 1987; Farrell, 1993; Sambrook *et al.*, 2001). In addition, Clontech offers several kits for the isolation of total RNA and subsequent isolation of poly A^+ RNA. Alternatively, you may wish to use one of our Premium Poly A^+ RNAs. For more information, 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. 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, respectively. The ratio of intensities of these bands should be 1.5–2.5:1. Intact mammalian poly A^+ RNA should appear as a smear (usually 0.5–12 kb) with faint 28S and 18S rRNA bands. The size distribution may be considerably smaller (0.5–3 kb) for nonmammalian species (e.g., plants, insects, yeast, and amphibians). For more information, see Sambrook *et al.* (2001).

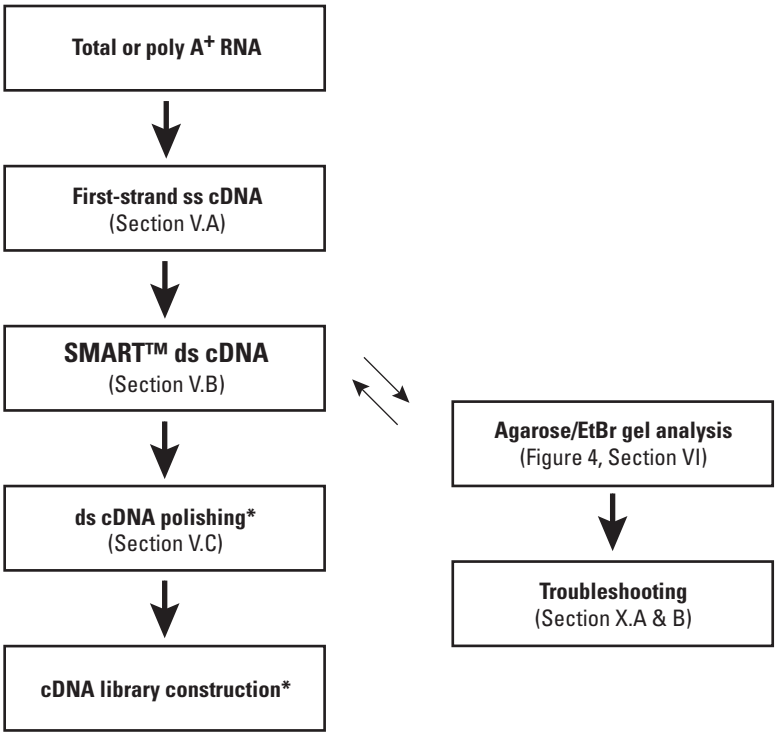
- Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.

IV. General Considerations *continued*

- The first time you use this kit, you should perform cDNA synthesis with the Control Human Placental Total RNA provided in the kit, in parallel with your experimental sample. Performing this control synthesis at least once will verify that all components (especially the reverse transcriptase) are working properly and will also help you troubleshoot any problems that may arise.
- The cycling parameters in this protocol have been optimized using an authorized hot-lid thermal cycler. 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.
- Vortex phenol:chloroform extractions to mix.
- 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. SMART™ cDNA Synthesis for Library Construction

Important: This protocol is designed for synthesizing SMART cDNA *for library construction.* For other applications, including Clontech PCR-Select cDNA subtraction, consult the protocol in Sections VII and VIII.



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

Figure 3. Protocol guide for SMART™ cDNA synthesis for library construction. If agarose/EtBr gel analysis of the ds cDNA indicates that more cycles are needed, simply return the reaction to the thermal cycler for a few more cycles, as described in the Troubleshooting Guide (Section X.B).

V. SMART cDNA Synthesis for Library... *continued*

A. First-Strand cDNA Synthesis

1. For each sample and control, combine the following reagents in a sterile 0.5-ml reaction tube:

   1–3 µl RNA sample*
   (0.025–0.5 µg of poly A^+ or 0.05–1 µg of total RNA)
   1 µl 3' SMART CDS Primer II A (12 µM)
   1 µl SMART II A Oligonucleotide (12 µM)
   x µl Deionized H_2O
   5 µl Total volume

   * For the control synthesis, add 1 µl (1 µg/µl) of Control Human Placental Total RNA.
2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 72°C for 2 min.
4. Cool the tube on ice for 2 min.
5. Centrifuge the tube briefly in a microcentrifuge to collect contents at the bottom.
6. Add the following to each reaction tube:
   2 µl 5X First-Strand Buffer
   1 µl DTT (20 mM)
   1 µl dNTP Mix (10 mM of each dNTP)
   1 µl PowerScript Reverse Transcriptase
7. Mix by gently pipetting and spin the tubes briefly in a microcentrifuge.
8. Incubate the tubes at 42°C for 1 hr in an air incubator.

   Note: If you use a water bath or thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.
9. Place the tube on ice to terminate first-strand synthesis.
10. If you plan to proceed directly to the PCR step (Section V.B), transfer a 2-µl aliquot from the first-strand synthesis to a clean, prechilled, 0.5-ml reaction tube. Place tube on ice. If you used mineral oil in your first-strand reaction tube, be careful to take the aliquot from the **bottom** of the tube to avoid the oil.
11. Any first-strand reaction mixture that is not used right away should be placed at –20°C. First-strand cDNA can be stored at –20°C for up to three months.

V. SMART cDNA Synthesis for Library... *continued*

B. cDNA Amplification by LD PCR

Table I provides guidelines for the optimal number of thermal cycles for a given amount of total or poly A$^+$ RNA used in the first-strand synthesis. These guidelines were developed using the Control Human Placental Total RNA and an authorized hot-lid thermal cycler; optimal parameters may vary with different templates and thermal cyclers. Use the **fewest** cycles possible; overcycling may yield nonspecific PCR products. If necessary, undercycling can be easily rectified by placing the reaction back in the thermal cycler for a few more cycles (see Troubleshooting Guide, Section X.B).

TABLE I: PCR CYCLING PARAMETERS (LIBRARY CONSTRUCTION)

Total RNA (µg)	Poly A$^+$ RNA (µg)	Number of Cycles
1.0–2.0	0.5–1.0	13–15
0.5–1.0	0.25–0.5	15–18
0.25–0.5	0.125–0.25	18–21
0.05–0.25	0.025–0.125	21–24

1. Preheat a thermal cycler to 95°C.
2. Prepare a Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

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

3. Mix well by vortexing and centrifuge the tube briefly in a microcentrifuge.
4. Aliquot 98 µl of the Master Mix into each reaction tube from Step A.10.
5. Mix contents by gently flicking the tubes. Centrifuge tubes briefly in a microcentrifuge.
6. Cap the tube, and place it in the preheated thermal cycler. If necessary, overlay the reaction mixture with 2 drops of mineral oil.

V. SMART cDNA Synthesis for Library... *continued*

7. Commence thermal cycling using the following program:
   - 95°C 1 min
   - x cycles*:
     95°C 15 sec
     65°C 30 sec
     68°C 6 min

   *Consult Table I for guidelines.
8. When the cycling is completed, electrophorese 5 µl of each sample on a 1.1% agarose/EtBr gel in 1X TAE buffer. For comparison, Figure 4 shows the characteristic gel profile of ds cDNA synthesized from the Control Human Placental Total RNA (Section VI).

C. ds cDNA Polishing

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

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

    Note: Do not chill the tube at –20°C or on ice before centrifuging. Chilling the sample will result in coprecipitation of impurities.
13. Carefully remove the supernatant.
14. Wash pellet with 80% ethanol.
15. Air dry the pellet (~10 min) to evaporate residual ethanol.
16. Add deionized H_2O to resuspend the pellet. The amount added will depend on your cDNA library construction protocol.

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

VI. Analysis of Results for Library Construction

Figure 4 shows a typical gel profile of ds cDNA synthesized using the Control Human Placental Total RNA and the SMART protocol outlined in Section V. The sample shown was taken after Step V.B.8 and represents "raw" cDNA before polishing. Typical results, indicative of a successful PCR, should have the following characteristics:

1. A moderately strong smear of cDNA from 0.5 to 6 kb

   Compare the intensity of the banding pattern of your PCR product to the 1-kb DNA ladder size marker (0.1 µg run on the same gel). For cDNA made from all mammalian RNA sources, the overall signal intensity (relative to the marker DNA) should be roughly similar to that shown for the control experiment in Figure 4. If the intensity of the cDNA smear is much stronger than that shown for the control (relative to 0.1 µg of size marker), especially if no bright bands are distinguishable, this may indicate that too many thermal cycles were used—that is, you have overcycled your PCR (see Troubleshooting Guide, Section X.B). If the smear is much fainter (relative to 0.1 µg of size marker) and the size distribution generally less than 4 kb, then too few thermal cycles (i.e., PCR undercycling) may be the problem (see Troubleshooting Guide, Section X.B).

2. Several bright bands corresponding to abundant transcripts

   The pattern of bright bands shown in Figure 4 is characteristic of the ds cDNA synthesized from the Control Human Placental Total RNA using the protocol outlined in Section V. As indicated by the arrow in Figure 5, you should observe a strong, distinct band at 900 bp. A very strong smear of cDNA in the control reaction without the characteristic bright bands may be indicative of PCR overcycling (see Troubleshooting Guide, Section X.B). If the characteristic bands are present but weak, this may be indicative of PCR undercycling (see Troubleshooting Guide, Section X.B). The number and position of the bands you obtain with your experimental RNA may differ from those shown for the control reaction. Furthermore, cDNA prepared from some mammalian tissue sources (e.g., human brain, spleen, and thymus) may not display any bright bands, due to the very high complexity of the poly A^+ RNA.

VI. Analysis of Results for Library Construction *continued*

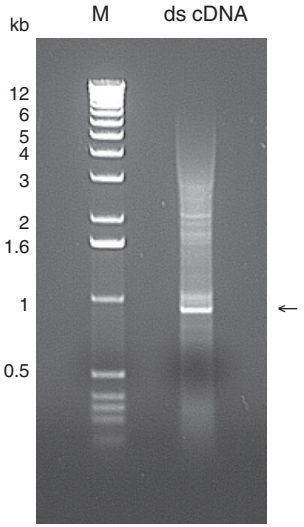


Figure 4. Analysis of ds cDNA synthesized for library construction. 1 µl (1.0 µg) of the Control Human Placental Total RNA provided in the kit was used as starting material in a first-strand cDNA synthesis. 2 µl of the ss cDNA then served as template for LD PCR-based second-strand synthesis using 15 thermal cycles, according to the protocol in Section V. A 5-µl sample of the PCR product (i.e., ds cDNA) was electrophoresed on a 1.1% agarose/EtBr gel. Lane M: 1-kb DNA ladder size markers, 0.1 µg loaded. The arrow indicates the strong band at 900 bp typically seen for human placental total RNA.

VII. SMART cDNA Synthesis Protocol

Important: This protocol is designed for synthesizing SMART cDNA for *applications other than library construction*, such as Clontech PCR-Select™ cDNA Subtraction or Virtual Northern Blots (See Appendix). To synthesize SMART cDNA for library construction, use the protocol in Sections V and VI.

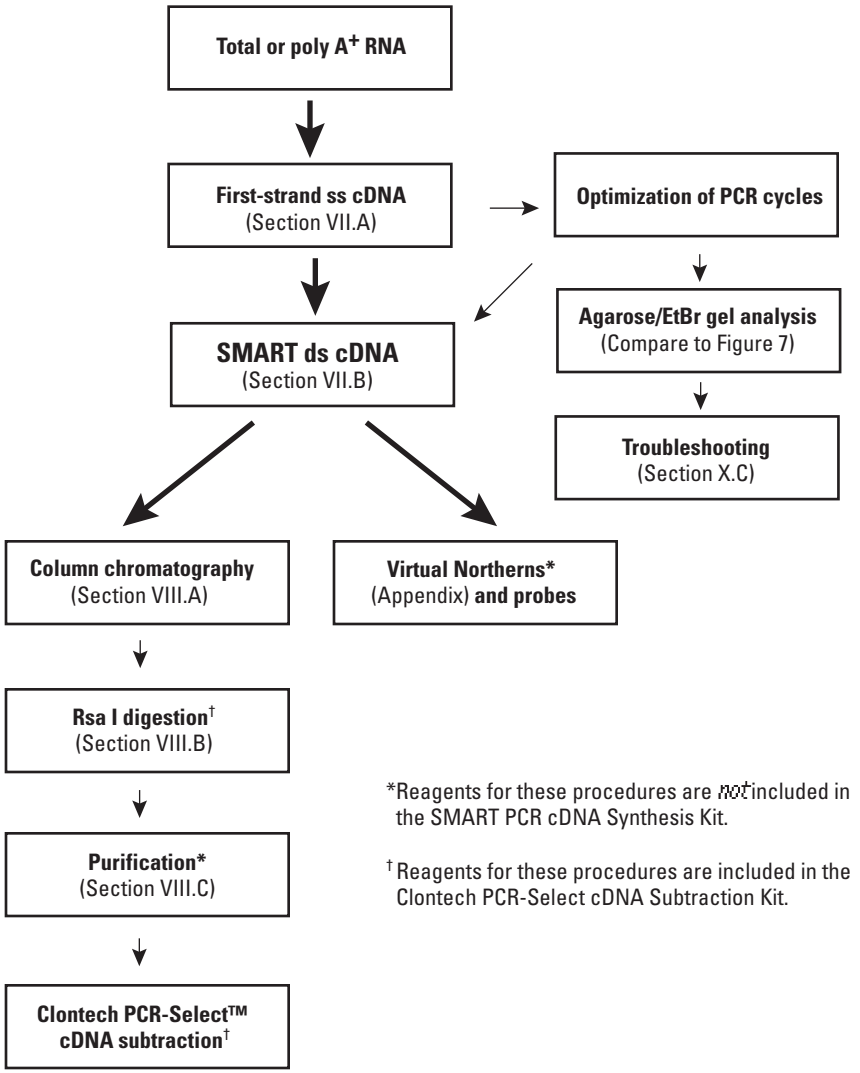


Figure 5. Protocol guide for SMART cDNA synthesis for PCR-Select cDNA subtraction and other applications.

VII. SMART cDNA Synthesis Protocol *continued*

Important: If you are planning to proceed with the Clontech PCR-Select cDNA Subtraction protocol, we recommend reading the User Manual for cDNA Subtraction before proceeding with first strand cDNA synthesis using the SMART method. The cDNA Subtraction Kit supplies a different RNA control that should be used to synthesize cDNA according to the PCR-Select User Manual (which describes 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 Section VIII.D of this User Manual.

A. First-Strand cDNA Synthesis

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

1–3 µl	RNA sample* (0.025–1 µg of poly A^+ or 0.05–1 µg of total RNA)
1 µl	3' SMART CDS Primer II A (12 µM)
1 µl	SMART II A Oligonucleotide (12 µM)
x µl	Deionized H_2O
5 µl	Total volume

*For the control synthesis, add 1 µl (1 µg/µl) of Control Human Placental Total RNA.

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 70°C in a thermal cycler for 2 min.
4. Spin the tube briefly in a microcentrifuge to collect contents at the bottom. Keep tube at room temperature.
5. Add the following to each reaction tube:

2 µl	5X First-Strand Buffer
1 µl	DTT (20 mM)
1 µl	dNTP Mix (10 mM of each dNTP)
1 µl	PowerScript Reverse Transcriptase

6. Gently vortex and spin the tubes briefly in a microcentrifuge.
7. Incubate the tubes at 42°C for 1 hr in an air incubator.

   Note: If you use a water bath or thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.
8. Dilute the first-strand reaction product by adding the appropriate volume of TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA):
   - Add 40 µl of TE buffer if you used total RNA as starting material.
   - Add 450 µl of TE buffer if you used more than 0.2 µg of poly A^+ RNA as starting material.
   - Add 90 µl of TE buffer if you used less than 0.2 µg of poly A^+ RNA as starting material.

VII. SMART cDNA Synthesis Protocol *continued*

9. Heat tubes at 72°C for 7 min.
10. Samples can be stored at –20°C for up to three months.

B. cDNA Amplification by LD PCR

Table II provides guidelines for optimizing your PCR, depending on the amount of total or poly A^+ RNA used in the first-strand synthesis. These guidelines were determined using the Control Human Placental Total RNA and an authorized 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, and 24 cycles (Figure 6).

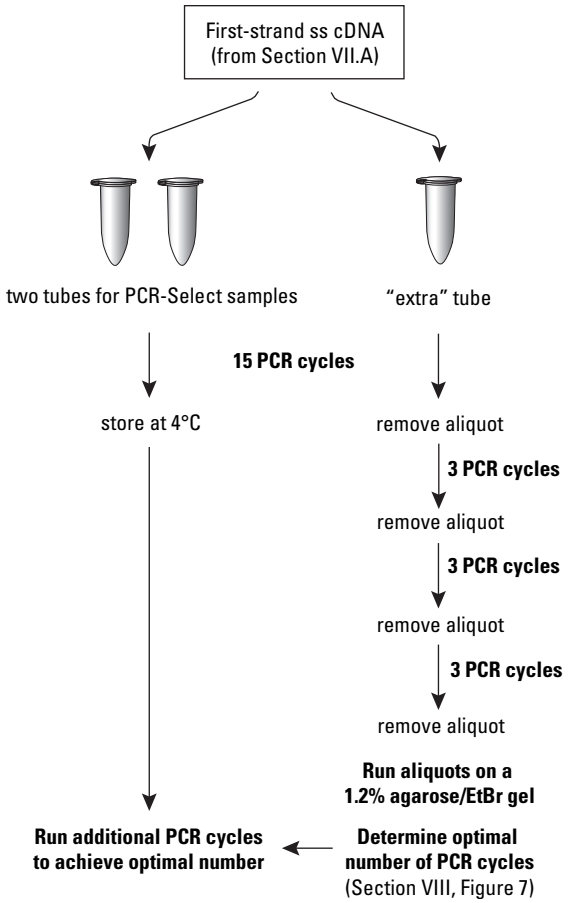


Figure 6. Optimizing PCR parameters for SMART™ cDNA synthesis. Note that for samples not used for cDNA subtraction, you will only have two tubes per sample or control: one experimental sample tube and one "extra" tube.

VII. SMART cDNA Synthesis Protocol *continued*

For each sample and control, set up an extra reaction tube to determine the optimal number of PCR cycles. If you plan to use your SMART cDNA for Clontech PCR-Select cDNA subtraction, you should set up a total of three tubes for each tester and driver sample (Figure 6). In our experience, each 100-µl reaction typically yields 1–3 µg of ds cDNA after the PCR and purification steps (Section VIII). Subtraction usually requires 2 µg of driver cDNA, so two tubes of SMART cDNA should be sufficient; two 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 a thermal cycler to 95°C.
2. For each reaction, aliquot the appropriate volume (see Table II, below) of each diluted cDNA into a labeled 0.5-ml reaction tube. If necessary, add deionized H_2O to adjust the volume to 10 µl.

TABLE II: GUIDELINES FOR SETTING UP PCR

Total RNA (µg)	Volume of diluted ss cDNA* for PCR (µl)	Typical optimal No. of PCR cycles
~1.0	1 µl	17–19
~0.5	2 µl	17–19
~0.25	4 µl	17–19
~0.1	10 µl	17–19
~0.05	10 µl	19–21
Poly A⁺ RNA (µg)	**Volume of diluted ss cDNA* for PCR (µl)**	**Typical optimal No. of PCR cycles**
~1.0	1 µl	16–18
~0.5	2 µl	16–18
~0.1–0.25	4 µl	16–18
~0.05	8 µl	16–18
~0.025	10 µl	17–19

*From Step VII.A.10.

VII. SMART cDNA Synthesis Protocol *continued*

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

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

4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 90 µl of the PCR Master Mix into each tube from Step 2.
6. Cap the tube, and place it in the preheated thermal cycler. If necessary, overlay the reaction mixture with two drops of mineral oil.
7. Commence thermal cycling using the following program:
   - 95°C 1 min
   - x cycles*:
     95°C 15 sec
     65°C 30 sec
     68°C 6 min

   *Consult Table II for guidelines. **Subject *all* tubes to 15 cycles**. Then, use the extra tube for each reaction to determine the optimal number of PCR cycles, as described in Step 8 (below). Store the other tubes at 4°C.
8. For each extra PCR tube, determine the optimal number of PCR cycles (see Figure 6):
   a. Transfer 15 µl from the 15-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   b. Run three additional cycles (for a total of 18) with the remaining 85 µl of the PCR mixture.
   c. Transfer 15 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 70 µl of PCR mixture.
   e. Transfer 15 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 55 µl of PCR mixture.
9. Electrophorese 5 µl of each aliquot of each PCR reaction alongside 0.1 µg of 1-kb DNA size marker on a 1.2% agarose/EtBr gel in 1X

VII. SMART cDNA Synthesis Protocol *continued*

TAE buffer. Determine the optimal number of cycles required for each experimental and control sample (see Figure 7, Section IX).

10. Retrieve the 15-cycle 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 marker on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 7 to confirm that your reactions were successful.
12. Add 2 µl of 0.5 M EDTA to each tube to terminate the reaction.
13. Transfer 7 µl of your 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 IX.B.

You now have SMART ds cDNA ready to use for applications such as Virtual Northern blotting or generation of cDNA probes. For PCR-Select cDNA subtraction, proceed with the following protocol (Step VIII.A, below).

VIII. Protocol for Clontech PCR-Select™ cDNA Subtraction

A. Column Chromatography (PCR-Select Users only!)

1. For every experimental sample and control, combine the two reaction tubes of PCR product (from Section VII.B) into a 1.5-ml microcentrifuge tube.
2. 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 mixture 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 5–7.

   Note: If your volume is <40 µl, add H_2O to the aqueous phase to adjust volume to 40–70 µl.

VIII. Protocol for Clontech 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 to the column.
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 IX.B.

B. ***Rsa*** **I Digestion** (PCR-Select Users only!)

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

Before proceeding with *Rsa* I digestion, set aside another 10 µl of purified

VIII. Protocol for Clontech PCR-Select... *continued*

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 VIII.A.21):

10X *Rsa* I restriction buffer	36 µl
Rsa I (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 *Rsa* I digestion was successful, electrophorese 10 µl of uncut ds cDNA (Sample D) and 10 µl of *Rsa* I-digested cDNA on a 1.2% agarose/EtBr gel in 1X TAE buffer (see Section IX.C in this User Manual and Section V.B in the Clontech 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 IX.D.

C. Purification of Digested cDNA (PCR-Select Users only!)

You may purify your digested cDNA using any silica matrix-based PCR purification system, such as those offered by Clontech (see Related Products, Section XII). Alternatively, a phenol:chloroform extraction may be performed; however, this may decrease the efficiency of the cDNA subtraction. The following purification procedure has been optimized using SMART ds cDNA and the NucleoTrap PCR Kit (Cat. No. 636020; not included with PCR-Select Kit).

Before you start: Add 64 ml of 95% ethanol to the Buffer NT3 for a final concentration of approximately 85%. The appropriate volume is also listed on the Buffer NT3 bottle.

1. Aliquot the *Rsa* I-digested cDNA (Section VIII.B.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.

VIII. Protocol for Clontech PCR-Select... *continued*

6. Add 680 µl of Buffer NT2 to the pellet. Mix gently to resuspend. Centrifuge at 10,000 x g for 1 min at room temperature. Remove the supernatant completely and discard.
7. Add 680 µl of Buffer NT3 to the pellet. Mix gently to resuspend. Centrifuge the sample at 10,000 x g for 1 min at room temperature. Remove the supernatant completely and discard.
8. Repeat Step 7.
9. 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 this incubation step.
12. Centrifuge the sample at 10,000 x g for 30 sec at room temperature. Transfer the supernatant, containing the pure DNA fragment, 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 IX.D.
15. To precipitate the DNA, add 1/2 volume of 4 M ammonium acetate (e.g., 50 µl for a 100-µl sample), then add 2.5 volumes of 95% ethanol (e.g., 375 µl for 150 µl sample + ammonium acetate) 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.

VIII. Protocol for Clontech 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 IX.D.
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 Clontech 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 the Clontech PCR-Select cDNA Subtraction Kit (Cat. No. 637401). Be sure to read Section VIII.D below for important cDNA subtraction control procedures.

D. Controls for Clontech 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 the driver, and mix another portion with a small amount of the control Hae III-digested ϕX174 DNA from the PCR-Select Kit as the 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 placental total RNA** (included in the SMART kit)

   Use the SMART kit to amplify the Control Human Placental Total RNA; then, perform a mock subtraction as described for Control No. 1: use a portion of the human placental cDNA as the driver, and mix another portion with a small amount of the control Hae III-digested ϕX174 DNA from the PCR-Select Kit as the tester. If Control No. 1 works, but Control No. 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 VIII.C).

IX. Analysis for Clontech™ PCR-Select Subtraction

Figure 7 shows a typical gel profile of ds cDNA synthesized using the Control Human Placental Total 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 poly A^+ RNA. For nonmammalian species, the size distribution may be smaller (see Section X.A.2 for more details).

A. Determining the Optimal Number of PCR Cycles (Step VII.B.8)

For best results, you must optimize the PCR cycling parameters for your experiment, as described in Section VII.B (Figure 6). 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.

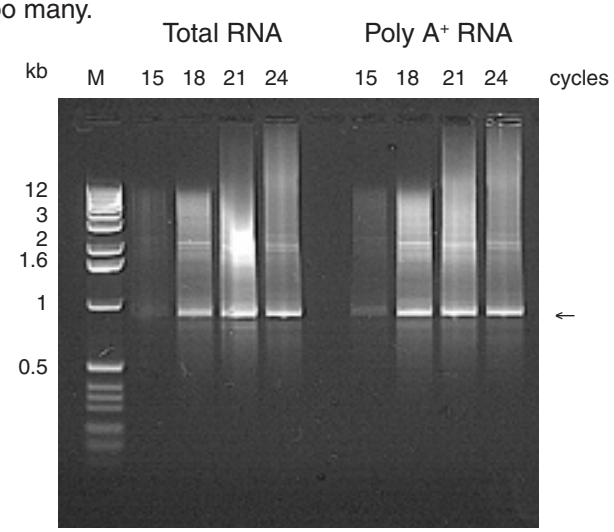


Figure 7. Analysis for optimizing PCR parameters. 5 µl of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1X TAE buffer following the indicated number of PCR cycles. The optimum number of cycles determined in this experiment was 17. Lane M: 1-kb DNA ladder size markers, 0.1 µg loaded. The arrow indicates the strong band at 900 bp typically seen for human placental total RNA.

IX. Analysis for Clontech PCR-Select Subtraction... *cont.*

We have optimized the PCR cycling parameters presented in this User Manual using an authorized hot-lid thermal cycler and the Advantage® 2 PCR Kit (Cat. Nos. 639206 & 639207). These parameters may vary with different polymerase mixes, templates, and thermal cyclers. We strongly recommend that you optimize the number of PCR cycles with your experimental sample(s) and the Control Human Placental 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 7 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.

B. Column Chromatography (Section VIII.A)

To analyze the ds cDNA after column chromatography, electrophorese 3 µl of the unpurified PCR product (Sample A, from Section VII.B.13) alongside 10 µl of the PCR product purified by column chromatography (Sample B, from Section VIII.A.19) and 10 µl of the second fraction (Sample C, from Section VIII.A.20) 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 percent. If your yield is <30 percent, 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 VIII.B. Otherwise if the cDNA is not present in Sample C, repeat the PCR and column chromatography steps.

C. *Rsa* I Digestion (Section VIII.B)

To confirm that *Rsa* I digestion was successful, electrophorese 10 µl of uncut ds cDNA (Sample D, from Section VIII.B) alongside 10 µl of *Rsa* I-digested cDNA (from Section VIII.B.4) on a 1.2% agarose/EtBr gel. Compare the profiles of both samples. Before *Rsa* I 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 *Rsa* I digestion, the smear should range from 0.1–2 kb. This result will be similar to that shown in the User Manual for the PCR-Select Kit.

IX. Analysis for Clontech PCR-Select Subtraction... *cont.*

D. Purification of Digested cDNA (Section VIII.C)

To analyze the yield of purified SMART cDNA, electrophorese 10 µl of *Rsa* I-digested cDNA before purification (Sample E, from Step VIII.B.6) alongside 10 µl of purified diluted cDNA before ethanol precipitation (Sample F, Step VIII.C.14) and 1.8 µl of purified diluted cDNA after ethanol precipitation (Sample G, from Step VIII.C.22) on a 1.5% agarose/EtBr gel. Compare the intensities of the samples and estimate what percentage of *Rsa* I-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.

X. Troubleshooting Guide

A. First-Strand cDNA Synthesis and SMART PCR Amplification (Sections V.A–B & VII.A–B)

1. Low molecular weight (size distribution <3 kb), poor yield, or no PCR product observed for the Control Human Placental Total RNA
   a. Proper template switching, which is essential to the SMART technology, requires the use of an MMLV RNase H^- point mutant reverse transcriptase such as PowerScript Reverse Transcriptase (included with each SMART Kit).
   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°C. Your working area, equipment, and solutions must be free of contamination by RNase A.
   c. 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.
   d. 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. If your PCR reaches its plateau after 24 cycles or more, the conditions of your PCR may not be optimal. Check the protocol and repeat the PCR using a fresh 2-µl aliquot of the first-strand product.
2. Poor yield or truncated PCR product from your experimental RNA

   If the reaction with the Control Human Placental 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.
   b. Your experimental RNA has been partially degraded (by contaminating RNases) before or during first-strand synthesis.

X. Troubleshooting Guide *continued*

Repeat the experiment using a fresh lot or preparation of RNA. Check the stability of your RNA by incubating a small sample 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, re-isolate the RNA using a different technique, such as one employed by our RNA isolation kits (see Related Products for ordering information). Several additional rounds of phenol:chloroform extraction may dramatically increase RNA stability.

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 one employed by our RNA isolation kits (see Related Products for ordering information).

B. Special Considerations for Library Construction (Sections V & VI)

1. Low yield of PCR product

a. Too few thermal cycles were used in the PCR step. Another indication of PCR undercycling is a cDNA size distribution <3 kb if the mRNA source was mammalian. (For some sources, such as many insect species, the normal mRNA size distribution may be <2–3 kb.) If you suspect that undercycling is the problem, incubate the PCR mixture for two more cycles and recheck the product. If you already used the maximum recommended number of cycles indicated in Table I, increase by three more cycles. If increasing the number of cycles does not improve the yield of PCR product, repeat the PCR using a fresh 2-µl aliquot of the first-strand product.

b. If you still obtain a low yield of PCR product, it may be due to a low yield of first-strand cDNA. Possible problems with the first-strand reaction include a mistake in the procedure (such as using a suboptimal incubation temperature or omitting a component) or not using enough RNA in the reaction. It is also possible that the RNA has been partially degraded (by contaminating RNases) before or during the first-strand synthesis. Reminder: problems with the first-strand cDNA synthesis can be more easily diagnosed if you perform parallel reactions using the Control RNA provided in the kit. If good results were obtained with the Control RNA but not with your experimental RNA, then there may be a problem with your RNA.

The easiest way to check the quality of the first-strand cDNA is by using a small sample of it as a PCR template with 3' and 5' gene-specific primers, such as Human β-Actin Control Amplimers (Cat. No. 639001, Cat. No. 639002). If the first-strand synthesis

X. Troubleshooting Guide *continued*

has been successful, a PCR product of the expected size will be generated.

2. No bright bands distinguishable in the PCR product

   For most mammalian RNA sources, there should be several bright bands distinguishable against the background smear when a sample of the PCR product is run on a gel. If bright bands are expected but are not visible, and the background smear is very intense, you may have overcycled your PCR. If you suspect that your problem is due to overcycling, then the PCR step (Section V.B) must be repeated with a fresh 2-µl sample of first-strand cDNA, using 2–3 fewer cycles.

C. Preparation for Clontech PCR-Select™ cDNA Subtraction (Sections VII–IX)

For troubleshooting the actual PCR-Select subtraction procedure, please refer to the User Manual for the Clontech PCR-Select™ cDNA Subtraction Kit. Here, we provide a troubleshooting guide for preparing SMART cDNA for subtraction (described in Section VII and VIII).

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

   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 *Rsa* I digestion (Section VIII.B)

   If the size distribution of your sample and/or control cDNA is not reduced after *Rsa* I 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 *Rsa* I digestion.

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

   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 under-cycled). Perform more PCR cycles. Optimize the number of cycles as described in Section IX.

XI. References

Barnes, W. M. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91**:2216–2220.

Chenchik A., Moqadam, F. & Siebert, P. (January 1995) Marathon cDNA amplification: A new method for cloning full-length cDNAs. *Clontechniques* **X**(1):5–8.

Chenchik, A., Moqadam, F. & Siebert, P. (1996) A new method for full-length cDNA cloning by PCR. In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis.* Ed. Krieg, P. A. (Wiley-Liss, Inc.), pp. 273–321.

Chenchik, A., Zhu, Y. Y., Diatchenko, L., Li, R., Hill, J. & Siebert, P. D. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *Gene Cloning and Analysis by RT-PCR* (BioTechniques Books, MA), pp. 305–319.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.

Diatchenko, L., Lau, Y.-F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D. & Siebert, P. D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**:6025–6030.

Endege, W. O., Steinmann, K. E., Boardman, L. A., Thibodeau, S. N. & Schlegel, R. (1999) Representative cDNA libraries and their utility in gene expression profiling. *BioTechniques* **26**:542–550.

Farrell, Jr., R. E. (1993) *RNA Methodologies—A Lab Guide for Isolation and Characterization* (Academic Press, San Diego, CA).

Gurskaya, N. G., Diatchenko, L., Chenchik, A., Siebert, P. D., Khaspekov, G. L., Lukyanov, K. A., Vagner, L. L., Ermolaeva, O. D., Lukyanov, S. A. & Sverdlov, E. D. (1996) Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: Cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. *Anal. Biochem.* **240**:90–97.

Kellogg, D. E., Rybalkin, I., Chen, S., Mukhamedova, N., Vlasik, T., Siebert, P. & Chenchik, A. (1994) TaqStart Antibody: Hotstart PCR facilitated by a neutralizing monoclonal antibody directed against *Taq* DNA polymerase. *BioTechniques* **16**:1134–1137.

Lukyanov, S. A., Gurskaya, N. G., Tarabykin, V. S. & Sverdlov, E. D. (1994) Highly efficient subtractive hybridization of cDNA. *Biorganic Chem.* (Russian) **20**:701–704.

Matz, M., Lukyanov, S., Bogdanova, E., Diatchenko, L., & Chenchik, A. (1999) Amplification of cDNA ends based on template-switching effect and step-out PCR. *Nucleic Acids Res.* **27**:1558–1560.

Morgan, J. G., Dolganov, G. M., Robbins, S. E., Hinton, L. M. & Lovett, M. (1992) Selective isolation of novel cDNAs encoded by the region surrounding the human IL-4 and -5 genes. *Nucleic Acids Res.* **20**:5173–5179.

Pietu, G., Alibert, O., Guichard, V., Lamy, B., Bois, F., Leroy, E., Mariage-Sampson, R., Houlgatte, R., Soularue, P. & Auffray, C. (1996) Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridization of a high density cDNA array. *Genome Res.* **6**:492–503.

Sambrook, J., & Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Siebert, P.D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A. & Lukyanov, S. A. (1995) An improved method for walking in uncloned genomic DNA. *Nucleic Acids Res.* **23**:1087–1088.

SMART RACE cDNA Amplification Kit (January 1999) Clontechniques **XIV**(1):4–6.

Zhu, Y.Y.,Machleder, E. M.,Chenchik, A., Li, R. & Siebert, P. M. (2001) Reverse transcriptase template switching: A SMART™ approach for full-length cDNA library construction. *BioTechniques* **30**:892–897.

XII. Related Products

For a complete listing of all Clontech products, please visit **www.clontech.com**

	Cat. No.
• Clontech PCR-Select™ cDNA Subtraction Kit	637401
• Clontech PCR-Select™ Differential Screening Kit	637403
• SMART™ cDNA Library Construction Kit	634901
• SMART™ RACE cDNA Amplification Kit	634914
• Advantage® 2 PCR Kit	639206 639207
• Advantage® 2 Polymerase Mix	639201 639202
• Sprint Advantage® Single Shots	639553 639554 639556
• Sprint Advantage® 96 Plate	639550
• PowerScript™ Reverse Transcriptase	639500 639501
• NucleoTrap® mRNA Mini Purification Kit	636022
• NucleoSpin® RNA II Kit	635990
• Premium Total RNAs	many
• Premium Poly A^+ RNAs	many
• TaqStart™ Antibody	639250
• Amplimer Sets	many
• Marathon®-Ready cDNAs	many
• MTN® Multiple Tissue Northern Blots	many

Appendix: 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 *et al.*, 1989). At Clontech, we use the TurboBlotter equipment and protocol from Schleicher & Schuell. Figure 8 shows how Virtual Northern blots can be used to confirm differential expression of subtracted cDNAs.

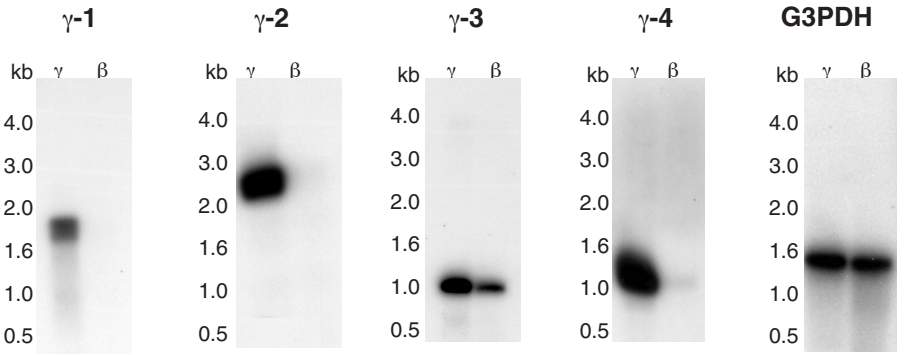


Figure 8. Virtual Northern blot analysis of cDNA fragments expressed in cells producing γ-globin. Clontech PCR-Select cDNA subtraction was performed to isolate cDNAs that were preferentially expressed in cells producing γ-globin. 1 µg of total RNA from cells producing γ-globin was used as the tester; 1 µg of total RNA from cells producing β-globin was used as the 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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