User Manual

SMARTer™ Pico Fluorescent Probe Amplification Kit User Manual

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I. List of Components

Storage Conditions

- Store Control Total RNA and SMARTer II A Oligonucleotide at –70°C.
- Store NucleoSpin Extract II Kits, Spin Filter/Collection Tubes and Glass Approved DMSO at room temperature.
- Store QuickClean Resin at 4°C.
- Store all other reagents at –20°C.

The following reagents are suitable for 10 probe synthesis & labeling reactions.

The SMARTer Pico Fluorescent Probe Amplification Kit (Cat. No. 634931) includes:

1 SMARTer™ Pico PCR cDNA Synthesis Kit Components (Cat. No. 634927; not sold separately**)**

Box 1

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

Box 2

- 70 µl **3′ SMART CDS Primer II A** (12 µM)
  5′–AAGCAGTGGTATCAACGCAGAGTACT(30)N-1N–3′
  Rsa I
  (N = A, C, G, or T; N-1 = A, G, or C)
- 250 µl **5X First-Strand Buffer** (RNase-Free)
  250 mM Tris-HCl (pH 8.3)
  375 mM KCl
  30 mM MgCl2
- 200 µl **5′ PCR Primer II A** (12 µM)
- 250 µl **dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
- 50 µl **Dithiothreitol** (DTT; 100 mM)
- 55 µl **RNase Inhibitor** (40 U/µl)
- 55 µl **SMARTScribe™ Reverse Transcriptase** (100 U/µl)
- 2x1 ml **Deionized H_2O**

I. List of Components, *continued*

2 NucleoSpin® Extract II (2 x Cat. No. 740609.10; 10 preps each)

1 Probe Labeling Components (Cat. No. 634932**;** not sold separately)

Box 1

- 35 µl **Random Primer Mix (N-15)** (1 µg/µl)
- 60 µl **20X Labeling dNTP Mix**
- 340 µl **Sodium Acetate** (3M)
- 100 µl **2X Fluorescent Labeling Buffer**
- 50 µl **Klenow Fragment** (4 U/µl)
- 200 µl **10X Klenow Fragment Buffer**

Box 2

- 30 each **Spin Filter/Collection Tubes** (0.22 µm)

1 QuickClean Enzyme Removal Resin (Cat. No. 631770)

- 0.5 ml **QuickClean Resin**

1 Glass Approved DMSO (Cat. No. 634705)

- 500 µl **Glass Approved DMSO**

II. Additional Materials Required

The following reagents are required but not supplied:

For Isolation and Analysis of RNA:

- **RNase Blaster** (Cat. No. 636839)
  RNase Blaster is a high-efficiency, near-neutral pH cleaning solution designed to make the lab working environment RNase-free in a matter of minutes. RNase Blaster has been designed to protect the integrity of your RNA sample as well as eliminate RNase contamination in reaction vessels.
- **NucleoSpin® RNA II Kit** (Cat. Nos. 740955.10, 740955.20, 740955.50 & 740955.250)
  We strongly recommend use of the NucleoSpin RNA II Kit for RNA purification. We have found that cells or tissues frozen in RA1 buffer are better preserved, resulting in the isolation of higher quality RNA. The RA1 buffer, which contains guanidinium isothiocyanate, protects cellular RNA by inactivating RNases. The higher quality of RNA reduces the number of cycles required to reach the optimal SMARTer cDNA amplification level, resulting in a more representative cDNA pool. Additional Buffer RA1 (Cat. No. 740961) can be purchased separately.
- **Tris-EDTA Buffer for measuring O.D.** (store at –20°C)

  10 mM Tris (pH 7.5)
  0.1 mM EDTA (pH 7.5)
- **Formamide** (Sigma Cat. No. F9037)
- **12.3 M formaldehyde** (Sigma Cat. No. F8775)
- **10X MOPS buffer** (Autoclave to sterilize; solution may turn yellow)

  0.4 M MOPS (pH 7.0)
  0.1 M NaOAc (pH 7.0)
  10 mM EDTA (pH 7.0)
- **Ethidium bromide** (10 mg/ml)

II. Additional Materials Required

For Probe Synthesis:

- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
  We strongly recommend use of the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) for PCR amplification. This kit includes the Advantage 2 Polymerase Mix, which has been specially formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). The Advantage 2 Polymerase Mix is formulated to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).
- **Fluorescent dye**

  The fluorescent labeling protocol is optimized for use with the following dyes:

  Cy3 Mono-Reactive Dye Pack (GE Healthcare P/N = PA23001)

  Cy5 Mono-Reactive Dye Pack (GE Healthcare P/N = PA25001)

  Each pack of GE Healthcare dye contains five vials.

  Other monofunctional, N-hydroxysuccinimide-activated fluorescent dyes are also compatible with this labeling kit. Please note that we have supplied an adequate quantity of Glass Approved DMSO to prepare stock solutions of GE Healthcare dyes. If you choose to use a dye from another supplier, you may require additional DMSO. Please order Glass Approved DMSO (Cat. No. 634705). **Do not substitute your own DMSO**.
- **0.5 M EDTA** (pH 8.0)
- **100% Ethanol** (Avoid denatured alcohol; we recommend Spectrum Cat. No. E1028)
- **70% Ethanol**
- **Quartz cuvettes**
- **UV/Vis spectrophotometer** (Optional: a scanning spectrophotometer will assist you in troublesooting probe quality. See Section VIII.)

For ds cDNA Purification and Probe Purification:

- **NucleoSpin® Extract II** (Cat. Nos. 740609.10, 740609.50 & 740609.250)

III. Introduction

The **SMARTer™ Pico Fluorescent Probe Amplification Kit** provides reagents for synthesizing and purifying high-quality fluorescently labeled cDNA array probes from minimal starting material. With the method used in this kit, 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 SMARTer Fluorescent Probe Amplification Kit allows you to generate array probes starting with 1 ng of total RNA, or the equivalent of 10 cells. The reagents and protocol in this kit are compatible with any microarray system.

SMARTer™ cDNA Synthesis Technology

SMARTer cDNA synthesis is a novel, PCR-based method for producing high-quality cDNA from nanogram quantities of total RNA or mRNA. The cornerstone of SMARTer cDNA synthesis is SMART (Switching Mechanism At 5′ End of RNA Transcript) technology. SMART technology is especially useful for researchers who have limited starting material, such as RNA derived from laser-capture microscopy samples, cells sorted by flow cytometry, or other extremely small samples. SMART is a unique technology that allows the efficient incorporation of known sequences at both ends of cDNA during first strand synthesis, without adaptor ligation. The unparalleled sensitivity and efficiency of the single-step SMART process ensures that full-length cDNA is generated and amplified.

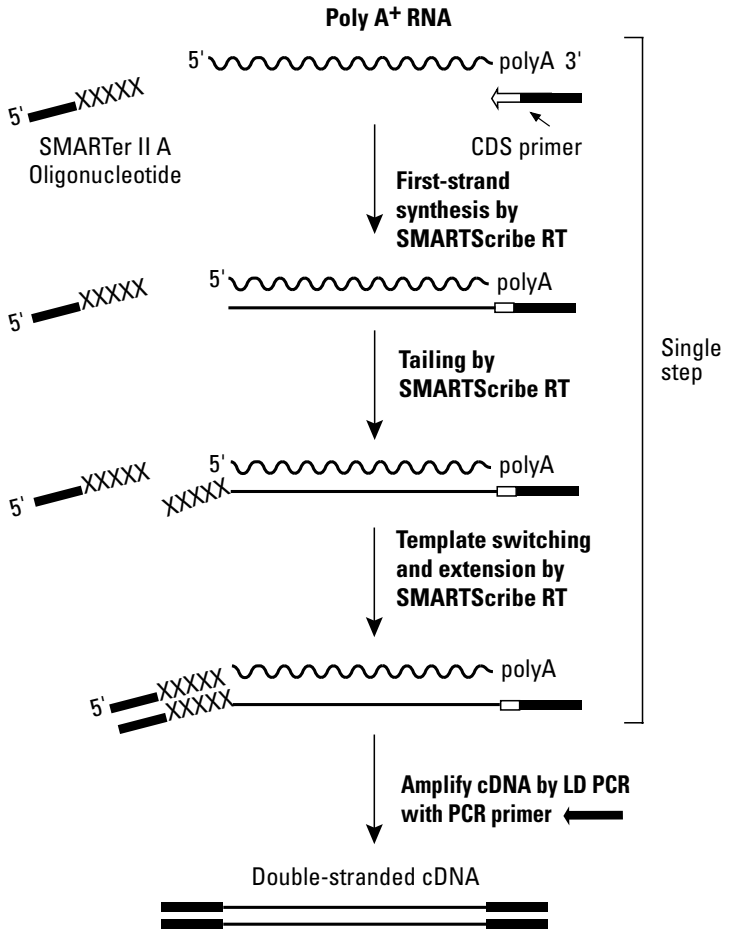


Figure 1. Flowchart of SMARTer cDNA synthesis. The SMARTer II A Oligonucleotide, 3′ SMART CDS Primer II A, and 5′ PCR Primer II A all contain a stretch of identical sequence (see Section I for sequence information).

III. Introduction, *continued*

SMARTer cDNA synthesis starts with nanogram amounts of total RNA or mRNA. A modified oligo(dT) primer (the 3′ SMART CDS Primer II A) primes the first-strand synthesis reaction (Figure 1). When SMARTScribe™ RT reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3′ end of the cDNA. The SMARTer Oligonucleotide (base-pairs with the non-template nucleotide stretch, creating an extended template. SMARTScribe RT then switches templates and continues replicating to the end of the oligonucleotide (Chenchik et al., 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5′ end of the mRNA, as well as sequences that are complementary to the SMARTer Oligonucleotide. In cases where the RT pauses before the end of the template, the addition of nucleotides is much less efficient than with full-length cDNA-RNA hybrids, thus the overhang needed for base-pairing with the SMARTer Oligonucleotide is absent. The SMARTer anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences, such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly A^- RNA, will not be exponentially amplified. However, truncated RNAs that are present in poor quality RNA starting material will be amplified, and will contaminate the final cDNA library.

If you have a sufficient amount of RNA, we recommend using the SMARTer Fluorescent Probe Amplification Kit (Cat. No. 634930). The SMARTer Fluorescent Probe Amplification Kit allows you to generate probes starting with 2 ng of total RNA at a concentration ≥ 0.6 ng/µl.

Advantage® 2 PCR Kit for long-distance PCR

We strongly recommend use of the Advantage 2 PCR Kits (Cat. Nos. 639206 & 639207) for cDNA amplification. These kits include the Advantage 2 Polymerase Mix, formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). This polymerase mix provides automatic hot-start PCR (Kellogg et al., 1994)—and efficiently amplifies full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).

Use of PCR amplification with array technology

Amplification of cDNA by the method described in this User Manual has been shown to maintain the relative representation of each transcript in the original sample (*Clontechniques*, July 2002; Ohtsu et al., 2008; Spirin et al., 1999; Wang et al., 2000). Using the optimized guidelines in Table 1 (Section V) also ensures that messages will not be amplified to saturation. In addition, since array analysis compares the expression levels of individual genes between two samples, minor differences in amplification efficiency for different transcripts will be equivalent across samples and will have a negligible effect on the overall analysis.

Two-Step Labeling

The SMARTer Pico Fluorescent Probe Amplification Kit uses an indirect, "two-step" procedure for probe labeling. First, primary aliphatic amino groups are incorporated through random-primed labeling using an optimized dNTP mix, which includes the dTTP analog, aminoallyl-dUTP. Then, monofunctional, N-hydroxysuccinimide-activated fluorescent dye (Cy3, Cy5, or other; not included) is coupled to the cDNA by reaction with the amino functional groups. Because labeling is independent of the efficiency of incorporation of dye-dNTP analogs, the two-step procedure yields equivalent molarities of labeled probe regardless of the dye used.

III. Introduction, *continued*

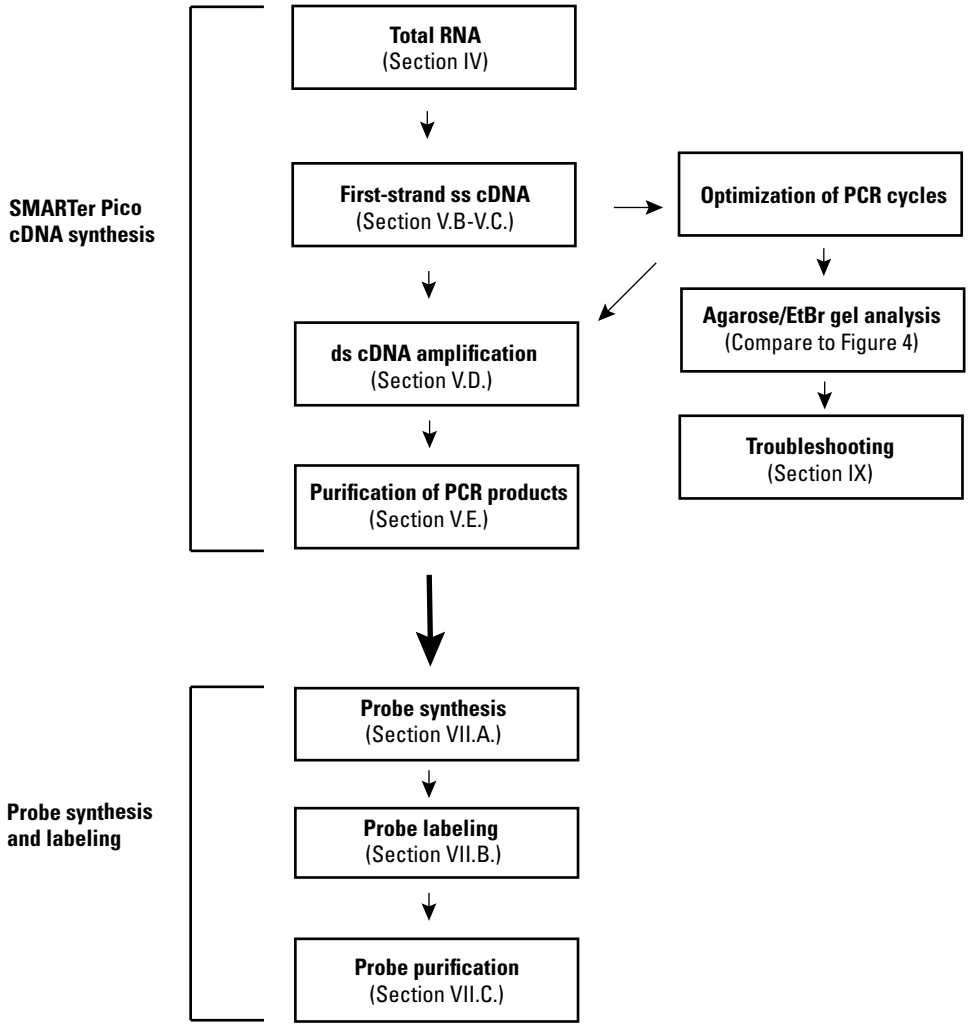


Figure 2. Overview of the SMARTer Pico Fluorescent Probe Amplification Procedure.

IV. RNA Preparation & Handling

A. General Precautions

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

- Wear gloves throughout the procedure to protect your RNA samples from degradation by nucleases.
- Prepare all solutions using deionized H_2O (e.g., MilliQ-grade or equivalent) that has not been treated with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H_2O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips.
- Ethidium bromide is a carcinogen. Use appropriate precautions when handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manual* by Sambrook & Russell (2001).

IV. RNA Preparation & Handling, *continued*

B. RNA Isolation

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

C. RNA Yield

If you have sufficient RNA you may perform the steps below to determine yield and analyze sample quality. The yield of total RNA will vary depending on the tissue or cells from which itis obtained.

1. Thoroughly mix your RNA. Measure the total RNA sample volume.
2. Transfer 2–5 µl of your total RNA sample to a 1.5-ml tube.
3. Bring volume up to 400 µl with Tris-EDTA buffer (See Section III: Additional Materials Required) and mix by pipetting.
4. Transfer contents to a 1-ml glass cuvette with a 1-cm path length.
5. Measure A_{260} and A_{280} using Tris-EDTA buffer as a reference blank.
6. Calculate RNA yield as follows:

   RNA constant for 1-cm path length = One A_{260} unit of RNA = 40 µg/ml

   - Total A_{260} = (A_{260} of dilute sample) x (dilution factor)
   - Concentration (µg/ml) = (total A_{260}) x (40 µg/ml)
   - Yield (µg) = (total sample volume) x (concentration)
7. Calculate the A_{260}/A_{280} ratio. Pure RNA exhibits a ratio of 1.9–2.1.

D. RNA Purity

EXAMPLE CALCULATIONS:

The RNA sample volume was 0.5 ml. A 2-µl sample aliquot was diluted to 400 µl in Tris-EDTA buffer. The following spectrophotometric readings were taken: A_{260}= 0.231; A_{280}= 0.115

- Total A_{260} = (0.231) x (200) = 46.2
- Concentration = (46.2) x (40) = 1,848 µg/ml
- RNA yield = (0.5 ml) x (1,848 µg/ml) = 924 µg
- Purity = 0.231/0.115 = 2.01

IV. RNA Preparation & Handling, *continued*

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

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

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

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

E. Assessing the Quality of the RNA Template

Methods for Assessing Total RNA Integrity

1. RNA/cDNA Quality Assay:

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

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

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

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

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

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

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

Methods for Assessing mRNA Integrity

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

V. SMARTer Pico cDNA Synthesis

A. General Considerations

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

B. Protocol: First-Strand cDNA Synthesis

This protocol has been optimized for total RNA. The minimum amount of starting material for cDNA synthesis is 1 ng of total RNA. However, if your RNA sample is not limiting, we recommend that you start with 20–1,000 ng of total RNA for cDNA synthesis. Please note that if you are starting from >100 ng of total RNA, you must follow the guidelines in Table II to dilute your first-strand cDNA product before proceeding with ds cDNA amplification (Section D).

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

IMPORTANT:

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

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

1–50 µl	**RNA** (1-1,000 ng of total RNA)*
7 µl	**3' SMART CDS Primer II A** (12 µM)
x µl	**Deionized H_2O**
57 µl	**Total Volume**

*For the control synthesis, add 10 ng of Control Total RNA.

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

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

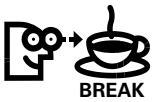
V. SMARTer Pico cDNA Synthesis, *continued*

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

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

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

5. Aliquot 49 µl of the Master Mix into each reaction tube. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
6. Incubate the tubes at 42°C for 90 min.
7. Terminate the reaction by heating the tubes at 70°C for 10 min.

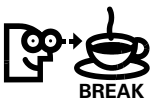


8. If necessary, cDNA samples can be stored at –20°C (for up to three months) until you are ready to proceed with spin-column purification (Section C).

C. Protocol: Column Purification of ss cDNA using NucleoSpin® Extract II

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

1. Add 350 µl of Binding Buffer NT to each cDNA synthesis reaction; mix well by pipetting.
2. Place a NucleoSpin Extract II Column into a 2 ml collection tube. Pipette the sample into the column. Centrifuge at 8,000 rpm for 1 min. Discard the flowthrough.
3. Return the column to the collection tube. Add 600 µl of Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
4. Return the column to the collection tube. Add 250 µl of Wash Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard the flowthrough.
5. Place the column back into the collection tube. Centrifuge at 14,000 rpm for 2 min to remove any residual Wash Buffer NT3.
6. Transfer the NucleoSpin Columns into a fresh 1.5 ml microcentrifuge tube. Add 50 µl of sterile Milli-Q H_2O to the column. Allow the column to stand for 2 min with the caps open.
7. Close the tube and centrifuge at 14,000 rpm for 1 min to elute the sample.
8. Repeat elution with 35 µl of sterile Milli-Q H_2O in the same 1.5 ml microcentrifuge tube. The 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.



9. Proceed with ds cDNA amplification (Section D). If necessary, samples can be stored at –20°C (for up to three months) until you are ready to proceed.

V. SMARTer Pico cDNA Synthesis, *continued*

PROTOCOL

D. Protocol: ds cDNA Amplification by LD PCR

Table I 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 total RNA and a hot-lid thermal cycler; optimal parameters may vary with different templates and thermal cyclers. Additional guidelines, based on the amount of starting material, are also provided in Table II.

In our experience, when starting with the minimum amount of RNA (1 ng) each 200 µl reaction typically yields at least 1–2 µg of ds cDNA after the PCR and purification steps (Sections D and E). Yield will vary depending on the amount of RNA input. To ensure that you have sufficient cDNA for your application, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

Table I: Guidelines for Setting Up PCR Reactions

Total RNA (ng)	Volume of Diluted ss cDNA[a] for PCR (µl)	Volume of H_2O (µl)	Typical Optimal No. of PCR Cycles*
1000	5	75	18–20
250	20	80	18–20
100	50	30	18–20
50	80	0	18–20
20	80	none	20–22
5	80	none	22–24
1	80	none	24–27

[a]From Step V.C.9.

Attention

***IMPORTANT**:
Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles: 15, 18, 21, 24, 27 and 30 cycles.

1. Preheat the PCR thermocycler to 95°C.
2. Prepare a Master Mix for two 100-µl PCR reactions per sample. Combine the following reagents in the order shown:

Recipe

80	µl	**First-strand cDNA**
88	µl	**Deionized H_2O**
20	µl	**10X Advantage 2 PCR Buffer**
4	µl	**50X dNTP Mix** (10 mM; in Advantage 2 PCR Kit)
4	µl	**5′ PCR Primer II A** (12 µM)
4	µl	**50X Advantage 2 Polymerase Mix**
200	µl	**Total Volume**

3. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
4. Aliquot the Master Mix into two PCR reaction tubes (100 µl per tube).

V. SMARTer Pico cDNA Synthesis, *continued*

5. Cap each tube, and place in the preheated thermal cycler. If you are NOT using a hot-lid thermal cycler, overlay the reaction mixture with two drops of mineral oil.

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

Table II: Cycling Guidelines Based on Starting Material

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

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

6. Commence thermal cycling using the following program:

   - 95°C 1 min
   - X cycles[a]:

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

     [a]Consult Tables I & II for guidelines. **Subject all tubes to 15 cycles.** Then, divide the PCR reaction mix between the "Experimental" and "Optimization" tube, using the Optimization tube to determine the optimal number of PCR cycles, as described in Step 7.

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

7. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30 µl from one tube to a second reaction tube labeled "Optimization". Store the "Experimental" tubes at 4°C. Using the Optimization PCR tube, determine the optimal number of PCR cycles (see Figure 3):

   a. Transfer 5 µl from the 15 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   b. Return the Optimization tube to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.
   c. Transfer 5 µl from the 18 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   d. Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.
   e. Transfer 5 µl from the 21 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   f. Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.
   g. Transfer 5 µl from the 24 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   h. Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.
   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.

V. SMARTer Pico cDNA Synthesis, *continued*

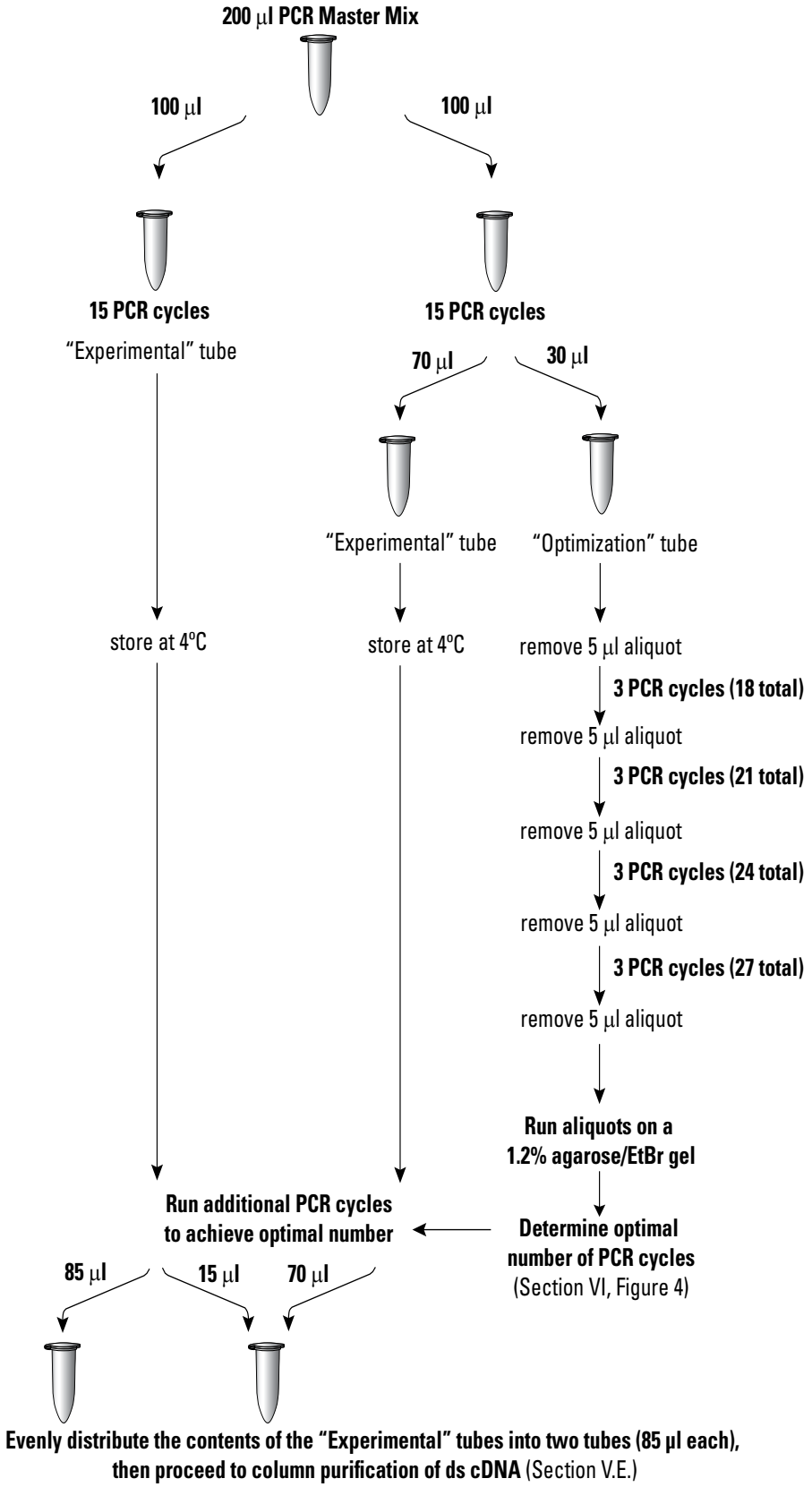


Figure 3. Optimizing PCR parameters for SMARTer Pico cDNA synthesis.

V. SMARTer Pico cDNA Synthesis, *continued*

8. Electrophorese each 5 µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Determine the optimal number of cycles required for each experimental and control sample (see Figure 4, Section VI).
9. 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.
10. When the cycling is completed, analyze a 5 µl sample of each PCR product alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 4 to confirm that your reactions were successful.
11. Add 2 µl of 0.5 M EDTA to each tube to terminate the reaction.
12. Evenly distribute the contents of the Experimental PCR tubes into two tubes (85 µl each; see Figure 3), then proceed to Section V.E. Column Purification of ds cDNA using NucleoSpin Extract II.

E. Protocol: Column Purification of ds cDNA using NucleoSpin® Extract II

We recommend using the NucleoSpin Extract II Kit (Cat. No. 740609.10) for PCR product purification. If you will not be using NucleoSpin Extract II, follow the manufacturer's protocol for your purification columns.

1. Add 500 µl Binding Buffer NT to each 85 µl PCR reaction. Mix well by pipetting.
2. Place two NucleoSpin Extraction spin columns into two 2-ml collection tubes and label them 1 and 2.
3. Pipet samples 1 and 2 into the appropriately labeled spin columns.
4. Centrifuge at 8,000 rpm for one minute. Discard the flow through.
5. Insert the NucleoSpin columns back into the 2-ml collection tubes and add 600 µl of Wash Buffer NT3 to each column.
6. Centrifuge at 14,000 rpm for one minute. Discard the flow through.
7. Repeat steps 5-6.
8. Empty the collection tubes and centrifuge at 14,000 rpm for one minute.
9. Place the NucleoSpin columns into fresh 1.5-ml microcentrifuge tubes. Add 35 µl of Elution Buffer NE to the spin filter and stand for 3 minutes. Centrifuge at 14,000 rpm for one minute to elute sample.
10. Add a second 25 µl of Elution Buffer NE to each of the spin filters and stand for 2 minutes. Centrifuge the tubes at 14,000 rpm for 1 minute.
11. After elution, discard the NucleoSpin columns and centrifuge the eluted cDNA at maximum speed for an additional 3 min. Transfer both supernatants to a new tube.
12. Pipet sample onto a 0.22 µm spin filter and centrifuge at 14,000 rpm for 1 minute.

VI. Analysis of cDNA Amplification Results

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

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

Figure 4 provides an example of how your analysis should proceed. In the experiment with human placental total RNA (Panel A), the PCR reached its plateau after 25 cycles for the 1 ng experiment and 21 cycles for the 20 ng experiment; that is, the yield of PCR products stopped increasing. After 25 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 24 for the 1 ng experiment and 20 for the 20 ng experiment. In the experiment with mouse liver total RNA (Panel B), the optimal number of cycles was 24 for the 1 ng experiment and 20 for the 20 ng experiment.

We have optimized the PCR cycling parameters presented in this User Manual using both hot-lid and non-hot-lid thermal cyclers and the Advantage® 2 PCR Kit (Cat. No. 639207). These parameters may vary with different polymerase mixes, templates, and thermal cyclers. We strongly recommend that you optimize the number of PCR cycles with your experimental sample(s) and the Control 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 1XTAE buffer.

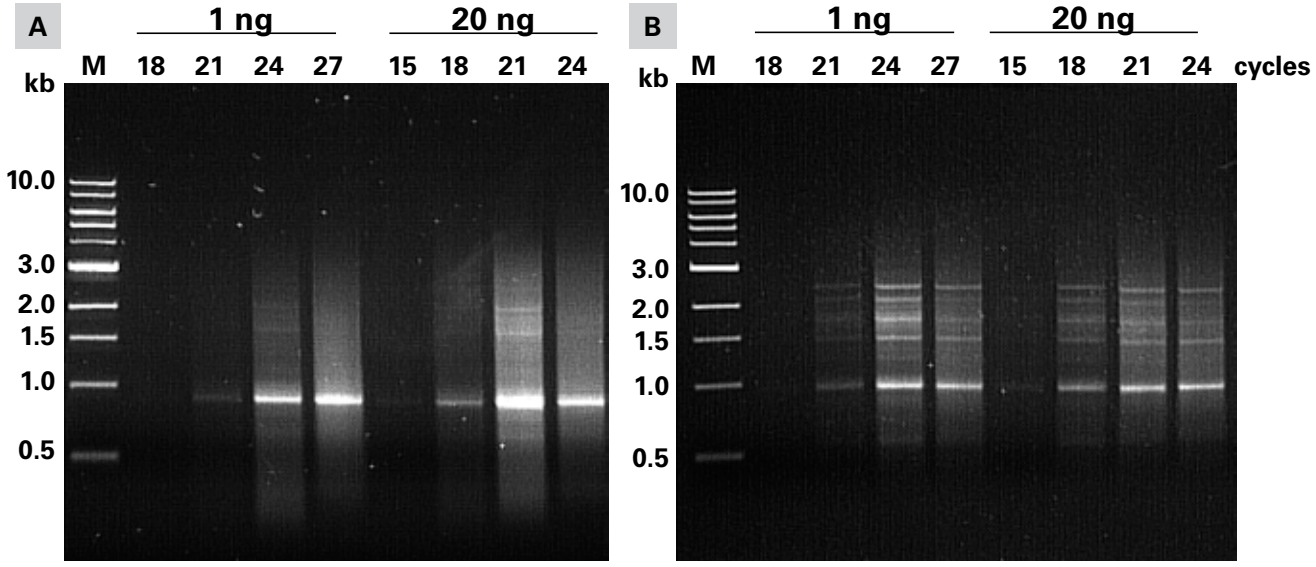


Figure 4. Analysis for optimizing PCR parameters. 1 ng or 20 ng of control human placental total RNA (Panel A) or control mouse liver total RNA (Panel B) was subjected to first-strand cDNA synthesis and purification as described in the protocol. 80 µl was used for PCR amplification. A range of PCR cycles were performed (15, 18, 21, 24, and 27). 5 µl 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 for the human placental total RNA experiment (Panel A) was 24 for the 1 ng reaction, and 20 for the 20 ng reaction. A strong band at 900 bp is typically seen for human placental total RNA. The optimal number of cycles determined for the experiment with mouse liver total RNA (Panel B) was 24 for the 1 ng reaction and 20 for the 20 ng reaction. Lanes M: 1 kb DNA ladder size markers.

VII. Probe Synthesis Procedure

A. Protocol: Probe Synthesis

Follow the steps below to synthesize amino-modified cDNA, then remove protein contaminants using the supplied QuickClean Resin and 0.22-µm Spin Filters—a rapid substitute for phenol/chloroform extraction. Read ahead to prepare an appropriate thermal cycler program before beginning. Before beginning, please be sure you have read Section II: Additional Materials Required for important information regarding the use of fluorescent dyes from manufacturers other than GE Healthcare.

1. Estimate the yield of SMARTer cDNA from Section VI by UV spectrophotometry.
2. In a 0.5-ml microcentrifuge tube, dilute 2 µg of purified cDNA to a final volume of 77.5 µl using sterile H_2O. Then, mix with 2.5 µl of Random Primer Mix (N-15).
3. Denature samples by incubating at 95°C for 5 min, then incubate at 37°C for 2 min.
4. While samples are being denatured, prepare a Master Labeling Mix sufficient for the number of reactions plus one extra reaction to ensure sufficient volume. Combine the following reagents in a 0.5-ml microcentrifuge tube at room temperature, and mix well:


Recipe

10 µl	**10X Klenow Fragment Buffer**
5 µl	**20X Labeling dNTP Mix**
5 µl	**Klenow Fragment***
20 µl	**Total Volume Master Labeling Mix per reaction****

*Do not add the Klenow Fragment to the Master Labeling Mix until just prior to use in Step 5.

**Multiply the quantities in the Master Labeling Mix by the total number of reactions.

5. After completion of the incubation at 37°C (Step 3), add 20 µl of Master Labeling Mix to each reaction tube.
6. Vortex, then spin tube(s) briefly in a microcentrifuge to collect contents.
7. Incubate tubes in the PCR thermal cycler at 37°C for 90 min.
8. Add 0.5 µl of 0.5 M EDTA (pH 8.0) and 10 µl QuickClean Resin; vortex for 1 min. Spin tube(s) briefly in a microcentrifuge to collect contents.
9. Insert a 0.22-µm Spin Filter into its accompanying collection tube, and pipet the sample into the Spin Filter. You need only ensure complete transfer of the liquid portion. Spin at maximum speed in a microcentrifuge for 1 min.
10. Remove and discard the Spin Filter. Add 11 µl 3 M Sodium Acetate, and vortex.
11. Add 275 µl of ice-cold 100% ethanol to the sample. Vortex, then spin tube briefly in a microcentrifuge to collect contents.
12. Place tube in a –20°C freezer for 1 hr to precipitate the cDNA.

    NOTE: For maximum yield, precipitate cDNA overnight at –20°C.

13. Spin tube at maximum speed in a microcentrifuge for 20 min at room temperature or colder.
14. Carefully pipet off supernatant, and wash pellet once in 70% ethanol.

NOTE: If desired, you may stop at this point and store cDNA, covered with ethanol, overnight at –20°C. For storage, do not remove ethanol and freeze the dry pellet. After overnight storage, centrifuge sample to ensure complete recovery of cDNA.

15. Air dry pellet and dissolve in 5 µl of 2X Fluorescent Labeling Buffer. Do not substitute a different buffer. Proceed **directly** with Section B.

VII. Probe Synthesis Procedure, *continued*

B. Protocol: Fluorescent Dye Coupling

Proceed with the steps below to couple fluorescent dye to your amino-modified first-strand cDNA. Follow the steps precisely. Do not use a protocol supplied with your dye.

IMPORTANT: Use only the DMSO provided. Do not substitute your own.

1. Prepare a 5 mM stock solution of fluorescent dye by adding the appropriate quantity of DMSO directly to the dye container. If you are using GE Healthcare Cy3 or Cy5 reactive dye, open one pouch of dye. Each pouch contains one tube of dye residue sufficient for four labeling reactions. Add 45 µl DMSO directly to the dye vial. Vortex and briefly spin down.
2. Add 5 µl of the DMSO/dye mixture to your 5-µl cDNA sample. Mix well and place the tube at room temperature in the dark or wrapped in aluminum foil. Incubate at room temperature for 60 min.

NOTE: The remaining DMSO/dye solution can be stored tightly capped at –20°C for at least 1–2 months without noticeable degradation.

3. Add 1 µl of 3 M Sodium Acetate and 25 µl of 100% ethanol. Vortex, then spin tube briefly in a microcentrifuge to collect contents.
4. Place tube in a –20°C freezer for 2 hr to precipitate the labeled probe.
5. Spin tube at maximum speed in a microcentrifuge for 20 min.
6. Carefully pipet off supernatant, and wash pellet once in 70% ethanol.
7. Dissolve pellet in 100 µl Deionized H_2O. Proceed with Section C.

C. Protocol: Probe Purification with NucleoSpin® Extract II

Probe purification is best achieved through chromatography with silica-gel membrane spin columns (not included). We recommend using the NucleoSpin Extract II Kit (Cat. No. 740609.10). If you will not be using NucleoSpin Extract II, follow the manufacturer's protocol for your purification columns, then assess your probe's quality and yield as described in Section VIII.

NOTE: Removal of unreacted dye is essential, as it can cause high levels of background.

1. Add 500 µl of Binding Buffer NT to the probe and mix by inverting several times.
2. Briefly centrifuge the tube to collect the contents.
3. Place the NucleoSpin Extraction column into 2-ml collection tube.
4. Transfer the binding buffer and probe mix to the spin filter.
5. Centrifuge the tube at room temperature for 1 minute at 8,000 rpm. Discard the flow through
6. Insert the NucleoSpin column back into the 2-ml collection tube and add 600 µl of Wash Buffer NT3 to the spin filter.
7. Centrifuge at 14,000 rpm for one minute. Discard the flow through.
8. Repeat Steps 6-7 two times.
9. Centrifuge the tubes one more time at room temperature for one minute at 14,000 rpm to remove excess ethanol wash from the spin filter.
10. Place the NucleoSpin column into a fresh 1.5-ml microcentrifuge tube and add 50 µl of Elution Buffer NE to the spin filter. Allow the columns to stand for three minutes.

VII. Probe Synthesis Procedure, *continued*

11. Centrifuge the tubes at room temperature for 1 minute at 14,000 rpm.
12. Add another 50 µl of Elution Buffer NE to each tube and allow the columns to stand for 3 minutes.
13. Centrifuge the tubes at room temperature for 1 minute at 14,000 rpm.
14. After elution, discard the NucleoSpin column and centrifuge the eluted probe at maximum speed for additional 3 min. Transfer the supernatant to a new tube.
15. Pipet probe onto a 0.22-µM spin filter and centrifuge at 14,000 rpm for 1 min.
16. Following purification, assess your probe's quality as described in Section VIII.

VIII. Analysis and Troubleshooting of Probe Quality

To assess the quality of your labeled probe, follow the steps below to analyze your entire, **undiluted** probe using UV/Vis spectrophotometry. Ensure that your cuvettes and spectrophotometer can accommodate the small volume of labeled probe (~100 µl). The measurements described in this section assume the use of cuvettes having a 10-mm path length (such as Sigma Cat. No. C1918 or 9917). For the most rigorous analysis, we recommend using a scanning instrument to read the full absorbance spectrum from 200–800 nm for both Cy3 and Cy5 probes, using ddH_2O as a blank. Ensure that the baseline correction function is activated, if available on your spectrophotometer. The resulting data will be useful in troubleshooting problems with probe quality. Once you have performed these scans, perform the specific analyses described below. If a scanning instrument is not available, you may make the direct measurements indicated.

A. Absorbance measurements for Cy3 and Cy5 Probes

Obtain the absorbance readings of your probe at each of the wavelengths indicated in Table III (Cy3 probes) or Table IV (Cy5 probes). After reading the blank sample, insert the cuvette with your probe, and "re-zero" the instrument at 800 nm. If absorbance values or absorbance ratios do not meet the expected values, please refer to Section IX for troubleshooting tips.

NOTE: The absorbances and absorbance ratios described in this section are representative of typical results. Values will vary depending on the type and quality of RNA. These values are different from those typically obtained for non-amplified probes.

Table III: CY3 Probe Expected Measurements

Probe Component	Wavelength (nm) or Wavelength Ratio	Expected Absorbance	Expected Absorbance Ratio
Nucleic acid	260	0.6–1.2	-
Cy3/nucleic acid baseline[a]	450	<15% of A_{550}	-
Cy3	550	0.15–0.35	-
Cy3 baseline[b]	650	~0	-
Nucleic acid purity	A_{260}/A_{280}	-	1.8–2
Nucleic acid/Cy3 ratio[c]	$A_{260}/(A_{550} - A_{650})$	-	3–7

[a] The A_{450} measurement provides a broad-spectrum "baseline adjustment" for both nucleic acid absorbance at 260 nm and Cy3 absorbance at 550 nm.

[b] The A_{650} measurement, which is generally minimal, provides a baseline adjustment for analyzing Cy3 absorbance at 550 nm. In some cases, this value may be < 0.

[c] If $A_{650} < 0$, subtract the negative value from A_{550} in this calculation.

VIII. Analysis and Troubleshooting of Probe Quality, *continued*

Table IV: CY5 Probe Expected Measurements			
Probe Component	**Wavelength (nm) or Wavelength Ratio**	**Expected Absorbance**	**Expected Absorbance Ratio**
Nucleic acid	260	0.6–1.2	-
Cy5/nucleic acid baseline[a]	520	<15% of A_{650}	-
Cy5	650	0.15–0.4	-
Cy5 baseline[b]	750	~0	-
Nucleic acid purity	A_{260}/A_{280}	-	1.8–2
Nucleic acid/Cy5 ratio[c]	$A_{260}/(A_{550} - A_{650})$	-	3–7

[a] The A_{520} measurement provides a broad-spectrum "baseline adjustment" for both nucleic acid absorbance at 260 nm and Cy5 absorbance at 650 nm.

[b] The A_{750} measurement, which is generally minimal, provides a baseline adjustment for analyzing Cy5 absorbance at 650 nm. In some cases, this value may be < 0.

[c] If $A_{750} < 0$, subtract the negative value from A_{650} in this calculation.

B. Troubleshooting Probe Quality

1. A_{260} (nucleic acid)

The A_{260} measurement provides a rough estimate of the cDNA synthesis yield. A precise measurement of cDNA quantity cannot be calculated from this value due to the presence of residual RNA fragments in the probe. If your A_{260} value is << 0.4, you will not have an adequate amount of probe for array analysis. Low probe yields can result from RNA degradation or failure of the cDNA synthesis reaction.

2. A_{450} (Cy3/nucleic acid baseline) or A_{520} (Cy5/nucleic acid baseline)

a. The A_{450} (Cy3/nucleic acid baseline) measurement is typically minimal and serves as a general baseline value for nucleic acid and Cy3 absorbance (measured at A_{260} and A_{550}, respectively). Excessive background observed at this baseline measurement may indicate probe contamination. If A_{450} is greater than 0.02, compare the Cy3/nucleic acid baseline with the Cy3 baseline by calculating the baseline ratio:

$$\text{Cy3 baseline ratio} = \frac{A_{450} - A_{650}}{A_{550} - A_{650}}$$

NOTE: If $A_{650} < 0$, do not round A_{650} off to zero. Instead, subtract the negative value as indicated in the formula.

If the baseline ratio is > 0.15, your probe may be contaminated by particulate matter, which can lead to a high level of non-specific background during hybridization. In this case, purify your probe from insoluble material using one of the supplied 0.22-µm Spin Filters, and reanalyze the baseline values. Ensure that cuvettes are clean and that the cuvette compartment is free of dust.

b. The A_{520} (Cy5/nucleic acid baseline) is used in the same manner as the Cy3/nucleic acid baseline, described above. If A_{520} is greater than 0.02, compare the Cy5/nucleic acid baseline with the Cy5 baseline by calculating the baseline ratio:

$$\text{Cy5 baseline ratio} = \frac{A_{520} - A_{750}}{A_{650} - A_{750}}$$

NOTE: If $A_{650} < 0$, do not round A_{650} off to zero. Instead, subtract the negative value as indicated in the formula.

If the baseline ratio is > 0.15, your probe may be contaminated by particulate matter, which can lead to a high level of non-specific background during hybridization. In this case, purify your probe from insoluble material using one of the supplied 0.22-µm Spin Filters, and reanalyze the baseline values. Ensure that cuvettes are clean and that the cuvette compartment is free of dust.

VIII. Analysis and Troubleshooting of Probe Quality, *continued*

3. A_{550} (Cy3 absorbance) and A_{650} (Cy5 absorbance)

a. 550 nm and 650 nm are the absorbance maxima for Cy3 and Cy5, respectively.

- If either value is significantly above the range indicated in Table III or IV, it is likely that there is a significant amount of pure, unincorporated dye in your sample, and you may or may not have sufficient labeled cDNA. In this case, you should reprecipitate your probe using 1/10 volume 3 M Sodium Acetate and 2.5 volumes of 100% ethanol as described in Section VII. Then, reanalyze the spectrum using the appropriate reference solution. If A_{550} or A_{650} does not change after reprecipitation, this may indicate too much dye was incorporated. In this case, hybridization to the array may not be successful because very high levels of dye incorporation decrease hybridization efficiency. Probe synthesis should be repeated.
- If either value is significantly below the range indicated in Table III or IV, it is possible that the labeling reaction was not successful. Possible reasons include: the supplied Glass Approved DMSO was not used, the RNA was degraded, or the dye quality was suboptimal.

4. A_{650} (Cy3 baseline) or A_{750} (Cy5 baseline)

a. The A_{650} (Cy3 baseline) measurement is generally minimal for Cy3 probes and serves as a baseline value for the A_{550} Cy3 absorbance. If A_{650} is >> 0.01, your baseline may not be compensated correctly. Ensure that you have used the same buffer for the blank as was used for the probe elution. Record the blank and sample again and ensure that baseline correction functionality is activated on your spectrophotometer, if it is an automated instrument. Additionally, ensure that after inserting the cuvette with your probe, you "re-zero" the instrument at 800 nm before reading the spectrum.

b. The A_{750} (Cy5 baseline) measurement is generally minimal for Cy5 probes and serves as a baseline value for the A_{650} Cy5 absorbance. If A_{750} is >> 0.01, your baseline may not be compensated correctly. Ensure that you have used the same buffer for the blank as was used for the probe elution. Record the blank and sample again and ensure that baseline correction functionality is activated on your spectrophotometer, if it is an automated instrument. Additionally, ensure that after inserting the cuvette with your probe, you "re-zero" the instrument at 800 nm before reading the spectrum.

5. A_{260}/A_{280} (nucleic acid purity)

a. If the A_{260}/A_{280} ratio is less than 1.8, your probe may be contaminated with protein or particulate matter or the baseline may not be compensated correctly. Follow the steps below, as necessary, and reanalyze your probe.

- To check for correct baseline compensation, ensure you have used the same buffer for the blank as was used for the probe elution. Record the blank and sample again and ensure that baseline correction functionality is activated on your spectrophotometer, if it is an automated instrument.
- To purify your probe from insoluble material, use one of the supplied 0.22-µm Spin Filters, and reanalyze the baseline values. Ensure that cuvettes are clean and that the cuvette compartment is free of dust.
- To remove protein contaminants, repurify your probe using QuickClean resin and reanalyze A_{260}/A_{280}. To purify with QuickClean, use your entire volume of labeled probe to repeat Steps VII.A.8–VII.A.14. After completing the purification, air dry the pellet, and resuspend the probe in 100 µl of buffer. Reanalyze the probe spectrum.

b. If the A_{260}/A_{280} ratio is greater than 2.0, your probe may be contaminated with particulate matter. To purify your probe from insoluble material, use one of the supplied 0.22-µm Spin Filters, and reanalyze the baseline values. Ensure that cuvettes are clean and that the cuvette compartment is free of dust.

IX. Troubleshooting SMARTer Pico First-Strand Synthesis & PCR Amplification

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

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

X. References

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

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