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Instruction Manual

3´ RACE System for Rapid Amplification of cDNA Ends

Catalog no. 18373-019

Version E
6 December 2004
10448

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1

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2 Overview

2.1 Principles of RACE

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3´ or the 5´ end of the mRNA (1). This methodology of amplification with single-sided specificity has been described as “one-sided” PCR (2) or “anchored” PCR (3). PCR requires two sequence-specific primers that flank the sequence to be amplified (4,5). However, to amplify and characterize regions of unknown sequences, this requirement imposes a limitation (3).

3´ RACE takes advantage of the natural poly(A) tail found in mRNA as a generic priming site for PCR. In this procedure, mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. Specific cDNA is then amplified by PCR using a gene-specific primer (GSP) that anneals to a region of known exon sequences and an adapter primer that targets the poly(A) tail region. This permits the capture of unknown 3´-mRNA sequences that lie between the exon and the poly(A) tail.

5´ RACE uses an antisense gene specific primer for the synthesis of specific cDNA by reverse transcriptase. Prior to PCR, a TdT-tailing step attatches an adapter sequence to the unknown 5´ sequences of the cDNA. Specific cDNA is then amplified by PCR using a GSP that anneals in a region of known exon sequences and an adapter primer that targets the 5´ terminus.

RACE has been used for amplification and cloning of rare mRNAs (6) and may be applied to existing cDNA libraries (7). Additionally, RACE products can be directly sequenced without any intermediate cloning steps (8,9), or the products may be used to prepare probes (10). Products generated by the 3´ and 5´ RACE procedures may be combined to generate full-length cDNAs (10,11). Lastly, the RACE procedures may be utilized in conjunction with exon-trapping methods (12) to enable amplification and subsequent characterization of unknown coding sequences.

2.2 Summary of the 3´ RACE System

The 3´ RACE procedure is summarized in figure 1. First strand cDNA synthesis is initiated at the poly(A) tail of mRNA using the adapter primer (AP). After first strand cDNA synthesis, the original mRNA template is destroyed with RNase H, which is specific for RNA:DNA heteroduplex molecules. Amplification is performed, without intermediate organic extractions or ethanol precipitations, using two primers: one is a user-designed GSP that anneals to a site located within the cDNA molecule; the other is a universal amplification primer that targets the mRNA of the cDNA complementary to the 3´ end of the mRNA. Two universal amplification primers are provided with the system. The universal amplification primer (UAP) is designed for the rapid and efficient cloning of RACE products using the uracil DNA glycosylase (UDG) cloning method (13–16). The abridged universal amplification primer (AUAP) is homologous to the adapter sequence used to prime first strand cDNA synthesis.

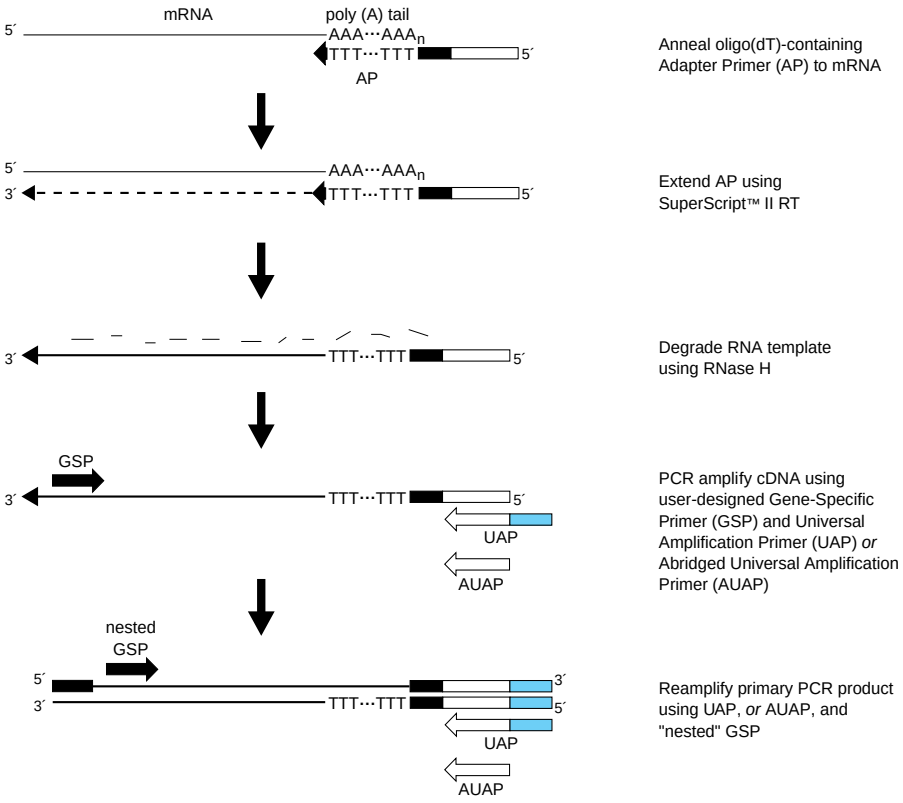

Figure 1. Summary of the 3´ RACE System procedure.

Since the 3´ RACE System utilizes the poly(A) tail region as an initial priming site, multiple amplification products may be synthesized, depending on the degree of specificity conferred by the GSP. To generate a specific amplification product, the user may find it advantageous to design a second "nested" GSP, as recommended by Frohman *et al.* (10) and reamplify the RACE products; this procedure is discussed in greater detail at the end of this chapter.

2.3 Isolation of Total RNA

One of the most important factors preceding the synthesis of substantially full-length cDNA is the isolation of intact RNA. The quality of the RNA dictates the maximum amount of sequence information that can be converted into cDNA. Thus, it is important to optimize the isolation of RNA from a given biological source and to prevent adventitious introduction of RNases (17) and inhibitors of reverse transcriptase such as guanidinium salts, SDS and EDTA (18). RNA can be isolated using a variety of methods. The recommended method for 3´ RACE is the guanidine isothiocyanate/acid-phenol method originally described by Chomzynski and Sacchi (19). The TRIzol® Reagent method is an improvement of the original single-step method of Chomczynski and Sacchi (20) and can be used for the preparation of RNA from as little as 10^3 cells or milligram quantities of tissue (21). Total RNA isolated with TRIzol® Reagent is undegraded and essentially free of protein and DNA contamination. The TRIzol® RNA isolation protocol is described in Section 6.2. For the isolation of RNA from small quantities of sample (<10^6 cells or <10 mg tissue) without using phenol, the GlassMAX RNA Microisolation Spin Cartridge System is recommended (22).

Total RNA isolated by these methods may contain small amounts of genomic DNA that may subsequently be amplified along with the target cDNA. The presence of this DNA is not likely to cause problems because it lacks the poly(A) region present in the mRNA analyte. As a precaution, however, we recommend performing a control experiment without reverse transcriptase to determine whether a given fragment is of genomic DNA or of cDNA origin. Products generated in the absence of RT are of genomic origin. If your application requires removal of all genomic DNA from your RNA preparation, refer Section 5.3, *DNase I Digestion of RNA Preparation.*

2.4 First Strand cDNA Synthesis from Total RNA

The first strand cDNA synthesis reaction is catalyzed by SuperScript™ II RT. This enzyme is a mutant of M-MLV RT that has been engineered to reduce RNase H activity, resulting in greater yields and more full-length synthesis (23,24,25). The enzyme exhibits increased thermal stability and may be used at temperatures up to 50°C. In addition, SuperScript™ II RT is not inhibited significantly by ribosomal and transfer RNA and may be used to synthesize first strand cDNA from a total RNA preparation. The RNA template is removed from the cDNA:RNA hybrid molecule by digestion with RNase H after cDNA synthesis to increase the sensitivity of PCR (26).

The AP (see figure 2), which primes first strand cDNA synthesis, has been engineered to contain three restriction endonuclease sites and a *Not* I half-site. Inclusion of these sequences in the primer may facilitate post-amplification cloning using either a restriction endonuclease-based (27) or a T4 DNA polymerase-based (28) method. Because the AP initiates cDNA synthesis at the poly(A) region of the mRNA, it effectively selects for polyadenylated mRNAs; thus, oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary although incorporating this step may facilitate the detection of rare mRNA transcripts.

2.5 Amplification of a Target cDNA

Amplification of a target cDNA requires priming with two oligonucleotides and *Taq* DNA polymerase. The sense amplification primer is the user-provided GSP,

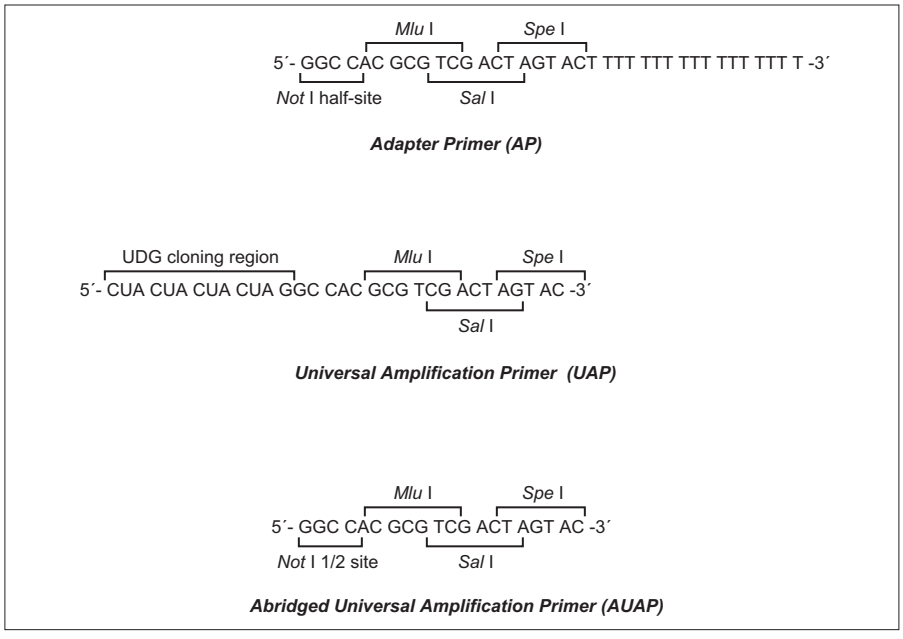

Figure 2. AP, UAP, and AUAP primer sequences.

which is specific for the particular gene or sequence of interest and may be designed to include sequence elements that facilitate subsequent cloning steps. The antisense amplification primer is one of the two universal amplification primers (see figure 2) provided with the system. The AUAP contains a restriction endonuclease site sequence (adapter region) homologous to the adapter region of the AP. The UAP is composed of the same adapter region plus a dUMP-containing sequence at the 5´ end of the primer required for UDG-mediated cloning. The UAP should not be used to prime DNA synthesis with any archaeobacterial polymerase (*e.g., Pyrococcus furiosus, Pyrococcus woesei,* etc.) or any long PCR enzyme mixture (*e.g.,* Elongase® Enzyme Mix) that contains one of these enzymes due to the inhibition of polymerase activity by dUMP-containing DNA. Both the AUAP and the UAP will function in PCR at annealing temperatures up to 68°C.

2.6 Design of the Gene-Specific Primer

Efficient and specific PCR amplification is highly dependent on primer design. This is especially true for RACE applications since the PCR is carried out with only a single GSP. In general, effective primers form stable duplexes with their target sequences, are highly specific for their target sequences, and are free of secondary structure such as hairpin loops and dimers (29–31). Additionally, the complementarity of primer 3´-termini must be minimized since primer-dimer artifacts may significantly reduce PCR efficiency. Therefore, dimer formation with the AUAP or UAP primer, as well as itself, should be reduced. Computer algorithms that have been developed (32–35) and are commercially available often facilitate this analysis. Discussion of primer design for RACE applications may be found in Frohman (11) and Loh (6). It should be noted that in cases where only limited peptide sequence information is available, a degenerate GSP may be prepared.

The AUAP and UAP included with the system have been engineered to function at PCR annealing temperatures up to 68°C and to facilitate the cloning step. The user-defined GSPs need to be compatible with the cloning method. Add the following to the 5' end of the GSP:

for UDG cloning:	5´–CAU CAU CAU CAU–3´	(use with UAP)
for T4 DNA polymerase cloning:	5´–CGA–3´	(use with AUAP)

2.7 Nested Amplification

The AP is designed to synthesize first strand cDNA from all polyadenylated mRNAs. The sequence specificity in the amplification reaction is therefore derived solely from the GSP. Often, a second "nested" GSP may be utilized in conjunction with the AUAP or UAP in a second amplification reaction to give the 3´ RACE procedure the specificity of a second primer (9). The nested GSP can anneal immediately adjacent to the first GSP or at sequences within the cDNA further downstream. The nested amplification reaction may be conveniently conducted using a plug of agarose from the gel analysis of the initial 3´ RACE reaction (see Section 5.5, *Nested Amplification from an Agarose Plug*). Ultimately, the 3´ RACE procedure should produce a single, prominent band on an agarose gel. When performing 3´ RACE with a nested primer, sequences specific for subsequent cloning manipulations (see Section 2.6, *Design of the Gene-Specific Primer*) must be designed into the nested GSP.

2.8 Cloning of Amplification Products

Conventional cloning methods that typically involve end-repair and blunt-end cloning can be problematic for amplified products (36–38). An alternative is a rapid and efficient method involving the use of UDG (13–16). This method requires that the user design a GSP containing containing a 5'-$(CAU)_4$ sequence. Incorporation of dUMP into the GSP may be accomplished on most automated synthesizers or with Invitrogen Custom Primers (see Section 2.6, *Design of the Gene-Specific Primer*). The product of the 3´ RACE reaction primed with the UAP and the dUMP-containing GSP is treated with UDG, which converts dUMP residues to abasic sites (39,40), to generate 3´ overhangs. The directional nature of the UDG cloning process can be exploited to lend an added level of specificity to the RACE procedure. Only amplification product that results from priming by both the UAP and the appropriately designed GSP are efficient substrates for UDG cloning.

Another alternative to conventional cloning methods uses the 3´ to 5´ exonuclease activity of T4 DNA polymerase as the basis for cloning as described by Stoker (28). In this procedure, the AUAP is used in the amplification reaction, and the 3´ RACE products are treated with T4 DNA polymerase to generate a *Not* I 5´ overhang. Similarly, the user may design a site into the GSP (see Section 2.6, *Design of the Gene-Specific Primer*).

Another approach to cloning is to digest the 3´ RACE product using one of the restriction endonuclease sites designed into the AUAP (figure 2; ref. 1). The user may also design unique restriction sites into the GSP, exploit a site present in the cDNA sequence or end-repair the 3´ RACE product prior to restriction endonuclease digestion (37).

Methods

3

3.1 Components

Components are provided in sufficient quantities to perform 20 separate reactions, each converting 1-5 µg of total RNA into first strand cDNA. A control RNA and amplification primers are included in the system to verify performance of the first strand cDNA synthesis reaction and subsequent amplification (see Chapter 5 for specific protocols). **Note:** The 3´ RACE System does not include *Taq* DNA polymerase or the reagents required for cloning. Store the 3´ RACE System at –20°C.

Component	Amount
10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	500 µl
25 mM $MgCl_2$	500 µl
10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)	100 µl
0.1 M DTT	100 µl
SuperScript™ II Reverse Transcriptase (RT, 200 units/µl)	20 µl
adapter primer (AP, 10 µM)	20 µl
universal amplification primer (UAP, 10 µM)	20 µl
abridged universal amplification primer (AUAP, 10 µM)	20 µl
E. coli RNase H (2 units/µl)	20 µl
DEPC-treated water	1.2 ml
control RNA (50 ng/µl)	10 µl
control gene-specific primer (GSP, 10 µM)	20 µl

3.2 Advance Preparations

Please review the protocols before using this system. You will need the following items *not included* in the system:

- sterilized 0.5-ml microcentrifuge tubes;
- automatic pipets capable of dispensing 1 to 20 µl and 20 to 200 µl;
- sterilized, RNase-free disposable tips for automatic pipets;
- disposable latex gloves;
- sterilized, distilled water;
- GSP (user-defined, appropriately engineered);
- microcentrifuge capable of generating a relative centrifugal force of 14,000 × *g;*
- 37°C, 42°C, and 70°C water baths or heat blocks;
- *Taq* DNA polymerase;
- chloroform;
- mineral oil; and
- thin-walled PCR tubes.

3.3 Protocol 1. First Strand cDNA Synthesis

This procedure converts 1 to 5 µg of total RNA into first strand cDNA. Poly(A)$^+$ RNA may be used in this protocol, but is typically not necessary. A control RNA is included in the 3´ RACE System as an aid in verifying that the system performs in your hands. If you decide to use the control RNA as a template for first strand synthesis, simply substitute 2 µl of control RNA (100 ng) in the first strand reaction for your total RNA. Please see Chapter 5 for additional information.

Note: For the control RNA, use 2 µl (100 ng). See chapter 4 for additional information.

Note: If you have >5 µg of total RNA, increase reaction volumes and amount of SuperScript™ II RT proportionately. If you have <1 µg of total RNA, no changes to the protocol are necessary. 50 to 500 ng of poly(A)$^+$ RNA may be substituted for total RNA in this protocol.

1. Mix and quickly centrifuge each component before use.
2. Combine 1-5 µg of total RNA or 50 ng of poly(A)$^+$ RNA and DEPC-treated water to a final volume of 11 µl in a 0.5-ml microcentrifuge tube.
3. Add 1 µl of the 10 µM AP solution, mix gently, and collect reaction by brief centrifugation.
4. Heat the mixture to 70°C for 10 min and chill on ice for at least 1 min. Collect the contents of the tube by brief centrifugation and add the following:

Component	Volume (µl)
10X PCR buffer	2
25 mM $MgCl_2$	2
10 mM dNTP mix	1
0.1 M DTT	2

Final composition of the reaction:

20 mM Tris-HCl (pH 8.4 at 22°C)
50 mM KCl
2.5 mM $MgCl_2$
10 mM DTT
500 nM AP
500 µM each dATP, dCTP, dGTP, dTTP
1-5 µg (≤50 ng/µl) of RNA

5. Mix gently and collect the reaction by brief centrifugation. Equilibrate the mixture to 42°C for 2 to 5 min.
6. Add 1 µl of SuperScript™ II RT. Incubate the tube in a 42°C water bath or heat block for 50 min.
7. Terminate the reaction by incubating at 70°C for 15 min.
8. Chill on ice. Collect the reaction by brief centrifugation. Add 1 µl of RNase H to the tube, mix, and incubate for 20 min at 37°C before proceeding to Protocol 2.

Note: You may stop at the end of step 9.

9. The reaction mixture may be stored at –20°C.

3.4 Protocol 2. Amplification of the Target cDNA

Optimal conditions for amplification are dependent on the nature of each particular primer and target sequence used. Alteration of the magnesium ion, dNTP, or primer concentration, as well as the thermocycling protocol, may be required. The optimal free magnesium concentration for efficient amplification is reported to be between 0.7 to 0.8 mM (29). Since magnesium binds deoxyribonucleoside triphosphates, this factor is affected by both primer and dNTP concentration. In general, lower concentrations of dNTP (50 to 200 µM), $MgCl_2$ (1 to 1.5 mM), and primer (0.1 to 0.2 µM) promote higher fidelity and specificity (41). Higher nucleotide concentration, however, can be used to improve product yield as well as to promote 3´-terminal T-mismatches (42). For a detailed discussion of parameters affecting PCR, please refer to Innis and Gelfand (43) or Saiki (29,30).

The addition of either *Taq* DNA polymerase, dNTPs, or $MgCl_2$ after reactions have been equilibrated at 75°C to 80°C has been reported to improve the specificity of the reaction (44,45). This "hot start" practice reduces nonspecific binding and the extension of primers during the initial denaturation process. A practical alternative to this classic "hot start" method is to set up reactions on ice then place complete PCR mixtures in a thermal cycler equilibrated to 80°C to 90°C.

1. To a fresh 0.5-ml microcentrifuge tube, add the following:

Component	Volume (µl)
10X PCR buffer	5
25 mM $MgCl_2$	3
autoclaved, distilled water	36.5
10 mM dNTP mix	1
GSP (prepared as 10 µM solution)	1
AUAP (10 µM) or UAP (10 µM)	1
Taq DNA polymerase (2 to 5 units/µl)	0.5

Note: Control cDNA should be diluted prior to amplification, see Section 4.2.2.

Final composition of the reaction:

20 mM Tris-HCl (pH 8.4)
50 mM KCl
1.5 mM $MgCl_2$
200 nM GSP
200 nM AUAP or UAP
200 µM each dATP, dCTP, dGTP, dTTP
0.04 to 0.1 unit/µl *Taq* DNA polymerase

Note: Mineral oil is necessary only when using thermal cyclers that require this barrier.

2. Add 2 µl from the cDNA synthesis reaction to the tube. Mix gently and layer 75 µl of mineral oil over the reaction. Collect the reaction briefly by centrifugation.
3. Incubate the reaction at 94°C for 3 min. (Extended preamplification denaturation times may impair the efficiency of long (>2 kb) target sequences (46).
4. Perform 20 to 35 cycles of PCR, using the protocol accompanying *Taq* DNA polymerase.

Note: Step 5 is unnecessary if no mineral oil was used in step 2.

5. Following amplification, extract the sample with 50 µl of chloroform. Transfer the aqueous layer to a fresh tube.

Note: If sequences are available for use as internal probes, it is strongly recommended that Southern blot analysis be used to confirm the identity of specific product bands. Specific product can also be identified using a diagnostic restriction endonuclease digestion if the amplified cDNA sequence contains a known restriction site.

6. Analyze 10 to 20 µl of the amplified sample, using agarose gel electrophoresis and ethidium bromide staining, and the appropriate molecular size standards. If the positive control RNA was used, a single 720-bp band will be visible (see Section 4.2, *Testing the 3´ RACE System*).

4 Troubleshooting Guide

4.1 General Suggestions

Problem	Possible Cause	Suggested Remedy
No bands after electrophoretic analysis of amplified products	Procedural error in first strand cDNA synthesis or PCR	Use the control RNA to verify the efficiency of the first strand reaction (see Section 4.2, *Testing the 3´ RACE System).*
	RNase contamination	Add the control RNA to the sample to determine if RNase is present in the first strand reaction.
		Maintain aseptic conditions to prevent RNase contamination (see Section 4.3, *Minimizing RNase Contamination*).
		Use RNase inhibitor during first strand cDNA synthesis.
	Inhibitors of RT present	Remove inhibitors by ethanol precipitation of the mRNA preparation before the first strand reaction. Include a 70% (v/v) ethanol wash of the mRNA pellet.
		Notes: Inhibitors of RT include sodium dodecyl sulfate (SDS), EDTA, guanidinium salts, glycerol (>35%), sodium pyrophosphate, and spermidine (18).
		Test for the presence of inhibitors by mixing 1 µg of sample RNA ± control RNA and compare yields of first strand cDNA or PCR product.
	Polysaccharide and small RNA (tRNA and 5SRNA) coprecipitation with mRNA	Ethanol-precipitate the RNA preparation; treat the pellet as described in Section 5.4, *Lithium Chloride Purification of RNA Preparation* (17).
	Target mRNA contains strong transcriptional pauses	Maintain an elevated temperature after the annealing step and increase the temperature of first strand reaction (up to 50°C) (see Section 5.1, *Alternative Protocol for First Strand cDNA Synthesis of Transcripts with High GC Content*).
	Too much first strand reaction was used in the PCR	Dilute cDNA reaction 10- to 100-fold. Use no more than 10% of the first strand product in PCR.
	Polymerase from an archaeobacteria was used with dUMP primers	Use AUAP and non-dUMP GSP in PCR using Elongase® or an archaeobacterial polymerase. Use *Taq* DNA polymerase for PCR with the UAP and dUMP containing GSP.
Unexpected bands after electrophoretic analysis of “nested” amplification products	Contamination by genomic DNA	To test if products were derived from genomic DNA, perform first strand reaction without SuperScript™ II RT.
		Pretreat RNA as described in Section 5.3, *DNase I Digestion of RNA Preparation.*
	Spurious priming in the PCR	Vary the parameters of the PCR according to *Taq* DNA polymerase instructions and/or use hot start for PCR. Incorporate a preamplification heating step (44,45).

Problem	Possible Cause	Suggested Remedy
Poor cloning efficiency	Inefficient ligation	Increase the incubation time in the ligation reaction; decrease the temperature.
		Ensure the removal of dNTPs prior to ligation by chromatographic separation.
		Ensure the removal of vector stuffer fragments by chromatographic separation.
	Poor restriction endonuclease digestion due to residual bound *Taq* DNA polymerase	Treat the PCR products with proteinase K (47) (see Section 5.8, *Proteinase K Treatment of 3´ RACE Products)*.
	Restriction endonuclease does not digest at the end of molecule	Add nucleotides to 5´ end of primer. Use a different restriction endonuclease.

4.2 Testing the 3´ RACE System

The control RNA provided with the 3´ RACE System can be used to trouble-shoot both the first strand reaction and the amplification reaction. Use the following protocols, as needed, to troubleshoot for particular problems.

To perform the 3´ RACE procedure using the control RNA, you may need the following items, *in addition to* those listed in Section 3.2, *Advance Preparations*.

- [α-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml)
- glass-fiber filters
- heat lamp
- 10% (w/v) TCA containing 1% (w/v) sodium pyrophosphate
- 95% ethanol

4.2.1 First Strand cDNA Synthesis

1. Label two autoclaved 0.5-ml microcentrifuge tubes “A” and “B.” Tube A will have an addition of radioisotope to determine the efficiency of first strand synthesis. An aliquot from tube B will be used for amplification.
2. Prepare the RNA:primer mixture in a sterile 0.5-ml tube:

Note: Up to 1 µg of your total RNA preparation may be added to this mixture in order to assess the effect of this RNA on first strand synthesis and subsequent amplification.

	Volume (µl)	
Component	**Tube A**	**Tube B**
control RNA (50 ng/µl)	2	2
AP (10 µM)	1	1
DEPC-treated water	8	9
final volume	11	12

3. Incubate at 70°C for 10 min and place on ice for 1 min. Collect the contents of each tube by brief centrifugation and add the following to each tube:

	Volume (µl)	
Component	**Tube A**	**Tube B**
10X PCR buffer	2	2
25 mM $MgCl_2$	2	2
10 mM dNTP mix	1	1
0.1 M DTT	2	2
SuperScript™ II RT (200 units/µl)	1	1

Troubleshooting Guide

4. Add 1 µl of [α-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml) to tube A. Final volume will be 20 µl.
5. Mix gently and collect the reaction by brief centrifugation.
6. Incubate for 42°C for 50 min.
7. Terminate both reactions by incubating at 70°C for 15 min. Place on ice for 10 min.
8. Collect each reaction by brief centrifugation. Add 1 µl of RNase H to tube B and incubate for 20 min at 37°C. Proceed with tube B to Section 4.2.2, *Amplification of the Control-Synthesized cDNA.*
9. Add 80 µl of distilled water to tube A and mix gently.
10. Remove two 5-µl aliquots from tube A and spot the aliquots onto glass-fiber filters. Dry one of the filters under a heat lamp or at room temperature. This filter will be used to determine the specific activity of the dCTP reaction.
11. Wash the other filter three times in sequence, for 5 min each time, with 50 ml of ice-cold, 10% (w/v) TCA containing 1% (w/v) sodium pyrophosphate. Wash the filter once with 50 ml of 95% ethanol at room temperature for 2 min. Dry the filter under a heat lamp or at room temperature. This filter will be used to determine the yield of first strand cDNA.
12. Count both filters in standard scintillant to determine the amount of ^{32}P in the reaction, as well as the amount of ^{32}P that was incorporated.
13. Using equation 1, determine the specific activity (SA) of the dCTP in the first strand reaction from the counts obtained from the *unwashed* filter:

$$\text{SA (cpm/pmol dCTP)} = \frac{\text{cpm/5 µl}}{\text{500 pmol dCTP/5 µl}}$$

14. Using equation 2, determine the yield of cDNA from the counts obtained from the *washed* filter and the specific activity calculated from the unwashed filter:

$$\text{Amount of cDNA (µg)} = \frac{(\text{cpm}) \times (100\ \text{µl}/5\ \text{µl}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(\text{SA}) \times (3{,}030\ \text{pmol dNTP/µg cDNA})}$$

The yield calculated for the labeled cDNA in tube A can be assumed as equivalent to that of the unlabeled cDNA in tube B.

15. Following first strand cDNA synthesis from the control RNA, you may wish to analyze the remaining cDNA in tube A by alkaline agarose gel electrophoresis or denaturing polyacrylamide gel electrophoresis (PAGE).

Note: The first strand yield using the control RNA should be 20% to 50%.

Note: Electrophoretic analysis should yield a prominent 891-bp band representing 25% to 50% of the cDNA synthesized.

4.2.2 Amplification of the Control-Synthesized cDNA

Following first strand cDNA synthesis from the control RNA, the control GSP (figure 3) and the UAP can be used to troubleshoot the amplification reaction. Electrophoretic analysis of DNA products amplified using the control primers should yield a prominent 720-bp band.

5´–CAU CAU CAU CAU GAC CGT TCA GCT GGA TAT TAC–3´

Figure 3. Sequence of the control GSP.

1. In each of three fresh 0.5-ml microcentrifuge tubes, prepare a PCR mixture as in step 1 of Protocol 2, substituting 1 µl of the control GSP for the user-defined GSP and using the UAP amplification primer. Place tubes on ice.
2. In separate 1.5-ml microcentrifuge tubes, prepare serial dilutions of the control cDNA from tube B in sterile, distilled water. Dilute 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. Use the three highest dilutions:

   control 1: $1:10^4$ ($\sim 10^6$ input RNA molecules/µl)
   control 2: $1:10^5$ ($\sim 10^5$ input RNA molecules/µl)
   control 3: $1:10^6$ ($\sim 10^4$ input RNA molecules/µl)

3. Add 2 µl from each control dilution to a separate reaction tube from step 1. Mix and layer 75 µl of mineral oil over each reaction. Collect each reaction by brief centrifugation.

Note: Refer to Protocol 2, Section 3.4, for additional information.

4. Perform steps 3 through 6 in Protocol 2. Amplify with 30 cycles of 94°C for 45 s, 55°C for 25 s, and 72°C for 3 min. Following agarose gel electrophoresis and ethidium bromide staining, you should be able to visualize a 720-bp band for all three concentrations of input cDNA (see figure 4).

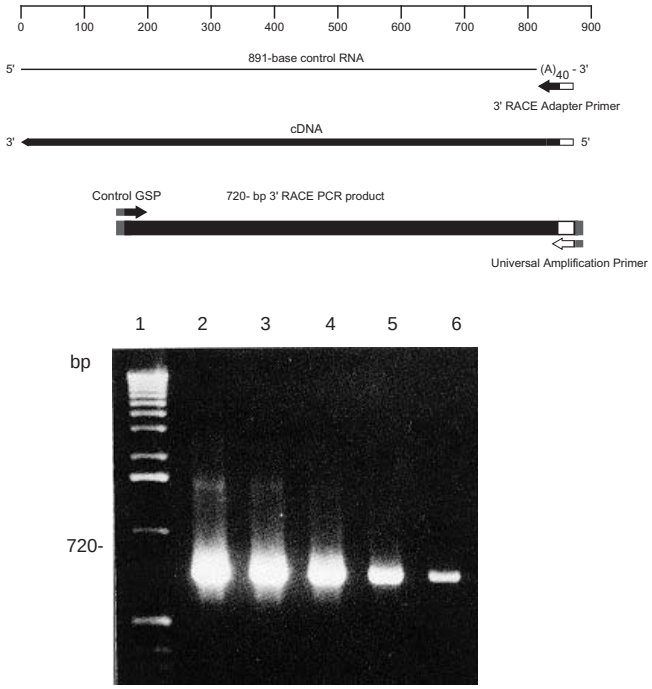

Figure 4. Agarose gel analysis of 3´ RACE products using control RNA. A 100-ng sample of control RNA was used for first strand cDNA synthesis as described in this section. The resulting cDNA was diluted 10^3 to 10^7 fold. 2 µl of each dilution was amplified according to Protocol 2 (Section 3.4) and one-tenth (5 µl) of the reaction product was analyzed by 1% agarose gel electrophoresis in the presence of 0.5 µg/ml of ethidium bromide. Lane 1: 1 Kb DNA Ladder. Lane 2: 2×10^7 input RNA molecules per reaction. Lane 3: 2×10^6 input RNA molecules per reaction. Lane 4: 2×10^5 input RNA molecules per reaction. Lane 5: 2×10^4 input RNA molecules per reaction. Lane 6: 2×10^3 input RNA molecules per reaction.

Troubleshooting Guide

4.3 Minimizing RNase Contamination

Successful cDNA synthesis demands an RNase-free environment at all times, which will generally require the same level of care used to maintain aseptic conditions when working with microorganisms. Several additional guidelines should be followed:

1. Never assume that anything is RNase-free. Use sterile pipets, centrifuge tubes, culture tubes, or any similar labware that is explicitly stated to be sterile. **Note:** Wear latex gloves for all manipulations involving RNA.
2. Dedicate a separate set of automatic pipets for manipulating RNA and the buffers and enzymes used to synthesize cDNA.
3. Avoid using any recycled glassware unless it has been specifically rendered RNase-free by rinsing with 0.5 N NaOH followed by copious amounts of sterilized, distilled water. Alternatively, bake glassware at 150°C for 4 h.
4. Microcentrifuge tubes can generally be taken from an unopened box, autoclaved, and used for all cDNA work. RNase-free microcentrifuge tubes can be purchased from several suppliers. If it is necessary to decontaminate untreated tubes, soak the tubes overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC), rinse with sterilized, distilled water, and autoclave.
5. If made with RNase-free labware, most solutions can be made from reagent-grade materials and distilled water, and then autoclaved. Prepare heat-sensitive solutions using sterilized, distilled water, and filtering through a 0.2 µm using sterilized, disposable unit.
6. Most aqueous buffer solutions can be treated with 0.01% (v/v) DEPC and autoclaved. **Note:** Buffers containing primary amines (such as tris) cannot be effectively treated by this method.
7. Use aerosol-resistant pipet tips.

Additional Protocols

5

5.1 First Strand cDNA Synthesis of Transcripts with High GC Content

Problems with 3´ RACE due to secondary structure of the target mRNA may be overcome by increasing the volume and temperature of the RT reaction. To avoid secondary RNA structure, shift the primer/RNA mix directly from 70°C to 50°C and prewarm the complete 2X reaction mix to 42°C before adding it to the primer and RNA. Use of a thermocycler simplifies the multiple temperature shifts in RT PCR and can help to prevent formation of secondary structure in RNA.

First Strand Synthesis Conditions
- 20 mM Tris-HCl (pH 8.4 at 22°C)
- 50 mM KCl
- 2.5 mM $MgCl_2$
- 10 mM DTT
- 500 µM each dATP, dCTP, dGTP, and dTTP
- 500 nM AP
- ≤1 µg total RNA
- 200 units SuperScript™ II RT
- Reaction volume: 50 µl

1. Mix and briefly centrifuge each component before use.
2. Combine up to 1 µg of total or 50 ng of poly(A)-selected RNA and DEPC-treated water to a final volume of 24 µl in a 0.5-ml microcentrifuge tube.
3. Add 1 µl of the 10 µM AP solution, mix gently, and collect by brief centrifugation.
4. Heat each sample to 70°C for 10 min and immediately transfer to 50°C.
5. In a separate tube, assemble the following reaction mixture, adding each component in the indicated order:

Component	Volume (µl)
DEPC-treated water	7.5
10X PCR buffer	5
25 mM $MgCl_2$	5
10 mM dNTP mix	2.5
0.1 M DTT	5

6. Prewarm the reaction mix to 42°C.
7. Add the prewarmed reaction mixture and 1 µl (200 units) of SuperScript™ II RT to the RNA/primer mixture, mix gently, and continue to incubate at 50°C for 50 min.
8. Terminate the reaction by incubating the tubes at 70°C for 15 min. Chill on ice.
9. Collect the reaction by brief centrifugation. Add 1 µl of RNase H and incubate for 20 min at 37°C before proceeding to Protocol 2 (see Section 3.4).

Additional Protocols

5.2 TRIzol® Reagent Isolation of Total RNA

TRIzol® Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a monophasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (19,20). During sample homogenization or lysis, TRIzol® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. The RNA is recovered from the aqueous phase by precipitation with isopropanol. The basic protocol is below. More details are found on the product information provided with the TRIzol® Reagent.

Note: Unless otherwise stated, the procedure is carried out at room temperature, and reagents are at room temperature.

Materials Required

- TRIzol® Reagent,
- chloroform,
- isopropanol,
- DEPC-treated water, and
- 75% ethanol (prepared using DEPC-treated water).

1. HOMOGENIZATION (see notes 1 and 2)
   a. *Tissues*

      Homogenize tissue samples in 1 ml of TRIzol® Reagent per 50 to 100 mg of tissue using a glass-Teflon® or power homogenizer. The sample volume should not exceed 10% of the volume of TRIzol® Reagent used for homogenization.

   b. *Cells Grown in Monolayer*

      Lyse cells directly in a culture dish by adding 1 ml of TRIzol® Reagent to a 3.5-cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIzol® Reagent added is based on the area of the culture dish (1 ml per 10 cm^2).

   c. *Cells Grown in Suspension*

      Pellet cells by centrifugation. Lyse cells in TRIzol® Reagent by repetitive pipetting. Use 1 ml of the reagent per 5×10^6 to 10×10^6 of animal, plant or yeast cells, or per 1×10^7 bacterial cells. Washing cells before addition of TRIzol® Reagent should be avoided to minimize mRNA degradation. Disruption of yeast and bacterial cells may require a homogenizer.

2. Incubate the homogenized samples for 5 min at room temperature. Add 0.2 ml of chloroform per 1 ml of TRIzol® Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 s and incubate for 2 to 3 min. Centrifuge the samples at no more than $12{,}000 \times g$ for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol® Reagent used for homogenization.
3. Transfer the aqueous phase to a fresh tube. Add 0.5 ml of isopropanol per 1 ml of TRIzol® Reagent used for the initial homogenization. Incubate samples at room temperature for 10 min and centrifuge at no more than $12{,}000 \times g$ for 10 min.

4. Remove the supernate. Resuspend the RNA pellet once in 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × *g* for 5 min at 4°C. The RNA precipitate can be stored in 75% ethanol at 4°C for at least one week, or at least one year at –20°C.

Note: Use only DEPC-treated water to resuspend RNA.

5. Briefly dry the RNA pellet (air-dry or vacuum-dry for 5 to 10 min). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an $A_{260}/_{280}$ ratio <1.6. Dissolve RNA in DEPC-treated water and incubate for 10 min at 55°C to 60°C.

Notes:

1. To facilitate isolation of RNA from small quantities of sample (10^6 cells or <10 mg tissue) perform homogenization (or lysis) of samples in 0.8 ml of TRIzol® Reagent, and add 5 to 10 µg of glycogen as carrier.
2. An additional isolation step may be required for samples with a high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × *g* for 10 min at 4°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernate contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. Transfer the clear supernate to a fresh tube and proceed with the phase separation.

5.3 DNase I Digestion of RNA Preparation

If amplification products are detected from the PCR in the absence of SuperScript™ II RT, it may be necessary to eliminate residual genomic DNA from the RNA sample. After confirming the efficiency of the first strand synthesis reaction with the control RNA, use the following protocol to remove genomic DNA from the total RNA preparation.

1. Add the following to a 0.5-ml microcentifuge tube on ice:

Component	Amount
total RNA	1–2 µg
10X reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 20 mM $MgCl_2$]	1 µl
Amplification grade DNase I (1 unit/µl)	1 µl
DEPC-treated water	to 10 µl

Note: The procedure requires careful pipetting of all solutions so that the concentration of divalent metal cation (Mg^{2+} and Ca^{2+}) is precise. Because the DNase I must be heated to 65°C to inactivate the enzyme, the concentration of free divalent metal ion must be low enough (<1 mM) after the addition of the EDTA to prevent chemical hydrolysis of the RNA.

2. Incubate at room temperature for 15 min.
3. Add 1 µl of 25 mM EDTA.
4. Incubate for 15 min at 65°C to heat inactivate the DNase I, then place on ice for 1 min. Collect the reaction by brief centrifugation. This mixture can be used directly for reverse transcription.

5.4 Lithium Chloride Purification of RNA Preparation

If you obtained poor first strand cDNA synthesis from your RNA preparation, the RNA may have coprecipitated with polysaccharides or small RNAs (tRNA and 5SRNA) during the RNA isolation procedure. You may be able to recover highly purified mRNA using the following protocol adapted from Sambrook *et al*. (17).

1. To the RNA sample in DEPC-treated water, add 0.1 volume 8 M LiCl (RNase-free) and vortex. Incubate on ice for 2 h.

2. Centrifuge at 14,000 × *g* for 30 min at 4°C.
3. Remove the supernate, being careful not to disturb the pellet (the pellet may be difficult to see). Dissolve the pellet in 200 µl of DEPC-treated water by drawing the pellet in and out of a sterilized pipet tip.
4. Repeat steps 1 through 3.
5. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol (–20°C). Place the tube at –20°C for 30 min. Centrifuge at 14,000 × *g* for 30 min at 4°C.
6. Remove the supernate carefully. Overlay the pellet with 100 µl of 70% ethanol (–20°C) and centrifuge at 14,000 × *g* for 10 min at 4°C. Remove the supernate and air dry the RNA pellet at room temperature.
7. Dissolve the pellet in 10 to 100 µl of DEPC-treated water.
8. If your starting cell or tissue sample was small (10^3 to 10^5 cells), dissolve the pellet in 13 µl of DEPC-treated water and use this entire amount for first strand synthesis. If your starting sample was large (10^6 to 10^7 cells), spectrophotometrically determine the concentration of total RNA recovered in step 5 before proceeding to Protocol 1 (Section 3.3).

5.5 Nested Amplification from an Agarose Plug

This procedure is intended to provide added specificity to the amplification procedure prior to cloning the 3´ RACE products. The following protocol should be performed after completion of Protocol 2 (agarose gel electrophoretic analysis and RACE product visualization by ethidium bromide staining), but prior to performing the cloning procedures described in this chapter.

Note: Sequences specific for downstream cloning manipulations (see Section 2.6, *Design of Gene-Specific Primer*) must be designed into GSP2, not GSP1.

1. Using a capillary pipet, remove a small (up to 5 µl) agarose plug from the area of the gel containing the band of interest.
2. Prepare an amplification reaction mix as in step 1 of Protocol 2, *except* use a second GSP (“GSP2”) in place of the original GSP.
3. Perform steps 2 through 7 in Protocol 2 and proceed to subsequent cloning procedures.

5.6 Cloning Using T4 DNA Polymerase

1. Following amplification, remove dNTPs from the reaction by agarose gel electrophoresis. Resuspend 200 to 500 ng of the reaction in 34 µl of sterilized, distilled water.
2. To the purified amplification product, add the following:

Component	Volume (µl)
10X Tris-acetate buffer [0.33 M Tris-acetate (pH 8.0), 0.66 M potassium acetate, 0.1 M magnesium acetate, 5 mM DTT, 1 mg/ml BSA]	5
dTTP (1 mM)	5
dATP (1 mM)	5
T4 DNA polymerase (1 unit/µl)	1

3. Incubate the reaction at 37°C for 15 min. Heat inactivate the enzyme by incubating at 65°C for 10 min. Ethanol precipitate the reaction (17) and resuspend in 5 µl of TE Buffer.
4. Prepare pSPORT 1 or other appropriate vector for ligation by digestion with *Acc* I and *Not* I (17). Purify the vector from the excised fragment by agarose gel electrophoresis.
5. Combine ~100 ng of nondephosphorylated *Not* I-*Acc* I-cut vector with 500 ng of the T4 DNA polymerase-treated 3´ RACE product and appropriate ligation reaction buffer to a final volume of 20 µl. Add 1 or 2 units of T4 DNA ligase and incubate at 20°C to 25°C for 2 to 4 h.
6. Dilute the ligation reaction 1:10 in TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. Transform competent cells such as MAX Efficiency® or Library Efficiency® DH5α™ Competent Cells using 1 to 2 µl of the vector-ligated DNA.

Note: For additional information about ligation reactions using T4 DNA ligase, see reference 48 or further information on bacterial transformation (49).

5.7 Proteinase K Treatment of 3´ RACE Products

If you obtained poor cloning efficiency following amplification, residual bound *Taq* DNA polymerase may have hindered restriction endonuclease digestion, resulting in poor vector/insert ligation. Use the following protocol to ensure complete removal of *Taq* DNA polymerase after amplification (47).

1. Take sample from step 5 of protocol 2 (see Section 3.4) and add 0.1 volume of proteinase K solution [500 µg/ml proteinase K, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA].
2. Incubate for 30 min at 37°C and then for 10 min at 65°C.
3. Extract once with phenol:chloroform (1:1) and once with chloroform.
4. Ethanol-precipitate by addition of 0.1 volume of sodium acetate (pH 5.2) and 2 volumes of absolute ethanol (–20°C). Place tube at –20°C for 30 min.
5. Centrifuge at 13,000 × *g* for 30 min at 4°C. Remove supernate, rinse pellet with 70% ethanol, and air-dry the pellet at room temperature.
6. Resuspend the sample in 10 µl of TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA] before advancing to cloning procedures.

6

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7 Related Products

Product	Size	Cat. No.
Primers:		
Custom Primers		Please inquire
Abridged Universal Amplification Primer	3.1 µg	10541-019
Universal Amplification Primer	5 µg	18382-010
5´ RACE Abridged Anchor Primer	5.7 µg	10630-010
5´ RACE Anchor Primer	7.5 µg	18388-017
3´ RACE Adapter Primer	2.3 µg	10542-017
Products for Transformation and Cloning:		
MAX Efficiency® DH5α™ Competent Cells	1 ml (5 × 0.2 ml)	18258-012
MAX Efficiency® DH10B™ Competent Cells	1 ml (5 × 0.2 ml)	18297-010
Library Efficiency® DH5α™ Competent Cells	1 ml (5 × 0.2 ml)	18263-012
MAX Efficiency® STBL2™ Competent Cells	1 ml (5 × 0.2 ml)	10268-019
S.O.C. Medium	10 × 10 ml	15544-034
Enzymes for Amplification:		
Taq DNA Polymerase, Native	100 units 500 units 1,500 units (3 × 500 units)	18038-018 18038-042 18038-067
Taq DNA Polymerase, Recombinant	100 units 500 units 1,500 units (3 × 500 units)	10342-053 10342-020 10342-046
Platinum® *Taq* DNA Polymerase	250 units	10966-026
Elongase® Amplification System	100 reactions	10481-018
Elongase® Enzyme Mix	100 reactions 500 reactions	10480-010 10480-028
Other Modifying Enzymes:		
T4 DNA Ligase	100 units 500 units 2,000 units (4 × 500 units)	15224-017 15224-025 15224-090
T4 DNA Ligase High Concentration	250 units	15224-041
DNase I, Amplification Grade	100 units	18068-015
T4 DNA Polymerase	50 units 250 units	18005-017 18005-025

Product	Size	Cat. No.
Ribonuclease H	30 units	18021-014
	120 units	18021-071
SuperScript™ II Reverse Transcriptase	2,000 units	18064-022
	10,000 units	18064-014
	40,000 units (4 × 10,000 units)	18064-071
RNaseOUT™, Recombinant RNase Inhibitor	5,000 units	10777-019
Other Related Products:		
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0	10 reactions	18374-058
100 bp DNA Ladder	50 µg	15628-019
	250 µg	15628-050
1 Kb DNA Ladder	250 µg	15615-016
	1,000 µg	15615-024
DEPC-Treated Water	4 × 1.25 ml	10813-012
GlassMAX RNA Isolation Spin Cartridge System	50 reactions	18385-039

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