

Takara Bio USA, Inc.

SMARTer® RACE 5'/3' Kit User Manual

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(050421)

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Table of Contents

I. Introduction 4

II. List of Components 7

III. Additional Materials Required 8

IV. Primer Design 9

 A. Primer Sequence 9

 B. Additional Considerations for Design 10

 C. Location of Primer Sequences within Genes 10

 D. Nested Primers 10

V. Generating RACE-Ready cDNA 11

 A. General Considerations 11

 B. Preparation and Handling of Total and Poly A⁺ RNA 11

 C. Assessing RNA Template Quality 12

 D. Protocol: First-Strand cDNA Synthesis 13

VI. Rapid Amplification of cDNA Ends (RACE) 15

 A. Things You Should Know Before Starting RACE PCR Reactions 15

 B. Protocol: Rapid Amplification of cDNA Ends (RACE) 15

VII. Characterization of RACE Products 17

 A. Protocol: Gel Extraction with the NucleoSpin Gel and PCR Clean-Up Kit 17

 B. Protocol: In-Fusion Cloning of RACE Products 18

 C. Sequencing RACE Products 19

VIII. References 20

Appendix A. Troubleshooting Guide 21

 A. Troubleshooting Touchdown PCR 21

 B. Multiple Band RACE Products 23

 C. Other Specific Problems 25

Appendix B. Detailed Flow Chart of 5' RACE 27

Appendix C. Detailed Flow Chart of 3' RACE 28

Appendix D. 5'-RACE cDNA Amplification with Random Primers 29

 A. Protocol: First-Strand cDNA Synthesis with Random Priming 29

Table of Figures

Figure 1. Mechanism of SMARTer cDNA synthesis. 4
Figure 2. Overview of the SMARTer RACE procedure.. 6
Figure 3. The relationship of gene-specific primers to the cDNA template..... 9
Figure 4. 3'- and 5'-RACE sample results.22
Figure 5. Detailed mechanism of the 5'-RACE reactions.....27
Figure 6. Detailed mechanism of the 3'-RACE reactions.....28

Table of Tables

Table 1. Additional 5'-RACE Sequence Obtained with SMART Technology..... 5
Table 2. Setting up 5'- and 3'-RACE PCR Reactions15
Table 3. Troubleshooting Guide: Other Specific Problems.....25

I. Introduction

The SMARTer RACE 5'/3' Kit provides a method for performing both 5'- and 3'-rapid amplification of cDNA ends (RACE). The SMARTer RACE 5'/3' Kit includes our SMARTer II A Oligonucleotide and SMARTScribe™ Reverse Transcriptase, which provides better sensitivity, less background and higher specificity than previous kits. This powerful system allows you to amplify the complete 5' sequence of your target transcript from as little as 10 ng of total RNA. The cornerstone of SMARTer RACE cDNA synthesis 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 our technology for suppression PCR & step-out PCR to increase the sensitivity and reduce the background of the RACE reactions. You can use either poly A⁺ or total RNA as starting material for constructing full-length cDNAs, even of very rare transcripts.

The SMARTer RACE 5'/3' Kit is an improved version of our original SMARTer RACE cDNA Amplification Kit, designed to accommodate larger RNA input volumes and perform more efficiently on challenging targets (e.g., those that are long, GC-rich, etc.). RACE PCR products are amplified with our highly robust SeqAmp™ DNA Polymerase, and cloned into the linearized pRACE vector with In-Fusion® Cloning. The In-Fusion Cloning Kit, NucleoSpin Gel and PCR Clean-Up Kit, and Stellar™ Competent Cells are included for your convenience in cloning RACE products.

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. When the SMARTScribe RT reaches the 5' end of the RNA, its terminal transferase activity adds a few additional nucleotides to the 3' end of the first-strand cDNA (Figure 1).

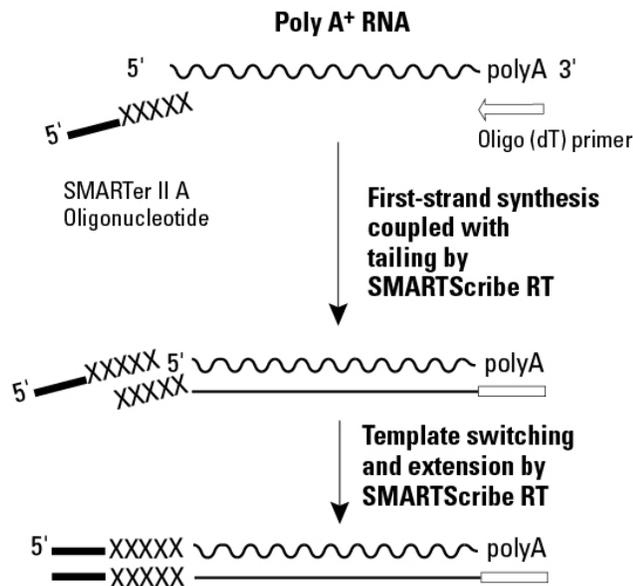


Figure 1. Mechanism of SMARTer cDNA synthesis. First-strand cDNA synthesis is primed using a modified oligo (dT) primer. After SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, it adds several nontemplated residues. The SMARTer II A Oligonucleotide anneals to the tail of the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

SMARTer® RACE 5'/3' Kit User Manual

The SMARTer II A Oligonucleotide 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. 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 1. Additional 5'-RACE Sequence Obtained with SMART Technology

Human gene	Size of mRNA (kb)	Additional sequence (bp)*	Matches genomic sequences
Piccolo presynaptic cytomatrix protein	20.29	+59	yes
Dynein, cytoplasmic 1, heavy chain 1	14.36	+36	yes
Polycystic kidney disease 1	14.14	+21	yes
Solute carrier family 1	12.02	+73	yes
Microtubule-associated protein 1A	10.54	+13	yes
Spectrin, beta, non-erythrocytic	10.24	+32	yes
Transferrin receptor	5.0	+25	yes
Interferon- α receptor	2.75	+17	yes
Smooth muscle g-actin	1.28	+31	yes

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 are used in combination with SMARTer technology to decrease background amplification in RACE PCR.

Requirements for SMARTer RACE cDNA Amplification

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.

Uses of SMARTer RACE cDNA Amplification

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.

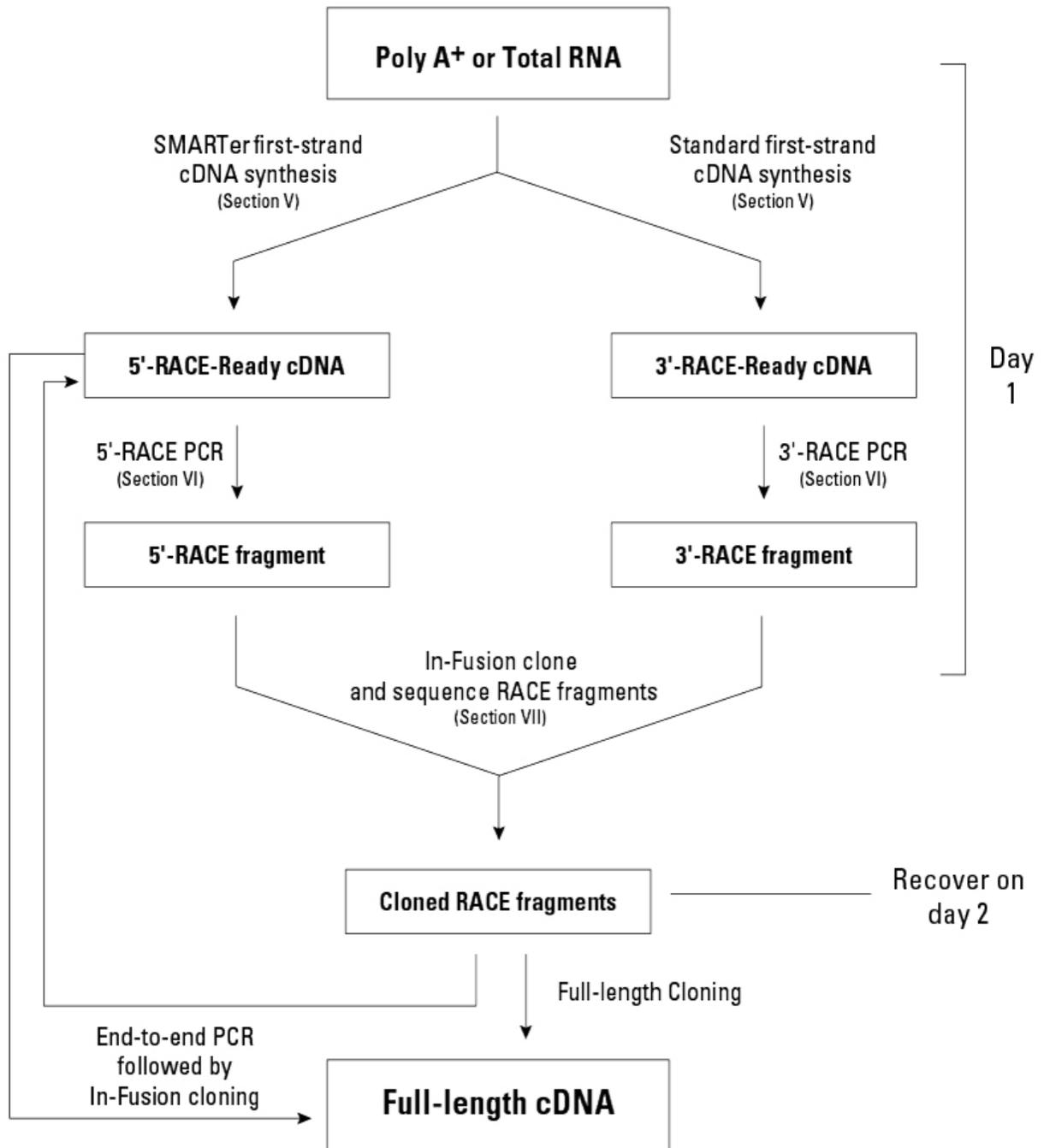


Figure 2. Overview of the SMARTer RACE procedure. Detailed flow charts of the SMARTer RACE mechanisms can be found in Appendices B & C. Alternatively, you can obtain the sequences of the extreme ends of the transcript by sequencing the 5' end of the 5' product and the 3' end of the 3' product. Using 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. 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, or design new GSPs to generate PCR products compatible with In-Fusion cloning.

II. List of Components

This section lists the components for Cat. No. 634858, a 10 reaction kit. The larger, 20 reaction kit (Cat. No. 634859) contains two of every item listed below.

SMARTer RACE 5'/3' Kit Components (Cat. No. 634860) (Not sold separately)

Store SMARTer II A Oligonucleotide and Control Mouse Heart Total RNA at -70°C . Store all other components at -20°C .

- First-Strand cDNA Synthesis
 - 10 μl SMARTer II A Oligonucleotide (24 μM)
 - 10 μl 3' RACE CDS Primer A (12 μM)
 - 10 μl 5' RACE CDS Primer A (12 μM)
 - 10 μl 10X Random Primer Mix (20 μM)
 - 40 μl 5X First-Strand Buffer (RNase-Free)
 - 5 μl Dithiothreitol (DTT) (100 mM)
 - 1 ml Deionized H₂O
 - 10 μl RNase Inhibitor (40 U/ μl)
 - 20 μl SMARTscribe Reverse Transcriptase (100 U/ μl)
 - 10 μl dNTP mix (20 mM)
- 5'- and 3'-RACE PCR
 - 400 μl 10X Universal Primer A Mix (UPM)
 - 50 μl Universal Primer Short (10 μM)
 - 5 μl Control Mouse Heart Total RNA (1 $\mu\text{g}/\mu\text{l}$)
 - 25 μl Control 5'-RACE TFR Primer (10 μM ; designed for compatibility with In-Fusion cloning)
 - 25 μl Control 3'-RACE TFR Primer (10 μM ; designed for compatibility with In-Fusion cloning)
- In-Fusion Cloning
 - 20 μl Linearized pRACE (50 ng/ μl)
- General Reagents
 - 2 tubes Tricine-EDTA Buffer (1 ml each)

SeqAmp DNA Polymerase (Cat. No. 638504)

Store all components at -20°C .

- 50 μl SeqAmp DNA Polymerase
- 1.25 ml SeqAmp PCR Buffer (2X)

In-Fusion Snap Assembly Master Mix (Cat. No. 638947)

Store all components at -20°C .

- 20 μl 5X In-Fusion Snap Assembly Master Mix
- 25 μl pUC19 Control Vector, linearized (50 ng/ μl)
- 50 μl 2 kb Control Insert (40 ng/ μl)

NucleoSpin Gel and PCR Clean-Up Kit (Cat. No. 740609.10) (Not sold separately)

Store all components at room temperature

- 10 ml Binding Buffer NTI
- 6 ml Wash Buffer NT3 (concentrate)
- 5 ml Elution Buffer NE (5 mM Tris/HCl, pH 8.5)
- 10 NucleoSpin Gel and PCR Clean-Up Columns (yellow rings)
- 10 Collection Tubes (2 ml)

Stellar Competent Cells (Cat. No. 636763)

Store Stellar Competent Cells at -70°C . Store all other components at -20°C .

- 10 tubes Stellar Competent Cells (100 μl /tube)
- 10 tubes SOC Medium (1 ml/tube)
- 10 μl pUC19 Vector (0.1 ng/ μl)

III. Additional Materials Required

If your RNA template is from a non-eukaryotic organism and lacks a polyadenylated tail, you can add one prior to first-strand 3'-cDNA synthesis using the following enzyme:

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

The following materials are required for In-Fusion cloning and transformation, but not supplied:

- Ampicillin (100 mg/ml stock) or other antibiotic required for plating the In-Fusion reaction
- LB (Luria-Bertani) medium (pH 7.0)
- LB/antibiotic plates

The following material is required for the NucleoSpin Gel and PCR Clean-Up Kit, but not supplied:

- 96–100% ethanol

IV. Primer Design

A. Primer Sequence

Gene-Specific Primers (GSPs) should:

- be 23–28 nt to ensure specific annealing
- be 50–70% GC
- have a $T_m \geq 65^\circ\text{C}$; best results are obtained if $T_m > 70^\circ\text{C}$, which enables the use of touchdown PCR. (T_m should be calculated based upon the 3' (gene-specific) end of the primer, NOT the entire primer.)
- **not** be complementary to the 3'-end of the Universal Primer Mix
 Long primer = 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'
 Short primer = 5'-CTAATACGACTCACTATAGGGC-3'
- be specific to your gene of interest
- both have 15 bp overlaps with the vector at their 5' ends (i.e., add the sequence **GATTACGCCAAGCTT** to the 5' ends of both GSPs' sequences; see details below)

The relationship of the primers used in the SMARTer RACE reactions to the template and resulting RACE products are 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 performing only 5'- or 3'-RACE, you will only need one GSP. In our experience, longer GSPs with annealing temperatures above 70°C give more robust amplification in RACE, particularly from difficult samples; however, there is generally no advantage to using primers with gene-specific sequence longer than 30 nt.

Successful In-Fusion cloning requires a 15 bp overlap with the linearized vector. Given this, you will need to add the sequence **GATTACGCCAAGCTT** to the 5'-end of your 5' and 3' GSPs to facilitate In-Fusion cloning of your RACE PCR products. This specific sequence is in addition to the 22 nt gene-specific sequence described above. The provided linearized pRACE vector already contains this overlap with the Universal Primer A Mix included for PCR, and adding this sequence to the 5'-end of your GSPs will complete the necessary overlap for the cloning reaction. **Please note that the In-Fusion User Manual contains only general primer recommendations that should not be used for this particular protocol.**

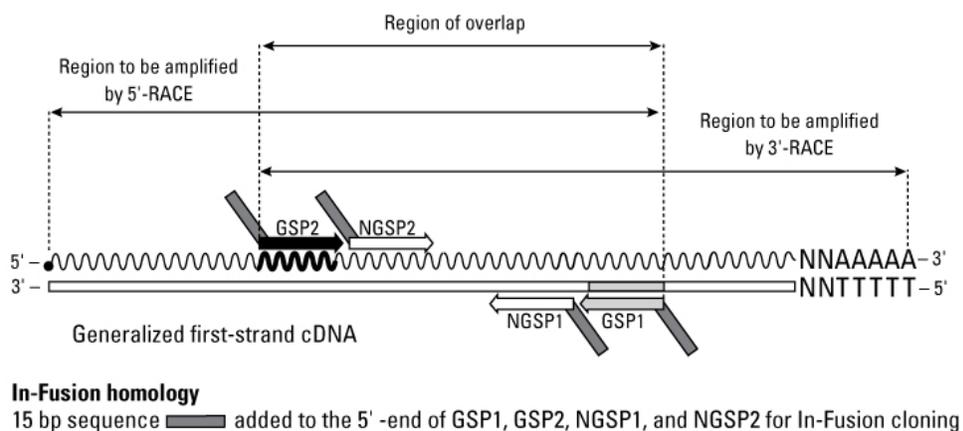


Figure 3. The relationship of gene-specific primers to the cDNA template. This diagram shows a generalized first-strand cDNA template. This RNA/DNA hybrid does not precisely represent either the 5'- or 3'-RACE-Ready cDNAs. For a detailed look at those structures, see Appendices B & C. Note that the gene-specific primers designed here contain tails with In-Fusion homology, and also 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.

B. Additional Considerations for Design

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. If no suitable restriction sites are available, you can alternately design new GSPs suitable for multi-fragment In-Fusion cloning. 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 an internal 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. The primers themselves can overlap (i.e., be complementary).

C. 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 range from 1–3 kb in length. If you are working with an annotated genome, we suggest using NCBI's Primer-BLAST to aid in your design for each transcript.

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, 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 re-amplified using the inner primers. The SMARTer RACE protocols include optional steps indicating where nested primers can be used. The Universal Primer Short (provided with the kit) can be used for both 5'- and 3'-RACE with nested primers.

Nested gene specific primers (NGSP) should be designed according to the same 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. Additionally, your nested primers should also contain the 15 bp overlap required for In-Fusion cloning.

V. Generating RACE-Ready cDNA

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

A. General Considerations

- We recommend using the Tricine-EDTA Buffer provided in the kit to resuspend and dilute your cDNA samples throughout the protocols in this user manual, because 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.
- Ethidium bromide (EtBr) 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 and Handling of Total and Poly A⁺ RNA

1. 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:

- Have a separate bench and/or pipette set dedicated to RNA work, free of RNase contamination.
- Wear gloves throughout to protect your RNA samples from nucleases.
- Use freshly deionized (e.g., MilliQ-grade) H₂O directly, without treatment with DEPC (diethyl pyrocarbonate). Takara Bio also offers RNase-Free Water (Cat. No. 9012).
- Use only single-use plastic pipettes and pipette tips. Filter tips are recommended.

2. RNA Isolation

We offer several kits for isolating total or poly A⁺ RNA from a variety of sources:

Purified Product	Starting Material	Product	Cat. #
Total RNA	Cells, tissues, or cell-free biological fluids	NucleoSpin RNA II	740955.50
Total RNA	Plant or fungal samples	NucleoSpin RNA Plant	740949.50
mRNA	Total RNA, cells, or tissues	NucleoTrap mRNA Mini	740655
mRNA	Total RNA derived from cultured cells or animal tissues	Magnosphere UltraPure mRNA Purification Kit	9186

Many procedures are available for the isolation of poly A⁺ RNA (Farrell, 1993; Sambrook *et al.*, 2001).

3. 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 can 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. 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.

Since RNA stability is a good indicator of RNA purity, we strongly recommend checking the stability of your RNA to ensure that it is free of contaminants.

Incubate a small portion of your RNA 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 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).

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 RNA Template Quality

1. Methods for Assessing Total RNA Integrity

- Detection with the Agilent 2100 BioAnalyzer (Agilent Technologies, CA):
This microfluidics-based technology, which provides an alternative to traditional gel-based analysis, requires only 2–7 ng of RNA per analysis. We recommend using RNA samples with an RNA Integrity Number (RIN) of 7 or higher. In addition to assessing RNA quality, this automated system provides a good estimate of RNA concentration.
- If you do not have access to an Agilent 2100 BioAnalyzer, you can visualize your RNA on a denaturing formaldehyde agarose gel under UV light. 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. When visualizing RNA using EtBr, you need at least 0.5–1 µg of total RNA. Alternatively, SYBR® Green II or SYBR Gold dyes (Molecular Probes; Eugene OR), allow you to detect as little as 1 or 2 ng of RNA on your gel, respectively.

2. 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 less than 1.5 kb, it could be an indication of degradation.

D. Protocol: First-Strand cDNA Synthesis

The two 20 µl reactions described in the 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.

NOTE: If your RNA template is from a non-eukaryotic organism and/or lacks a polyadenylated tail, follow the protocol for 5'-first-strand cDNA synthesis with random primers in Appendix D. For 3'-first-strand cDNA synthesis, add a poly(A) tail using Poly(A) Polymerase (Takara 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 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'- and 3'-RACE-Ready cDNA synthesis reactions plus 1 extra reaction to ensure sufficient volume. Mix the following reagents and spin briefly in a microcentrifuge, then set aside at room temperature until Step 6:

4.0 µl	5X First-Strand Buffer
0.5 µl	DTT (100 mM)
1.0 µl	dNTPs (20 mM)
<hr/>	
5.5 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	
1.0–10 µl	RNA*	1.0–11 µl	RNA*
1.0 µl	5'-CDS Primer A	1.0 µl	3'-CDS Primer A
0–9 µl	Sterile H ₂ O	0–10 µl	Sterile H ₂ O
<hr/>		<hr/>	
11 µl	Total Volume	12 µl	Total Volume

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

3. Mix contents and spin the tubes briefly in a microcentrifuge.

4. Incubate tubes at 72°C for 3 minutes, then cool the tubes to 42°C for 2 minutes. After cooling, spin the tubes briefly for 10 seconds at 14,000 x g to collect the contents at the bottom.

NOTE: This step can be performed in a thermal cycler. While the tubes are incubating, you can prepare the Master Mix in Step 6.

5. **To just the 5'-RACE cDNA synthesis reaction(s)**, add 1 µl of the SMARTer II A Oligonucleotide per reaction.
6. Prepare enough of the following Master Mix for all 5'- and 3'-RACE-Ready cDNA synthesis reactions. Mix these reagents at room temperatures in the following order:

5.5 µl	Buffer Mix from Step 1
0.5 µl	RNase Inhibitor (40 U/µl)
2.0 µl	SMARTScribe Reverse Transcriptase (100 U)
8.0 µl	Total Volume

7. Add 8 µl of the Master Mix from Step 6 to the denatured RNA from Step 4 (3'-RACE cDNA) and Step 5 (5'-RACE cDNA), for a total volume of 20 µl per cDNA synthesis reaction.
8. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
9. Incubate the tubes at 42°C for 90 minutes 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 cDNA synthesis.

10. Heat tubes at 70°C for 10 minutes.
11. Dilute the first-strand cDNA synthesis reaction product with Tricine-EDTA Buffer:
 - Add 10 µl if you started with <200 ng of total RNA.*
 - Add 90 µl if you started with >200 ng of total RNA.*
 - Add 240 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 10 µl. If you have 200 ng of total RNA and the gene of interest is highly abundant, dilute with 90 µl.

12. You now have 3'- and 5'-RACE-Ready cDNA samples. 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.

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.

A. Things You Should Know Before Starting RACE PCR Reactions

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.

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 (Appendix A) for suggestions on optimizing PCR conditions.

NOTE: This is a RACE-specific protocol. It differs from the general SeqAmp protocol in many regards.

B. 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 and UPM. Although the Universal Primer Short (UPM short) is provided, nested PCR is generally not necessary in SMARTer RACE reactions.

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:

15.5 µl	PCR-Grade H ₂ O
25.0 µl	2X SeqAmp Buffer
1.0 µl	SeqAmp DNA Polymerase
41.5 µl	Total Volume
2. Prepare PCR reactions as shown below in Table 2. Add the components to 0.5 ml PCR tubes in the order shown and mix gently.

Table 2. Setting up 5'- and 3'-RACE PCR Reactions

Component	5'- or 3'-RACE Sample	UPM only (– control)	GSP only (– control)
5'- or 3'-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl
10X UPM	5 µl	5 µl	—
5' or 3' GSP (10 µM)	1 µl	—	1 µl
H ₂ O	—	1 µl	5 µl
Master Mix (Step 1)	41.5 µl	41.5 µl	41.5 µl
Total Volume	50 µl	50 µl	50 µl

3. Commence thermal cycling using one of the following PCR 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.

NOTES ON CYCLING: You may need to determine the optimal cycling parameters for your gene empirically, because the number of cycles necessary depends on the abundance of the target transcript. 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 minutes; for 2–4 kb amplicons, we extend for 3 minutes; and for 5–10 kb amplicons, we extend for up to 10 minutes.

NOTE: The T_m should be calculated based upon the 3' (gene-specific) end of the primer, and NOT the entire primer.

Program 1 (touchdown PCR—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 minute for each additional 1 kb.

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 minute for each additional 1 kb.

4. [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 secondary, or “nested” PCR reaction using the Universal Primer Short (UPM short; supplied) and a NGSP (See the discussion in Section IV.D.) This result is more common for transcripts that are less abundant. The suggested secondary PCR will most likely result in the expected distinct band(s).
 - a. Dilute 5 µl of the primary PCR product into 245 µl of Tricine-EDTA buffer.
 - b. Repeat Steps 1–3 above, using:
 - i. 5 µl of the diluted primary PCR product in place of the RACE-Ready cDNAs.
 - ii. 1 µl of the Universal Primer Short and 1 µl of your nested GSPs.
 - iii. 15–20 cycles of Program 2.

NOTE: The Troubleshooting Guide (Appendix A) discusses several control reactions that will help you troubleshoot your RACE reactions if yields are suboptimal.

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 multi-gene family. Multiple bands are more common with 5'-RACE than with 3'-RACE. Multiple transcriptional start sites tend to create a number of different transcripts, and there's a good chance these multiple bands are real variants and not artifacts.

We provide you with the materials necessary for the suggested method of characterizing RACE products via cloning and sequencing (Sections B & C, below).

A. Protocol: Gel Extraction with the NucleoSpin Gel and PCR Clean-Up Kit

For more details on the included NucleoSpin Gel and PCR Clean-Up Kit, please download its User Manual from our website at takarabio.com/manuals.

Before you start: Add 24 ml of 96–100% ethanol to Wash Buffer NT3. Mark the label of the bottle to indicate that ethanol was added. Wash Buffer NT3 is stable at room temperature (18–25°C) for at least one year.

1. Electrophorese your RACE 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.

NOTE: Minimize UV exposure time to avoid damaging the DNA.

3. Measure the weight of the gel slice and transfer it to a clean 1.5 ml microcentrifuge tube.
4. For each 100 mg of agarose, add 200 µl Buffer NTI.
5. Incubate the sample for 5–10 minutes at 50°C. Vortex every 2–3 minutes until the gel slice is completely dissolved.

6. Place a NucleoSpin Gel and PCR Clean-Up Column into a Collection Tube (2 ml) and load up to 700 µl of sample. Centrifuge for 30 seconds at 11,000 x g. Discard flow-through and place the column back into the collection tube. Load remaining sample if necessary and repeat the centrifugation step.
7. Add 700 µl Buffer NT3 to the column. Centrifuge for 30 seconds at 11,000 x g. Discard flow-through and place the column back into the collection tube.
8. Centrifuge for 1 minute at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and collection tube.

NOTE: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 minutes at 70°C prior to elution (Step 9).

9. Place the column into a new 1.5 ml microcentrifuge tube (not provided). Add 15–30 µl Buffer NE and incubate at room temperature (18–25°C) for 1 minute. Centrifuge for 1 minute at 11,000 x g to elute DNA.

NOTE: DNA recovery of larger fragments (>1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70°C, and incubation for 5 minutes.

B. Protocol: In-Fusion Cloning of RACE Products

For more details on the included In-Fusion Snap Assembly Master Mix, please download its User Manual from our website at takarabio.com/manuals.

1. Combine:

1 µl	Lineareized pRACE vector (provided with SMARTer RACE 5'/3' Kit Components)
7 µl	Gel-purified RACE product (Section VII.A, Step 9)
2 µl	In-Fusion Snap Assembly Master Mix

10 µl Total Volume

2. Incubate for 15 minutes at 50°C and transfer to ice.
3. Follow the protocol provided with your Stellar Competent Cells to transform the cells with 2.5 µl of the In-Fusion reaction mixture.

IMPORTANT: DO NOT add more than 5 µl of the reaction to 50 µl of competent cells. More is not better. Using too much of the reaction mixture inhibits the transformation.
4. Place 1/100–1/5 of each transformation reaction into separate tubes and bring the volume to 100 µl with SOC medium. Spread each diluted transformation on a separate LB plate containing 100 µg/ml of ampicillin.
5. Centrifuge the remainder of each transformation at 6,000 rpm for 5 minutes. Discard the supernatant and resuspend each pellet in 100 µl fresh SOC medium. Spread each sample on a separate LB plate containing the appropriate antibiotic. Incubate all of the plates overnight at 37°C.
6. The next day, pick individual isolated colonies from each experimental plate. Isolate plasmid DNA using a standard method of your choice (e.g. miniprep). To determine the presence of your RACE

insert, analyze the DNA by PCR screening (with your GSPs) or restriction digest (with EcoRI and HindIII, which flank the cloning site).

NOTE: 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 in Section C, below).

C. Sequencing RACE Products

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.

NOTE: The provided pRACE vector is a pUC19-based vector, and is compatible with M13 sequencing primers for characterization of your cloned insert(s). Because In-Fusion cloning is directional, you can preferentially use the M13F primer to sequence into the UPM end, and the M13R primer to sequence into the gene-specific end.

The UPM contains a T7 priming site which can be used for Sanger sequencing, but we recommend using M13 primers to get full clean reads of your experimental sequence. The T7 priming sites are too close to the 5'- and 3'-cloning sites to ensure complete coverage in the sequencing trace.

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 SeqAmp 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.

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:

- 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 a template.
- By cloning overlapping 5'- and 3'-RACE fragments using a restriction site in the overlapping region (if available). If no suitable restriction sites are available, you can alternately design new GSPs suitable for multi-fragment In-Fusion cloning.

NOTE: Details on multi-fragment In-Fusion cloning can be found in our tech note [In-Fusion Multiple Fragment Cloning](#).

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 multi-gene 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 SeqAmp DNA Polymerase and In-Fusion Cloning.

If you do not wish to characterize your RACE products via cloning and sequencing, you may choose from the following alternative methods:

- **Comparison of RACE Products Obtained with GSPs & NGSPs**

For the 5'- and 3'-RACE reactions, compare the products of primary amplifications performed with the UPM Mix and GSP to the secondary products obtained using the UPM and NGSP. If multiple bands are observed, bands representing real transcripts should be slightly smaller in the reaction using NGSPs. The difference in size should correspond to the positions of the outer and inner (nested) GSPs in the cDNA structure. Multiple bands that are the result of nonspecifically-primed PCR should disappear upon amplification with UPM and NGSPs.

- **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. Larger RACE products that do not hybridize to gene-specific probes are generally due to nonspecific priming. Smaller bands that do 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 multi-gene family.

VIII. References

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

A. 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.

If increasing the number of cycles does not solve your problem, try diluting your template in a smaller amount of Tricine-EDTA buffer (Section V.D., Step 12), thus raising the concentration of template in the RACE reaction.

Positive Control RACE PCR Experiment

Also consider running a positive-control reaction, using RACE-ready cDNA generated from the included Control Mouse Heart Total RNA during first-strand cDNA synthesis (Section V.D.). Carry this control reaction through touchdown PCR using the provided Control 5'- or 3'-RACE TFR Primers. Perform the positive control reaction as stated in Section VI.B, Steps 1–3. Replace your experimental sample cDNA with 2.5 µl control 5'- or 3'-cDNA, and your GSPs with 1 µl each 5'- and 3'-RACE TFR Primers. Analyze 5 µl on a 1.2% agarose/EtBr gel.

Expected Results

The 3'-RACE control reaction should produce a 3.1 kb band (Figure 4, Lane 1). The 5'-RACE control reaction should produce a 2.1 kb band (Figure 4, Lane 2). Before you attempt 5'- and 3'-RACE with your primers and experimental cDNA, we recommend you optimize your positive control reactions so that they produce distinct bands of the correct size.

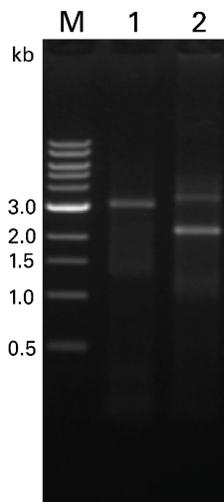


Figure 4. 3'- and 5'-RACE sample results. The gel shows the 3'- and 5'-RACE amplifications of transferrin receptor starting with mouse heart total RNA. Lane M: 1 kb DNA marker. Lanes 1 & 2: transferrin receptor (TFR). The 3' product will be 3.1 kb; the 5' product will be 2.1 kb. As seen here, minor products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

Control PCR Reactions

There are several control reactions that will help you troubleshoot your RACE reactions if yields are suboptimal. These include:

- **Control Reaction 1:** 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.
- **Control Reaction 2:** 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 UPM Short and NGSP.
- **Control Reaction 3 (if working with mouse RNA):** 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.
- **Control Reaction 4:** 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.

If your control reactions provide appropriate bands, but you still have trouble with your experimental samples, go back and check the quantity and quality of your RNA template (Section V.C.). Rare transcripts may be especially difficult to amplify from your RNA sample.

B. 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.

Even if you produce multiple products, in some cases you can and should proceed with generating full-length cDNA (see Section VII). If multiple fragments persist, you should generally start with the largest fragment from each RACE reaction, because it is most likely to be a true, complete RACE product.

1. 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 multi-gene 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.

2. 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.

- **Incomplete fragments**, which are generated from correctly primed sites, can be due to degradation of the RNA template. Generally, degraded RNA used as starting material causes multiple 5'-RACE products.
- **Nonspecific RACE products** arise from nonspecific binding of the primer to multiple sites in the ds cDNA or primer-dimer artifacts.

3. Troubleshooting Suggestions for Multiple-Band RACE Products

A secondary, or “nested” PCR reaction using the UPM Short and an NGSP is discussed in Section IV.D and Section VI.B., Step 4. This secondary PCR will most likely result in the expected distinct band(s). If this does not solve the problem of multiple RACE products, proceed with the suggestions below:

- 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 include the Positive Control RACE PCR Experiment (Appendix A, Section A).

- 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, re-purify your RNA and repeat cDNA synthesis.
- **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 (calculated based upon the gene-specific end of the primer only) and use the cycling parameters for touchdown PCR. Remember, the T_m ...
 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 re-amplified 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.D.
 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.
- **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 none of these methods improve your RACE reactions, it is most likely due to a problem with RNA quality.

C. Other Specific Problems

Table 3. Troubleshooting Guide: Other Specific Problems

Problem	Possible Explanation	Solution
<p>Using your experimental cDNA sample, no 5'- or 3'-RACE bands are produced, but the positive control RACE reactions give the expected products.</p>	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 an 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.
<p>RACE cDNA product is smeared.</p> <p>NOTE: Some SMARTer RACE reactions produce very complex patterns of bands that appear almost as smears.</p>	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 re-purifying 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 B of the Troubleshooting Guide.	

Problem	Possible Explanation	Solution
No band is observed using GSP1 + GSP2 with your experimental sample, but a band is seen with your positive control.	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.	
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 first-strand cDNA synthesis reactions.
No band is observed in positive control amplification of overlapping region of RACE products with GSP1 + GSP2.	Your cDNA synthesis reaction may have failed.	Repeat the first-strand cDNA synthesis reaction.

Appendix B. Detailed Flow Chart of 5' RACE

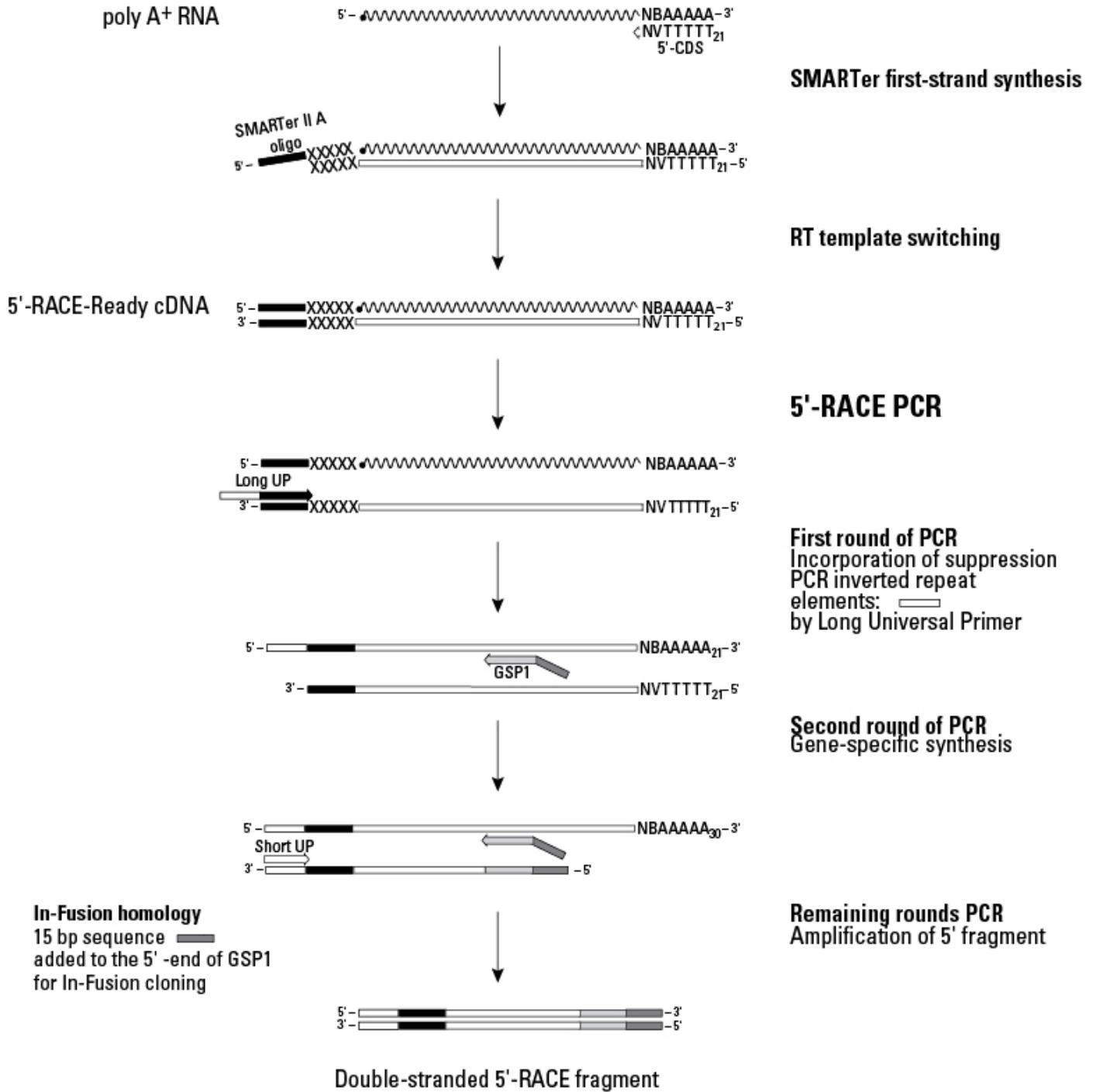


Figure 5. Detailed mechanism of the 5'-RACE reactions.

Appendix C. Detailed Flow Chart of 3' RACE

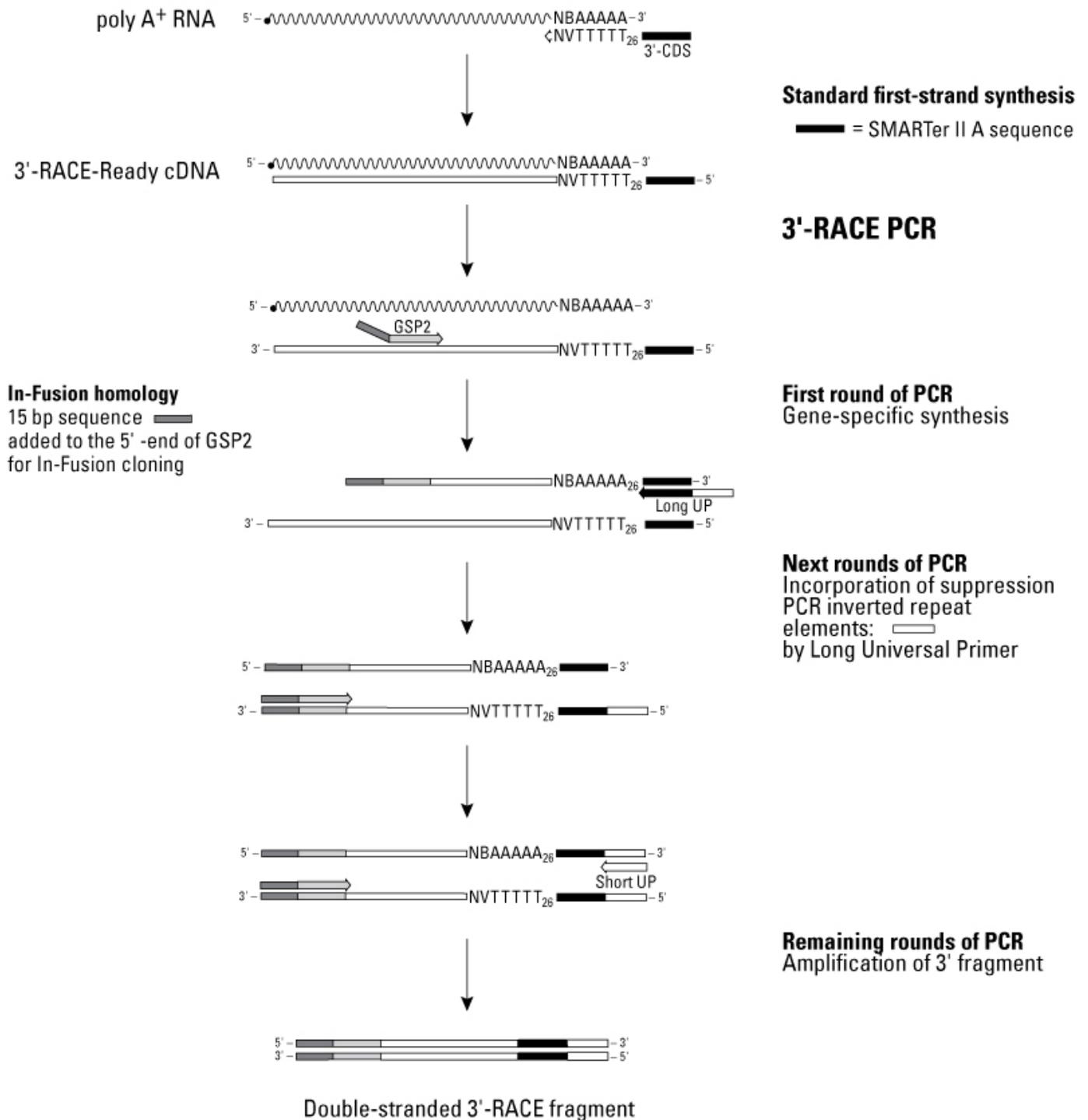


Figure 6. Detailed mechanism of the 3'-RACE reactions.

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 with Random Priming

1. Prepare enough of the following Buffer Mix for all of the 5'-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 6:

4.0 µl 5X First-Strand Buffer

0.5 µl DTT (100 mM)

1.0 µl dNTPs (20 mM)

5.5 µl Total Volume

2. Combine the following reagents in separate microcentrifuge tubes:

**For preparation of
5'-RACE-Ready cDNA**

1.0–10 µl RNA*

1.0 µl 10X Random Primer Mix

0–9 µl Sterile H₂O

11 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 tubes briefly in a microcentrifuge.
4. Incubate tubes at 72°C for 3 minutes, then cool the tubes to 42°C for 2 minutes. After cooling, spin the tubes briefly for 10 seconds at 14,000 x g to collect the contents at the bottom.

NOTE: This step can be performed in a thermal cycler. While the tubes are incubating, you can prepare the Master Mix in Step 6.

5. To the 5'-RACE cDNA synthesis reaction(s), add 1 µl of the SMARTer II A Oligonucleotide per reaction.
6. Prepare enough of the following Master Mix for all 5'-RACE-Ready cDNA synthesis reactions. Mix these reagents at room temperatures in the following order:

5.5 µl Buffer Mix from Step 1

0.5 µl RNase Inhibitor (40 U/µl)

2.0 µl Reverse Transcriptase (100 U)

8.0 µl Total Volume

7. Add 8 µl of the Master Mix from Step 6 to the denatured RNA from Step 5 (5'-RACE cDNA), for a total volume of 20 µl.
8. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.

9. Incubate the tubes at 42°C for 90 minutes 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 cDNA synthesis.

10. Heat tubes at 70°C for 10 minutes.

11. Dilute the first-strand reaction product with Tricine-EDTA Buffer:

- Add 10 µl if you started with <200 ng of total RNA.*
- Add 90 µl if you started with >200 ng of total 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 10 µl. If you have 200 ng of total RNA and the gene of interest is highly abundant, dilute with 90 µl.

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

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