FirstChoice® RACE-Ready cDNA Kit

(Part Number AM3200–AM3209, AM3250–AM3271)

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For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the FirstChoice® RACE-Ready cDNA Kit.

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I. Introduction

A. Background

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction-based technique which facilitates the cloning of full-length cDNA sequences when only a partial cDNA sequence is available. Traditionally, cDNA sequence is obtained from clones isolated from plasmid or phage libraries. Frequently these clones lack sequences corresponding to the 5' ends of the mRNA transcripts. The missing sequence information is typically sought by repeatedly screening the cDNA library in an effort to obtain clones that extended further towards the 5' end of the message. The nature of the enzymatic reactions employed to produce cDNA libraries limits the probability of retrieving extreme 5' sequence even from libraries that are very high quality.

Classic 5' RACE

Classic 5' RACE protocols vary slightly in design, but are essentially equivalent. The major limitation of the procedure is that there is no selection for amplification of fragments corresponding to the actual 5' ends of mRNA: all cDNAs are acceptable templates in the reaction. Additionally, the PCR step selects the most efficient amplicons (e.g., the smallest), favoring amplification of less than full-length products. 5' RACE usually produces a heterogeneous population of amplified products.

RLM-RACE

RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) represents a major improvement to the classic RACE technique (Maruyama and Sugano, 1994, Shaefer, 1995). RLM-RACE is designed to amplify cDNA ***only*** from full-length, capped mRNA, usually producing a single band after PCR. Ambion® uses only the highest quality, triple oligo(dT) selected, DNA-free, RNA as template for RACE-Ready cDNA.

B. RACE-Ready cDNA Procedure Overview

5' RACE using RACE-Ready cDNA

To make Ambion FirstChoice® RACE-Ready cDNA, we start with high quality total RNA, which is then enriched for mRNA content by 3 rounds of oligo(dT) cellulose selection. This RNA is treated with Calf Intestine Alkaline Phosphatase (CIP) to remove free 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, and tRNA. The cap structure found on intact 5' ends of mRNA is not affected by CIP. The RNA is then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. A synthetic RNA Adapter oligonucleotide is ligated to the RNA population using T4 RNA ligase. The adapter cannot ligate

to dephosphorylated RNA because these molecules lack the 5'-phosphate necessary for ligation. During the ligation reaction, the majority of the full length, decapped mRNA acquires the adapter sequence as its 5' end. The treated RNA is then reverse transcribed using random-primers and Ambion's exclusive ArrayScript™ reverse transcriptase (patent pending). ArrayScript is engineered to produce higher yields of first strand cDNA than wild type enzymes, and it results in nearly full-length cDNA. The last steps in producing RACE-Ready cDNA are RNase treatment to remove the mRNA template and column chromatography to remove adapters, primers, and low molecular weight synthesis products (<200 nt).

Ambion provides a pair of partially overlapping primers corresponding to the 5' RACE Adapter sequence for use in nested PCR. The user supplies two nested antisense primers specific to the target gene. Guidelines for the design of gene-specific primers can be found in section II.B on page 5.

3' RACE using RACE-Ready cDNA

A second population of cDNA is included in the RACE-Ready cDNA sample. This cDNA is synthesized from the same high quality starting material, but is primed using an anchored poly(T) adapter-primer: 5'-GCGAGCACAGAATTAATACGACTCACTATAGGT$_{12}$VN. This cDNA can be used in 3' RACE to amplify the 3' end of an mRNA. 3' RACE is generally an easier task than 5' RACE, and it may not require a nested PCR. Ambion provides a pair of nested primers which are complimentary to the anchored adapter, in case nested PCR is determined to be necessary. Keep in mind that the 5' adapter and the 3' anchored oligo(dT) primer are NOT found on the same population of molecules. So the population of cDNA ***cannot*** be amplified using a 5' adapter primer and a 3' adapter primer as PCR primers.

RACE-Ready cDNA can be used for amplification and isolation of either the 5' or the 3' end of a cDNA molecule. RLM-RACE selects for true 5' ends, while the anchored poly(T) adapter insures true 3' end cloning without a long stretch of poly(A) in the clone.

C. Materials Provided with the Kit and Storage Conditions

Store the RACE-Ready cDNA in a non frost-free freezer. Avoid contaminating any reagent with nuclease. Thaw and briefly microfuge the cDNA each time before use. Reagents and cDNA for 30 Primary 5' or 3' RACE reactions and 60 nested 5' or 3' RACE reactions including control reactions are included.

Amount	Component	Storage
30 µL	RACE ready cDNA, 0.5 ng/µL	–20°C
60 µL	5' RACE Outer Primer, 10 µM 5'-GCTGATGGCGATGAATGAACACTG-3'	–20°C
120 µL	5' RACE Inner Primer, 10 µM 5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'	–20°C
60 µL	3' RACE Outer Primer, 10 µM 5'-GCGAGCACAGAATTAATACGACT-3'	–20°C
120 µL	3' RACE Inner Primer, 10 µM 5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	–20°C
25 µL	5' RACE Outer Control Primer, 10 µM mouse 5'-GATCACCAATCCATTGCCGACTAT-3' human 5'-TCTTGGGTTGGTCCTTTCACTT-3'	–20°C
25 µL	5' RACE Inner Control Primer, 10 µM mouse 5'-GAAGTAGATGGTGGGCAGGAAGAT-3' human 5'-CGACACAGTGGAGTACGTCATT-3'	–20°C
25 µL	3' RACE Control Primer, 10 µM mouse 5'-AGCAGTTGGTTGGAGCAAACATC-3' human 5'-CAGGGACATTTTCCAGCAAATTC-3'	–20°C
25 µL	5' PCR Control Primer, 10 µM mouse 5'-GCAGCAGGTAGCAGTGAC-3' human 5'-CAAGTCTGGTTCTTCTCCTCTT-3'	–20°C

D. Materials Not Provided with the Kit

RLM-RACE specific reagents:

- Gene-specific PCR primer(s). See section II.A starting on page 5 for details
- Thermostable DNA polymerase—recommended: Ambion SuperTaq™, recombinant thermostable DNA polymerase or SuperTaq™ Plus Extended Range Taq polymerase.
- Thermal cycler (e.g., Applied Biosystems GeneAmp® PCR System 9700 and the Veriti™ 96-Well Thermal Cycler)
- A method to clone PCR products: either a linearized cloning vector (see sections II.D on page 6 and V.A on page 11) or a 'quick cloning system' like the TA cloning kit from Invitrogen

General reagents:

- Disposable, RNase-free, pipette tips, polypropylene 1.5 mL micro-centrifuge tubes and thin wall microfuge tubes for PCR
- Materials and equipment for gel electrophoresis
- Reagent grade isopropanol
- Reagent grade ethanol
- Cold 70% ethanol made with reagent grade ethanol

E. Related Products Available from Ambion

*SuperTaq™ P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
†SuperTaq™ Plus P/N AM2054, (50U) P/N AM2056, (250U)	Extended Range Thermostable DNA Polymerase Super Taq Plus has a proof reading activity, and produces significantly higher yields of PCR products than ordinary Taq polymerase (includes 10X buffers and dNTPs)
Electrophoresis Reagents See catalog for P/Ns	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.

* Use of this product is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims), no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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II. PCR Primer Design

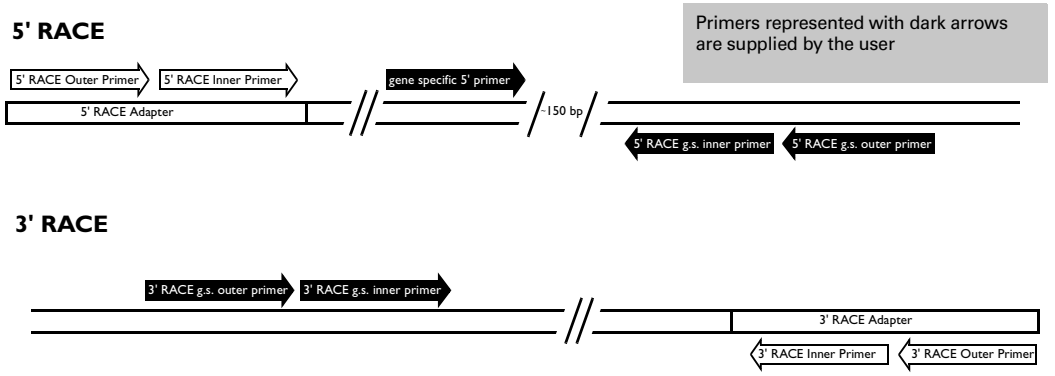
A. General PCR Primer Design Suggestions

Use the following PCR primer design recommendations:

- 20–24 bases in length
- 50% G:C content, with no secondary structure
- Avoid placing more than 3 G or C residues in the 3'-most 5 bases
- Avoid primers with a G as the 3'-terminal base
- Avoid sequences with 3' ends that can self-hybridize or hybridize to the 3' ends of the other primer in the PCR (forming primer dimers)
- Finally, using primer design software, evaluate your gene-specific primers in combination with the corresponding RACE Primer.

Figure 1 shows where the gene-specific primers should lie, and where the primers supplied with the kit are positioned.

Figure 1. Primer positions for 5' and 3' RACE



B. Primer Design for 5' RLM-RACE

1. Nested gene-specific downstream (3' or antisense) primers

The sequences of the 5' RACE Outer and Inner Primers are shown in the list of materials provided with the kit on page 3. The inner primers include a BamH1 site at the 5' end. The 5' RACE Primers work well in PCR using an annealing temperature of 55–65°C (they are typically used at ~60°C). Use primer design software to choose two nested sequences of similar length and melting temperature as PCR primers for your gene. If the distance to the 5' end of the RNA is known, your primers should be designed to anneal no closer than 150–200 bases downstream of the beginning of the RNA transcript to produce a large enough PCR product to evaluate by gel electrophoresis. If the distance

to the 5' end of the RNA is unknown, position the gene-specific primers as far 5' as possible, leaving room to design an upstream primer as a positive control (i.e., ~150 bp downstream of the 5' end of the known sequence). 5' RACE gene-specific primers must be in the reverse complement orientation to the coding sequence of the mRNA so that they prime the antisense strand in PCR. The spacing between the inner and outer nested primers is not critical, although placing them 50–100 base pairs apart will produce PCR fragments that can be easily distinguished by size. If the RACE products will be cloned using restriction sites, design the inner gene-specific primer with a restriction enzyme site at its 5' end (see section II.D on page 6).

2. Gene-specific 5' (upstream or sense) primer

To assist in the analysis and optimization of your reactions, we recommend synthesizing an upstream (sense-strand) gene specific primer positioned so that it produces a resolvable (≥150 bp) product in PCR when used with the 5' RACE gene-specific outer primer. Choose a sequence that is compatible in standard PCR with the corresponding gene-specific primers. This upstream primer can be used in conjunction with your gene specific 5' RACE primers to verify the presence of the target in the cDNA preparation, and to evaluate RLM-RACE products (as described in section VI.A.3 on page 15).

C. Primer Design for 3' RACE

The sequences of the 3' RACE Inner and Outer Primers are shown in the list of materials provided with the kit on page 3. Basic PCR primer design considerations as discussed above should be followed. If the distance from your primers to the 3' end of the target is larger than 1 kb or is unknown, Ambion recommends using an extended-range Taq polymerase to have the best chance of success. The 3' RACE protocol describes nested PCR, however 3' RACE reactions may produce significant product after a single round of PCR. You can try a PCR with a gene specific primer and the 3' RACE Outer Primer, if enough product is amplified, the inner nested reaction may be omitted.

D. Cloning RACE products

The 5' RACE Inner Primer and the 3' RACE Inner Primer have BamH1 sites at their 5' end (CGCGGATCC). If the inner gene specific primers also have restriction sites at their 5' end, PCR fragments generated in the "inner" PCR reactions can be ligated into a digested plasmid vector using standard cloning techniques. We recommend using a restriction site other than BamH1 on inner gene specific primers, so that fragments can be directionally cloned into a double-digested vector. Alternatively, one of the 'quick PCR cloning' kits which use Topoisomerase or T/A overhangs to facilitate cloning can be used to clone reaction products without restriction enzyme sites.

III. 5' RLM-RACE Protocol

A. Nested PCR for 5' RLM-RACE

Minus-template control

It is always a good idea to include a minus-template control in any PCR. This control should include all of the PCR components used in the experimental samples except template. If anything amplifies in this reaction, it indicates that one or more of the PCR reagents is contaminated with DNA.

1. Outer 5' RLM-RACE PCR

a. Assemble the components in PCR tubes on ice:

IMPORTANT

Ambion recommends a hot start for PCRs. At a minimum, assemble reactions on ice, preheat thermal cycler to 94°C, and then place the tubes in the thermal cycler.

Amount	Component
1 µL	RACE-Ready cDNA
5 µL	10X PCR Buffer
4 µL	dNTP Mix (2.5 mM each dNTP)
2 µL	5' RACE gene-specific outer primer (10 µM)
2 µL	5' RACE Outer Primer
to 50 µL	Nuclease-free Water
1.25 U	thermostable DNA polymerase* (0.25 µL of 5U/µL)

* We strongly recommend using an extended range thermostable DNA polymerase, such as SuperTaq-Plus, for targets over 1 kb.

NOTE

Thermal cyclers with very short ramp times, may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, will probably require 1 minute at each temperature in the cycle.

b. Mix gently, flick tube or spin briefly to return the contents to the bottom of the tube.

c. Cycle as follows:

	Stage	Reps	Temp	Time
Initial denaturation	1	1	94°C	3 min
Amplification	2	35	94°C	30 sec
			60°C*	30 sec
			72°C	30 sec
Final extension	3	1	72°C	7 min

* The 5' RACE Outer Primer works well in PCR using a annealing temperature from 55 to 65°C. Therefore, an annealing temperature of 60°C is probably a reasonable starting point. The optimal temperature for your primer and template combination may have to be determined empirically.

For targets longer than 1 kb, add 1 minute to the 72°C extension time for each kilobase. For example, the 35 cycles for a 3 kb target would be: 94°C – 30 sec, 60°C – 30 sec, 72°C – 3 minutes

2. Inner 5' RLM-RACE PCR

a. Assemble the components in PCR tubes on ice:

Amount	Component
1–2 µL	Outer PCR (from the previous step – III.A.1)
5 µL	10X PCR Buffer
4 µL	dNTP Mix (2.5 mM each dNTP)
2 µL	5' RACE gene specific inner primer (10 µM)
2 µL	5' RACE Inner Primer
to 50 µL	Nuclease-free Water
1.25 U	thermostable DNA polymerase (0.25 µL of 5U/µL)

b. Mix gently, flick tube or spin briefly to return the contents to the bottom of the tube.

c. Use the same PCR cycling profile as in the outer 5' RLM-RACE PCR.

B. Gel Analysis of Products and Expected Results

After the PCR is complete, run 5–10 µL of each sample in a 2% high resolution agarose gel containing 1 µg/mL ethidium bromide and visualize on a UV transilluminator. A sample of the outer PCR can also be run for evaluation since a product is sometimes visible after the primary PCR. There should be one to a few bands from the nested PCR from the experimental samples, and the minus-template control sample should have no visible PCR product. In the event no bands are present in the experimental samples, or if there is an unexpectedly complicated pattern (e.g. a smear), optimization of the procedure, as described in section VI.B on page 16 may be beneficial.

IV. 3' RLM-RACE Protocol

A. PCR for 3' RLM-RACE

Often, a single PCR will amplify enough product from 3' RACE. In case a second PCR is necessary to provide more material or greater specificity, two nested primers for the 3' RACE Adapter sequence are provided with this kit. Do an outer PCR first, and then do the inner PCR only if necessary.

Minus-template control

It is always a good idea to include a minus-template control in any PCR. This control should include all of the PCR components used in the experimental samples except template. If anything amplifies in this reaction, it indicates that one or more of the PCR reagents is contaminated with DNA.

1. Outer 3' RLM-RACE PCR

a. Assemble the components in PCR tubes on ice:

IMPORTANT

Ambion recommends a hot start for PCR reactions. At a minimum, assemble reactions on ice, preheat thermal cycler to 94°C, and then place the tubes in the thermal cycler.

Amount	Component
1 µL	RACE-Ready cDNA
5 µL	10X PCR Buffer
4 µL	dNTP Mix (2.5 mM each dNTP)
2 µL	3' RACE gene-specific outer primer (10 µM)
2 µL	3' RACE Outer Primer
to 50 µL	Nuclease-free Water
1.25 U	thermostable DNA polymerase* (0.25 µL of 5U/µL)

* We strongly recommend using an extended range thermostable DNA polymerase, such as SuperTaq-Plus, for targets over 1 kb.

b. Mix gently, flick tube or spin briefly to return the contents to the bottom of the tube.

NOTE

Thermal cyclers with very short ramp times, may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, will probably require 1 minute at each temperature in the cycle.

c. Cycle as follows:

	Stage	Reps	Temp	Time
Initial denaturation	1	1	94°C	3 min
Amplification	2	35	94°C	30 sec
			60°C*	30 sec
			72°C	30 sec
Final extension	3	1	72°C	7 min

* The 3' RACE Outer Primer works well in PCR using a annealing temperature from 55 to 65°C. Therefore, an annealing temperature of 60°C is probably a reasonable starting point. The optimal temperature for your primer and template combination may have to be determined empirically.

For targets longer than 1 kb, add 1 min to the 72°C extension time for each kilobase. For example, the 35 cycles for a 3 kb target would be: 94°C – 30 sec, 60°C – 30 sec, 72°C – 3 minutes

2. Inner 3' RLM-RACE PCR (optional)

Do this PCR if the outer PCR yield is low, or if the outer PCR yields a smear of products instead of a discrete band(s).

a. Assemble the components in PCR tubes on ice:

Amount	Component
1 µL	Outer 3' RACE PCR (from previous step – IV.A.1)
5 µL	10X PCR Buffer
4 µL	dNTP Mix (2.5 mM each dNTP)
2 µL	3' RACE gene-specific inner primer (10 µM)
2 µL	3' RACE Inner Primer
to 50 µL	Nuclease-free Water
1.25 U	thermostable DNA polymerase (0.25 µL of 5U/µL)

b. Mix gently, flick tube or spin briefly to return the contents to the bottom of the tube.

c. Use the same PCR cycling profile as in the outer 3' RLM-RACE PCR.

B. Gel Analysis of Products and Expected Results

Run 5–10 µL of each PCR in a 2% agarose gel containing 1 µg/mL EtBr and visualize on a UV transilluminator. If you have done both the inner and outer PCRs, run samples from both reactions to compare the products. There should be one to a few bands from the PCR. If no bands are present, or if there is an unexpectedly complicated pattern (e.g. a smear), optimization of the procedure, as described in section VI.B on page 16 may help.

V. Cloning and Sequence Analysis of Products

A. Cloning

RACE products can be cloned into suitable plasmid vectors using standard techniques. Both the 5' RACE Inner Primers and 3' RACE Inner Primers have BamH1 sites at their 5' ends. So, RACE products amplified with a gene-specific primer that also has a restriction site can be cloned into an appropriately digested plasmid vector using standard cloning techniques (see Current Protocols in Molecular Biology). Alternatively, one of the 'quick PCR cloning' kits can be used to clone RACE products without using restriction enzymes. Before sequencing a clone, do a diagnostic restriction digest to confirm the presence of the expected size insert.

If the nested PCR produced several bands, this may indicate alternative transcriptional start sites, polyadenylation sites, or splicing products. Alternatively, it may be an indication that the PCRs should be thermal cycled at higher stringency. The pattern of bands may be greatly simplified by raising the annealing temperature to 60°C or higher. If you want to analyze all the products, they can be cloned en masse and sorted out by restriction digest of individual bacterial colonies, or each band can be gel-purified, and cloned individually.

B. Sequence Analysis

As with any cloning experiment, it is a good idea to check insert size by restriction digest before going to the expense and trouble of sequencing.

5' RLM-RACE

5' RLM-RACE products should contain a clean splice at the junction of the 5' RACE Adapter and the mRNA. When analyzing the sequence of 5' RLM-RACE products, it is advisable to sequence more than one clone.

The synthetic RNA adapter oligonucleotide used to prepare the RACE-Ready cDNA will add 45 bp to your experimental outer PCR product, and 36 bp to your experimental inner PCR product. The sequence that will be added to your product after the inner PCR (using the 5' RACE Inner Primer and your gene-specific primer) is the following (assuming that no spurious rearrangement or cloning artifact has occurred):

CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAA–your sequence

(bold sequence is the BamH1 site)

3' RLM-RACE

3' RACE products should contain either the 3' RACE Outer Primer or the 3' RACE Inner Primer sequence at the junction of the anchored poly(T) adapter used to prepare the RACE-Ready cDNA and the mRNA, depending on which was used in the final PCR (assuming that no spurious rearrangement or cloning artifact has occurred).

- 3' RACE Outer Primer used in final PCR:

5'-**GCGAGCACAGAATTAATACGACT**CACTATAGGT$_{12}$VN your sequence

(bold sequence represents the 3' RACE Outer Primer sequence.)

- 3' RACE Inner Primer used in final PCR:

5'-CGC**GGATCC**GAATTAATACGACTCACTATAGGT$_{12}$VN–your sequence

(bold sequence is the BamH1 site)

When analyzing the sequence of RACE products, it is advisable to sequence more than one clone.

VI. Troubleshooting

A. Using the Positive Controls

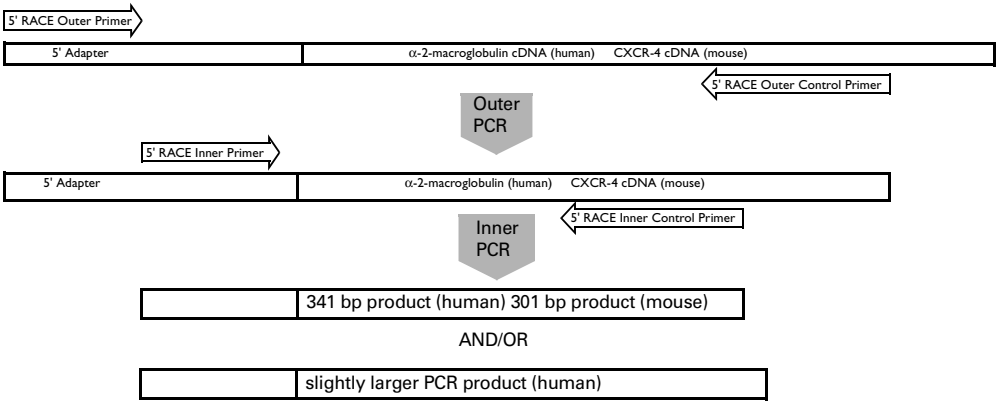
A set of control primers is included with the RACE-Ready cDNA kit to test both 5' and 3' RACE, and PCR.

1. 5' RACE control

a. Purpose of the control

Nested primers for α-2-macroglobulin (human), or CXCR-4 (mouse) are provided to verify that the RACE-Ready cDNA components are functioning properly.

Figure 2. 5' RACE Control



b. Nested PCR for 5' RACE

For the PCRs, amplify 0.5 µL of RACE-Ready cDNA using the 5' RACE Outer Primer with the 5' RACE Outer Control Primer in PCR using an annealing temperature of 60°C (instructions in section III.A.1 on page 7).

Use 1 µL of the outer PCR as template in the nested PCR with the 5' RACE Inner Primer with the 5' RACE Inner Control Primer. The annealing temperature should be 60°C (instructions in section III.A.2 on page 8).

c. Analysis and expected result

Analyze the results by running 5–10 µL of each sample in a 2% high resolution agarose gel containing 1 µg/mL EtBr and visualizing on a UV transilluminator.

Mouse RACE-Ready cDNA:

The inner 5' RACE control PCR should generate a 301 bp PCR product.

Human RACE-Ready cDNA:

The α-2-macroglobulin gene has major and minor transcription start sites. As a result, the 5' RACE control reaction for human cDNAs can produce 1 or both of 2 different reaction products depending on which start site(s) is used in the tissue from which the RACE-Ready cDNA was produced. In the Inner PCR, most RACE-Ready cDNAs will generate a 341 bp PCR product derived from the major start site. Some RACE-Ready cDNAs will generate a doublet in the Inner PCR, and RACE-Ready cDNAs from a few tissues will produce only the PCR product derived from the minor start site (<10% larger).

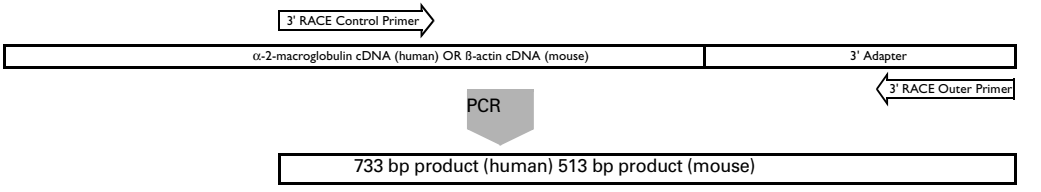
If the correctly-sized product(s) is not generated in this reaction, identify whether there is a problem with the PCR by doing the PCR control described in section VI.A.3 on page 15.

2. 3' RACE control

a. Purpose of the control

An upstream 3' RACE Control Primer is included in with the RLM RACE kit to perform 3' RACE on the RACE-Ready cDNA to confirm functioning of the kit. This primer will be used in conjunction with the 3' RACE Outer Primer to amplify the 3' end of the ß-actin gene.

Figure 3. 3' RACE Control



b. 3' RACE PCR

Use 0.2 µL of RACE-Ready cDNA in PCR with the 3' RACE Control Primer and the 3' RACE Outer Primer. Follow the setup and cycling instructions in section IV.A on page 9; the annealing temperature should be 60°C.

c. Analysis and expected result

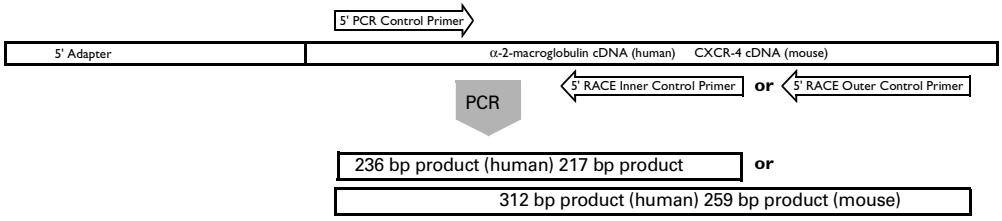
Analyze the results by running 10 µL of each sample in a 2% high resolution agarose gel containing 1 µg/mL EtBr and visualizing on a UV transilluminator. There should be a predominant product from the PCR: 733 bp for human and 513 bp for mouse. We observe additional bands if the PCR yielded a large amount of product; this can usually be eliminated by using only 10–50% as much starting cDNA, or by raising the annealing temperature by a few degrees. If no bands are present, this indicates a problem with the kit or with your PCR protocol.

3. PCR control

a. Purpose of the control

An upstream 'sense strand' primer for α-2-macroglobulin (human), or CXCR-4 (mouse) is provided so that the PCR reaction can be evaluated independently of RACE. (The tube label reads: 5' PCR Control Primer.) This control reaction tests for the proper functioning of the PCR reagents. You can use this primer in a control reaction to troubleshoot your PCR reagents if the 5' and 3' RACE control reactions gave negative results.

Figure 4. PCR Control



b. Set-up and cycling

Set up two PCRs, each with 0.2 µL of cDNA template:

- One reaction should use the 5' PCR Control Primer with the 5' RACE Outer Control Primer,
- The other reaction should use the 5' PCR Control Primer with the 5' RACE Inner Control Primer.

Use an annealing temperature of 60°C and the cycling conditions described in section III.A on page 7.

c. Analysis and expected result

Analyze the results by running 10 µL of each sample in a 2% high resolution agarose gel containing 1 µg/mL EtBr and visualizing on a UV transilluminator. The expected fragment from the outer PCR is 312 bp for human and 259 bp for mouse, from the inner PCR, it is 236 bp for human and 217 bp for mouse. There should be a single band from the PCR (sometimes we observe a second band if the amplification yielded a large amount of product or the annealing temperature was a little low).

If the predicted fragment is not seen, there is a problem with the template, the PCR components, the experimental technique, or the thermal cycler. It is unlikely that RLM-RACE will be successful if these PCRs do not work.

B. Optimization of RLM-RACE

As part of the development of this kit, the importance of each variable in every step of the RLM-RACE protocol were evaluated. The variable that had the most significant impact on the outcome of our experiments was optimization of PCR annealing temperatures. Using SuperTaq-Plus or another extended range Taq polymerase (in lieu of SuperTaq) for PCRs can also dramatically improve yield.

1. Possible causes of ambiguous results

Without optimization, nested PCR may produce no band, a single band, several bands, or a complicated pattern of bands (a smear). Smearing or failure to amplify could be caused by absence of the target in the RNA used for RLM-RACE or suboptimal stringency in the PCR annealing step.

A complicated pattern of discrete bands may be due to multiple initiation sites for transcription of the target gene, or primer homology to several members of a multi-gene family. In some cases, primers can be designed to hybridize only with specific targets, but this is not possible without extensive sequence information.

Since it may not be possible to rule out all of the possible causes of confusing results such as no bands, several bands, or a smear of bands, we recommend trying to optimize the experiment using the suggestions in the following sections.

2. Test the gene-specific PCR primers

A useful control reaction is to test the inner and outer gene specific 5' RLM RACE primers by using each one of them in a PCR with a gene specific 5' primer and a 0.2 µL aliquot of the RACE-Ready cDNA as template (as described in section *VI.A.3. PCR control* on page 15). Each reaction should produce a single band of the appropriate size. Failure to produce the appropriate bands in these control reactions is a strong indication that the cycling conditions are inappropriate, or that the gene specific primers should be redesigned. Sometimes reducing the concentration of the primers in the PCR by 50% reduces background significantly.

If the expected bands are produced in these control PCRs, optimize the RACE PCR by varying the annealing temperature as described below. Sometimes a complicated pattern (or no pattern at all) will resolve into a single band with an increase in stringency of the PCR.

3. Optimization of PCR annealing temperature

More than any other variable, optimization of the PCR annealing temperature will provide the greatest improvement to the outcome of the FirstChoice RLM-RACE protocol. There is ample experimental material for thorough optimization. Each reverse transcription reaction can provide template for 20 PCRs and 5' RACE Inner and Outer Primers

for 100 PCRs are included in the kit. If you need more 5' RACE Inner and Outer Primers, their sequence is provided in section I.C. on page 3. In general, the annealing temperature in the outer PCR is less critical, and should be 55–65°C. The annealing temperature of the inner, nested PCR may need to be higher than predicted by calculation or by primer design software to achieve the required selectivity in the amplification. If the PCR fails to give the expected results, repeat the experiment using a higher (try 2°C) annealing temperature.

4. Protocol modifications for long targets

Lack of a specific RLM-RACE product may be dependent on the distance between your nested primers and the 5' end of the target. The 72°C extension step of the amplification cycle should be extended 1 minute for each kilobase of target over 1 kb. Larger RACE products are more difficult to amplify in general. SuperTaq™ Plus (Ambion P/N AM2054 & AM2056, or similar products from other companies) has a proofreading activity, providing greater fidelity and processivity than ordinary thermostable DMA polymerase. In routine use, PCRs using extended range polymerases have higher yields, especially when the target amplicon is larger than 1 kb. The only drawback to using a 'long-Taq' instead of ordinary thermostable DNA polymerase is that PCR products might not be clonable using the T/A method. Otherwise, the robustness of most reactions will be improved by this simple substitution.


NOTE

SuperTaq Plus™ (P/N AM2054, AM2056) ***is*** *compatible with T/A cloning.*

VII. Appendix

A. References

Maruyama K and Sugano S. (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138**:171-174

Shaefer, B. (1995) Revolution in rapid amplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-length cDNA ends. *Analytical Biochem.* **227**:255-273.

B. Quality Control

Functional testing

All components are functionally tested in RLM-RACE following this protocol. PCR products are assessed on a 2% agarose gel.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 40 ng labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.

C. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds

- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.