User Manual

SMARTer™ RACE cDNA Amplification Kit User Manual

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I. List of Components

Cat. No. 634923 10 rxns	Cat. No. 634924 20 rxns	
First-Strand cDNA Synthesis		
• 10 µl	2x10 µl	**SMARTer II A Oligonucleotide** (12 µM) 5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3' (X = undisclosed base in the proprietary SMARTer oligo sequence)
• 10 µl	20 µl	**3'-RACE CDS Primer A** (3'-CDS; 12 µM) 5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30 V N–3' (N = A, C, G, or T; V = A, G, or C)
• 10 µl	20 µl	**5'-RACE CDS Primer A** (5'-CDS; 12 µM) 5'–$(T)_{25}$V N–3' (N = A, C, G, or T; V = A, G, or C)
• 10 µl	20 µl	**10X Random Primer Mix (N-15)** (20 µM)
• 40 µl	80 µl	**5X First-Strand Buffer (RNAse-Free)** 250 mM Tris-HCl (pH 8.3) 375 mM KCl 30 mM $MgCl_2$
• 20 µl	40 µl	**Dithiothreitol** (DTT; 20 mM)
• 1 ml	1 ml	**Deionized H_2O**
• 10 µl	10 µl	**RNase Inhibitor** (40 U/µl)
• 12 µl	25 µl	**SMARTScribe™ Reverse Transcriptase** (100 U/µl)
5'- & 3'-RACE PCR		
• 400 µl	800 µl	**10X Universal Primer A Mix (UPM)** Long (0.4 µM): 5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3' Short (2 µM): 5'–CTAATACGACTCACTATAGGGC–3'
• 50 µl	100 µl	**Nested Universal Primer A** (NUP; 10 µM) 5'–AAGCAGTGGTATCAACGCAGAGT–3'Control Reagents
• 5 µl	5 µl	**Control Mouse Heart Total RNA** (1 µg/µl)
• 25 µl	50 µl	**Control 5'-RACE TFR Primer** (10 µM)
• 25 µl	50 µl	**Control 3'-RACE TFR Primer** (10 µM)
General Reagents		
• 100 µl	200 µl	**dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
• 2x1 ml	2x1 ml	**Tricine-EDTA Buffer** 10 mM Tricine-KOH (pH 8.5) 1.0 mM EDTA

I. List of Components, *continued*

Cat. No. 634923 10 rxns	Cat. No. 634924 20 rxns	
NucleoTrap® Gel Extraction Kit		
• 100 µl	2x100 µl	**NucleoTrap Suspension**
• 6 ml	2x6 ml	**Buffer NT1**
• 6 ml	2x6 ml	**Buffer NT2**
• 7 ml	2x7 ml	**Buffer NT3** (concentrate)
• 5 ml	2x5 ml	**Buffer NE**

Storage Conditions

- Store Control Mouse Heart Total RNA and SMARTer II A Oligonucleotide at –70°C.
- Store the NucleoTrap Gel Extraction Kit at room temperature.
- Store all other reagents at –20°C.

II. Additional Materials Required

The SMARTer RACE cDNA Amplification Kit does not include a PCR polymerase.

We recommend that you perform your SMARTer RACE PCR reactions using the following kit or polymerase mix:

- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
- **Advantage 2 Polymerase Mix** (Cat. Nos. 639201 & 639202)

  Advantage 2 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. Advantage 2 technology enables you to perform long distance PCR (LD PCR) reactions with confidence that your products will have high fidelity to the original sequences (Barnes, 1994; Cheng *et al.*, 1994).

The following reagents are optional:

For efficient amplification of GC-rich templates, we recommend:

- **Advantage GC 2 PCR Kit** (Cat. Nos. 639119 & 639120)

For applications in which the highest fidelity product is desired, we recommend:

- **Advantage HF 2 PCR Kit** (Cat. Nos. 639123 & 639124)

If your RNA template is from a non-eukaryotic organism and lacks a polyadenylated tail, you can add one prior to first-strand cDNA synthesis using the following enzyme:

- **Poly(A) Polymerase** (Takara Bio Cat. No. 2180A)

III. Introduction & Protocol Overview

The **SMARTer™ RACE cDNA Amplification Kit** provides a method for performing both 5′- and 3′-rapid amplification of cDNA ends (RACE). The SMARTer RACE cDNA Amplification Kit is an improved version of our original SMART RACE Kit, with a new, SMARTer II A oligonucleotide and **SMARTScribe™ Reverse Transcriptase** included; it provides better sensitivity, less background and higher specificity. This powerful system allows you to isolate the complete 5′ sequence of your target transcript from as little as 10 ng of total RNA. The cornerstone of the SMARTer RACE cDNA Amplification Kit is SMART technology, which eliminates the need for problematic adaptor ligation and lets you use first-strand cDNA directly in RACE PCR, a benefit that makes RACE far less complex and much faster (Chenchik *et al.*, 1998). Additionally, the SMARTer RACE Kit exploits Clontech's patented suppression PCR & step-out PCR to increase the sensitivity and reduce the background of the RACE reactions. As a result you can use either poly A^+ or total RNA as starting material for constructing full-length cDNAs of even very rare transcripts.

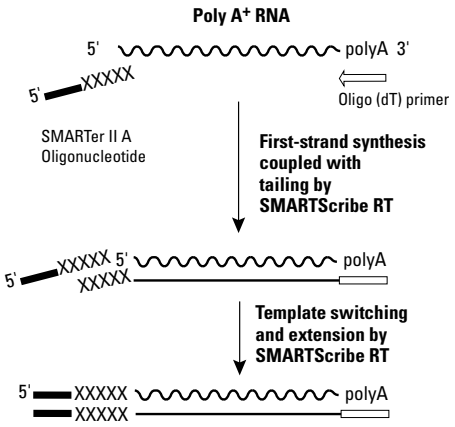


Figure 1. Mechanism of SMARTer cDNA synthesis. First-strand synthesis is primed using a modified oligo(dT) primer. After SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, it adds several nontemplate residues. The SMARTer II A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for SMARTScribe RT.

SMART technology provides a mechanism for generating full-length cDNAs in reverse transcription reactions (Zhu *et al.*, 2001). This is made possible by the joint action of the SMARTer II A Oligonucleotide and SMARTScribe Reverse Transcriptase (a variant of MMLV RT). The SMARTScribe RT, upon reaching the end of an RNA template, exhibits terminal transferase activity, adding 3–5 residues to the 3′ end of the first-strand cDNA (Figure 1). The SMARTer oligo contains a terminal stretch of modified bases that anneal to the extended cDNA tail, allowing the oligo to serve as a template for the RT. SMARTScribe RT switches templates from the mRNA molecule to the SMARTer oligo, generating a complete cDNA copy of the original RNA with the additional SMARTer sequence at the end. Since the template switching activity of the RT occurs only when the enzyme reaches the end of the RNA template, the SMARTer sequence is typically only incorporated into full-length, first-strand cDNAs. For more information about SMART technology, read "Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR" (Chenchik *et al.*, 1998). This process guarantees that the use of high quality RNA will result in the formation of a set of cDNAs that have a maximum amount of 5′ sequence (Table I).

Table I: Additional 5′-RACE Sequence Obtained with SMART Technology

Human gene	Size of mRNA (kb)	Additional sequence (bp)*	Matches genomic sequences	Includes transcription start site
Transferrin receptor	5.0	+25	yes	yes
Smooth muscle g-actin	1.28	+31	yes	yes
Vascular smooth muscle α–actin	1.33	+17	yes	yes
Cytoskeletal γ-actin	1.9	+1	yes	yes
23 kDa HBP	0.67	+9	yes	yes
p53	2.6	+4	yes	yes
Interferon-γ receptor	2.06	+14	yes	yes
14-3-3 protein	1.03	+1	n/a	n/a
Interferon-α receptor	2.75	+17	yes	yes

n/a = not available
* Compared to GenBank cDNA sequence.

III. Introduction & Protocol Overview, *continued*

Following reverse transcription, SMART technology allows first-strand cDNA to be used directly in 5′- and 3′-RACE PCR reactions. Incorporation of universal primer binding sites in a single-step during first-strand cDNA synthesis eliminates the need for tedious second-strand synthesis and adaptor ligation. This simple and highly efficient SMARTer cDNA synthesis method ensures higher specificity in amplifying your target cDNA. Suppression PCR & step-out PCR techniques (described in detail in Appendix C) are used in combination with SMARTer technology to decrease background amplification in RACE PCR.

The only requirement for SMARTer RACE cDNA amplification is that you know at least 23–28 nucleotides (nt) of sequence information in order to design gene-specific primers (GSPs) for the 5′- and 3′-RACE reactions. (Additional sequence information will facilitate analysis of your RACE products.) This limited requirement makes SMARTer RACE ideal for characterizing genes identified through diverse methods, including cDNA subtraction, differential display, RNA fingerprinting, ESTs, library screening, and more.

SMARTer RACE cDNA amplification is a flexible tool—many researchers use this kit in place of conventional kits to amplify just the 5′ or 3′ end of a particular cDNA. Others perform both 5′- and 3′-RACE, and many then go on to clone full-length cDNAs using one of the two methods described in the latter part of this protocol. In many cases, researchers obtain full-length cDNAs without ever constructing or screening a cDNA library.

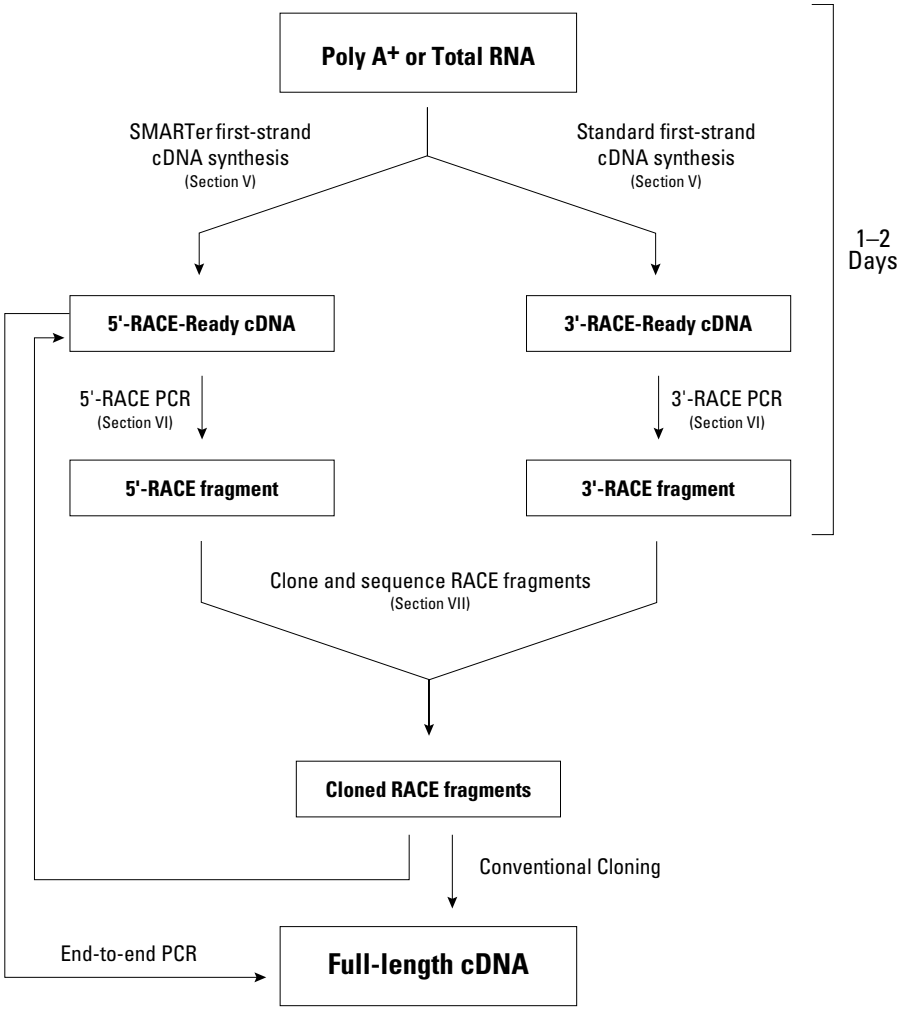


Figure 2. Overview of the SMARTer RACE procedure. Detailed flow charts of the SMARTer RACE mechanisms can be found in Appendices A & B. Note that with the cloned RACE fragments you can use a restriction site in an overlapping region to construct a full-length cDNA by subcloning. Alternatively, you can sequence the 5' end of the 5' product and the 3' end of the 3' product to obtain the sequences of the extreme ends of the transcript. Using this information, you can design 5' and 3' gene-specific primers to use in LD PCR with the 5'-RACE-Ready cDNA as template to generate the full-length cDNA.

III. Introduction & Protocol Overview, *continued*

The table below describes the major steps necessary to generate RACE-ready cDNA, perform 5′ & 3′ RACE PCR reactions, and characterize the final RACE products using the SMARTer RACE cDNA Amplification Kit. Please refer to the specified sections for details on performing each step. Detailed mechanisms of the RACE reactions are provided in Appendices A & B.

Table II: SMARTer RACE Protocol Overview

Step	Description	Section
Primer Design	You must design gene-specific primers for the 5′- and/or 3′-RACE reactions (GSP1 and GSP2, respectively). As described, nested primers (NGSP1 and NGSP2) will facilitate analysis of your RACE products. They can also be used for nested RACE PCR if necessary. Primer design is discussed in detail in Section IV; Figure 3 shows the relationship of primers and template used in SMARTer RACE reactions.	IV
Check the Quality of your RNA Template	The purity of RNA is the key factor for successful cDNA synthesis and SMARTer RACE. Prior to cDNA synthesis, you must **make sure that your RNA is intact and free of contaminants**.	V.C
RACE-Ready First-Strand cDNA Synthesis	Since the 5′ elongation benefits of SMART technology are only relevant for 5′-RACE, the SMARTer RACE Kit includes a protocol for the synthesis of two separate cDNA populations: 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA. The **5′-RACE cDNA** is synthesized using a modified lock-docking oligo(dT) primer and the SMARTer II A oligo as described above. The modified oligo(dT) primer, termed the 5′-RACE CDS Primer A (5′-CDS), has two degenerate nucleotide positions at the 3′ end. These nucleotides position the primer at the start of the poly A^+ tail and thus eliminate the 3′ heterogeneity inherent with conventional oligo(dT) priming (Borson *et al.*, 1994). The **3′-RACE cDNA** is synthesized using a traditional reverse transcription procedure, but with a special oligo(dT) primer. This 3′-RACE CDS Primer A (3′-CDS) primer includes the lock-docking nucleotide positions as in the 5′-CDS primer and also has a portion of the SMARTer sequence at its 5′ end. By incorporating the SMARTer sequence into both the 5′- and 3′-RACE-Ready cDNA populations, you can prime both RACE PCR reactions using the Universal Primer A Mix (UPM), which recognizes the SMARTer sequence, in conjunction with distinct gene-specific primers. Alternatively, **non-poly A^+ RNA can be primed with random primers using the protocol in Appendix D.**	V.D
Positive Control RACE Experiment	Prior to performing RACE with your template, we strongly recommend that you perform the positive control RACE experiment using the Control Mouse Heart Total RNA provided in the kit.	VI.B
5′ & 3′ RACE PCR Reactions	After you generate RACE-Ready cDNAs, you will have enough material to perform 5′– and 3′–RACE with different genes, simply by using different gene-specific primers. All PCR reactions in the SMARTer RACE protocol are optimized for use with the Advantage® 2 Polymerase Mix. Advantage 2 provides an automatic hot-start, and enables you to perform long distance PCR (LD PCR) reactions with confidence that your products will have high fidelity to the original sequences (Barnes, 1994; Cheng *et al.*, 1994). As a result, you will be able to amplify longer templates than were possible in traditional RACE procedures.	VI.D
Characterization of RACE Products	Before constructing your full-length cDNA, we strongly recommend that you confirm amplification of the desired target. You can characterize your RACE products by one or more of the following: (1) comparing PCR products generated with GSP1 and UPM to products generated with NGSP1 and UPM; (2) probing a Southern blot of your PCR products with an internal gene-specific probe (e.g., labeled NGSP1); and (3) cloning and sequencing your RACE products. In general, we recommend that you obtain at least some sequence information. Careful characterization of your RACE products at this point can prevent confusion and wasted effort in your subsequent experiments, even when both RACE reactions produce single major products. This analysis is especially important if you have multiple RACE products or suspect that you are working with a member of a multigene family.	VII

IV. Primer Design

A. Primer Sequence

Gene-Specific Primers (GSPs) should be:

- 23–28 nt
- 50–70% GC
- T_m ≥65°C; best results are obtained if T_m >70°C (enables the use of touchdown PCR)
- not complementary to the 3′-end of the Universal Primer Mix
- specific to your gene of interest

The relationship of the primers used in the SMARTer RACE reactions to the template and resulting RACE products is shown in detail in Figure 3. For the complete SMARTer RACE protocol, you will need at least two GSPs: an antisense primer for the 5′-RACE PCR and a sense primer for the 3′-RACE PCR. If you are doing only 5′- or 3′-RACE, you will only need one GSP. All primers should be 23–28 nt long; there is generally no advantage to using primers longer than 30 nt. The primers shown in Figure 3 will create overlapping 5′- and 3′-RACE products. If a suitable restriction site is located in the region of overlap, the fragments can subsequently be joined by restriction digestion and ligation to create the full-length cDNA. By designing primers that give a 100–200-bp overlap in the RACE products, you will also be able to use the primers together as a positive control for the PCR reactions. However, it is not absolutely necessary to use primers that give overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. Additionally, the primers themselves can overlap (i.e., be complementary).

GSPs should have a GC content of 50–70% and a T_m of at least 65°C; whenever possible the T_m should be greater than 70°C, as determined by nearest neighbor analysis (Freier *et al.*, 1986; we use the Primer Premier software to calculate T_m's). In our experience, longer primers with annealing temperatures above 70°C give more robust amplification in RACE, particularly from difficult samples. T_m's over 70°C allow you to use "touchdown PCR" (Section C below). Additionally, designing GSP1 and GSP2 so that they have similar T_m's will facilitate their use in the SMARTer RACE protocol. T_m's of GSP1 and GSP2 can be calculated or determined experimentally by performing PCR at different temperatures. Avoid using self-complementary primer sequences, which can fold back and form intramolecular hydrogen bonds.

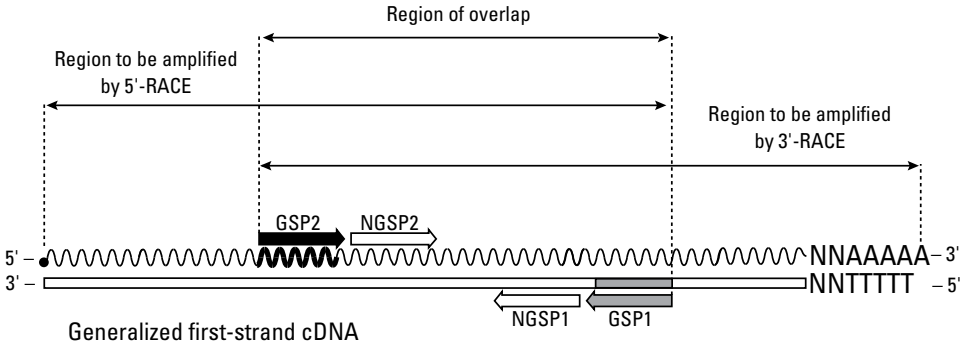


Figure 3. The relationship of gene-specific primers to the cDNA template. This diagram shows a generalized first-strand cDNA template. This RNA/DNA hybrid does not precisely represent either the 5′-RACE-Ready or 3′-RACE-Ready cDNAs. For a detailed look at those structures, see Appendices A & B. Note that the gene-specific primers designed here produce overlapping RACE products. This overlap permits the use of the primers together in a control PCR reaction. Additionally, if a suitable restriction site is located within this region, it will be possible to construct the full-length cDNA by subcloning.

IV. Primer Design, *continued*

B. Location of Primer Sequences within Genes

We have had good success using the SMARTer RACE Kit to amplify 5′ and 3′ cDNA fragments that extend up to 6.5 kb from the GSP binding sites. Nevertheless, for optimum results we recommend choosing your primers so that the 5′- and 3′-RACE products will be 2 kb or less.

C. Touchdown PCR

We have found that touchdown PCR (Don *et al.*, 1991; Roux, 1995) significantly improves the specificity of SMARTer RACE amplification. Touchdown PCR uses an annealing temperature during the initial PCR cycles that is higher than the T_m of the Universal Primer. If the T_m of your GSP is >70°C, only gene-specific synthesis occurs during these cycles, allowing a critical amount of gene-specific product to accumulate. The annealing temperature is then reduced to a level compatible with the UPM, permitting efficient, exponential amplification of the gene-specific template. (See Appendices A–C for more details.)

As noted above, we recommend using primers with T_m's >70°C to allow you to use the touchdown cycling programs in the protocol. (Non-touchdown cycling programs are also included for use with primers with T_m's <70°C.)

D. Nested Primers

We recommend that you do not use nested PCR in your initial experiments. The UPM Primer and a GSP will usually generate a good RACE product with a low level of nonspecific background. However, Southern blotting with nested GSPs (NGSP1 and NGSP2) as probes is useful for characterizing your RACE products. Furthermore, nested PCR may be necessary in some cases where the level of background or nonspecific amplification in the 5′- or 3′-RACE reaction is too high with a single GSP. In nested PCR, a primary amplification is performed with the outer primers and, if a smear is produced, an aliquot of the primary PCR product is reamplified using the inner primers. The SMARTer RACE protocols include optional steps indicating where nested primers can be used. The Nested Universal Primer A (provided with the kit) can be used for both 5′- and 3′-RACE.

Nested gene specific primers should be designed according to the guidelines discussed above. If possible, nested primers should not overlap with the outer gene-specific primers; if they must overlap due to limited sequence information, the 3′ end of the inner primer should have as much unique sequence as possible.

V. Generating RACE-Ready cDNA

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Use the following protocol for generating RACE-Ready cDNA using Clontech's simple and highly efficient SMARTer™ technology. General considerations for safety, success & RNA handling are provided in Sections A & B. Detailed instructions for assessing the quality of your RNA template prior to first-strand cDNA synthesis are provided in Section C, followed by the protocol for first-strand cDNA synthesis in Section D.

A. General Considerations

- We recommend the Tricine-EDTA Buffer provided in the kit for resuspending and diluting your cDNA samples throughout the protocols in this user manual. Tricine buffers maintain their pH at high temperature better than Tris-based buffers. Tris-based buffers can lead to low pH conditions that degrade DNA.
- Resuspend pellets and mix reactions by gently pipetting the solution up and down or by flicking the bottom of the tube. Always spin tubes briefly prior to opening to collect the contents at the bottom of the tubes.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last.
- Use the recommended amounts of enzyme. These amounts have been carefully optimized for the SMARTer RACE amplification protocol and reagents.
- Ethidium bromide is a carcinogen. Use appropriate precautions when handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manual* by Sambrook & Russell (2001).

B. Preparation & Handling of Total and Poly A$^+$ RNA

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 to protect your RNA samples from nucleases.
- Use freshly deionized (e.g., MilliQ-grade) H_2O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H_2O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips.

RNA Isolation

Clontech offers several kits for isolating total or poly A$^+$ RNA from a variety of sources. The **NucleoBond® RNA/DNA Kit** (Cat. No. 740650) contains AX-R tips to isolate total RNA from tissue or cells without using phenol or chloroform. With the **NucleoSpin® RNA II Kit** (Cat. No. 740955.20), you can isolate highly pure total RNA from cells, tissues, or cell-free biological fluids without phenol chloroform extractions. The **NucleoTrap® mRNA Mini Kit** (Cat. No. 740665) combines a spin-column filter with oligo(dT)-latex bead technology to isolate high-quality mRNA from total RNA in less than 30 minutes. Many procedures are available for the isolation of poly A$^+$ RNA (Farrell, 1993; Sambrook *et al.*, 1989).

V. Generating RACE-Ready cDNA, *continued*

RNA Purity

The purity of RNA is the key factor for successful cDNA synthesis and SMARTer RACE. The presence of residual organics, metal ions, salt or nucleases in your RNA sample could have a large impact on downstream enzymatic 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 V.C., below, 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.

C. Assessing the Quality of the RNA Template

Methods for Assessing Total RNA Integrity

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

   The integrity of total RNA can be visually assessed by the ratio of 28S:18S RNA on a denaturing formaldehyde agarose gel by staining with EtBr. The theoretical 28S:18S ratio for eukaryotic RNA is approximately 2:1. If the 28S:18S ratio of your RNA is less than 1, your RNA template is not suitable for SMARTer RACE. You need at least 0.5 –1 µg of total RNA for this method for better visualization.

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

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

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

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

Methods for Assessing mRNA Integrity

All of the 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. Generating RACE-Ready cDNA, *continued*

D. PROTOCOL: First-Strand cDNA Synthesis

The two 10 µl reactions described in this protocol convert 10 ng–1 µg of total or poly A+ RNA into RACE-Ready first-strand cDNA.

We recommend that you use poly A+ RNA whenever possible. However, if you have less than 50 µg of total RNA we do not recommend purification of poly A+ RNA because the final yield will be too small to effectively analyze the RNA quantity and quality.

We strongly recommend that you perform a positive control cDNA synthesis using the included Mouse Heart Total RNA in addition to your experimental reactions. This cDNA will be used in the positive control RACE reactions in Section VI.B.

NOTE: If your RNA template is from a non-eukaryotic organism and/or lacks a polyadenylated tail, follow the protocol for first-strand cDNA synthesis with random primers in Appendix D. Alternatively, you can add a poly(A) tail using Poly(A) Polymerase (Takara Bio USA Cat. No.2180A), and proceed with the following protocol.

IMPORTANT:

- Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants (see Section V.C. Assessing the Quality of the RNA Template).
- Do not change the size (volume) of any of the reactions. All components have been optimized for the volumes specified.

1. Prepare enough of the following Buffer Mix for all of the 5′- & 3′-RACE-Ready cDNA synthesis reactions plus 1 extra reaction to ensure sufficient volume. For each 10 µl cDNA synthesis reaction, mix the following reagents and spin briefly in a microcentrifuge, then set aside at room temperature until Step 7:

2.0 µl	**5X First-Strand Buffer**
1.0 µl	**DTT** (20 mM)
1.0 µl	**dNTP Mix** (10 mM)
4.0 µl	**Total Volume**

2. Combine the following reagents in separate microcentrifuge tubes:

For preparation of 5'-RACE-Ready cDNA	**For preparation of 3'-RACE-Ready cDNA**
1.0–2.75 µl **RNA***	1.0–3.75 µl **RNA***
1.0 µl **5'-CDS Primer A**	1.0 µl **3'-CDS Primer A**

*For the control synthesis, use 1 µl of Control Mouse Heart Total RNA (1 µg/µl)

3. Add sterile H_2O to the tubes from Step 2 for a final volume of 3.75 µl for 5′ RACE and 4.75 µl for 3′ RACE.
4. Mix contents and spin the tubes briefly in a microcentrifuge.
5. Incubate the tubes at 72°C for 3 min, then cool the tubes to 42°C for 2 min. After cooling, spin the tubes briefly for 10 seconds at 14,000 g to collect the contents at the bottom.

NOTE: This step can be performed in a thermocycler. While the tubes are incubating, you can prepare the Master Mix in Step 7.

6. To just the 5' RACE cDNA synthesis reaction(s), add 1 µl of the SMARTer IIA oligo per reaction.

V. Generating RACE-Ready cDNA, *continued*

7. Prepare enough of the following Master Mix for all 5′- & 3′-RACE-Ready cDNA synthesis reactions. Mix these reagents at room temperature in the following order:

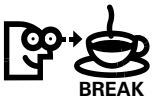
4.0 µl	**Buffer Mix from Step 1**
0.25 µl	**RNase Inhibitor** (40 U/µl)
1.0 µl	**SMARTScribe™ Reverse Transcriptase** (100 U)
5.25 µl	**Total Volume**

8. Add 5.25 µl of the Master Mix from Step 7 to the denatured RNA from Step 5 (3'-RACE cDNA) and Step 6 (5' RACE cDNA), for a total volume of 10 µl.
9. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
10. Incubate the tubes at 42°C for 90 min in an air incubator or a hot-lid thermal cycler.

NOTE: Using a water bath for this incubation may reduce the volume of the reaction mixture (due to evaporation), and therefore reduce the efficiency of first-strand synthesis.

11. Heat tubes at 70°C for 10 min.
12. Dilute the first-strand reaction product with Tricine-EDTA Buffer:
    - Add 20 µl if you started with ≤200 ng of total RNA.*
    - Add 100 µl if you started with ≥200 ng of total RNA.*
    - Add 250 µl if you started with poly A^+ RNA.

    *The copy number of your gene of interest should be the determining factor for diluting your sample. If you have 200 ng of total RNA but your gene of interest has low abundance, dilute with 20 µl. If you have 200 ng of total RNA and the gene of interest is highly abundant, dilute with 100 µl.



13. Samples can be stored at –20°C for up to three months.

VI. Rapid Amplification of cDNA Ends (RACE)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Things you should know prior to starting RACE PCR (Section A), a positive control RACE PCR Experiment (Section B), and details about the control reactions (Section C) are outlined below. The protocol for RACE is provided in Section D.

At this point, you have 3′- and 5′-RACE-Ready cDNA samples. The RACE reactions in this section use only a fraction of this material for each RNA of interest. There is sufficient single-stranded cDNA for PCR amplification of multiple genes.

If you intend to use LD PCR to construct your full-length cDNA after completing 5′- and 3′-RACE, be sure to set aside an aliquot of the 5′-RACE-Ready cDNA to use as a template in the PCR reaction.

A. Things You Should Know Before Starting RACE PCR Reactions

The cycling parameters throughout this protocol were optimized with an authorized hot-lid thermal cycler, Advantage 2 Polymerase Mix, and the reagents and TFR controls provided in the SMARTer RACE Kit. The optimal cycling parameters may vary with different polymerase mixes, templates, gene-specific primers, and thermal cyclers. Prior to performing 5′- and 3′-RACE with your experimental sample, you should perform the positive control PCR experiment (Section B). These reactions, which use cDNA generated from the Control Mouse Heart Total RNA and the Control 5′- and 3′- RACE TFR Primers, will help determine if you need to alter the PCR program for your thermal cycler.

Please note that the efficiency of RACE PCR depends on the abundance of the mRNA of interest in your RNA sample. Additionally, different primers will have different optimal annealing/extension temperatures. Refer to the Troubleshooting Guide (Section XI) for suggestions on optimizing PCR conditions.

You must use some form of hot start in the 5′-RACE and 3′-RACE PCR reactions (Section D). The following protocols were optimized using the Advantage 2 Polymerase Mix which contains TaqStart Antibody for automatic hot start PCR (Kellogg *et al.*, 1994). Hot start can also be performed using wax beads (Chou *et al.*, 1992) or manually (D'Aquila *et al.*, 1991).

B. PROTOCOL: Positive Control RACE PCR Experiment

Prior to performing 5′- and 3′-RACE reactions with your cDNA, we strongly recommend that you perform the following positive control RACE PCR experiment using the RACE-Ready cDNAs generated from the Control Mouse Heart Total RNA. These reactions will amplify the ends of the transferrin receptor (TFR) cDNA. This procedure can save you considerable time by ensuring that the SMARTer RACE protocol works with your thermal cycler. If problems arise later in the protocol, the results of this experiment will help you determine immediately if the problem is with your RACE reaction (e.g., from the use of a different thermal cycler) or with your cDNA.

We recommend that you first perform SMARTer RACE PCR reactions using the Advantage 2 Polymerase Mix (Cat. Nos. 639206 & 639207). If your cDNA of interest has a high GC content you can use the Advantage GC 2 Polymerase Mix (Cat. No. 639114) or PCR Kit (Cat. Nos. 639119 & 639120) for subsequent analysis. For applications in which the highest fidelity product is desired, the Advantage HF 2 PCR Kit (Cat. Nos. 639123 & 639124) can amplify templates of up to 3.5 kb. For more information, see Section VIII (Troubleshooting Guide).

1. Prepare enough Master Mix for all of the PCR reactions plus 1 extra reaction to ensure sufficient volume. For each 50 µl PCR reaction, mix the following reagents:

34.5 µl	**PCR-Grade Water**
5.0 µl	**10X Advantage 2 PCR Buffer**
1.0 µl	**dNTP Mix** (10 mM; in SMARTer RACE or Advantage 2 PCR Kit)
1.0 µl	**50X Advantage 2 Polymerase Mix**
41.5 µl	**Total Volume**

VI. Rapid Amplification of cDNA Ends (RACE), *continued*

2. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
3. Prepare PCR reactions as shown in Table III. Add the components to PCR tubes in the order shown and mix gently.

Table III: Setting Up the Positive Control RACE Experiment

Component	Tube No. & Description			
	1 5′ RACE Control	2 3′ RACE Control	3 Internal Control (5′-cDNA)	4 Internal Control (3′-cDNA)
Control 5′-RACE-Ready cDNA	2.5 µl	—	2.5 µl	—
Control 3′-RACE-Ready cDNA	—	2.5 µl	—	2.5 µl
5′-RACE TFR Primer (10 µM)	1 µl	—	1 µl	1 µl
3′-RACE TFR Primer (10 µM)	—	1 µl	1 µl	1 µl
UPM (10X)	5 µl	5 µl	—	—
H_2O	—	—	4 µl	4 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final Volume	50 µl	50 µl	50 µl	50 µl
Expected Product Size	2.1 kb	3.1 kb	0.38 kb	0.38 kb

NOTE: If you are NOT using a hot-lid thermal cycler, overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

4. Commence thermal cycling using the following program for touchdown PCR:

   - 5 cycles:

     94°C 30 sec
     72°C 3 min

   - 5 cycles:

     94°C 30 sec
     70°C 30 sec
     72°C 3 min

   - 27 cycles:

     94°C 30 sec
     68°C 30 sec
     72°C 3 min

5. Analyze 5 µl of each sample on a 1.2 % agarose/EtBr gel. Store the remaining 45 µl of each reaction at 4°C until you are sure the control experiment has worked.

VI. Rapid Amplification of cDNA Ends (RACE), *continued*

Expected Results

The 5′-RACE control reaction should produce a 2.1 kb band (Figure 4, Lane 1). The 3′-RACE control reaction should produce a 3.1 kb band (Figure 4, Lane 2). If you do not observe these bands, return the tube(s) to your thermal cycler and try cycling the remaining portion of the reaction for 5 additional cycles. If you still do not see the desired product, consult Section VIII for troubleshooting. Before you attempt 5′- and 3′-RACE with your primers and experimental cDNA, we recommend that the positive control reactions produce single strong bands of the correct size in 42 or fewer total cycles (5 cycles annealing at 72°C + 5 cycles at 70°C + 32 cycles at 68°C).

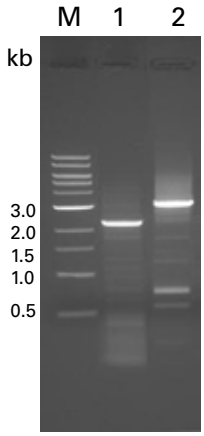


Figure 4. 5′- and 3′-RACE sample results. The gel shows the 5′- and 3′-RACE amplifications of transferrin receptor starting with mouse heart total RNA. Lane M: 1 kb DNA marker. Lanes 1& 2: transferrin receptor (TFR). The 5′ product will be 2.1 kb; the 3′ product will be 3.1 kb. As seen here, minor products will occasionally be generated in transferrin receptor 3′-RACE.

C. Control PCR Reactions

Tables IV and V in Section VI.D describe several control reactions that will help you troubleshoot your RACE reactions if yields are suboptimal. These include:

- **Tube No. 2:** 5′- or 3′-RACE PCR using the positive control TFR Primer, the UPM Primer Mix, and the 5′- and 3′-RACE-Ready cDNA made from your experimental RNA. Figure 4 (above) shows the expected results of 5′- and 3′- RACE using these positive controls.
- **Tube No. 3:** An additional positive control using both GSPs to amplify the overlapping segment of your 5′- and 3′-RACE fragments (if available). This reaction should give a single band corresponding to the overlap between the primers and confirms that your target cDNA is present in, and can be amplified from, your RACE-Ready cDNA. If you do not have suitable 5′- and 3′-GSPs (i.e., GSPs that create overlapping 5′- and 3′-RACE products), use the control 5′- and 3′-RACE TFR Primers with 5 µl of your positive control RACE-Ready cDNAs (if human).
- **Tube No. 4:** A negative control using the UPM alone to amplify your cDNA. With fewer than 40 cycles, this reaction should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters or perform a secondary amplification using the Nested Universal Primer A.
- **Tube No. 5:** A negative control using each GSP by itself. This control should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters, perform a secondary amplification using nested primers, or redesign your original primers.

VI. Rapid Amplification of cDNA Ends (RACE), *continued*

D. PROTOCOL: Rapid Amplification of cDNA Ends (RACE)

This procedure describes the 5′-RACE and 3′-RACE PCR reactions that generate the 5′ and 3′ cDNA fragments. We recommend that you also perform positive control 5′- and 3′-RACE using the TFR primers, UPM, and control RACE-Ready cDNAs as described in Section VI.B. Although the Nested Universal Primer A (NUP) is provided, nested PCR is generally not necessary in SMARTer RACE reactions.

Please note that all RACE PCR reactions have been optimized for use with the Advantage 2 Polymerase Mix.

1. Prepare enough PCR Master Mix for all of the PCR reactions plus one extra reaction to ensure sufficient volume. The same Master Mix can be used for both 5′- and 3′-RACE reactions. For each 50 µl PCR reaction, mix the following reagents:

34.5 µl	**PCR-Grade Water**
5.0 µl	**10X Advantage 2 PCR Buffer**
1.0 µl	**dNTP Mix** (10 mM; in SMARTer RACE or Advantage 2 PCR Kit)
1.0 µl	**50X Advantage 2 Polymerase Mix**
41.5 µl	**Total Volume**

2. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
3. **For 5′-RACE:** prepare PCR reactions as shown in Table IV.
   For 3′-RACE: prepare PCR reactions as shown in Table V.
   Add the components to 0.5 ml PCR tubes in the order shown and mix gently.

Table IV: Setting Up the 5′-RACE PCR Reactions

Component	Tube No. & Description				
	1 5′-RACE Sample	2 5′-TFR* (+ Control)	3 GSP1 + GSP2† (+ Control)	4 UPM only (– Control)	5 GSP1 only (– Control)
5′-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	5 µl	5 µl	—	5 µl	—
GSP1 (10 µM)	1 µl	—	1 µl	—	1 µl
GSP2 (10 µM)	—	—	1 µl	—	—
Control 5′-RACE TFR Primer (10 µM)	—	1 µl	—	—	—
H_2O	—	—	4 µl	1 µl	5 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final Volume	50 µl	50 µl	50 µl	50 µl	50 µl

* Skip this reaction if your RNA is nonmouse.
† Skip this reaction if your GSPs will not create overlapping RACE fragments.
For detailed descriptions of the control reactions, see Section VI.C.

VI. Rapid Amplification of cDNA Ends (RACE), *continued*

Table V: Setting Up the 3′-RACE PCR Reactions

Component	Tube No. & Description				
	1 3′-RACE Sample	2 3′-TFR* (+ Control)	3 GSP1 + GSP2† (+ Control)	4 UPM only (– Control)	5 GSP1 only (– Control)
3′-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	5 µl	5 µl	—	5 µl	—
GSP1 (10 µM)	—	—	1 µl	—	—
GSP2 (10 µM)	1 µl	—	1 µl	—	1 µl
Control 3′-RACE TFR Primer (10 µM)	—	1 µl	—	—	—
H_2O	—	—	4 µl	1 µl	5 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final Volume	50 µl	50 µl	50 µl	50 µl	50 µl

Recipe

* Skip this reaction if your RNA is nonmouse.

† Skip this reaction if your GSPs will not create overlapping RACE fragments.

For detailed descriptions of the control reactions, see Section VI.C.

NOTE

NOTE: If you are NOT using a hot-lid thermal cycler, overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

4. Commence thermal cycling using one of the following programs (both programs 1 and 2 work with the positive control 5′- and 3′-RACE TFR and UPM Primers). Be sure to choose the correct number of cycles (as noted) based on whether you started with poly A⁺ or total RNA.

NOTE

NOTES on cycling: Because the necessary number of cycles depends on the abundance of the target transcript, you may need to determine the optimal cycling parameters for your gene empirically. Run 20 or 25 PCR cycles first as described and analyze 5 µl from each tube, along with appropriate DNA size markers, on a 1.2% agarose/EtBr gel. If you see weak bands or no bands, return the tube(s) to your thermal cycler and perform five additional cycles (according to the third set of cycles for touchdown PCR). The optimal extension time depends on the length of the desired amplicon. For 0.2-2 kb amplicons, we typically extend for 2 min; for 2-4 kb amplicons, we extend for 3 min; and for 5-10 kb amplicons, we extend for up to 10 min.

Program 1 (preferred; use if GSP T_m >70°C)

- 5 cycles:

  94°C 30 sec
  72°C 3 min*

- 5 cycles:

  94°C 30 sec
  70°C 30 sec
  72°C 3 min*

- 20 cycles (Poly A⁺ RNA) OR 25 cycles (Total RNA):
  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

  *If fragments >3 kb are expected, add 1 min for each additional 1 kb.

VI. Rapid Amplification of cDNA Ends (RACE), *continued*

Program 2 (use if GSP T_m = 60–70°C)

- 20 cycles (Poly A^+ RNA) OR 25 cycles (Total RNA):
  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

  *If fragments >3 kb are expected, add 1 min for each additional 1 kb.

5. [OPTIONAL] If the primary PCR reaction fails to give the distinct band(s) of interest or produces a smear, you may wish to perform a Southern blot using:

   a. A cDNA Probe

   b. A nested primer as a probe

   Or, you may wish to perform a secondary, or "nested" PCR reaction using the NUP primer supplied and a NGSP (See the discussion in Section IV.)

   a. Dilute 5 µl of the primary PCR product into 245 µl of Tricine-EDTA buffer.

   b. Repeat Steps 1–5 above, using:

      - 5 µl of the diluted primary PCR product in place of the RACE-Ready cDNAs.
      - 1 µl of the NUP primer and 1 µl of your nested GSPs.
      - 15–20 cycles of Program 2.

VII. Characterization of RACE Products

At this point, we recommend that you characterize your RACE fragments and confirm that you have amplified the desired product. This procedure can prevent confusion and wasted effort when you generate the full-length cDNA, even if you have single major products from both the 5′- and 3′-RACE reactions. Characterization is especially important if you have multiple bands or if you suspect that you are working with a member of a multigene family.

We describe three methods for characterizing RACE products: (Section A) Comparison of RACE products obtained with GSPs and NGSPs; (Section B) Southern blotting; and (Section D) Cloning and sequencing. Options A and B require nested GSPs for analyzing 5′- and 3′-RACE products. For more detailed blotting and cloning protocols, see Sambrook & Russell (2001) or other appropriate laboratory manuals.

A. Comparison of RACE Products Obtained with GSPs & NGSPs

For the 5′-RACE reaction, compare the products of primary amplifications performed with the UPM Mix and GSP1 to the products obtained using the UPM and NGSP1. (For 3′-RACE, compare the products obtained from amplifications with the UPM and GSP2 to those obtained with the UPM and NGSP2.) This analysis will help determine if any multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If the bands are real (i.e., the result of correct priming), they should be slightly smaller in the reaction using the nested gene-specific primers. The difference in the mobility of the products should correspond to the positions of the outer and inner (nested) gene-specific primers in the cDNA structure. If you have multiple bands with UPM and GSP1 (or GSP2), some may disappear upon amplification with UPM and NGSP1 (or NGSP2).

B. Southern Blot Analysis

You can obtain stronger confirmation of your RACE products by probing a Southern blot with an internal gene-specific probe (usually one of your other GSPs or NGSPs). This method can be particularly useful for determining which bands are real when RACE produces multiple bands. Multiple bands are more common with 5′-RACE than with 3′-RACE.)

1. Examine your RACE products on an agarose/EtBr gel.
2. Photograph the gel, then transfer the DNA to a nylon membrane using standard blotting procedures.
3. Prepare a hybridization probe that does not have sequences in common with GSP1 (or GSP2). The probe can be end-labeled NGSP1 (or NGSP2). Alternatively, if your GSPs define overlapping 5′ and 3′ fragments, GSP2 can be used as a probe to characterize your 5′-RACE products, and GSP1 can be used as a probe to characterize your 3′-RACE products. Nick-translated or random-primed internal restriction fragments from a previously cloned partial cDNA can also be used.
4. Hybridize the probe to the Southern blot, wash under moderate-to-high stringency conditions, and expose x-ray film.
5. Compare the hybridization pattern to the photograph of the agarose/EtBr gel. Generally, you will want to isolate the RACE product(s) that correspond(s) to the largest band(s) on the Southern blot. There may be larger RACE products that appear on the agarose gel but that do not hybridize to the gene-specific probe. These bands are generally due to nonspecific priming. Smaller bands that hybridize to your probe may be the result of incomplete reverse transcription; however, you cannot exclude the possibility that some of these shorter bands are real and correspond to alternatively spliced transcripts, transcripts derived from multiple promoters, or other members of a multigene family.
6. Once you have pinpointed the band(s) of interest, isolate the DNA from the gel using the NucleoTrap® Gel Extraction Kit provided, and proceed with your experiments.

VII. Characterization of RACE Products, *continued*

C. PROTOCOL: NucleoTrap® Gel Extraction

The NucleoTrap Gel Extraction protocol includes "standard" and "high-speed" procedures. The high-speed procedure is specially designed for the purification of fragments that are 0.4–5 kb in length. It can reduce procedure time by 50 percent, while only leading to a 10 percent lower recovery rate. Use the standard procedure if you wish to purify fragments smaller than 400 bp or larger than 5 kb, or when you need to recover as much of a fragment as possible. Differences between the "standard" and "high-speed" procedures are listed as Notes for the appropriate steps.

Before you start: Add 28 ml of 95% ethanol to Buffer NT3. This volume is also printed on the Buffer NT3 bottle.

1. Electrophorese your DNA sample on an agarose/EtBr gel. We recommend using a buffer system containing either TAE (40 mM Tris-acetate [pH 8], 1 mM EDTA) or TBE (45 mM Tris-borate [pH 8], 1 mM EDTA).
2. Locate the position of your fragment under UV light. Use a clean scalpel or razor blade to excise the DNA fragment of interest. Cut close to the fragment to minimize the surrounding agarose. Estimate the amount of DNA present in the gel slice.
3. Measure the weight of the gel slice and transfer it to a clean 1.5 ml microcentrifuge tube or a 15 ml conical tube.
4. For every 100 mg of agarose, add 300 µl of Buffer NT1. For gels containing >2% agarose, add 600 µl NT1 for every 100 mg of agarose.
5. Vortex the NucleoTrap Suspension thoroughly until the beads are completely resuspended.
6. For each 1 µg of DNA to be purified, add 4 µl of NucleoTrap Suspension.

NOTE: A minimum of 10 µl of NucleoTrap Suspension is needed to ensure a high binding efficiency.

7. Incubate the sample at 50°C for 5–15 min. Vortex briefly every 2–3 min during the incubation period.

NOTE: [HIGH SPEED PROTOCOL] For DNA fragments between 400 bp and 5 kb, incubate the sample at 50°C for 6 min. Vortex briefly every 2 min.

8. Centrifuge the sample at 10,000 x g for 30 sec at room temperature. Discard the supernatant.
9. Add 500 µl of Buffer NT2 to the pellet. Vortex briefly.
10. Centrifuge at 10,000 x g for 30 sec at room temperature. Remove the supernatant completely.
11. [STANDARD PROTOCOL ONLY] Repeat Steps 9–10.
12. Add 500 µl of Buffer NT3 to the sample. Vortex briefly.
13. Centrifuge the sample at 10,000 x g for 30 sec at room temperature. Remove the supernatant completely.
14. Repeat Steps 12–13.
15. Centrifuge the pellet again at 10,000 x g for 30 sec at room temperature. Air dry the pellet for 10–15 min.

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

16. Add 20–50 µl of Buffer NE or another low-salt buffer to the pellet. Resuspend the pellet by vortexing.

NOTE: Expected recovery rates range from 60% (eluting in 20 µl) to 80% (eluting in 50 µl).

17. Elute the DNA by incubating the sample at room temperature for 10–15 min. Vortex the mixture 2–3 times during the incubation step.

NOTES:
• Incubate the sample at 55°C for 10 min if the DNA fragment to be eluted is ≥5 kb.
• [HIGH SPEED PROTOCOL] For DNA fragments between 400 bp and 5 kb, incubate the sample at 50°C for 5 min.

VII. Characterization of RACE Products, *continued*

18. Centrifuge the sample at 10,000 x g for 30 sec at room temperature. Transfer the supernatant, containing the purified DNA fragment, to a clean 1.5 ml microcentrifuge tube.

NOTES:
• Repeating Steps 16–18 can increase yields approximately 10%.
• HIGH SPEED PROTOCOL: For DNA fragments between 400 bp and 5 kb, repeating steps 16–18 can increase yields up to 15%.

D. Cloning & Sequencing RACE Products

NOTE: The Universal Primer contains a T7 priming site. Using a cloning vector that contains a T7 site will generate multiple sequencing products if using a T7 primer.

1. Gel-purify the RACE product(s) of interest using the NucleoTrap Gel Extraction Kit. Then, clone the isolated fragment(s) directly into a T/A-type PCR cloning vector.

2. After you have TA-cloned your RACE products, identify clones containing gene-specific inserts by colony hybridization using a ^{32}P-end-labeled NGSP as a probe or by sequencing from your GSP. **For 5′-RACE products, we recommend picking at least 8–10 different independent clones in order to obtain the maximum amount of sequence at the 5′ end** (see the note on full-length cDNA below).

   Once you have identified the clones containing the largest gene-specific inserts, obtain as much sequence data as you can. Ideally, you will be able to sequence the entire open reading frame, as well as the 5′ and 3′ untranslated regions.

A note on full-length cDNA

No method of cDNA synthesis can guarantee a full-length cDNA, particularly at the 5′ end. Determining the true 5′ end requires some combination of RNase protection assays, primer extension assays, and cDNA or genomic sequence information. Many SMARTer RACE cDNAs include the complete 5′ end of the cDNA; however, severe secondary structure may block the action of RT and/or *Taq* DNA polymerase in some instances. In our experience, SMARTer RACE products and full-length cDNAs compare favorably in this regard with cDNAs obtained by conventional RACE or from libraries. **To obtain the maximum possible amount of 5′ sequence, we recommend that you sequence the 5′ end of 8–10 separate clones of the 5′-RACE product.**

Options for generating full-length cDNA

After the RACE products have been characterized by partial or complete sequencing, you can generate the full-length cDNA by one of two methods:

1. By long distance PCR (LD PCR) using primers designed from the extreme 5′ and 3′ ends of your cDNA and the 5′-RACE-Ready cDNA as template.

2. By cloning overlapping 5′- and 3′-RACE fragments using a restriction site in the overlapping region (if available).

In general, the LD PCR method is more direct and less subject to complications or artifacts. With cloning, it is possible to join 5′ and 3′ cDNA fragments derived from two different transcripts; this could occur with two different forms of a polymorphic RNA or with transcripts from a multigene family. In contrast, with end-to-end PCR, the 5′ and 3′ end primers will amplify a single cDNA, without the possibility of generating a hybrid. Virtually all cDNAs are within the range of LD PCR.

If you are going to use your cloned RACE products for further analysis, we recommend that you generate your full-length cDNA using the Advantage HF 2 PCR Kit (Cat. No. 639123). This kit is designed to yield products of less than 3.5 kb with fidelity comparable to that of the leading high-fidelity polymerase. Again, the initial RACE reactions should be performed using the Advantage 2 Polymerase Mix to confirm that the product is present and that the GSPs work well.

VIII. Troubleshooting Guide

Optimizing your 5′- and 3′-RACE reactions is generally advisable and often necessary. This process usually consists of improving the yield of your desired fragment(s), while decreasing the amount of background or nonspecific and/or incomplete bands in your RACE reactions. The cDNA synthesis protocols contained in this User Manual typically produce enough 5′- and 3′-RACE-Ready cDNA for 100 or more RACE PCR reactions. Thus, there is plenty of material for optimizing your RACE amplifications.

Table VI: Troubleshooting Guide

SECTION A. GENERAL PCR PROBLEMS
Troubleshooting GC-Rich Templates **If the PCR product, especially your 5′-RACE product, is not the expected size or is absent, the cause may be a GC-rich template.** Clontech offers the Advantage GC 2 Polymerase Mix (Cat. No. 639114) and PCR Kit (Cat. Nos. 639119 & 639120) for efficient amplification of GC-rich templates. However, the master mixes will need to be modified and PCR parameters may need to be optimized for these templates. For more information, please see the Advantage GC 2 PCR User Manual (PT3316-1). Perform the initial RACE reactions with the Advantage 2 Polymerase Mix. If you are unable to amplify the expected PCR product due to high GC content, perform the RACE reactions using the Advantage GC 2 Polymerase Mix.
Troubleshooting Touchdown PCR When troubleshooting touchdown PCR, begin by modifying the final set of cycling parameters (i.e., the 20–25 cycles performed with annealing at 68°C). **If you do not observe an amplified product after the minimum number of cycles at 68°C**, return your tube(s) to the thermal cycler and run five additional cycles. If the product still does not appear, add an additional 3–5 cycles at 68°C. If you are still unsuccessful, run a new PCR experiment, changing the annealing temperature in the third set of cycles from 68°C to 65°C. This last program is especially useful if your GSP has a T_m close to 70°C.
SECTION B. FIRST-STRAND CDNA SYNTHESIS PROBLEMS
The RT reaction described in the First-Strand cDNA Synthesis Protocol (Section V.D.) has been optimized, however, in some situations, like with long, rare transcripts in a small amount of RNA, reducing the amount of SMARTScribe Reverse Transcriptase from 1 µl to 0.5 µl in the first-strand cDNA synthesis reaction can increase the sensitivity of the SMARTer 5′-RACE reaction.
Analyzing the Quality of First-Strand cDNA If you suspect that problems amplifying your RACE fragments may be due to a failure of the reverse transcription reaction, you can check the quality of first-strand cDNA (if generated from poly A^+ RNA) using a ^{32}P-labeling procedure. To do this, repeat the first-strand synthesis, substituting 1 µl of 0.1 µCi/µl [α-^{32}P] dATP or dCTP for 1 µl of water. Run the reaction products on an alkaline agarose gel, and examine the banding pattern by autoradiography. If the first-strand reaction was successful, you should see a banding pattern similar to that produced by your RNA. Mammalian poly A^+ RNA typically produces a smear from 0.5–12 kb. Mammalian total RNA usually exhibits two bright bands at 1.9 kb and 4.5 kb.
SECTION C. MULTIPLE BAND RACE PRODUCTS
In some cases, your initial experiments will produce **multiple 5′- and/or 3′-RACE products**. You will have to determine which products are real and which are artifacts. While the following guidelines will help you eliminate artifacts, confirmation of real and complete bands requires additional studies such as mapping of transcription start sites, intron/exon structure and polyadenylation sites, and genomic sequencing. Multiple fragments do not mean you cannot proceed with generating the full-length cDNA. However, you may save time in the long run if you try to eliminate nonspecific fragments by troubleshooting the reactions. If multiple fragments persist and you want to proceed, you should generally start with the largest fragment from each RACE reaction, because it is most likely to be a true, complete RACE product.

VIII. Troubleshooting Guide, *continued*

SECTION C. MULTIPLE BAND RACE PRODUCTS, continued

Sources of "Real" Multiple RACE Products

Individual genes can give rise to multiple transcripts of different sizes—and hence to multiple RACE fragments—via at least three mechanisms:

- Alternative splicing can cause multiple products in 5′- or 3′-RACE.
- Different transcription initiation sites cause multiple 5′-RACE products.
- Different polyadenylation sites cause multiple 3′-RACE products.

Alternatively, the gene may be a member of a multigene family, in which case your "gene-specific" primers may simultaneously amplify several highly homologous cDNAs.

Distinguishing true polymorphic forms of an RNA is a matter for scientific investigation. However, you may be able to find an alternative source of RNA in which one form is more abundant than others.

Sources of Artifacts

Multiple bands often do not correspond to actual, complete transcripts. These artifact RACE products can be divided into two classes—incomplete and nonspecific.

There are several possible sources of incomplete fragments, which are generated from correctly primed sites:

- Premature termination of first-strand cDNA synthesis caused by RT pausing generally causes multiple 5′-RACE products. This problem is common with larger RNAs, and is difficult to overcome because it is due to an intrinsic limitation of the RT.
- Degradation of the RNA used as starting material generally causes multiple 5′-RACE products.
- Difficulty in amplifying certain genes can cause multiple products in either 5′- or 3′-RACE and is often a result of high GC content.

Nonspecific RACE products arise from nonspecific binding of the primer to multiple sites in the ds cDNA or primer-dimer artifacts.

Troubleshooting Suggestions for Multiple Band RACE Products

- If you have not already done so, repeat your RACE reactions with all of the recommended controls. In particular, be sure that your GSPs do not give bands when used alone, and that they give a single band when used together. If either GSP alone gives persistent bands, we recommend altering the cycling parameters or designing nested primers as discussed below. Also repeat the Positive Control RACE PCR Experiment (Section VI.B).
- Repeat your reactions using 5 µl of a 5–10-fold dilution of the RACE-Ready cDNA.
- If you have not already done so, examine the size distribution of your RNA starting material as discussed in Section V.C. If your RNA looks smaller than expected, repurify your RNA and repeat cDNA synthesis.
- **If multiple bands persist, try altering the PCR cycling parameters:**
  1. Increase the stringency of your PCR by raising the annealing temperature in increments of 2–5°C. In many cases, bands arising from nonspecific priming will disappear while real or incomplete products will persist.
  2. Reduce the cycle number. Again, bands arising from nonspecific priming may disappear, while real or incomplete products will persist.
  3. Reduce the extension time.
  4. In the case of large RACE products, increasing the extension time may help eliminate extra bands.
- **If multiple bands persist, try designing a new set of primers:**
  1. Redesign your primers so that they have a T_m greater than 70°C and use the cycling parameters for touchdown PCR.
  2. We recommend that you design new primers that will give RACE products that are slightly different in size than those expected with the original primers. These new primers can either be used by themselves or in combination with the original primers in "nested PCR." In nested PCR, the product of a PCR reaction is reamplified using a second set of primers that is internal to the original primers. This often greatly reduces the background and nonspecific amplification seen with either set of primers alone. The design of nested primers is discussed in Section IV.
  3. Prior to performing nested RACE PCR, we recommend that you perform two separate primary amplifications with the UPM and either the GSP1 or NGSP1. This test will help show if multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If the multiple bands are real (i.e., the result of correct priming), they should be present in both reactions, but slightly smaller in the reaction using the nested primers. The difference in the mobility of the products should correspond to the positions of the GSP and NGSP in the cDNA structure.

VIII. Troubleshooting Guide, *continued*

SECTION D. OTHER SPECIFIC PROBLEMS		
PROBLEM	**POSSIBLE CAUSE**	**SUGGESTED ACTION/SOLUTION**
No band is observed in positive control amplification of the overlapping region of RACE products (either with GSP1 + GSP2 or TFR1 + TFR2).	There may be a problem with your polymerase mix.	If you are not using the Advantage 2 Polymerase Mix, consider switching. The SMARTer RACE protocol was optimized with the Advantage 2 Polymerase Mix. Be sure to perform the positive control PCR experiment in Section VI.B.
	Your cDNA synthesis reaction may have failed.	Repeat the first-strand synthesis reaction. You may wish to analyze the quality of your first-strand cDNA using the procedure described in Section B of the Troubleshooting Guide.
No band is observed using TFR1 + TFR2 with your experimental cDNA, but the correct product is seen with cDNA made from the positive control placental RNA.	Your RNA may be partially degraded or may contain impurities.	Check the quality of your RNA against the criteria described in Section V.B. You may need to use new RNA.
No band is observed using GSP1 + GSP2, but the correct product is seen using TFR1 + TFR2.	This problem can be caused by the impeding of RT by strong secondary structure and/or high GC content in your gene. This is especially indicated if the 3′-RACE works but, the 5′-RACE does not, and the positive control (GSP1 + GSP2) does not produce the expected fragment.	See Section B of the Troubleshooting Guide for help with GC-rich templates. Additionally, you may wish to analyze the quality of your first-strand cDNA using the procedure described in Section B of the Troubleshooting Guide.
	Your gene may be expressed weakly or not at all in your starting RNA.	You may have to find a new source of RNA. The efficiency of both 5′- and 3′-RACE amplifications depends on the abundance of the target transcript.
	There is a problem with your primers. This could be due to either poor primer design or poor primer preparation.	First, try lowering your annealing/extension temperatures. If this does not work, you may need to design new primers or repurify your GSPs.
	You may be able to obtain more information by amplifying the internal fragment (with GSP1 and GSP2) using genomic DNA as the template. If the expected band is produced, your primers are suitable and the problem is either (a) the target RNA is a poor template for the RT; or (b) the RNA is not expressed in the tissue source you have chosen. Note, however, that this test is not conclusive, since your primers may be separated by an intron in the genomic DNA. If this is the case, amplification of genomic DNA will give a larger fragment than expected or no fragment at all.	
The 3′-RACE works, but the 5′-RACE does not in both experimental and TFR amplification.	This is often the result of a failure in full-length cDNA synthesis and/or the template switching reaction.	Try repeating the first-strand synthesis reaction. You may wish to analyze the quality of your first-strand cDNA using the procedure described in Section B of the Troubleshooting Guide.
	Your RT may be degraded. This can happen if the enzyme is not kept on ice at all times, or if it is not returned to the freezer promptly after use.	Try repeating first-strand synthesis with a fresh RT.

VIII. Troubleshooting Guide, *continued*

SECTION D. OTHER SPECIFIC PROBLEMS, continued		
PROBLEM	**POSSIBLE CAUSE**	**SUGGESTED ACTION/SOLUTION**
No bands are observed in any RACE reactions using either gene-specific or positive control primers with either experimental or control RNA samples.	You may have to optimize your PCR cycling parameters.	If you still do not observe RACE products after 25–30 cycles of PCR (especially in both 5′- and 3′-RACE reactions), return the tubes to your thermal cycler and perform 5 additional cycles.
	The cDNA synthesis and/or template switching reaction has failed.	In this case try repeating the cDNA synthesis reactions. You may wish to analyze the quality of your first-strand cDNA using the procedure described in Section B of the Troubleshooting Guide.
Using your experimental cDNA sample, no 5′- or 3′-RACE bands are produced, but the TFR positive control RACE reactions (Tube No. 2) give the expected products.	Your gene may not be abundant in your RNA sample.	Perform 5 more PCR cycles at the 68°C annealing temperature. Repeat these additional cycles until your RACE fragments appear, but do not exceed 50 cycles for touchdown PCR or 40 cycles for non-touchdown PCR. If you still fail to produce the expected products, you may have to find a new source of RNA in which your gene is more abundant.
	The annealing temperature is too high for your primers.	Lower the annealing temperature by increments of 2°C.
	Your primers are not suitable for PCR.	Check your primers against the criteria in Section IV, and design new ones if necessary.
	Extensive secondary structure and/or high GC-content prevent efficient amplification of your gene of interest.	Try redesigning your primers closer to the ends of the cDNA, or try to avoid GC-rich regions if they are known. For additional tips in troubleshooting GC-rich sequences, see Section A of the Troubleshooting Guide.
RACE cDNA product is smeared. **NOTE:** Some SMARTer RACE reactions produce very complex patterns of bands that appear almost as smears.	In most cases of true smearing, a problem has occurred prior to the RACE reaction, especially if the 3′-RACE reaction produces a smear.	In these cases we recommend repeating the entire procedure after repurifying your RNA (or confirming that your RNA is intact and clean). See Section V.B. for more details.
	Smearing of only the 5′-RACE reaction products may indicate a difficult template for reverse transcription or degraded RNA.	
	Smearing of both reactions is a strong indication of contamination of your starting RNA or a problem in reverse transcription.	
	Your gene-specific primer was not specific.	Redesign your gene-specific primer.
	If smearing is apparently not due to a problem that occurred prior to RACE, try optimizing your RACE reactions using the troubleshooting suggestions for multiple band RACE products in Section C of the Troubleshooting Guide.	

IX. References

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

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Appendix A: Detailed Flow Chart of 5′ RACE

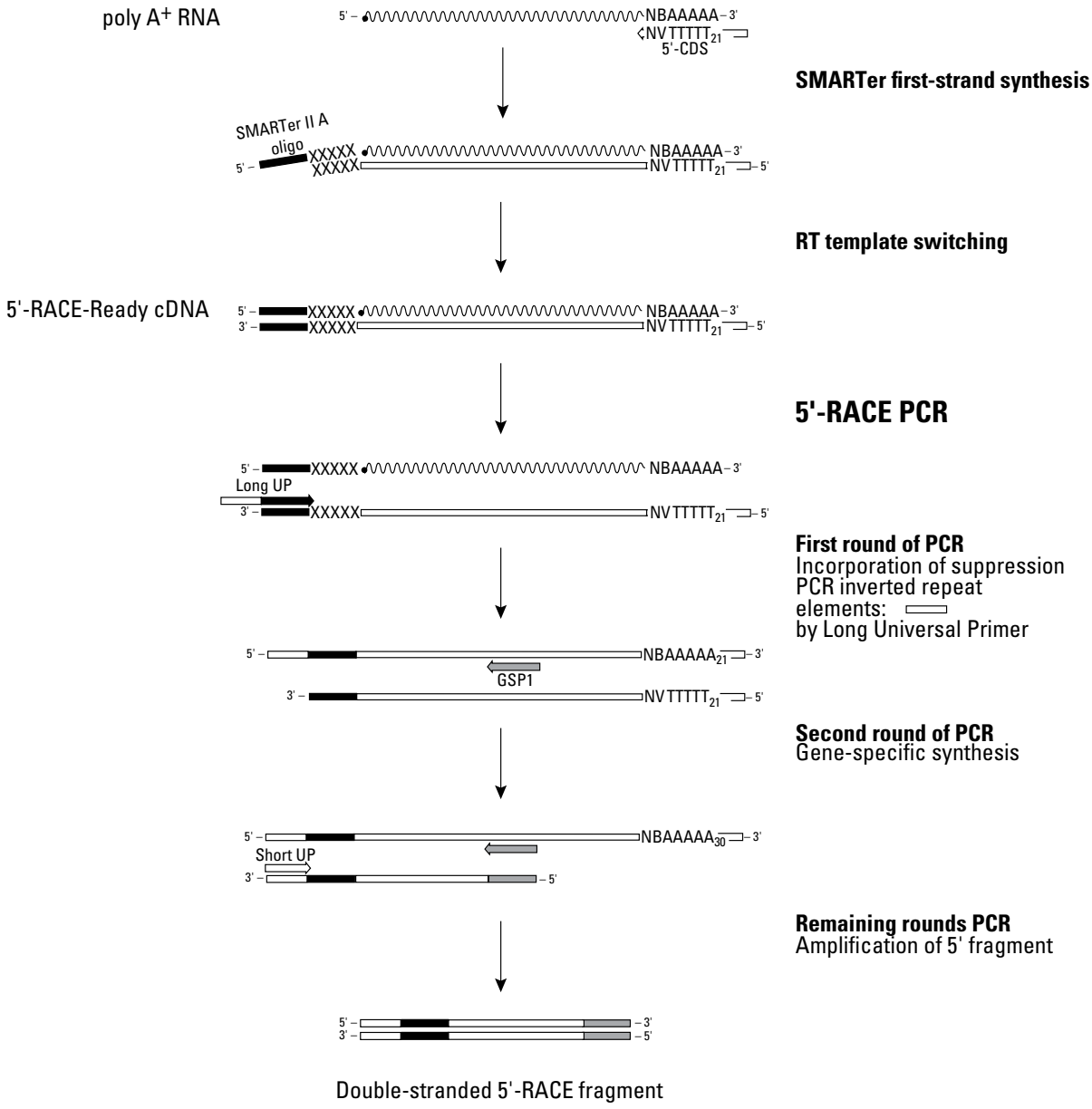


Figure 5. Detailed mechanism of the 5′-RACE reactions.

Appendix B: Detailed Flow Chart of 3′ RACE

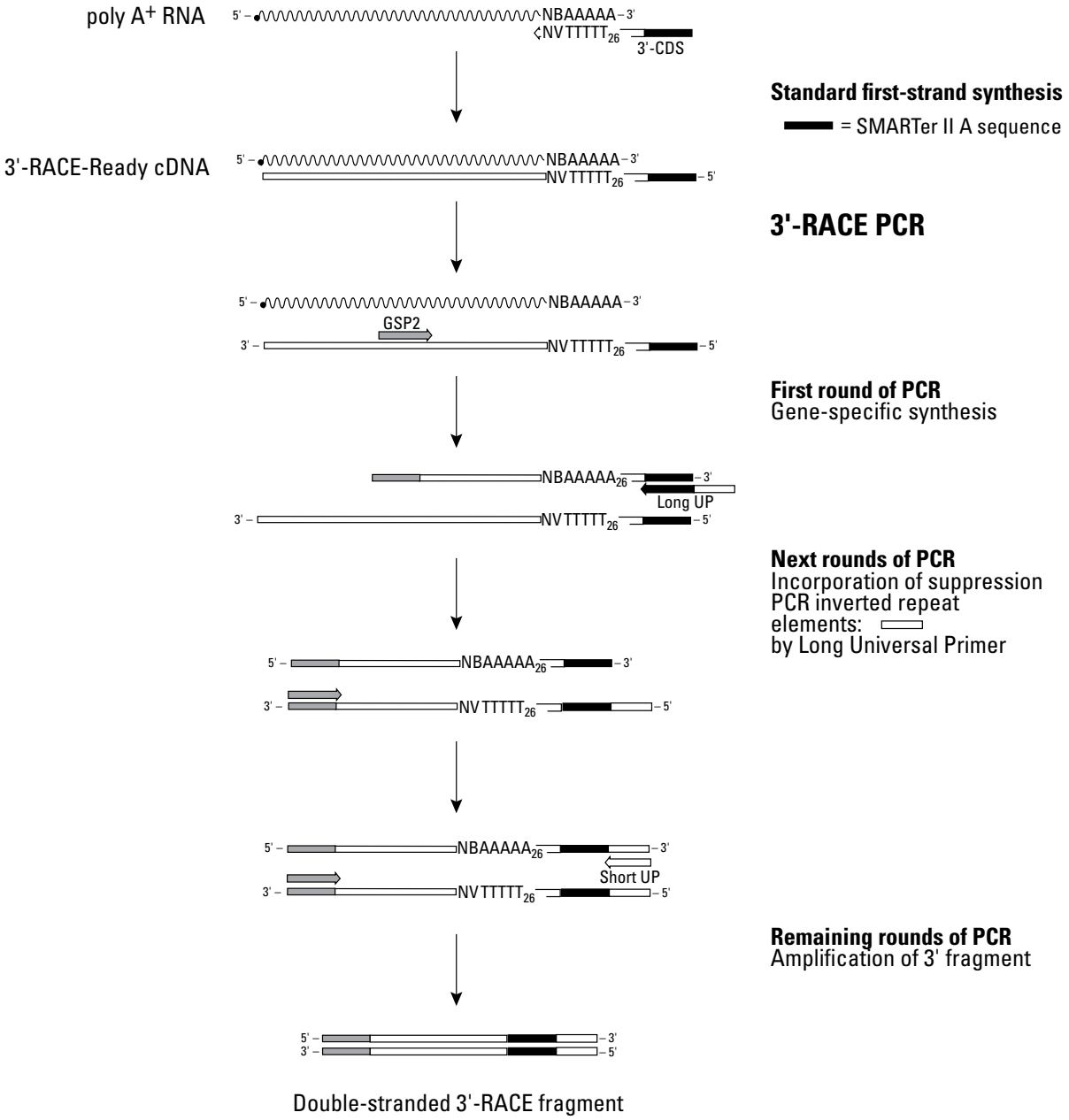


Figure 6. Detailed mechanism of the 3′-RACE reactions.

Appendix C: Suppression PCR and Step-Out PCR

In our initial SMARTer-based 5′-RACE experiments, we tended to observe heavy background amplification. We determined that undesired bands were produced by the nonspecific binding of the SMARTer II A oligo during synthesis of the 5′-RACE-Ready cDNA. When reverse transcription and template switching are completed, these cDNAs contain the SMARTer sequence at both ends (Figure 7). As a result, RACE PCR using primers based on the SMARTer sequence amplifies these cDNAs in addition to the desired gene-specific fragment. This problem was overcome with the use of suppression PCR and step-out PCR.

In suppression PCR (Siebert *et al.*, 1995), an inverted repeat is incorporated into the ends of DNA sequences to prevent amplification during PCR. The suppression effect occurs when these inverted repeats anneal intramolecularly to form panhandle structures which cannot be amplified by PCR (see the bottom of the flow chart in Figure 7). The SMARTer RACE Kit uses the technique of step-out PCR to add these inverted repeats and thus suppress the amplification of cDNA species that were synthesized by SMARTer II A oligo priming during reverse transcription. Step-out PCR uses a mixture of two primers to incorporate additional sequence at the end/s of template DNA (Matz *et al.*, 1999). One of these primers is exceptionally long and contains the additional sequence as a non-annealing overhang. The overhang sequence is incorporated into template DNA ends in the early rounds of PCR. After overhang addition, the second primer, which is only complementary to the overhang sequence, takes over and serves as an efficient primer for PCR amplification. This short primer is essential because the bulky incorporation primer is inadequate for effective amplification. The short primer is included at a higher concentration than the long primer so that it out-competes the long primer in annealing to template DNA during PCR.

In this same manner, the Universal Primer A Mix adds suppression PCR inverted repeat elements to ends of cDNAs in SMARTer RACE. One of the primers in the mix, the "Long" Universal Primer (UP), is identical to the SMARTer sequence at its 3′ end and also has a 5′ heel of 20 bp which contains the suppression sequence (Figure 7). During the early rounds of RACE PCR, this primer incorporates the suppression sequence on the 5′ side of all SMARTer sequences present in the cDNA population. As a result, all cDNAs that were correctly primed by oligo(dT) and have only one SMARTer sequence at the 3′ end of the first-strand cDNA will contain one suppression sequence at that end. Conversely, all cDNAs that were primed by the SMARTer II A oligo, and which were consequently flanked by the SMARTer sequence, become flanked again by the inverted repeat and are subject to suppression PCR. Therefore, cDNAs that have the SMARTer sequence on only one end and the gene specific sequence will be amplified exclusively. As described above, the "Short" UP, which is present at five times the concentration of the Long UP, only contains the 5′-heel sequence of the Long UP, and simply serves as an efficient PCR primer after incorporation of the inverted repeat.

Appendix C: Suppression PCR and Step-Out PCR, *continued*

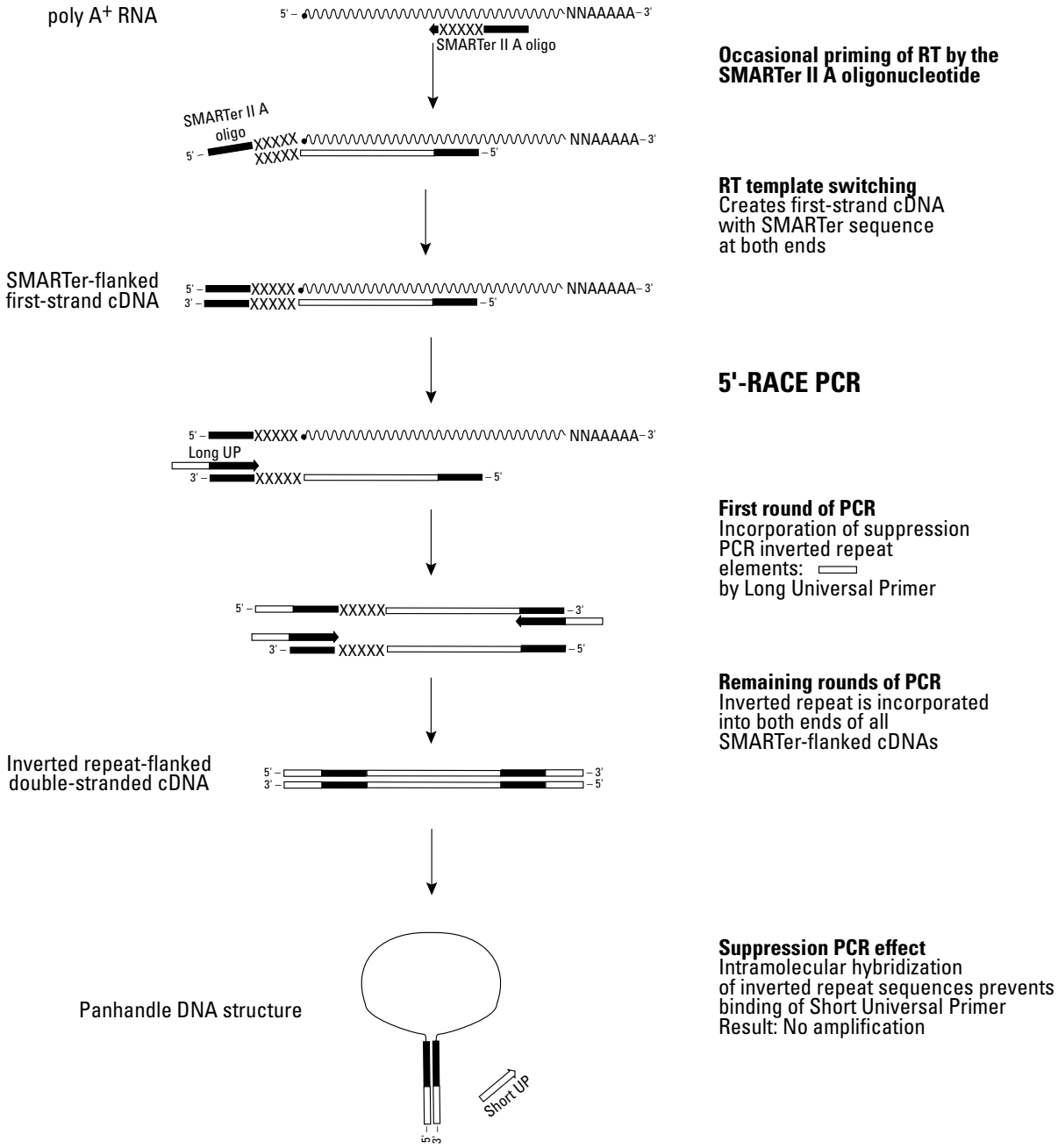


Figure 7. Mechanisms of suppression PCR and step-out PCR. On occasion, a reverse transcription reaction can be "nonspecifically" primed by the SMARTer II A oligonucleotide. This will result in the synthesis of a cDNA containing the SMARTer sequence at both ends. Through the technique of step-out PCR, suppression PCR inverted repeat elements are incorporated next to all SMARTer sequences. During PCR, these inverted repeats anneal to each other intramolecularly. This rapid first-order reaction out-competes the second-order binding of the Short Universal Primer to the cDNA. As a result, panhandle-like structures, which cannot be amplified, are formed.

Appendix D: 5′-RACE cDNA Amplification with Random Primers

If your RNA template is from a non-eukaryotic organism and/or lacks a polyadenylated tail, use the following protocol for first-strand cDNA synthesis with random priming.

A. PROTOCOL: First-Strand cDNA Synthesis

1. Preheat a thermal cycler to 72°C.
2. For each sample and control, combine the following reagents in a sterile 0.5 ml reaction tube:

1–2.75	µl	**RNA** (50 ng–1 µg)*
1	µl	**10X Random Primer Mix (N-15)** (20 µm)
x	µl	**Deionized H_2O**
3.75	**µl**	**Total Volume**

* For the control synthesis, use 1 µl of Control Mouse Heart Total RNA (1 µg/µl).

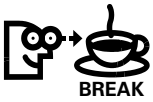
3. Mix contents and spin the tube briefly in a microcentrifuge.
4. Incubate the tube at 72°C for 3 min, then reduce to room temperature (22°C) for 2 min.
5. While the tubes from Step 4 are incubating, you can prepare this Master Mix at room temperature. Mix the reagents in the following order:

2	µl	**5X First-Strand Buffer**
1	µl	**DTT** (20 mM)
1	µl	**dNTP Mix** (10 mM)
0.25	µl	**RNase Inhibitor**
1	µl	**SMARTer II A Oligonucleotide** (12 µm)
1	µl	**SMARTScribe™ Reverse Transcriptase** (100 U/µl)†
6.25	**µl**	**Total Volume**

† Add the reverse transcriptase to the master mix just prior to use.

6. Add 6.25 µl of the Master Mix from Step 5 to the denatured RNA from Step 4. Mix well by pipetting and spin the tube briefly in a microcentrifuge.
7. Incubate the reaction tube at room temperature for 10 min, then place the tube at 42°C for 90 min.
8. Terminate the reaction by heating at 70°C for 10 min.
9. Dilute the first-strand reaction product with Tricine-EDTA Buffer:
   - Add 20 µl if you started with ≤200 ng of total RNA.‡
   - Add 100 µl if you started with ≥200 ng of total RNA.‡
   - Add 250 µl if you started with poly A^+ RNA.

   ‡The copy number of your gene of interest should be the determining factor for diluting your sample. If you have 200 ng of total RNA but your gene of interest has low abundance, dilute with 20 µl. If you have 200 ng of total RNA and the gene of interest is highly abundant, dilute with 100 µl.



10. Samples can be stored at –20°C for up to three months.

B. Positive Control RACE PCR Experiment

Perform the positive control 5′-RACE PCR experiment according to Section VI.B. of the User Manual.

C. Rapid Amplification of cDNA Ends (RACE)

Perform the 5′-RACE PCR experiment according to Section VI.D. of the User Manual.

Notes

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