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FirstChoice™ RLM-RACE Kit

USER GUIDE

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For Research Use Only. Not for use in diagnostic procedures.

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Revision	Date	Description
A	9 June 2025	New baseline document for the FirstChoice™ RLM-RACE Kit.

The information in this guide is subject to change without notice.

Contents

1 Product information

Product description

The FirstChoice™ RLM-RACE Kit is designed to only amplify cDNA from full-length, capped mRNA through RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE). The FirstChoice™ RLM-RACE Kit contains reagents and enzymes to produce 6 RLM-RACE-ready cDNA preparations, primers and nested RACE adapter primers for 100 PCR reactions. Each kit also include control RNA and primers to test kit performance. The kit helps to ensure the amplification of only full-length transcripts by eliminating ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA during the amplification process. Each step of the FirstChoice™ RLM-RACE procedure is optimized so that enzymatic reactions can be completed, and PCR started in less than 5 hours.

How the FirstChoice™ RLM-RACE Kit works

RLM-RACE is an improvement to the standard RACE technique designed to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR.

In addition, RLM-RACE avoids the bias in amplification of less than full length products of first strand cDNA synthesis. First strand cDNA molecules that do not extend all the way to the 5' end of the adapter will not yield product during PCR because such targets lack adapter-specific primer binding sites.

The FirstChoice™ RLM-RACE Kit can be used to perform traditional 5'-RACE reactions, and 3'-RACE reactions.

About 5' RLM-RACE

Total or poly(A) selected RNA is treated with FastAP™ Thermosensitive Alkaline Phosphatase to remove free 5'-phosphates from ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA. Intact mRNAs are protected from dephosphorylation due to the presence of a 5' end cap structure.

The RNA is then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the 5' end cap structure from intact full-length mRNA, leaving a 5'-monophosphate.

A 45 base 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 full length, decapped mRNA acquire the adapter sequence as its 5' end.

A random-primed reverse transcription reaction and nested PCR then amplifies the 5' end of a specific transcript. This kit includes two nested primers corresponding to the 5' RACE adapter sequence, while the user supplies two nested antisense primers specific to their target gene. RNA and PCR primers for mouse α-2-macroglobulin are included for use in control reactions.

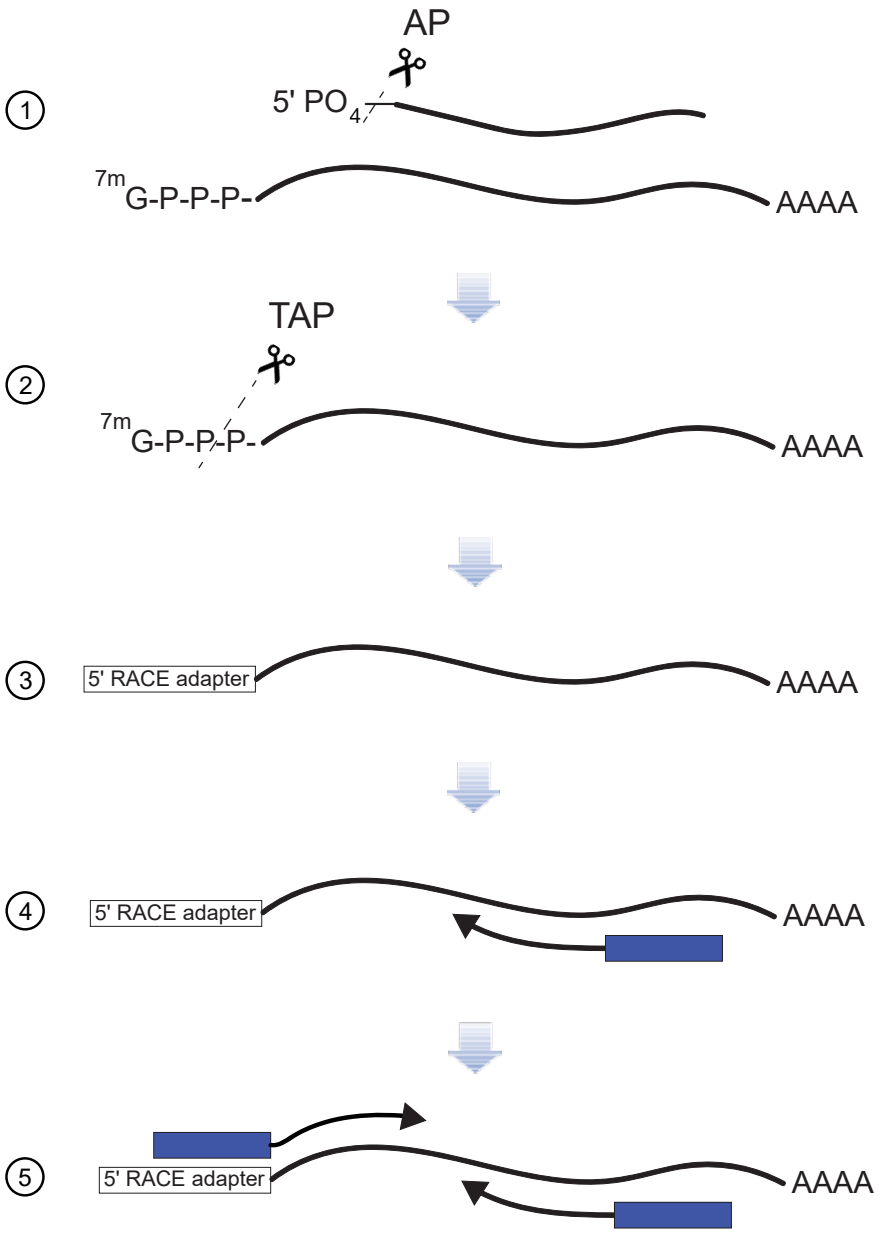


Figure 1 Overview of 5' RLM-RACE procedure

① FastAP™ Thermosensitive Alkaline Phosphatase (AP) treatment to remove free 5'-phosphates.
② Tobacco acid pyrophosphatase (TAP) treatment to remove 5' end cap structure.
③ Ligate 5' RACE adapter to decapped mRNA.
④ Reverse transcription of mRNA with 5' RACE adapter.
⑤ PCR amplification of transcript.

About 3' RLM-RACE

The FirstChoice™ RLM-RACE Kit can also be used to amplify and clone sequence at the 3' end of an mRNA using the 3' RACE technique. First strand cDNA is synthesized from either total RNA or poly(A)-selected RNA, using a supplied 3' RACE adapter.

The cDNA then undergoes PCR using a 3' RACE primer which is complimentary to the anchored adapter and a user-supplied primer for the gene of interest.

3' RACE may not require nested PCR to be performed, but a pair of nested primers for the adapter sequence are provided if nested PCR is determined to be necessary.

A 3' RACE control primers for mouse β-actin is included for use in control reactions.

Note: Using the 3' RACE adapter primer as the first strand primer for 5' RACE is not recommended because this would require the reverse transcriptase to transcribe through the entire mRNA to reach the 5' adapter sequence.

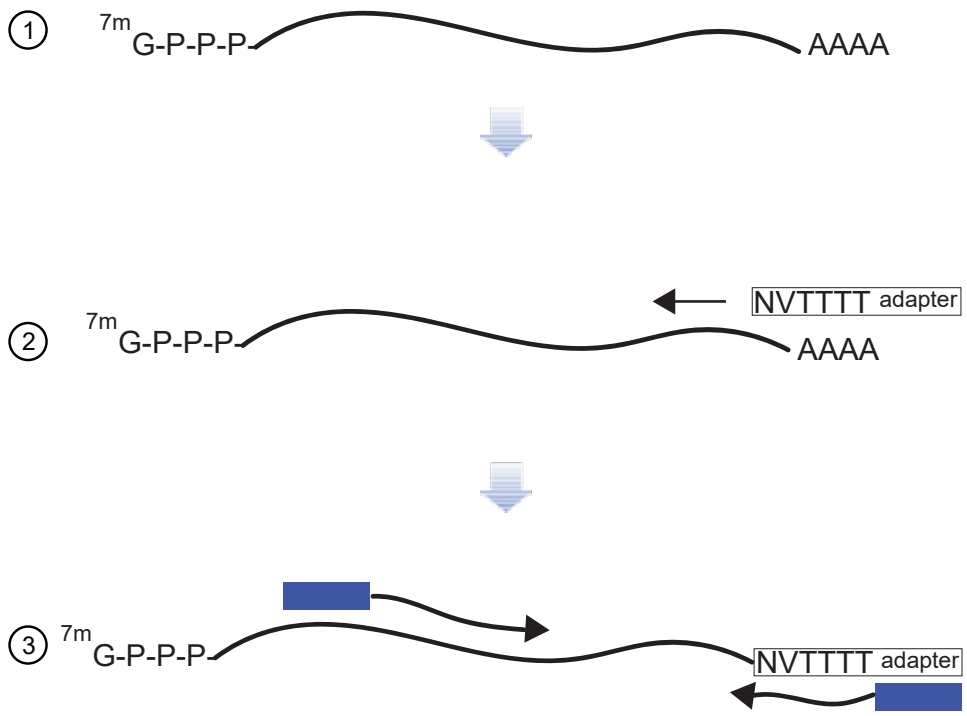


Figure 2 Overview of 3' RLM-RACE procedure

① Select for intact full length mRNA.
② Reverse transcription of mRNA with 3' RACE adapter.
③ PCR amplification of transcript.

Contents and storage

The FirstChoice™ RLM-RACE Kit contains reagents for 6 dephosphorylation, TAP, ligation, and reverse transcription reactions. Primers for 100 nested PCR reactions are included. Take measures to prevent contaminating reagents with nucleases.

Component	Amount	Storage
FastAP™ Thermosensitive Alkaline Phosphatase	12 µL	–20°C (non-frost-free freezer)
10X FastAP™ Buffer	50 µL	
Tobacco Acid Pyrophosphatase (TAP)	12 µL	
10X TAP Buffer	50 µL	
T4 RNA Ligase	12 µL	
10X T4 RNA Ligase Buffer	50 µL	
5' RACE Adapter (0.3 µg/µL) 5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′	10 µL	
3' RACE Adapter 5′-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3′	10 µL	
5' RACE Outer Primer (10 µM) 5′-GCTGATGGCGATGAATGAACACTG-3′	200 µL	
5' RACE Inner Primer (10 µM) 5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′	200 µL	
3' RACE Outer Primer (10 µM) 5′-GCGAGCACAGAATTAATACGACT-3′	200 µL	
3' RACE Inner Primer (10 µM) 5′-CGCGGATCCGAATTAATACGACTCACTATAGG-3′	200 µL	
Ammonium Acetate Stop Solution	1 mL	
M-MLV Reverse Transcriptase	10 µL	
dNTP Mix (2.5 mM each dNTP)	500 µL	
10X RT Buffer	50 µL	
Random Decamers (50 µM)	12 µL	
RNase Inhibitor (10 U/µL)	10 µL	
Mouse Thymus RNA (1 mg/mL)	10 µL	

(continued)

Component	Amount	Storage
5' RACE Outer Control Primer (10 µM) 5′-GATCACCAATCCATTGCCGACTAT-3′	25 µL	–20°C (non-frost-free freezer)
5' RACE Inner Control Primer (10 µM) 5′-GAAGTAGATGGTGGGCAGGAAGAT-3′	25 µL	
5' PCR Control Primer (10 µM) 5′-GCAGCAGGTAGCAGTGAC-3′	25 µL	
3' RACE Control Primer 5′-AGCAGTTGGTTGGAGCAAACATC-3′	25 µL	
Nuclease-free Water	1.75 mL	–20°C, 4°C, or room temperature

Required materials not supplied

- Gene-specific PCR primer(s).
- Thermostable DNA polymerase (e.g., Platinum™ II Hot-Start PCR Master Mix, Cat. Nos. 14001012).
- Thermocycler (e.g., Applied Biosystems™ GeneAmp™ PCR System 9700, Veriti™ Thermal Cycler, ProFlex™ PCR System).
- Materials for cloning PCR products (e.g., a linearized cloning vector or a cloning kit (see “Guidelines for cloning RACE products” on page 12).
- Disposable, RNase-free, pipette tips, polypropylene 1.5-mL microcentrifuge tubes, and thin wall microcentrifuge tubes for PCR.
- Materials and equipment for gel electrophoresis.
- (*Optional*) Isopropanol.
- (*Optional*) Cold 70% ethanol.

2 Methods

Guidelines for RNA preparation

- Ensure that the mRNA target is expressed in the RNA that is being considered for use in RLM-RACE. If there is any doubt, test RNA samples from several tissue sources by Northern hybridization, ribonuclease protection assay, or RT-PCR to identify an RNA source that contains the highest proportion of the target RNA.
- Use high quality total or poly(A) selected RNA for RLM-RACE. It is essential that the starting RNA be of the best possible quality. Only full-length, capped message will be amplified using the RLM-RACE procedure.
- It is not necessary to use poly(A) selected RNA as template in the RLM-RACE procedure. The 5' ends of rare targets have been successfully amplified starting with total cellular RNA using the FirstChoice™ RLM-RACE Kit. However, using poly(A) RNA in RLM-RACE, can increase the likelihood of success for amplification of rare or difficult to amplify targets.

  Regardless of the method used to purify the RNA, if there is any question about the cleanliness of the prep (e.g. low A_{260}/A_{280} ratio), the RNA should be further purified with an organic extraction and alcohol precipitation.
- Starting with poly(A) RNA can yield a 20–50 fold enrichment of the target. Purified poly(A) RNA suitable for RLM-RACE can be obtained using the RiboPure™ RNA Purification Kit (Cat. No. AM1924), the MagMAX™ Sequential DNA/RNA Kit (Cat. No. A65309), or the RNAqueous™ Total RNA Isolation Kit (Cat. No. AM1912).
- RNA prepared with single step RNA isolation procedures, e.g., TRI Reagent™ Solution (Cat. No. AM9738) can also be used.
- Evaluate RNA for integrity by running a sample on an Agilent™ 2100 Bioanalyzer™ Instrument or on a denaturing agarose gel. Features that indicate high quality RNA include having a 28S ribosomal RNA band that is twice the intensity of the 18S band, and having both bands resolve tightly, with no smearing.

Guidelines for RACE PCR primer design

- Access free primer design tools at **OligoPerfect Primer Designer**
- 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.

5' RLM-RACE primer design

The sequences of the 5′ RACE Outer and Inner Primers are shown in the list of materials provided with the kit (see “Contents and storage” on page 8).

- The inner primers include a BamHI site at the 5' end.
- 23–28 nucleotides in length to increase specificity of binding.
- 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 (**OligoPerfect Primer Designer**) 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 .

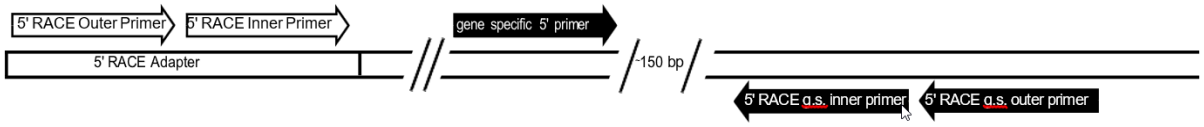


Figure 3 Primer positions for 5' RACE

Note: Primers represented with dark arrows are supplied by the user.

Gene specific 5' (upstream or sense) primer design

- To assist in the analysis and optimization of your reactions, synthesizing an upstream (sense-strand) gene specific primer positioned so that it produces a resolvable (≥150 bp) PCR product when used with the 5' RACE gene-specific outer primer is recommended.
- 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 an RNA preparation, and to evaluate RLM-RACE products.

3' RLM-RACE primer design

The sequences of the 3′ RACE Outer and Inner Primers are shown in the list of materials provided with the kit (see “Contents and storage” on page 8).

- Basic PCR primer design considerations as previously mentioned should be followed.
- The 3' RACE protocol describes nested PCR, however 3' RACE reactions can produce significant product after a single round of PCR.
- PCR with a gene specific primer and the 3' RACE Outer Primer can be tested. If enough product is amplified, the inner nested reaction can be omitted.

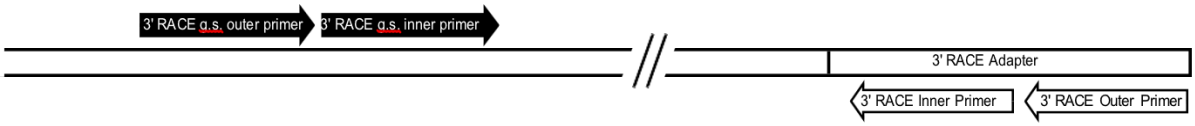


Figure 4 Primer positions for 3' RACE

Note: Primers represented with dark arrows are supplied by the user.

Guidelines for cloning RACE products

The 5' RACE Inner Primer and the 3' RACE Inner Primer have BamHI sites at their 5' end (CGC**G^GATCC**). 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 traditional cloning by restriction digestion and ligation of the fragment into desired vector.

Alternatively, depending on the DNA polymerase used, the Zero Blunt™ TOPO™ PCR Cloning Kit for Sequencing (Cat. No. 450159) can be used for fast blunt-end fragment cloning, or the TOPO™ TA Cloning™ Kit for Sequencing (Cat. No. 450030) can be used for PCR products with 3′ A-overhangs.

For directional cloning, use a restriction site other than BamHI on inner gene specific primers, so that fragments can be directionally cloned into a double-digested vector. Alternatively, seamless cloning techniques, such as the GeneArt™ Gibson Assembly® HiFi Master Mix (Cat. No. A46628) can be used to assemble two or more fragments simultaneously.

Perform 5' RLM-RACE

Process the RNA

The FastAP™ dephosphorylation, TAP, and ligase reactions are robust and typically do not require user optimization. Exceeding the recommended RNA concentration in the TAP or ligation reactions however, may compromise the reaction(s). In particular, if poly(A) RNA is used as template, limit the amount of RNA in the TAP reaction to 250 ng as specified in the protocol.

It is recommended that the minus-TAP control reaction be run, and that PCR annealing temperatures be optimized. The minus-TAP reaction can be used to assess whether the products produced by the procedure are true 5' RACE products. To successfully perform RACE reaction, some optimization of cycling parameters and PCR annealing temperature might be needed to generate discrete RACE products with minimal background. Alternatively, Platinum II PCR Master Mixes could be used with universal annealing temperature. The RLM-RACE kit contains primers for 100 PCRs; this is more than enough to optimize most PCR protocols.

This protocol is optimized for starting with 10 µg of total RNA, or 250 ng of poly(A)-selected RNA. Using these amounts of RNA will be referred to as the ***standard*** reaction. This provides extra material in case of partial sample loss or if a downstream reaction must be repeated.

If only an extremely limited amount of RNA is available, the reaction can be scaled down to start with 1 µg or less total RNA. Modifications to the protocol for the use of only 1 µg of total RNA as template are referred to as ***small*** reactions.

Dephosphorylate the RNA

1. Assemble the following reagents in an RNase-free microcentrifuge tube.

Reagent	Amount	
	Standard reaction	Small reaction
Total RNA	10 µg	1 µg
poly(A) RNA	250 µg	—
10X FastAP™ Buffer	2 µL	
FastAP™ Thermosensitive Alkaline Phosphatase	1–2 µL	
Nuclease-free Water	fill to 20 µL	

2. Mix gently, then centrifuge briefly.
3. Incubate at 37°C for 10 minutes.
4. Incubate at 75°C for 5 minutes to inactivate FastAP™ Thermosensitive Alkaline Phosphatase.
5. (*Optional*) Prepare a 1 µL aliquot of RNA at –20°C for a 'minus-TAP' control reaction.
6. Proceed to "Decap the RNA" on page 14, or "(Optional) Concentrate the dephosphorylated RNA" on page 14.

(*Optional*) Concentrate the dephosphorylated RNA

If the amount of starting material is low or dilute, perform concentration by precipitation. For small amounts of starting material, follow the protocol for small reaction volumes.

1. Add the following reagents to the tube containing the dephosphorylated RNA.

Reagent	Amount
Nuclease-free Water	115 µL
Ammonium Acetate Solution	15 µL
Isopropanol	150 µL

2. Vortex thoroughly, then chill on ice for 10 minutes.
3. Centrifuge at maximum speed for 20 minutes, then remove the solution carefully.
4. Rinse the pellet with 0.5 mL of cold 70% ethanol, then centrifuge for 5 minutes at maximum speed. Remove the ethanol carefully and discard it.
5. Allow pellet to air dry, then resuspend the RNA.

Standard reaction	Small reaction
1. Resuspend the RNA pellet in 11–20 µL Nuclease-free Water. 2. (*Optional*) Prepare a 1 µL aliquot of RNA at –20°C for a 'minus-TAP' control reaction.	1. Prepare 10 µL of 1X TAP Buffer. 2. Resuspend the RNA sample in 4 µL of 1X TAP Buffer.

Decap the RNA

1. Assemble the following reagents in an RNase-free microcentrifuge tube.

Reagent	Amount	
	Standard reaction	Small reaction
Dephosphorylated RNA	5 µL	4 µL
10X TAP Buffer	1 µL	—
Tobacco Acid Pyrophosphatase	2 µL	1 µL
Nuclease-free Water	2 µL	—

2. Mix gently, then centrifuge briefly.
3. Incubate at 37°C for 1 hour.
4. Store reaction at –20°C or proceed to ligation step (page 15).

Ligate 5' RACE Adapter

1. Assemble the following reagents in an RNase-free microcentrifuge tube.

Reagent	Amount	
	Standard reaction	Small reaction
Dephosphorylated, decapped RNA	2–6 µL	5 µL
5' RACE Adapter	1 µL	
10X RNA Ligase Buffer	1 µL	
T4 RNA Ligase (2.5 U/µL)	2 µL	
Nuclease-free Water	fill to 10 µL	1 µL

2. Mix gently, then centrifuge briefly.
3. Incubate at 37°C for 1 hour.
4. Store reaction at –20°C or proceed to reverse transcription (page 15).

Perform reverse transcription

1. Assemble the following reagents in an RNase-free microcentrifuge tube.

Reagent	Amount
Ligated RNA (or minus-TAP control)	2 µL
dNTP Mix	4 µL
Random Decamers	2 µL
10X RT Buffer	2 µL
RNase Inhibitor	1 µL
M-MLV Reverse Transcriptase	1 µL
Nuclease-free Water	fill to 20 µL

2. Mix gently, then centrifuge briefly.
3. Incubate at 42°C for 1 hour.
4. Store reaction at –20°C or proceed to nested PCR (page 16).

Perform nested PCR for 5' RLM-RACE

- Including a minus-template control is recommended for any PCR. The 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.
- Use a hot start polymerase for PCR.
- At a minimum, assemble reactions on ice, preheat the thermal cycler to 94°C, and then place the tubes in the thermal cycler.

Perform outer 5' RLM-RACE PCR

1. Add the following reagents in PCR tubes on ice.

Reagent	Amount
RT reaction	1 µL
2X Platinum™ II Hot-Start PCR Master Mix	25 µL
5' RACE gene-specific outer primer (10 µM)	2 µL
5' RACE Outer Primer (10 µM)	2 µL
Nuclease-free Water	fill to 50 µL

2. Mix gently by flicking the tube, then centrifuge briefly to bring the contents to the bottom of the tube.

3. Set the PCR amplification conditions according to the following table.

Step	Temperature	Time [1]	Cycles
Activation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	60°C [2]	30 seconds	
Extension	72°C	1 minute [3]	
Final extension	72°C	7 minutes	1
Final hold	4°C	Hold	1

[1] *Thermal cyclers with very short ramp times, may require slightly longer incubation times, e.g., 1 minute at each temperature in the cycle.*

[2] 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.

[3] For targets longer than 1 kb add 1 minute to extension time for each kilobase.

4. Store reaction at –20°C or proceed to PCR with inner primer (page 17).

Perform inner 5' RLM-RACE PCR

1. Add the following reagents in PCR tubes on ice.

Reagent	Amount
Outer PCR product	1–2 µL
2X Platinum™ II Hot-Start PCR Master Mix	25 µL
5' RACE gene-specific inner primer (10 µM)	2 µL
5' RACE Inner Primer (10 µM)	2 µL
Nuclease-free Water	fill to 50 µL

2. Mix gently by flicking the tube, then centrifuge briefly to bring the contents to the bottom of the tube.

3. Use the same PCR amplification profile as in “Perform outer 5' RLM-RACE PCR”.

Perform gel analysis of PCR products

- 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. Check the fragment size against a DNA length standard.
- A sample of the outer PCR can 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 may be necessary.

Expected result

- Mouse liver RNA and *Xenopus* stage 41 embryo RNA were used in the RLM-RACE kit.
- CXCR-4 is a moderately-expressed message, and the TGF-β related gene encodes a very rare message.

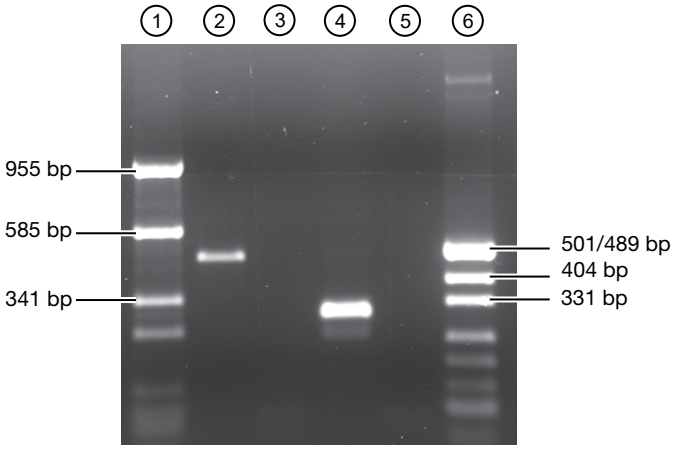


Figure 5 RLM-RACE for Mouse CXCR-4 Gene and *Xenopus* TGF-β Related Gene

① pUC19/Sau3A
② TGF-β related gene inner 5' RACE PCR
③ TGF-β related gene outer 5' RACE PCR
④ CXCR-4 inner 5' RACE PCR
⑤ CXCR-4 outer 5' RACE PCR
⑥ pUC19/HpaII

Perform 3' RLM-RACE

Perform reverse transcription

1. Assemble the following reagents in an RNase-free microcentrifuge tube.

Reagent	Amount
RNA (1 µg total RNA or 50 ng poly(A) RNA)	2 µL
dNTP Mix	4 µL
3' RACE Adapter	2 µL
10X RT Buffer	2 µL
RNase Inhibitor	1 µL
M-MLV Reverse Transcriptase	1 µL
Nuclease-free Water	fill to 20 µL

2. Mix gently, then centrifuge briefly.

3. Incubate at 42°C for 1 hour.

4. Store reaction at –20°C or proceed to PCR (page 19).

Perform PCR for 3' RLM-RACE

- A single PCR will typically amplify enough product from 3' RACE, but two nested primers for the 3' RACE Adapter sequence are provided with this kit to perform a second PCR if more material or greater specificity is required.

  Perform an outer PCR first, then if needed, perform the inner PCR.
- Including a minus-template control is recommended for any PCR. The 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.
- Use a hot start polymerase for PCR.
- At a minimum, assemble reactions on ice, preheat the thermal cycler to 94°C, and then place the tubes in the thermal cycler.

Perform outer 3' RLM-RACE PCR

1. Add the following reagents in PCR tubes on ice.

Reagent	Amount
RT reaction	1 µL
2X Platinum™ II Hot-Start PCR Master Mix	25 µL
3' RACE gene-specific outer primer (10 µM)	2 µL
3' RACE Outer Primer (10 µM)	2 µL
Nuclease-free Water	fill to 50 µL

2. Mix gently by flicking the tube, then centrifuge briefly to bring the contents to the bottom of the tube.

3. Set the PCR amplification conditions according to the following table.

Step	Temperature	Time [1]	Cycles
Activation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	60°C [2]	30 seconds	
Extension	72°C	30 seconds [3]	

(continued)

Step	Temperature	Time [1]	Cycles
Final extension	72°C	7 minutes	1
Final hold	4°C	Hold	1

[1] *Thermal cyclers with very short ramp times, may require slightly longer incubation times, e.g., 1 minute at each temperature in the cycle.*

[2] 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.

[3] For targets longer than 1 kb add 1 minute to extension time for each kilobase.

4. Store reaction at –20°C or proceed to PCR with inner primer (page 20).

(*Optional*) Perform inner 3' RLM-RACE PCR

1. Add the following reagents in PCR tubes on ice.

Reagent	Amount
Outer PCR product	1 µL
2X Platinum™ II Hot-Start PCR Master Mix	25 µL
3' RACE gene-specific inner primer (10 µM)	2 µL
3' RACE Inner Primer (10 µM)	2 µL
Nuclease-free Water	fill to 50 µL

2. Mix gently by flicking the tube, then centrifuge briefly to bring the contents to the bottom of the tube.

3. Use the same PCR amplification profile as in “Perform outer 3' RLM-RACE PCR”.

Perform gel analysis of PCR products

- 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. Check the fragment size against a DNA length standard.
- 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 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 may be necessary.

Cloning and sequencing your PCR product

Perform cloning of PCR products

RACE products can be cloned into plasmid vectors using standard techniques. The 5' and 3' RACE Inner Primers have BamHI sites at their 5' ends, allowiing RACE products amplified with a gene-specific primer containing a restriction site to be cloned into an appropriately cut plasmid vector using the standard restriction digestion and ligation cloning technique. Alternatively, TOPO cloning kits can be used for fast blunt-end fragment cloning (Cat. No. 450159) or TA cloning (Cat. No. 450030) depending on DNA polymerase used. For directional cloning, we recommend using a restriction site other than BamHI on inner gene specific primers, so that fragments can be directionally cloned into a double-digested vector. Alternatively, seamless cloning techniques with the GeneArt™ Gibson Assembly® HiFi Master Mix (Cat. No. A46628) can be used to assemble two or more fragments simultaneously.

Multiple bands from nested PCR may indicate alternative transcriptional start sites, polyadenylation sites, or splicing products. It can also indicate that the stringency of PCR thermal cycling conditions should be increased. Raising the annealing temperature to 60°C or higher can simplify the band pattern. To analyze all the PCR products, cloned them en masse and sort by restriction digestion of individual bacterial colonies, or gel-purify and clone each band individually.

Perform sequence analysis of 5' RLM-RACE products

- 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 5' RACE Adapter 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).

  CGC**GGATCC**GAACACTGCGTTTGCTGGCTTTGATGAAA-[your seque
  nce]

  (BamH1 site in bold)

Perform sequence analysis of 3' RLM-RACE products

- 3' RACE products should contain either the 3' RACE Outer Primer or the 3' RACE Inner Primer sequence at the junction of the 3' RACE Adapter 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 seq
  uence]-3'

  (3' RACE Outer Primer sequence in bold)
- 3' RACE Inner Primer used in final PCR.

  5'-CGC**GGATCC**GAATTAATACGACTCACTATAGGT$_{12}$VN-[your seq
  uence]-3'

  (BamH1 site in bold)
- When analyzing the sequence of RACE products, it is advisable to sequence more than one clone.

A Troubleshooting

Observation	Possible cause	Recommended action
PCR does not produce a band, or product appears as multiple or smeared bands	Poor quality RNA used for RLM-RACE.	Use only very pure, high quality RNA as starting material.
	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.	Ensure primers are designed to hybridize only with specific targets.
	Suboptimal annealing temperature.	Optimization of the PCR annealing temperature can provide the greatest improvement to the outcome of the RLM-RACE protocol. Increase stringency by testing different annealing temperatures to see which conditions produce a single band. Each reverse transcription reaction can provide template for 20 PCRs, and 5' RACE Inner and Outer Primers are sufficient for 100 PCRs. If additional primers are needed, their sequences are provided in section 4. In general, the annealing temperature for the outer PCR is less critical, and should be 55–65°C. The annealing temperature for 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 annealing temperature (change conditions by 2°C increments).
	Primer concentration is too high.	Reduce concentration of primers used in PCR by 50%.
	Larger RACE products are more difficult to amplify in general due to the distance between the nested primers and the 5' end of the target.	Increase the 72°C extension step by 1 minute for each kilobase of the target over 1 kb.

Observation	Possible cause	Recommended action
PCR does not produce a band, or product appears as multiple or smeared bands *(continued)*	Insufficient RNA denaturation during reverse transcription step.	GC-rich regions or other regions of stable secondary structure in RNA transcripts can present a problem for M-MLV Reverse Transcriptase at 42°C. By increasing the temperature of the reverse transcriptase reaction, secondary structure effects can be minimized. The M-MLV Reverse Transcriptase included in the RLM-RACE kit can be used at up to 50°C. Increasing the temperature of the synthesis reaction can facilitate read-through by the RT enzyme. In extreme cases, a thermostable reverse transcriptase can be used for first strand synthesis from very GC-rich sequences. Such enzymes can be purchased from several commercial sources. If such a thermostable reverse transcriptase is used, confirm that the manufacturer certifies that the enzyme is nuclease-free. Follow the recommended reaction conditions for the enzyme (e.g., use the specific reagents from the enzyme supplier). Substitution of a thermostable enzyme should be considered only if optimization using the supplied kit components fails to yield the desired products.
No or low yield of PCR product	Poor quality RNA used for RLM-RACE.	Use only very pure, high quality RNA as starting material.
	Absence or low abundance.of the target in the RNA used for RLM-RACE.	Use RNA from starting material known to contain the highest amount of target gene.
	Trying to amplify a long RACE products.	Use an extended range polymerase, especially when the target amplicon is larger than 1 kb.
	Insufficient RNA denaturation during reverse transcription step.	GC-rich regions or other regions of stable secondary structure in RNA transcripts can present a problem for M-MLV Reverse Transcriptase at 42°C. By increasing the temperature of the reverse transcriptase reaction, secondary structure effects can be minimized. The M-MLV Reverse Transcriptase included in the RLM-RACE kit can be used at up to 50°C. Increasing the temperature of the synthesis reaction can facilitate read-through by the RT enzyme. In extreme cases, a thermostable reverse transcriptase can be used for first strand synthesis from very GC-rich sequences. Such enzymes can be purchased from several commercial sources. If such a thermostable reverse transcriptase is used, confirm that the manufacturer certifies that the enzyme is nuclease-free. Follow the recommended reaction conditions for the enzyme (e.g., use the specific reagents from the enzyme supplier). Substitution of a thermostable enzyme should be considered only if optimization using the supplied kit components fails to yield the desired products.

Control experiments

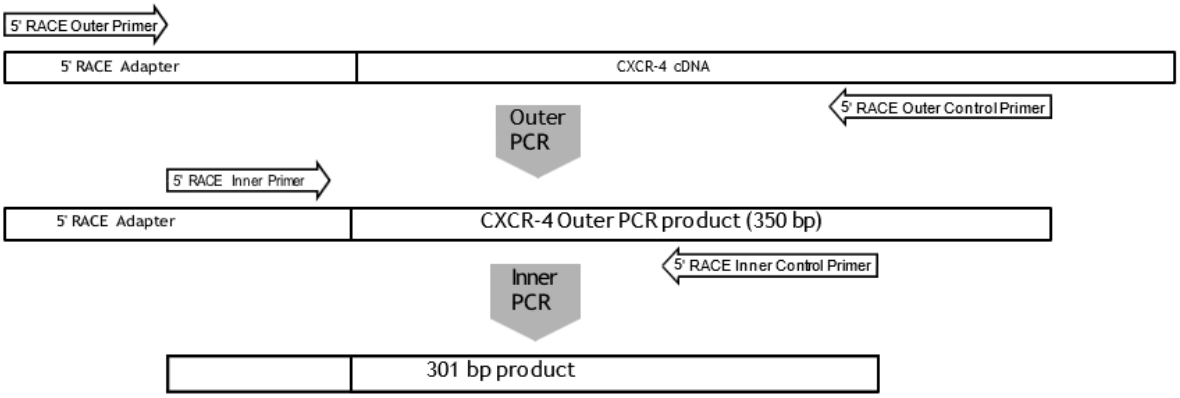
Positive control experiments

Mouse Thymus RNA and a set of control primers is included with the RLM-RACE kit to test both 5' and 3' RACE, and PCR.

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 an aliquot of the RLM-RACE reverse transcription reaction as template. 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 PCR amplification conditions are inappropriate, or that the gene specific primers should be redesigned.

5' RACE control

Nested primers for CXCR-4 are provided to verify that the RLM-RACE components are functioning properly. CXCR-4 is a g-protein–coupled chemokine receptor. It is over-expressed in glioblastoma and other brain tumors. CXCR-4 is also a co-receptor for T-tropic human immuno- deficiency virus type 1 (HIV-1).

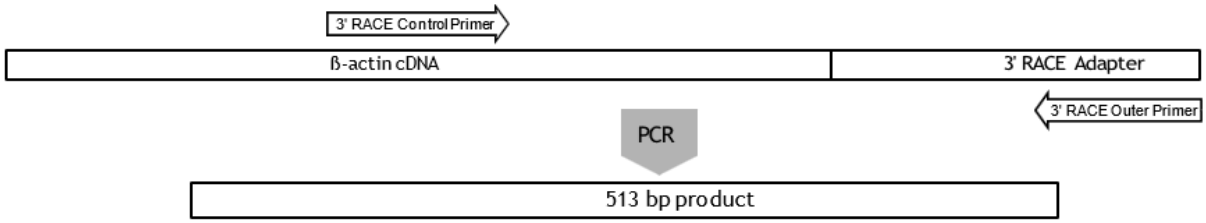


- Use 10 µg of the Mouse Thymus RNA in RLM-RACE following the instructions to test RNA processing and reverse transcription.
- Perform nested PCR for 5' RACE amplify 1 µL of the RT reaction from the previous step using the 5' RACE Outer Primer with the 5' RACE Outer Control Primer in PCR using an annealing temperature of 60°C. This produces a 350 bp product that is typically too faint to see when run on an agarose gel.

  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.
- 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. The inner 5' RACE control PCR should generate a 301 bp PCR product. The Control primers are positioned at position 305 (Outer Primer) and 263 (Inner Primer) in Genbank accession #**D87747** (CXCR-4) If the 301 bp product is not generated in this reaction, identify whether there is a problem with the RT-PCR or with the RNA processing steps by doing the RT-PCR control (see page 27). Note that a second band is sometimes seen if the PCR has a very high yield. The extra band can usually be eliminated by either reducing the PCR template amount to only 10–50% the amount used initially, or by increasing the annealing temperature in the PCR by 2–3°C.

3' RACE control

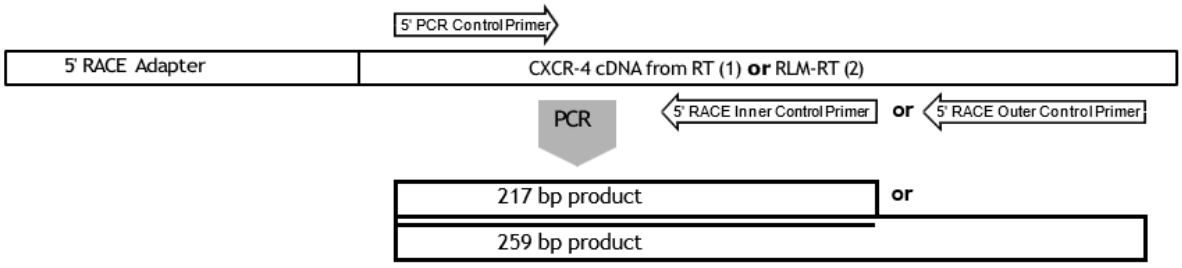
An upstream 3' RACE Control Primer for mouse ß-actin is included in with the RLM RACE kit to perform 3' RACE on the Mouse Thymus RNA (or other mouse RNA if desired) to confirm functioning of the kit. This primer will be used in conjunction with the 3' RACE Outer Primer to amplify the 3' end of the ß-actin gene. The 3' RACE Control Primer is at position 35165 (Genebank accesion AC139218.10).



- Use 1 µg of the Mouse Thymus RNA in RLM-RACE following the instructions to test RNA processing and reverse transcription.
- Perform 3' RACE PCR to amplify 1 µL of the RT fromthe previous step in PCR with the 3' RACE Control Primer and the 3' RACE Outer Primer. Follow the setup and cycling instructions in “Perform outer 3' RLM-RACE PCR” on page 19 with the annealing temperature at 60°C.
- 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 513 bp product from the PCR. 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.

RT-PCR control

An upstream ‘sense strand’ primer for CXCR-4 is provided so that the RT and PCR reactions can be evaluated independently of the dephosphorylation, TAP, and ligation reactions. (The tube label reads: 5' PCR Control Primer.) Do the control reactions described below if the 5' RACE control reaction gave unexpected results, or to check that the RT-PCR end of RLM-RACE is working properly.



- Set-up and cycling experiment should include two sets of RT-PCRs, with different input cDNA as described.
  - Template #1: An RT reaction should be performed on a 1 µg (2 µL) aliquot of the Mouse Thymus RNA provided in the kit. Follow the instructions in “Perform reverse transcription” on page 15. The resulting cDNA (1 µL) should be used as template in control PCRs.
  - Template #2: A 1 µL aliquot of the cDNA made from the 5' RACE control (see above) can be used directly in PCR.
- Do two PCRs on each PCR template, (for a total of 4 reactions). Use an annealing temperature of 60°C and the cycling conditions described in section
  - 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.
- 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 259 bp, from the inner PCR, it is 217 bp. 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).

  Each of the reactions using template #1 should produce the expected size fragment. 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. If you see specific products using template #1, but not with template #2, your RLM-RACE RNA may be degraded.

Minus-TAP control

An optional control consists of carrying a 'minus-TAP'–treated sample (optional 1 µL aliquot removed from "Dephosphorylate the RNA" on page 13 or "(Optional) Concentrate the dephosphorylated RNA" on page 14) through adapter ligation, reverse transcription and PCR. This will demonstrate that the products generated by RLM-RACE are specific to the 5' ends of decapped RNA .

A the end of the RLM-RACE procedure, the minus-TAP control RNA should **not** yield the same PCR products as the experimental RNA that underwent the entire RLM-RACE procedure. In theory, no bands should be produced since the RNA has either been dephosphorylated with FastAP™ Thermosensitive Alkaline Phosphatase, or it has an intact cap structure (because it was not treated with TAP) that cannot undergo ligation to the 5' RACE Adapter.

Note: Sometimes a smear of non-specific products is seen from the minus-TAP control RNA, this is not a concern.

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  cdc.gov/labs/bmbl
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  who.int/publications/i/item/9789240011311

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.