Instruction Manual

GeneRacer™ Kit

For full-length, RNA ligase-mediated rapid amplification of 5´ and 3´ cDNA ends (RLM-RACE)

Catalog nos. L1500-01; L1500-02; L1502-01; L1502-02

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Kit Contents and Storage

Shipping and Storage

Each GeneRacer™ Kit is shipped on dry ice. Upon receipt, store the components of the kit as follows:

Component	Storage Temperature
GeneRacer™ Module	-20°C
Cloned AMV RT Module	-80°C
SuperScript™ III RT Module	-20°C
S.N.A.P.™ Columns	Room Temperature
TOPO TA Cloning® Kit for Sequencing	-20°C
Zero Blunt® TOPO® PCR Cloning Kit for Sequencing	-20°C
One Shot® TOP10 Chemically Competent *E. coli*	-80°C

Types Of Kits

Four types of GeneRacer™ Kits are available. Each kit is available with a choice of reverse transcriptase and cloning kit. See the table below for details.

Catalog no.	Reverse Transcriptase	Cloning Kit
L1502-01	SuperScript™ III RT	TOPO TA Cloning® Kit for Sequencing
L1502-02	SuperScript™ III RT	Zero Blunt® TOPO® PCR Cloning Kit for Sequencing
L1500-01	Cloned AMV RT	TOPO TA Cloning® Kit for Sequencing
L1500-02	Cloned AMV RT	Zero Blunt® TOPO® PCR Cloning Kit for Sequencing

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GeneRacer™ Module

The GeneRacer™ Module contains reagents for five PCR-ready cDNA synthesis reactions, one control cDNA reaction, and primers for 50 PCRs. Reagents are included for dephosphorylating RNA, removing the mRNA cap structure, ligating the GeneRacer™ RNA Oligo to the mRNA, and reverse transcribing the mRNA. Primers are included for performing PCR on either the 5′ or 3′ end.

Note that the user must supply additional PCR reagents.

Component	Formulation	Amount
Sterile Water	Sterile, diethylpyrocarbonate (DEPC)-treated ("DEPC water")	2 x 1.5 ml
RNaseOut™	40 U/µl in: 20 mM Tris-HCl, pH 8 50 mM KCl 0.5 mM EDTA 8 mM DTT 50% glycerol (v/v)	24 µl
Calf Intestinal Phosphatase (CIP)	10 U/µl in: 25 mM Tris-HCl, pH 7.6 (+4°C) 1 mM $MgCl_2$ 0.1 mM $ZnCl_2$ 50% glycerol (w/v)	6 µl
10X CIP Buffer	0.5 M Tris-HCl, pH 8.5 (20°C) 1 mM EDTA	6 µl
Tobacco Acid Pyrophosphatase (TAP)	0.5 U/µl in: 10 mM Tris-HCl, pH 7.5 0.1 M NaCl 0.1 mM EDTA 1 mM DTT 0.01% Triton® X-100 50% glycerol (w/v)	6 µl
10X TAP Buffer	0.5 M sodium acetate, pH 6.0 10 mM EDTA 1% β-mercaptoethanol 0.1% Triton® X-100	6 µl

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GeneRacer™ Module, continued

Component	Formulation	Amount
GeneRacer™ RNA Oligo	Pre-aliquoted, lyophilized	6 x 250 ng
T4 RNA Ligase	5 U/µl in: 50 mM Tris-HCl, pH 7.5 0.1 M NaCl 0.1 mM EDTA 1 mM DTT 0.1% Triton® X-100 50% glycerol (w/v)	6 µl
10X T4 RNA Ligase Buffer	330 mM Tris-Acetate, pH 7.8 (25°C) 660 mM potassium acetate 100 mM magnesium acetate 5 mM DTT	6 µl
10 mM ATP	Nuclease-free water and neutralized to pH 7 with NaOH	6 µl
Phenol/Chloroform	Phenol:chloroform:isoamyl alcohol (25:24:1) 0.1% 8-Hydroxyquinoline	2 x 1 ml
Mussel Glycogen	10 mg/ml in DEPC water	36 µl
3 M Sodium Acetate	in DEPC water, pH 5.2	200 µl
GeneRacer™ 5′ Primer	10 µM in DEPC water (71.5 ng/µl)	225 µl
GeneRacer™ 5′ Nested Primer	10 µM in DEPC water (81.3 ng/µl)	225 µl
GeneRacer™ 3′ Primer	10 µM in DEPC water (76.9 ng/µl)	225 µl
GeneRacer™ 3′ Nested Primer	10 µM in DEPC water (71.1 ng/µl)	225 µl
Control HeLa Total RNA	500 ng/µl in DEPC water	20 µl
Control Primer A	10 µM in DEPC water (73.9 ng/µl)	15 µl
Control Primer B.1	10 µM in DEPC water (67.1 ng/µl)	15 µl

S.N.A.P.™ Columns

Ten S.N.A.P.™ columns are provided in the kit to gel-purify your PCR products prior to cloning. Store at room temperature.

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SuperScript™ III RT Module

The following reagents are included in the SuperScript™ III Reverse Transcriptase Module:

Component	Formulation	Amount
SuperScript™ III Reverse Transcriptase (RT)	200 U/µl in: 20 mM Tris-HCl, pH 7.5 100 mM NaCl 0.1 mM EDTA 1 mM DTT 0.01% Nonidet P-40 (v/v) 50% glycerol (w/v)	6 µl
5X First Strand Buffer	250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $MgCl_2$	24 µl
0.1 M DTT	in DEPC water	15 µl
RNase H	2 U/µl in: 20 mM Tris-HCl, pH 7.5 100 mM KCl 10 mM $MgCl_2$ 0.1 mM EDTA 0.1 mM DTT 50 µg/ml BSA 50% glycerol	6 µl
Random Primers (N_6)	100 ng/µl in DEPC water (54 µM)	6 µl
GeneRacer™ Oligo dT Primer	900 ng/µl in DEPC water (50 µM)	6 µl
dNTP Mix (10 mM each)	10 mM dATP 10 mM dGTP 10 mM dCTP 10 mM dTTP in 1 mM Tris-HCl, pH 7.5	6 µl

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Cloned AMV RT Module

The following reagents are included in the Cloned AMV RT Module:

Component	Formulation	Amount
Cloned Avian Myeloblastosis Virus Reverse Transcriptase (Cloned AMV RT)	15 U/µl in: 200 mM Potassium phosphate (pH 7.1) 1 mM DTT 50% (v/v) glycerol 0.2% (w/v) Triton® X-100	6 µl
5X RT Buffer	250 mM Tris acetate (pH 8.4) 375 mM potassium acetate 40 mM magnesium acetate Stabilizer 20 µg/ml BSA	24 µl
Random Primers (N_6)	100 ng/µl in DEPC water (54 µM)	6 µl
GeneRacer™ Oligo dT Primer	820 ng/µl in DEPC water (50 µM)	6 µl
100 mM dNTPs	25 mM dATP 25 mM dGTP 25 mM dCTP 25 mM dTTP in 200 mM Tris-HCl, pH 7.5	6 µl

GeneRacer™ RNA Oligo Sequence

5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′ (44 bases)

GeneRacer™ Oligo dT Primer Sequence

SuperScript™ III RT Module:

5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$-3′ (60 bases)

Cloned AMV RT Module:

5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$-3′ (54 bases)

TOPO TA Cloning® Kit for Sequencing

The TOPO TA Cloning® Kit for Sequencing contains cloning reagents and One Shot® TOP10 Chemically Competent *E. coli* to clone your GeneRacer™ PCR product with 3′-A overhangs for sequencing. The kit contains sufficient reagents to clone 10 GeneRacer™ PCR products.

To use the TOPO TA Cloning® Kit for Sequencing, please refer to the manual supplied with this kit. Please note that there are reagents in this kit that might not be needed when used in conjunction with the GeneRacer™ Kit.

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Zero Blunt® TOPO® PCR Cloning Kit for Sequencing

The Zero Blunt® TOPO® PCR Cloning Kit for Sequencing contains cloning reagents and One Shot® TOP10 Chemically Competent *E. coli* to clone your GeneRacer™ blunt-end PCR product for sequencing. The kit contains sufficient reagents to clone 10 GeneRacer™ PCR products.

To use the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing, please refer to the manual supplied with this kit. Please note that there are reagents in this kit that might not be needed when used in conjunction with the GeneRacer™ Kit.

PCR Primer Sequences

The table below lists the sequence of the PCR primers included in the GeneRacer™ Kit.

Primer	Sequence	Bases	Tm
GeneRacer™ 5′ Primer	5′-CGACTGGAGCACGAGGACACTGA-3′	23	74°C
GeneRacer™ 5′ Nested Primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′	26	78°C
GeneRacer™ 3′ Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′	25	76°C
GeneRacer™ 3′ Nested Primer	5′-CGCTACGTAACGGCATGACAGTG-3′	23	72°C
Control Primer A	5′-GCTCACCATGGATGATGATATCGC-3′	24	72°C
Control Primer B.1	5′-GACCTGGCCGTCAGGCAGCTCG -3′	22	76°C

Introduction

Overview

Introduction

The GeneRacer™ Kit provides a method to obtain full-length 5′ and 3′ ends of cDNA using known cDNA sequence from expressed sequence tags (ESTs), subtracted cDNA, differential display, or library screening. The kit ensures the amplification of only full-length transcripts via elimination of truncated messages from the amplification process. RACE PCR products can be quickly and easily cloned using either the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (blunt-end PCR products) or the TOPO TA Cloning® for Sequencing Kit (PCR products with 3′ A-overhangs).

Using the protocols provided, the cDNA ends of rare (30 copies/cell) and long (9 kb) transcripts can be amplified and sequenced starting from 1 μg of total RNA (Invitrogen, 2000).

Applications

The GeneRacer™ Kit can be used to:

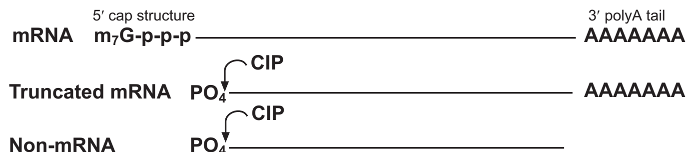
- Identify the 5′ and 3′ untranslated regions of genes
- Study heterogeneous transcriptional start sites
- Characterize promoter regions
- Obtain the complete cDNA sequence of a gene

Description

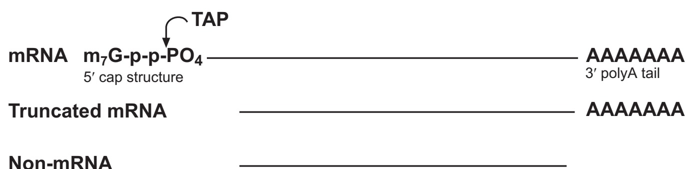
The GeneRacer™ method is described below. This technique is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods, and results in the selective ligation of an RNA oligonucleotide to the 5′ ends of decapped mRNA using T4 RNA ligase (Maruyama and Sugano, 1994; Schaefer, 1995; Volloch *et al.*, 1994).

Note: If you are only interested in the 3′ ends of mRNA, skip Steps 1–3 and proceed directly to Step 4, reverse transcription.

1. Treat total RNA or mRNA with calf intestinal phosphatase (CIP) to remove the 5′ phosphates. This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. **Note**: CIP has no effect on full-length, capped mRNA.



2. Treat dephosphorylated RNA with tobacco acid pyrophosphatase (TAP) to remove the 5′ cap structure from intact, full-length mRNA. This treatment leaves a 5′ phosphate required for ligation to the GeneRacer™ RNA Oligo.



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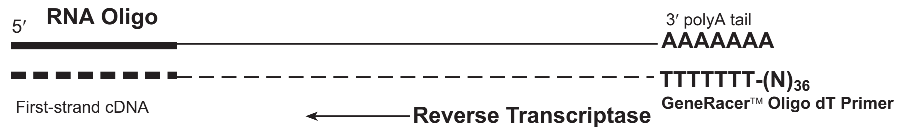
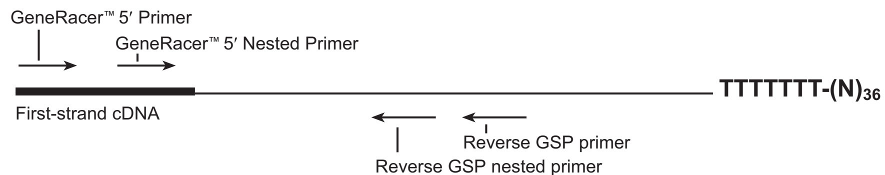
Description, continued

3. Ligate the GeneRacer™ RNA Oligo to the 5′ end of the mRNA using T4 RNA ligase. The GeneRacer™ RNA Oligo will provide a known priming site for GeneRacer™ PCR primers after the mRNA is transcribed into cDNA.

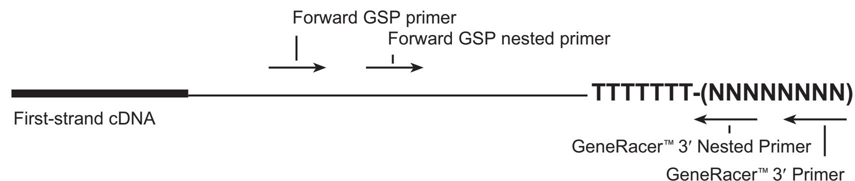
4. Reverse transcribe the ligated mRNA using Cloned AMV RT or SuperScript™ III RT and the GeneRacer™ Oligo dT Primer to create RACE-ready first-strand cDNA with known priming sites at the 5′ and 3′ ends. (If you are only interested in the 5′ ends, you can reverse transcribe using random primers or a gene-specific primer. If you are only interested in the 3′ ends, reverse transcribe the original, unligated mRNA or total RNA using the GeneRacer™ Oligo dT Primer.)



5. To obtain 5′ ends, amplify the first-strand cDNA using a reverse gene-specific primer (Reverse GSP) and the GeneRacer™ 5′ Primer (homologous to the GeneRacer™ RNA Oligo). Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5′ end AND is completely reverse transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.



6. To obtain 3′ ends, amplify the first-strand cDNA using a forward gene-specific primer (Forward GSP) and the GeneRacer™ 3′ Primer (homologous to the GeneRacer™ Oligo dT Primer). Only mRNA that has a polyA tail and is reverse transcribed will be amplified using PCR. If needed, perform additional PCR with nested primers.



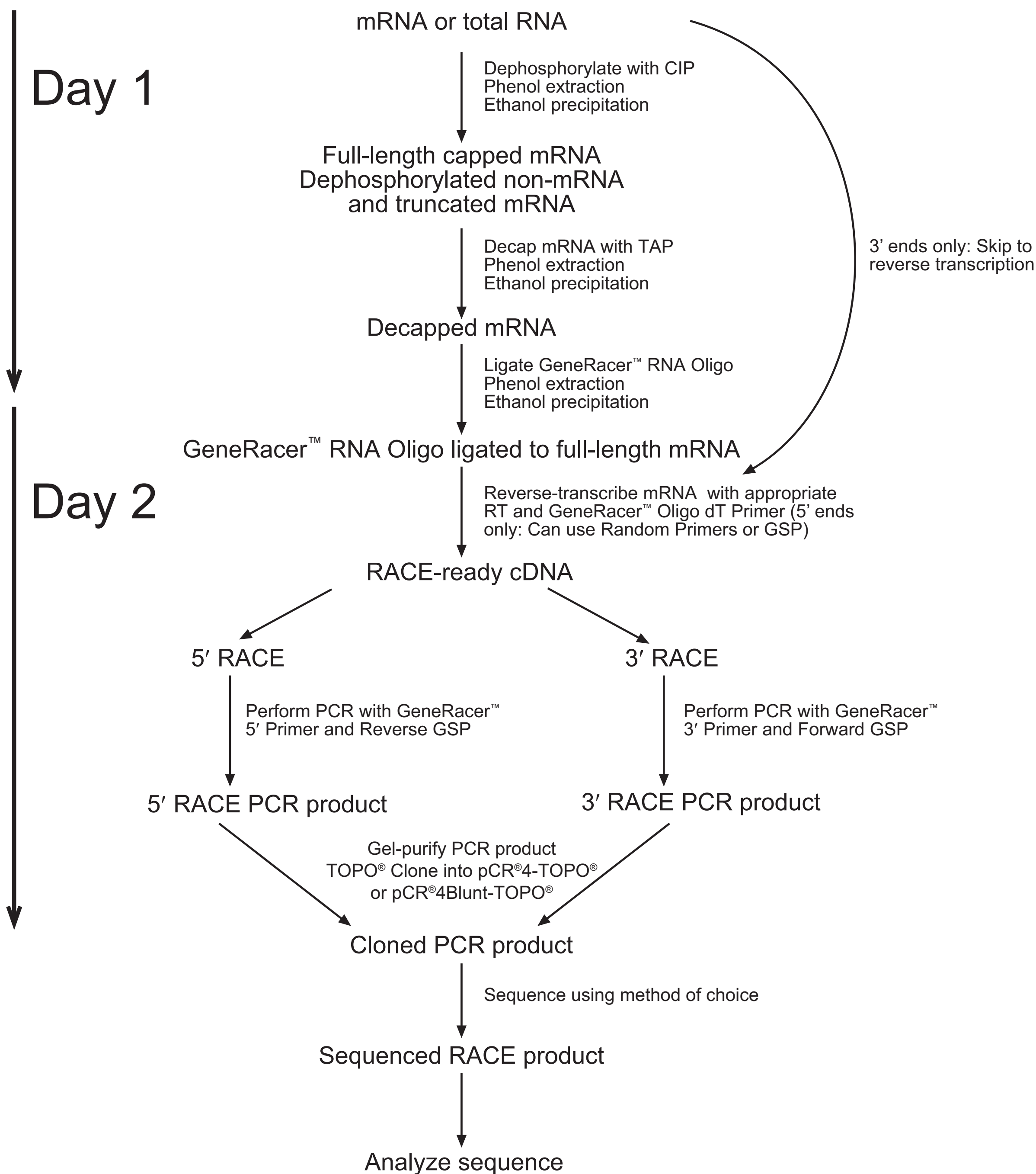
7. Purify RACE PCR products using the S.N.A.P.™ columns included in the kit.

8. TOPO® Clone into the pCR®4-TOPO® or pCR®4Blunt-TOPO® vector for sequencing (please refer to the appropriate manual for more information).

Experimental Outline

Flowchart

The flowchart below shows the GeneRacer™ experimental outline. Day 1 includes all the enzyme reactions up to ligating the GeneRacer™ RNA Oligo to decapped RNA. The ligated RNA can be stored in ethanol overnight and precipitated the next morning. For Day 2, you can perform the reverse transcriptase (RT) reaction, PCR, and TOPO® Clone the PCR products (if you do not have to perform nested PCR). By the morning of Day 3, you can have plasmid DNA containing your 5′ or 3′ PCR product ready for sequencing.



Methods

Preparing RNA

Introduction

You may use either 1-5 μg total RNA or 50-250 ng mRNA with the GeneRacer™ Kit. Using mRNA may increase your chances of obtaining PCR products from rare messages. You will need to isolate mRNA or total RNA using your method of choice prior to using the GeneRacer™ Kit.

Isolation of mRNA or Total RNA

It is very important to use the highest quality RNA possible to ensure the success. Check the integrity and purity of your RNA before starting (see below).

We recommend isolating mRNA using the Micro-FastTrack™ 2.0 (Catalog no. K1520-02) or FastTrack® 2.0 (Catalog no. K1593-02) mRNA Isolation Kits. To isolate total RNA, we recommend the Micro-to-Midi Total RNA Purification System (Catalog no. 12183-018) or TRIzol® Reagent (Catalog no. 15596-026). Other methods for isolating RNA are suitable.

Resuspend RNA in DEPC-treated water (0.1-1 μg/μl) before using the GeneRacer™ Kit.

Checking the RNA Integrity

To check the RNA for integrity, analyze 500 ng of your RNA by agarose/ethidium bromide gel electrophoresis. You may use a regular 1% agarose gel (e.g., E-Gels®, page 31) or a denaturing agarose gel (Ausubel *et al.*, 1994). For total RNA you should see the 28S and 18S rRNA bands. mRNA will appear as a smear from 0.5 to 12 kb. The 28S band should be twice the intensity of the 18S band. If you do not load enough RNA, the 28S band may appear to be diffuse. If you are using a denaturing gel, the rRNA bands should be very clear and sharp. The 28S band should run at 4.5 kb and the 18S band should run at 1.9 kb.

Degraded RNA

If you do not detect your RNA, you will need to isolate new RNA. Be sure to follow the recommendations listed below to prevent RNase contamination.

General Handling of RNA

When working with RNA:

- Use disposable, individually wrapped, sterile plasticware.
- Use only sterile, new pipette tips and microcentrifuge tubes.
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin.
- Always use proper microbiological aseptic technique when working with RNA.

You may use RNase *Away*™ Reagent, a non-toxic solution available from Invitrogen (Catalog no. 10328-011) to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, *et al.*, 1990 or Sambrook, *et al*, 1989.

Designing PCR Primers for RACE

Introduction

You need to know some sequence of your gene of interest to design gene-specific primers (GSPs) for use with the GeneRacer™ Kit. You will use your GSPs in conjunction with the primers in the kit to amplify the 5′ or 3′ end of your gene of interest. Guidelines for designing your GSPs are provided below.

Designing Gene-Specific Primers

You will need at least one GSP if you are performing either 5′ or 3′ RACE. You will need at least two GSPs if you are doing both 5′ and 3′ RACE. Gene-specific primers should have the following characteristics:

- 50–70% GC content to obtain a high annealing temperature (>72°C).
- 23–28 nucleotides in length to increase specificity of binding.
- Low GC content at 3′ ends to minimize extension by DNA polymerase at non-target sites (no more than two G or C residues in the last five bases).
- No self-complementary sequences within the primer or no sequence complementary to the primers supplied in the kit, especially at the 3′ end.
- Annealing temperature greater than 72°C (see below for annealing temperature calculation). Using primers with a high annealing temperature will improve the specificity of your PCR and will allow you to use touchdown PCR (see page 15).

Touchdown PCR (see page 15) and high annealing temperatures increase primer binding specificity and reduce non-specific amplification. For best results, design primers as close to the cDNA ends as possible to minimize the size of the RACE PCR product.

Nested Gene-Specific Primers

In most cases, one round of PCR is sufficient to generate a gene-specific RACE PCR product. However, if you do not obtain a distinct RACE PCR product or observe high background in the first PCR you may need to perform nested PCR with the GeneRacer™ Nested primers and nested GSPs. Follow the guidelines above to design your nested GSP. In addition, design your nested GSP so that the:

- Annealing temperature is similar to the annealing temperature for the GeneRacer™ Nested Primer.
- Nested GSP far enough from the original GSP so that you can distinguish the products of original and nested PCR by size.

Gene-Specific PCR Positive Control

You may want to design two GSPs that will amplify a short region (~500 bp) of your gene cDNA. Amplification of this region will serve as a PCR positive control and ensure that your gene cDNA is present in your RACE-ready cDNA.

Annealing Temperature

Use the following formula to approximate the annealing temperature for your primer:

$4 \times (G+C) + 2 \times (A+T)$ = Annealing temperature (approximate T_m), where G, C, A, or T represent the number of these bases in the primer sequence.

Dephosphorylating RNA

Introduction

In this step, you treat your total RNA or mRNA with calf intestinal phosphatase (CIP) to dephosphorylate non-mRNA or truncated mRNA. We have also included control HeLa total RNA as a positive control for all GeneRacer™ reactions.

Note

If you are only interested in the 3′ ends of your mRNA, skip this step and proceed directly to **Reverse Transcribing mRNA** on page 12.

Positive Control Reactions with HeLa Total RNA

HeLa total RNA is included as a positive control for each enzymatic step and PCR. We strongly recommend that you perform the entire procedure with the control RNA prior to or simultaneously with your RNA sample. This will save you time and ensure that the reactions work. Control primers to the β-actin gene are included for PCR. When the control primers are used in conjunction with either the GeneRacer™ 5′ primers or GeneRacer™ 3′ primers, the 5′ or 3′ end of the β-actin gene is amplified (see page 20 for a diagram).

Handling Enzymes

All enzymes are provided in a glycerol solution. Keep at -20°C at all times. Remove the enzyme at the time of use, briefly centrifuge, aliquot, and return to -20°C.

Before Starting

Be sure to have the following reagents and equipment on hand before starting:

- Total RNA (1-5 µg, or less) or mRNA (50-250 ng, or less) in DEPC water (approximately 0.1 to 1 µg/µl)
- Ice
- 1.5 ml sterile microcentrifuge tubes
- Heat block or water bath set at 50°C
- 95% ethanol
- Dry ice
- 70% ethanol
- Microcentrifuge at room temperature or +4°C

Using More RNA

Using amounts greater than 5 µg total RNA or 250 ng mRNA may require increasing the CIP in the **Dephosphorylation Reaction**, next page. Please scale up the reaction accordingly.

Continued on next page

Dephosphorylation Reaction

1. Set up on ice the following 10 µl dephosphorylation reaction in a 1.5 ml sterile microcentrifuge tube using the reagents in the kit. Use 1-5 µg total RNA or 50-250 ng mRNA. Use 2 µl (1 µg) of HeLa total RNA for the control reaction.

Reagent	Sample RNA	Control RNA
RNA	x µl	2 µl
10X CIP Buffer	1 µl	1 µl
RNaseOut™ (40 U/µl)	1 µl	1 µl
CIP (10 U/µl)	1 µl	1 µl
DEPC water	y µl	5 µl
Total Volume	10 µl	10 µl

2. Mix gently by pipetting and vortex briefly. Centrifuge to collect fluid.
3. Incubate at 50°C for 1 hour.
4. After incubation, centrifuge briefly and place on ice.

Precipitating RNA

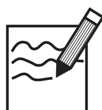
1. To precipitate RNA, add 90 µl DEPC water and 100 µl phenol:chloroform and vortex vigorously for 30 seconds.
2. Centrifuge at maximum speed in a microcentrifuge for 5 minutes at room temperature.
3. Transfer aqueous (top) phase to a new microcentrifuge tube (~100 µl).
4. Add 2 µl 10 mg/ml mussel glycogen, 10 µl 3 M sodium acetate, pH 5.2, and mix well. Add 220 µl 95% ethanol and vortex briefly.
5. Freeze on dry ice for 10 minutes. You may proceed to the next step or store at -20°C overnight.

   Note: Do not store the RNA in DEPC water. Store RNA in ethanol at -20°C.
6. To pellet RNA, centrifuge at maximum speed in a microcentrifuge for 20 minutes at +4°C.
7. Note the position of the pellet and remove the supernatant by pipet. Be careful not to disturb pellet.
8. Add 500 µl 70% ethanol, invert several times, and vortex briefly.
9. Centrifuge at maximum speed in a microcentrifuge for 2 minutes at +4°C.
10. Note the position of the pellet and carefully remove the ethanol using a pipet. Centrifuge again to collect remaining ethanol.
11. Carefully remove the remaining ethanol by pipet and air-dry the pellet for 1-2 minutes at room temperature.
12. Resuspend the pellet in 7 µl DEPC water. If you want to check the stability of RNA after the CIP reaction, resuspend the pellet in 8 µl DEPC water and analyze 1 µl by agarose gel electrophoresis. Proceed to **Removing the mRNA Cap Structure**, next page.

Removing the mRNA Cap Structure

Introduction

After dephosphorylating and precipitating the RNA, you are ready to remove the 5′ cap structure from full-length mRNA.



Note

If you are only interested in the 3′ ends of your mRNA, skip this step and proceed directly to **Reverse Transcribing mRNA** on page 12.

Before Starting

Be sure to have the following reagents and equipment on hand before starting:

- 1.5 ml sterile microcentrifuge tubes
- Ice
- Heat block or water bath at 37°C
- 95% ethanol
- 70% ethanol
- Dry ice
- Microcentrifuge at room temperature and +4°C

Using More RNA

Using amounts greater than 5 µg total RNA or 250 ng mRNA may require increasing the TAP in the **Decapping Reaction**, below. Please scale up the reaction accordingly.

Decapping Reaction

1. Set up on ice the 10 µl decapping reaction in a 1.5 ml sterile microcentrifuge tube using the reagents in the kit.

Dephosphorylated RNA	7 µl
10X TAP Buffer	1 µl
RNaseOut™ (40 U/µl)	1 µl
TAP (0.5 U/µl)	1 µl
Total Volume	10 µl

2. Mix gently by pipetting and vortex briefly. Centrifuge briefly to collect fluid.
3. Incubate at 37°C for 1 hour.
4. After incubation, centrifuge briefly and place on ice.

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Precipitating RNA

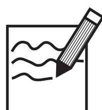
1. After incubation, add 90 µl DEPC water and 100 µl phenol:chloroform and vortex vigorously for 30 seconds.
2. Centrifuge at maximum speed in a microcentrifuge for 5 minutes at room temperature.
3. Transfer aqueous (top) phase to a new microcentrifuge tube (~100 µl).
4. Add 2 µl 10 mg/ml mussel glycogen, 10 µl 3 M sodium acetate, pH 5.2, and mix well. Add 220 µl 95% ethanol and vortex briefly.
5. Freeze on dry ice for 10 minutes. You may proceed to the next step or store at -20°C overnight.

   Note: Do not store the RNA in DEPC water. Store RNA in ethanol at -20°C.
6. To pellet RNA, centrifuge at maximum speed in a microcentrifuge for 20 minutes at +4°C.
7. Note the position of the pellet and remove the supernatant by pipet. Be careful not to disturb pellet.
8. Add 500 µl 70% ethanol, invert several times, and vortex briefly.
9. Centrifuge at maximum speed in a microcentrifuge for 2 minutes at +4°C.
10. Note the position of the pellet and carefully remove the ethanol using a pipet. Centrifuge again to collect remaining ethanol.
11. Carefully remove the remaining ethanol by pipet and air-dry the pellet for no more than 1-2 minutes at room temperature.
12. Resuspend the pellet in 7 µl DEPC water. If you want to check the stability of RNA after the TAP reaction, resuspend the pellet in 8 µl DEPC water and analyze 1 µl by agarose gel electrophoresis. Proceed directly to **Ligating the RNA Oligo to Decapped mRNA**, next page.

Ligating the RNA Oligo to Decapped mRNA

Introduction

Once you have decapped the mRNA, you are ready to ligate the GeneRacer™ RNA Oligo to the 5′ end of your mRNA.



Note

If you are only interested in the 3′ ends of your mRNA, skip this step and proceed directly to **Reverse Transcribing mRNA** on page 12.

GeneRacer™ RNA Oligo

This oligo is specifically designed to optimize ligation to decapped mRNA. In particular, it has:

- Minimal secondary structure to provide a free 3′ end for efficient ligation
- Adenines at the 3′ end to increase ligation efficiency (Uhlenbeck and Gumport, 1982)

In addition, it contains the priming sites for the GeneRacer™ 5′ Primer and the GeneRacer™ 5′ Nested Primer (see diagram below).

Before Starting

Be sure to have the following reagents and equipment on hand before starting:

- 1.5 ml sterile microcentrifuge tubes
- Heat block at 65°C
- Heat block or water bath at 37°C
- Ice
- 95% ethanol
- 70% ethanol
- Dry ice
- Microcentrifuge at room temperature and +4°C

Using More RNA

Using amounts greater than 5 µg total RNA or 250 ng mRNA may require increasing the GeneRacer™ RNA Oligo. Please scale up the reaction accordingly.

Continued on next page

Ligation Reaction

1. Add 7 µl of dephosphorylated, decapped RNA to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg). Pipet up and down several times to mix and resuspend RNA Oligo. Centrifuge briefly to collect the fluid in the bottom of the tube.
2. Incubate at 65°C for 5 minutes to relax the RNA secondary structure. **Note**: After the incubation, the total volume of this solution may decrease by 1 µl due to evaporation.
3. Place on ice to chill (~2 minutes) and centrifuge briefly.
4. Add the following reagents to the tube, mix gently by pipetting, and centrifuge briefly.

10X Ligase Buffer	1 µl
10 mM ATP	1 µl
RNaseOut™ (40 U/µl)	1 µl
T4 RNA ligase (5 U/µl)	1 µl
Total Volume	10 µl

5. Incubate at 37°C for 1 hour.
6. Centrifuge briefly and place on ice. Precipitate the RNA (see below).

Precipitating RNA

1. After incubation, add 90 µl DEPC water and 100 µl phenol:chloroform and vortex vigorously for 30 seconds.
2. Centrifuge at maximum speed in a microcentrifuge for 5 minutes at room temperature.
3. Transfer aqueous (top) phase to a new microcentrifuge tube (~100 µl).
4. Add 2 µl 10 mg/ml mussel glycogen, 10 µl 3 M sodium acetate, pH 5.2, and mix well. Add 220 µl 95% ethanol and vortex briefly.
5. Freeze on dry ice for 10 minutes. You may proceed to the next step or store at -20°C overnight.

   Note: Do not store the RNA in DEPC water. Store RNA in ethanol at -20°C.
6. To pellet RNA, centrifuge at maximum speed in a microcentrifuge for 20 minutes at +4°C.
7. Note the position of the pellet and decant supernatant or remove the supernatant by pipet. Be careful not to disturb pellet.
8. Add 500 µl 70% ethanol, invert several times, and vortex briefly.
9. Centrifuge at maximum speed in a microcentrifuge for 2 minutes at +4°C.
10. Note the position of the pellet and carefully remove the ethanol using a pipet. Centrifuge again to collect remaining ethanol.
11. Carefully remove the ethanol by pipet and air-dry the pellet for no more than 1-2 minutes at room temperature.
12. Resuspend the pellet in 10 µl DEPC water. If you want to check the stability of RNA after ligation, resuspend the pellet in 11 µl DEPC water and analyze 1 µl by agarose gel electrophoresis. Proceed to **Reverse Transcribing mRNA**, next page.

Reverse Transcribing mRNA

Introduction

After you have ligated the GeneRacer™ RNA Oligo to decapped, full-length mRNA, you are ready to reverse transcribe the mRNA into cDNA. Protocols are provided for Cloned AMV RT (below) and SuperScript™ III RT (next page).

3′ Ends Only

If you are only interested in the 3′ ends of your mRNA, perform this step on the original, unligated mRNA or total RNA.

GeneRacer™ Oligo dT Primer

This primer contains a dT tail of 24 nucleotides (SuperScript™ III RT Module) or 18 nucleotides (Cloned AMV RT Module) to prime the first-strand cDNA synthesis in the RT reaction. The sequence at the 5′ end contains the priming sites for the GeneRacer™ 3′ and the GeneRacer™ 3′ Nested primers (see the diagram below). Use 1 µl (50 µM) of this primer.

Other Primers

If you do not need sequence information for the 3′ end, you may use the Random Primers (100 ng) provided in the kit or a GSP (2 pmole) to synthesize cDNA. Furthermore, if your particular gene is greater than 4 or 5 kb, you may want to use the Random Primers or a GSP to obtain the 5′ end.

Before Starting

Be sure to have the following reagents and equipment on hand before starting:

- 1.5 ml sterile microcentrifuge tubes
- Heat block at 45°C, 65°C, 70°C and 85°C
- Ice

Cloned AMV RT Reaction

1. Add 1 µl of the desired primer and 1 µl of dNTP Mix (25 mM each) to the ligated RNA (10 µl, Step 12, page 11).
2. Incubate at 65°C for 5 minutes to remove any RNA secondary structure.
3. Chill on ice for 2 minutes and centrifuge briefly.
4. Add the following reagents to the 12 µl ligated RNA and primer mixture.

5X RT Buffer	4 µl
Cloned AMV RT (15 U/µl)	1 µl
Sterile water	2 µl
RNaseOut™ (40 U/µl)	1 µl
Total Volume	20 µl

   Note: If you are using Random Primers, incubate the reaction mix at 25°C for 10 minutes prior to performing Step 5 to allow efficient binding of the Random Primer to the template.

5. Mix well and incubate at 45°C for 1 hour.
6. Incubate at 85°C for 15 minutes to inactivate Cloned AMV RT.
7. Centrifuge briefly and use immediately for amplification or store at -20°C. You may use up to 2 µl of the RT reaction in each PCR reaction.

Continued on next page

SuperScript™ III RT Reaction

1. Add the following to the 10 µl of ligated RNA from Step 12, page 11:

Primers	1 µl
dNTP Mix	1 µl
Sterile, distilled water	1 µl

2. Incubate at 65°C for 5 minutes to remove any RNA secondary structure.
3. Chill on ice for at least 1 minute and centrifuge briefly.
4. Add the following reagents to the 13-µl ligated RNA and primer mixture:

5X First Strand Buffer	4 µl
0.1 M DTT	1 µl
RNaseOut™ (40 U/µl)	1 µl
SuperScript™ III RT (200 U/µl)	1 µl
Total Volume	20 µl

5. Mix well by pipetting gently up and down.

   Note: If you are using random primers, incubate the reaction mix at 25°C for 5 minutes prior to Step 6 to allow efficient binding of the random primers to the template.
6. Centrifuge briefly and incubate at 50°C for 30-60 minutes. If you are using gene-specific primers, increase the reaction temperature to 55°C.
7. Inactivate the RT reaction at 70°C for 15 minutes. Chill on ice for 2 minutes and centrifuge briefly at maximum speed in a microcentrifuge.
8. Add 1 µl of RNase H (2 U) to the reaction mix.
9. Incubate at 37°C for 20 minutes.
10. Centrifuge briefly and use immediately for amplification or store at -20°C. You may use up to 2 µl of the RT reaction in each PCR reaction.

- If you are having difficulty performing the RT reaction using Cloned AMV RT or SuperScript III™ RT, you can use any reverse transcriptase of choice.
- Thermoscript™ RT, an avian reverse transcriptase with reduced RNase H activity, offers exceptional performance for difficult templates with extensive secondary structure (Schwabe, *et al).* You can use Thermoscript™ RT at temperatures up to 70°C. See page 31 for ordering information.

Amplifying cDNA Ends

Introduction

You now have RACE-ready cDNA with known priming sites on each end that you can use to amplify the 5′ and 3′ ends for sequencing. Use appropriate polymerase to create blunt-end PCR products for cloning into pCR®4Blunt-TOPO® or 3′ A-overhang-containing PCR products for cloning into pCR®4-TOPO® (see below).

Before Starting

Be sure to have the following reagents and equipment on hand before starting:

- Thermocycler (see recommendations, below)
- Thermostable DNA polymerase (see below)
- Gene-specific primers for amplification (please see page 5 for criteria)
- 10X PCR Buffer for the thermostable polymerase
- dNTP solution (10 mM each dNTP) (not supplied)
- 1% agarose/ethidium bromide gel and apparatus

Thermocycler Recommendation

Be aware that different thermocyclers may yield different results depending on ramping times. The β-actin control reactions were optimized using GeneAmp® PCR System 9700 (PE Applied Biosystems). Similar results were obtained using PTC-200 DNA Engine (MJ Research). Depending on your PCR machine you may have to optimize your PCR parameters to obtain optimal results.

Thermostable DNA Polymerase

To clone into pCR®4-TOPO®, you must use a thermostable polymerase or a mixture that adds 3′ A-overhangs. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity (Catalog no. 11304-011).

To clone into pCR®4Blunt-TOPO®, you must use a thermostable proofreading polymerase that generates blunt-end products. We recommend Platinum® *Pfx* DNA polymerase (Catalog no. 11708-013) or ThermalAce™ DNA Polymerase (Catalog no. E0200). Other polymerases that generate blunt ends or 3′ A-overhangs may be suitable.

The protocols for setting up the PCR reaction and the cycling parameters for using Platinum® *Taq* DNA Polymerase High Fidelity and Platinum® *Pfx* DNA polymerase are provided on pages 17 and 18, respectively. If you are using ThermalAce™ DNA Polymerase, use the protocol described in the manual for setting up the PCR reactions. For any other thermostable DNA polymerase, please follow the manufacturer's recommendations.

PCR Recommendation

We recommend that you use a hot start and touchdown PCR to minimize the background (see next page). Annealing and extension times, and temperatures may need to be optimized for each individual primer/template combination. Use a 1 minute extension for each 1 kb of DNA. The number of cycles can be adjusted depending on the transcript abundance. After cycling, hold the reactions at +4°C or place on ice.

Continued on next page

Hot Start

The hot start PCR method minimizes mispriming and extension. Hot start PCR can be achieved using any of the following methods:

- Using Platinum® *Taq* DNA Polymerase High Fidelity or Platinum® *Pfx* DNA polymerase that provide an automatic hot start. A thermolabile inhibitor containing monoclonal antibodies to *Taq* DNA polymerase is bound to these polymerases. Initial denaturing step of the PCR results in denaturation of the inhibitor, releasing active polymerase into the reaction.
- Withholding a key component of the reaction (e.g. thermostable polymerase or magnesium) until the denaturation temperature is reached.

Touchdown PCR

Touchdown PCR increases specificity and reduces background amplification. We use a variation of touchdown PCR (Don *et al.*, 1991; Roux, 1995) that exploits the high annealing temperatures of the GeneRacer™ primers and your GSPs to selectively amplify your gene-specific cDNAs that are tagged with the GeneRacer™ RNA Oligo. By starting at a high annealing temperature, only gene-specific or GeneRacer™-tagged cDNA is amplified, allowing the desired product to accumulate. Decreasing the annealing temperature through the remaining PCR cycles permits efficient amplification of tagged, gene-specific template.

GeneRacer™ Primers

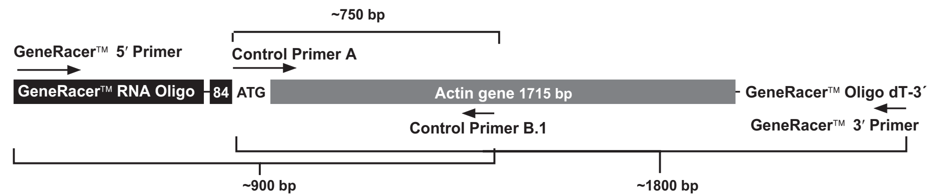
The GeneRacer™ Kit contains 4 primers specifically designed to amplify either the 5′ end or the 3′ end of full-length cDNA. Two primers are homologous to sequence in the GeneRacer™ RNA Oligo (see page 10) and the other two primers are homologous to sequence in the GeneRacer™ Oligo dT Primer (see page 12). The two Control Primers are homologous to the human β-actin gene (see next page). The table below summarizes other physical characteristics of the GeneRacer™ primers. Ordering information for primers is provided on page 31.

Name	Size	Homology	Tm
GeneRacer™ 5′ Primer	23-mer	Position 1-23 of GeneRacer™ RNA Oligo	74°C
GeneRacer™ 5′ Nested Primer	26-mer	Position 15-40 of GeneRacer™ RNA Oligo	78°C
GeneRacer™ 3′ Primer	25-mer	Position 1-25 of GeneRacer™ Oligo dT Primer	76°C
GeneRacer™ 3′ Nested Primer	23-mer	Position 14-36 of GeneRacer™ Oligo dT Primer	72°C
Control A Primer	24-mer	Position 67-90 of human β-actin (NM 001101.2)	72°C
Control B.1 Primer	22-mer	Position 793-814 of human β-actin (NM 001101.2)	76°C

Continued on next page

HeLa RNA RACE PCR Control

To analyze the RACE-ready cDNA created from the control HeLa total RNA, use GeneRacer™ 5′ Primer and Control Primer B.1 to amplify the 5′ end of the β-actin cDNA and GeneRacer™ 3′ Primer and Control Primer A to amplify the 3′ end of the β-actin cDNA (see diagram below).



Amount of Template

- Use 1-2 µl of the template (from Step 7, page 12 or Step 10, page 13) or you may dilute your cDNA with sterile water before using. Diluting your template may improve specificity and reduce background amplifications. The appropriate dilution will depend on the amount of starting RNA you used, the type of polymerase you are using, and the abundance of your transcript. We recommend testing various dilutions of the template. Do not use more than 10% (2 µl) of the RT reaction for PCR.
- Use 1 µl of control HeLa RT reaction template for PCR.

Continued on next page

PCR Setup with Platinum® *Taq* DNA Polymerase High Fidelity

Set up your reactions to amplify either the 5′ end or the 3′ end of your gene of interest. Use the table below to set up your sample reactions and positive control reactions using Platinum® *Taq* DNA Polymerase High Fidelity. Suggestions for negative controls are included on the next page. See page 5 for more details on gene-specific primers. See previous page for amount of the template to be used.

Reagent	5′ RACE	5′ RACE Control	3′ RACE	3′ RACE Control
GeneRacer™ 5′ Primer, 10 µ M	3 µl	3 µl	--	--
Reverse GSP, 10 µM	1 µl	--	--	--
Control Primer B.1, 10 µM		1 µl		
GeneRacer™ 3′ Primer, 10 µM	--	--	3 µl	3 µl
Forward GSP, 10 µM	--	--	1 µl	--
Control Primer A, 10 µM	--	--	--	1 µl
RT Template	1 µl		1 µl	
HeLa RT Template		1 µl		1 µl
10X High Fidelity PCR Buffer	5 µl	5 µl	5 µl	5 µl
dNTP Solution (10 mM each)	1 µl	1 µl	1 µl	1 µl
Platinum® *Taq* DNA Polymerase High Fidelity, 5U/µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
$MgSO_4$, 50 mM	2 µl	2 µl	2 µl	2 µl
Sterile Water	36.5 µl	36.5 µl	36.5 µl	36.5 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**	**50 µl**

Continued on next page

PCR Setup with Platinum® *Pfx* DNA Polymerase

Set up your reactions to amplify either the 5′ end or the 3′ end of your gene of interest. Use the table below to set up your sample reactions and positive control reactions using Platinum® *Pfx* DNA Polymerase. Suggestions for negative controls are included below. See page 5 for more details on gene-specific primers. See page 16 for amount of template to be used.

Reagent	5′ RACE	5′ RACE Control	3′ RACE	3′ RACE Control
GeneRacer™ 5′ Primer, 10 μ M	4.5 μl	4.5 μl	--	--
Reverse GSP, 10 μM	1.5 μl	--	--	--
Control Primer B.1, 10 μM		1.5 μl		
GeneRacer™ 3′ Primer, 10 μM	--	--	4.5 μl	4.5 μl
Forward GSP, 10 μM	--	--	1.5 μl	--
Control Primer A, 10 μM	--	--	--	1.5 μl
RT Template	1 μl		1 μl	
HeLa RT Template		1 μl		1 μl
10X *Pfx* Amplification Buffer	5 μl	5 μl	5 μl	5 μl
dNTP Solution (10 mM each)	1.5 μl	1.5 μl	1.5 μl	1.5 μl
Platinum® *Pfx* DNA Polymerase, 2.5U/μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl
$MgSO_4$, 50 mM	1 μl	1 μl	1 μl	1 μl
Sterile Water	35 μl	35 μl	35 μl	35 μl
Total Volume	**50 μl**	**50 μl**	**50 μl**	**50 μl**

Negative Controls

In addition, we recommend including the following negative controls:

- All components except template — Presence of a band or smear in this lane indicates contamination with DNA (see page 28).
- All components except your GSP — Presence of a band or smear indicates nonspecific binding of GeneRacer™ primer. Optimize PCR conditions (see page 29).
- All components except the GeneRacer™ 5′ or 3′ Primer — Presence of a band or smear indicates nonspecific binding of GSP primer. Optimize PCR conditions (see page 29) or redesign your GSP.

Performing these negative controls will save you time in identifying your specific RACE products. Nonspecific amplification should be obvious from the negative control reactions and can occur because of a number of reasons (see **Troubleshooting** section, pages 26-29). Performing nested PCR or optimizing your PCR should help you identify real RACE products.

Continued on next page

Cycling Parameters

You will have to determine the cycling parameters for your particular GeneRacer™ primer/GSP combination. Use a 1 minute extension for each 1 kb of DNA. Use 25-35 total PCR cycles depending on the transcript abundance. We do not recommend using more than 35 total cycles as it increases the background. To increase the yield and specificity of your RACE product, perform nested PCR (see page 22).

A table is provided below to help you design cycling parameters specific for your applications.

Temperature	Time	Cycles
94°C	2 minutes	1
94°C	30 seconds	5
72°C	1 min/ 1 kb DNA	
94°C	30 seconds	5
70°C	1 min/ 1 kb DNA	
94°C	30 seconds	20-25
60-68°C	30 seconds	
68-72°C	1 min/ 1 kb DNA	
68-72°C	10 minutes	1

We recommend using touchdown PCR (see page 15). If you are using Platinum® *Taq* DNA Polymerase High Fidelity or Platinum® *Pfx* DNA Polymerase, perform the extension at 68°C. If you are using any other thermostable DNA polymerase, use the extension temperature as recommended by the manufacturer. The optimal annealing temperature will depend on the Tm of your GSP. If the Tm of your GSP is > 72°C, use 65-68°C as the annealing temperature and if the Tm is < 72°C, use 60-65°C.

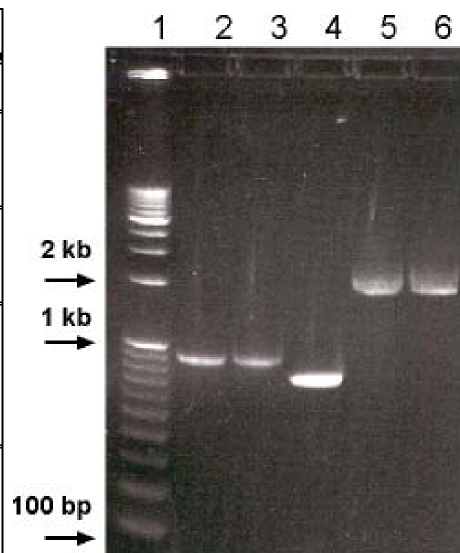
In some cases, using lower annealing temperatures (<65°C) may increase the chances of obtaining your RACE product. However, the background amplification also increases due to non-specific primer binding.

Continued on next page

Cycling Parameters for Positive Control

The cycling parameters used for the positive control reactions using Platinum® *Taq* DNA Polymerase High Fidelity or Platinum® *Pfx* DNA Polymerase are provided below. After cycling, hold the reactions at +4°C or place on ice. The gel shows the results using the control HeLa RACE-ready cDNA with appropriate primers (see legend below for more details).

Temperature	Time	Cycles
94°C	2 minutes	1
94°C	30 seconds	5
72°C	2 minutes	
94°C	30 seconds	5
70°C	2 minutes	
94°C	30 seconds	20
65°C	30 seconds	
68°C	2 minutes	
68°C	10 minutes	1



Legend for gel:

Lane 1: Mixed DNA Ladder (1 kb + 100 bp)

Lane 2: GeneRacer™ 5′ Primer + Control Primer B.1 + template (872 bp, 828 bp of β-actin gene + 44 bp of the GeneRacer™ RNA Oligo)

Lane 3: GeneRacer™ 5′ Nested Primer + Control Primer B.1 + template (858 bp, 828 bp of β-actin gene + 30 bp of the GeneRacer™ RNA Oligo)

Lane 4: Control Primer A + Control Primer B.1 + template (747 bp)

Lane 5: GeneRacer™ 3′ Primer + Control Primer A + template (~1800 bp, 1715 bp human β-actin + 60 bp of GeneRacer™ Oligo dT Primer + polyA tail)

Lane 6: GeneRacer™ 3′ Nested Primer + Control Primer A + template (~1800 bp, 1715 bp of β-actin + 41 bp of GeneRacer™ Oligo dT Primer + polyA tail)

Continued on next page

Analyzing RACE Products

After PCR, analyze 10-20 µl of the amplification reaction on a 1% agarose gel. We recommend that you verify the presence and identify your specific RACE product before you proceed with gel purification and sequencing of PCR products. Use the guidelines below to evaluate your PCR results.

- If you obtain a single, discrete band, proceed to **Gel-Purifying PCR Products** page 24.
- If you observe multiple bands or a smear and the negative controls are truly negative, proceed to **Optimizing PCR** (see page 29) or **Performing Nested PCR** next page.
- If you observe nonspecific products or a smear in any of the negative control reactions, then consider the options listed below.
  - If you have discrete bands in the negative control, refer to the **Troubleshooting** section on pages 26-29.
  - If you observe a smear, we recommend that you optimize your PCR (page 29).
- If you do not obtain a PCR product, please refer to the **Troubleshooting** section on pages 26-29.

Southern Blotting

Some researchers prefer to check their PCR products by Southern blotting (Ausubel *et al.*, 1994) to confirm that these products are real RACE products. Prepare an additional GSP and end-label it to probe your Southern blot. Compare your blot with the original gel to identify real RACE products.

Performing Nested PCR

Multiple RACE PCR Products

Multiple bands in the 5′ or 3′ RACE PCR may be real products or artifacts. We recommend that you take steps to eliminate the possibility of artifacts (see page 28) and then perform nested PCR to identify real products.

Authentic RACE PCR Products

Multiple RACE PCR products can result from the presence of real transcripts of various sizes. Multiple transcripts can result from the following:

- Multiple transcription initiation sites resulting in multiple 5′ PCR products
- Alternative mRNA splicing resulting in multiple 5′ or 3′ PCR products
- Different polyadenylation sites resulting in multiple 3′ PCR products
- Amplification of a multigene family resulting in multiple 5′ or 3′ PCR products

Nested PCR

Nested PCR is used to increase the specificity and sensitivity of RACE products for the 5′ or 3′ ends of your gene.

1. Use 1 µl of the original amplification reaction (page 16) as a template for nested PCR. Set up reactions as described below. You can use the GeneRacer™ Nested primers with the Control Primers A and B.1 to amplify a nested RACE PCR product from the β-actin gene as a positive control, if desired. After setting up your reactions, proceed to the next page.

Reagent	5′ RACE PCR	5′ Control PCR (Optional)	3′ RACE PCR	3′ Control PCR (Optional)
GeneRacer™ 5′ Nested, 10 µM	1 µl	1 µl	--	--
Reverse Nested GSP, 10 µM	1 µl	--	--	--
Control Primer B.1, 10 µM		1 µl		
GeneRacer™ 3′ Nested, 10 µM	--	--	1 µl	1 µl
Forward Nested GSP, 10 µM	--	--	1 µl	--
Control Primer A, 10 µM	--	--	--	1 µl
Initial PCR	1 µl		1 µl	
Control Initial PCR		1 µl		1 µl
10X PCR Buffer	5 µl	5 µl	5 µl	5 µl
dNTP Solution (10 mM each)	1 µl	1 µl	1 µl	1 µl
PCR Enzyme	x µl	x µl	x µl	x µl
$MgCl_2$ or $MgSO_4$	As required for enzyme	As required for enzyme	As required for enzyme	As required for enzyme
Sterile Water	y µl	y µl	y µl	y µl
Total Volume	**50 µl**	**50 µl**	**50 µl**	**50 µl**

Continued on next page

Nested PCR, continued

2. Use the following program for the control nested PCR reactions. Please see page 20 for a picture and sizes of the control nested PCR products. Optimize PCR parameters for your primer/template combination. Use 1 minute extension for each 1 kb of your DNA. Please refer to the guidelines on page 5 for annealing temperature.

   The cycling parameters provided below in the table are for the control nested PCR reaction using Platinum® Taq DNA Polymerase High Fidelity.

Temperature	Time	Cycles
94°C	2 minutes	1
94°C	30 seconds	15-25
65°C	30 seconds	
68°C	2 minute	
68°C	10 minutes	1

3. Analyze 10-20 µl on a 1% agarose/ethidium bromide gel. Nested PCR products will be shorter by the number of bases between the original primers and the nested primers. You may see one band or multiple bands. If you see multiple bands, your gene of interest may have transcripts of various lengths or be a member of a multigene family. Usually the largest product represents the most full-length message. Excise the band(s) as described on the next page.

Southern Blotting

Some researchers prefer to check their PCR products by Southern blotting to confirm that these products are real RACE products. Prepare an additional GSP and end-label it to probe your Southern blot. Compare your blot with the original gel to identify real RACE products.

Gel-Purifying PCR Products

Introduction

Reagents are included in the kit to help you purify your PCR product prior to cloning and sequencing.

Materials Supplied by the User

You will need the following reagents and equipment for gel purification.

- Autoclaved water or TE buffer
- New razor blade
- Microcentrifuge
- 1.5 ml sterile microcentrifuge tube

Nuclease Control

It is very important to minimize the presence of nucleases to ensure accurate sequencing. Please follow the guidelines listed below. While some guidelines may not appear as rigorous as others, they are sufficient for purifying PCR products.

- Wear gloves at all times
- Use clean plasticware and glassware
- Use a new razor to excise gel slice*
- Use new plastic wrap (e.g., Saran® Wrap) if needed

*The same razor may be used to excise different bands in the same gel if you are careful not to bring over pieces from an earlier excision.

PCR Product Purification

1. After electrophoresis, transfer the agarose gel to new Saran® Wrap. Visualize gel under UV light.
2. Using a new razor blade, carefully excise the PCR product from the gel. Be sure to make the gel slice as small as possible.

   Note: Razor blade may be rinsed with autoclaved water or TE prior to cutting the next band.
3. Transfer the gel slice to the S.N.A.P.™ column and place the column in a sterile microcentrifuge tube.
4. Centrifuge at maximum speed in a microcentrifuge for 1 minute at room temperature.
5. You should obtain between 15 and 60 µl, depending on the size of the gel slice. Proceed to **Cloning and Sequencing Your PCR Product**, next page or directly sequence your PCR product using the appropriate GSP or GeneRacer™ primer. PCR products can be stored at -20°C overnight, but after that the 3′ A-overhangs may start to degrade.

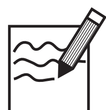
Cloning and Sequencing Your PCR Product

Cloning Your PCR Product

To clone and sequence your PCR products using either pCR®4Blunt-TOPO® or pCR®4-TOPO® vector, please refer to the appropriate manual included with this product. **Start with the TOPO® Cloning and Transformation section and use 4 µl of your purified PCR product in the TOPO® Cloning reaction.**

Once you have cloned your PCR products and selected transformants, you will isolate plasmid DNA using your method of choice. We recommend that you select 10-12 clones for sequencing to ensure full coverage of the 5′ end. Many genes have alternative start sites for transcription and splice variants.

When analyzing the 5′ RACE product, be sure the complete sequence of the GeneRacer™ RNA Oligo is present. This would indicate that the full-length message was ligated to the GeneRacer™ RNA Oligo. If you only have sequence through the GeneRacer™ 5′ Primer, then the PCR product is non-specific.



Note

Remember that PCR products clone bidirectionally, so be sure to sequence from both directions. M13 Forward (-20), M13 Reverse, T3 and T7 sequencing primers are provided in the kit.

Full-Length cDNA

You can use the sequence information obtained from 5′ and 3′ RACE to design primers for end-to-end amplification of the complete gene from your RACE-ready cDNA template.

Expression Studies

Once you obtain the complete sequence of your gene of interest, you are ready to perform expression studies. You can clone your gene of interest into a wide variety of vectors for expressing your gene of interest in bacterial, yeast, insect or mammalian systems. For more information on the different expression systems available from Invitrogen, please visit our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Troubleshooting

Introduction

mRNA quality and PCR conditions are the two primary factors that affect the outcome of your GeneRacer™ experiment. CIP, TAP, ligation, and RT reaction conditions are optimized to ensure the best results. To obtain a RACE product of your gene, you may need to optimize the RACE PCR conditions. Please review the information below to troubleshoot your experiments.

Positive Control Experiment

We recommend performing a positive control experiment using the HeLa total RNA provided in the kit. Obtaining RACE products using the control RNA demonstrates that the reagents are working properly.

Control RACE Reaction Failed

RNA degradation or failure of either the PCR or RT reaction may produce no 5′ or 3′ RACE PCR products from the control HeLa RNA. Please see the information below to troubleshoot the RNA stability, RT, and PCR reactions.

No RACE PCR Products

If you successfully amplified control β-actin RACE products from control HeLa RNA, but did not obtain any RACE PCR products from your gene of interest, use the table below to troubleshoot.

Cause	Solution
Your gene is in low abundance	Increase the number of PCR cycles. Perform nested PCR (see page 22).
Your gene is not expressed in this tissue	Amplify with two GSPs to assay for the presence of your gene's cDNA.
Your gene is too long for RT and the GeneRacer™ Oligo dT Primer to generate full-length cDNA	Perform RT with Random Primers or a GSP that hybridizes as close as possible to the 5′ end. You can also combine the Random Primers with the GeneRacer™ Oligo dT Primer to increase the chances of obtaining full-length cDNA.
The cDNA template is a difficult template for PCR	Optimize PCR parameters or reaction buffer. Lower the annealing temperature. Use 5-10% DMSO in the PCR to help read through GC-rich regions. Use a high-processivity, high-fidelity PCR enzyme (see page 14).
RT reaction failed	Cloned AMV RT and SuperScript™ III RT are capable of generating cDNA from rare and long templates. If you were unable to get any cDNA using either RT, you may use any other reverse transcriptase. For rare and GC-rich templates we recommend using Thermoscript™ RT (Catalog no. 12236-014).

Continued on next page

Low or No 3′ RACE PCR Product

If the amplification of 5′ ends is satisfactory, but you have difficulty obtaining 3′ RACE product, you can start with a new sample of mRNA or total RNA and proceed directly to reverse transcription with the GeneRacer™ Oligo dT Primer. See **Reverse Transcribing mRNA** on page 12.

mRNA Quality and Stability

The key factor for success is quality of the RNA. RNA degradation is the most likely reason for failure to obtain a correct RACE product. **We strongly recommend that you analyze a sample of your RNA on an agarose gel before starting to confirm RNA integrity (see page 4).**

The presence of RNaseOut™ RNase inhibitor ensures RNA stability during various enzymatic reactions. If you are concerned about RNA stability, you may check the stability of the RNA after each enzymatic reaction (CIP, TAP and ligation reaction) using agarose gel electrophoresis. Resuspend the RNA in DEPC water after enzymatic treatment in an appropriate volume (see pages 7, 9, and 11) and check 1 µl on an agarose gel. Compare with the same amount of untreated RNA to check for degradation.

RT Reaction

Review the following guidelines to optimize your RT reaction.

- Use half of the ligation reaction in the RT reaction in case you have to repeat the RT reaction
- You can use the β-actin control primers A and B.1 to confirm the presence of cDNA in your sample. Use the following cycling conditions if you are using Platinum® *Taq* DNA Polymerase High Fidelity:

Temperature	Time	Cycles
94°C	2 minutes	1
94°C	30 seconds	25
55°C	30 seconds	
68°C	2 minutes	
68°C	10 minutes	1

- If the primers successfully amplify the 750 bp β-actin gene, then the RT reaction worked and you need to examine the CIP, TAP, and ligation procedures to ensure that you performed each step correctly. Repeat the reactions with fresh RNA and check for RNA stability after each enzymatic step as described above in **mRNA Quality and Stability**.
- If the primers do not amplify the β-actin gene, then either the RNA was degraded or the RT reaction failed. You need to start over with fresh RNA. Perform a control RT reaction using the control HeLa total RNA to ensure that your RT reaction is working.
- Make sure that you inactivate RT prior to PCR by heating the first strand cDNA as described on pages 12 and 13.

Continued on next page

RACE PCR Artifacts

RACE PCR artifacts or non-specific PCR bands can result from one or more of the following.

- Non-specific binding of GSPs to other cDNAs resulting in the amplification of unrelated products as well as desired products.
- Non-specific binding of GeneRacer™ primers to cDNA resulting in PCR products with GeneRacer™ primer sequence on one end.
- RNA degradation
- Contamination of PCR tubes or reagents

Artifacts usually result from less than optimal PCR conditions and can be identified in negative control PCR. To perform **Negative PCR Controls** and **Optimize your PCR**, see below.

Negative PCR Controls

Perform the following negative PCR controls to eliminate non-specific amplification.

Negative Control	Result
Primers only, no template	The presence of a smear or bands indicates that the PCR cocktail or primers are contaminated with DNA. Clean your pipets and practice careful pipetting techniques.
One GeneRacer™ primer, plus template One GSP, plus template	The presence of a smear and/or distinct bands indicates non-specific binding of the primer on the opposite strand of cDNA. Compare results with those obtained from reactions containing both the GeneRacer™ primer and the GSP. Those bands common to both reactions are probably non-specific bands and should not be excised. To reduce or eliminate non-specific bands, reduce the amount of template or the number of amplification cycles. In addition, try to optimize your PCR using the recommendations on the next page.

Continued on next page

Optimizing PCR

To increase sensitivity and specificity:

- Perform nested PCR as described on page 22
- If you observe multiple RACE PCR products, try to eliminate non-specific amplification as suggested below. Perform nested PCR to identify real RACE products (see page 22)

To reduce or eliminate non-specific amplification and smearing:

- Reduce the amount of template or use 2-4 fold diluted RT template (see page 16)
- Reduce the number of PCR cycles
- Increase the annealing temperature to eliminate non-specific primer binding
- Re-design your GSPs to have an annealing temperature >70°C
- Use touchdown PCR (see page 15 for an example)
- Use a hot start for PCR or pre-heat the thermocycler to 94°C prior to placing your tubes in the machine
- Be sure to use high-quality RNA and include RNaseOut™ in all reactions using RNA to prevent RNA degradation. Check the RNA before use to ensure you are using high-quality RNA (see page 4) and after each enzymatic step
- Perform negative controls to ensure that your PCR is not contaminated (see table on the previous page)
- Perform nested PCR as described on page 22

Non-Full-Length 5′ RACE PCR Products

The GeneRacer™ method is designed to ensure that only full-length capped messages are ligated to the GeneRacer™ RNA Oligo and amplified. Be sure to sequence and analyze at least 10-12 clones to ensure that you isolate the longest message. It is possible that you might obtain PCR products that represent non-full-length message for your gene. PCR products that do not represent full-length message may be obtained because:

- RNA degradation after the CIP reaction creates new truncated substrates with a 5′ phosphate for ligation to the GeneRacer™ RNA Oligo. Be sure to take precautions to ensure that the RNA is not degraded.
- CIP dephosphorylation was incomplete. Increase the amount of CIP in the reaction or decrease the amount of RNA.
- PCR yielded a PCR artifact and not true ligation product. Optimize your PCR using the suggestions described above.

Appendix

Product Qualification

Introduction

Invitrogen qualifies the GeneRacer™ Kit as described below. To learn how we qualify the TOPO TA Cloning® Kit for Sequencing or the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing, please refer to the appropriate manual.

Functional QC

Invitrogen functionally qualifies the GeneRacer™ Kit using the control HeLa total RNA, the reagents in the kit, and the protocol described in this manual. The resulting RACE-ready cDNA is analyzed for the presence of 5′ and 3′ RACE PCR product of the β-actin gene using the GeneRacer™ primers and Control primers included in the kit.

No products are visible when the cDNA template is subjected to PCR with only one of the primers, or when both primers are included and the cDNA template omitted.

RNase Activity

Visualization of control RNA on a gel following CIP and TAP treatment must show no degradation when compared to unmodified RNA.

Accessory Products

Products

The table below lists some products that you may find helpful for use with the GeneRacer™ Kit.

Product	Amount	Catalog no.
GeneRacer™ 5′ Primer	10 µM, 60 µl	N150-50
GeneRacer™ 5′ Nested Primer	10 µM, 60 µl	N150-51
GeneRacer™ 3′ Primer	10 µM, 60 µl	N150-30
GeneRacer™ 3′ Nested Primer	10 µM, 60 µl	N150-31
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25
ThermalAce™ DNA Polymerase	200 units	E0200
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
Platinum® *Pfx* DNA polymerase	100 units	11708-013
Thermoscript™ RNase H^{-} Reverse Transcriptase	25 µl	12236-014
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions	C4040-50
	20 reactions	C4040-52
Zero Blunt® TOPO® PCR Cloning Kit for Sequencing	10 reactions	K2875-J10
	20 reactions	K2875-20
	40 reactions	K2875-40
TOPO TA Cloning® for Sequencing Kit	10 reaction	K4575-J10
	20 reactions	K4575-01
	40 reactions	K4575-40

E-Gels®

E-Gel® gels are self-contained, bufferless, pre-cast agarose gels that are designed to provide fast, convenient, and easy electrophoresis. Each E-Gel® gel contains agarose (0.8%, 1.2%, 2%, or 4%), electrodes, and ethidium bromide all packaged inside a dry, disposable, UV-transparent cassette. They run in a specially designed, inexpensive E-Gel® Base that connects directly to your power supply. They are perfect for quick analysis of RNA and cDNA. For more information, please contact Technical Service (page 32).

S.N.A.P.™ Gel Purification Kit

In addition to the S.N.A.P.™ columns, the S.N.A.P.™ Gel Purification Kit contains additional reagents to isolate pure DNA for other applications.

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