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Instruction Manual

5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0

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Notices to Customer

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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 unknown sequences at either the 3' or the 5' -end of the mRNA (1). This methodology of amplification with single-sided specificity has been described by others as “one-sided” PCR (2) or “anchored” PCR (3). In general, PCR amplification of relatively few target molecules in a complex mixture requires two sequence-specific primers that flank the region of sequence to be amplified (4,5). However, to amplify and characterize regions of unknown sequences, this requirement imposes a severe limitation (3). 3' and 5' RACE methodologies offer possible solutions to this problem.

3' RACE takes advantage of the natural poly(A) tail in mRNA as a generic priming site for PCR amplification. In this procedure, mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. Specific cDNA is then directly 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, or “anchored” PCR, is a technique that facilitates the isolation and characterization of 5' ends from low-copy messages. The method has been reviewed by both Frohman (6,8) and Loh (7). Although the precise protocol varies among different users, the general strategy remains consistent. First strand cDNA synthesis is primed using a gene-specific antisense oligonucleotide (GSP1). This permits cDNA conversion of specific mRNA, or related families of mRNAs, and maximizes the potential for complete extension to the 5' -end of the message. Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) is used to add homopolymeric tails to the 3' ends of the cDNA. In the original protocol, tailed cDNA is then amplified by PCR using a mixture of three primers: a nested gene-specific primer (GSP2), which anneals 3' to GSP1; and a combination of a complementary homopolymer-containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5'-end of the mRNA.

RACE procedures have been used for amplification and cloning of rare mRNAs that may escape, or prove challenging for, conventional cDNA cloning methodologies (7). Additionally, RACE may be applied to existing cDNA libraries (9). Random hexamer-primed cDNA has also been adapted to 5' RACE for amplification and cloning of multiple genes from a single first strand synthesis reaction (10). Products of RACE reactions can be directly sequenced without any intermittent cloning steps (11,12), or the products can be used for the preparation of probes (13). Products generated by the 3' and 5' RACE procedures may be combined to generate full-length cDNAs (6,13). Lastly, the RACE procedures may be utilized in conjunction with exon trapping methods (14) to enable amplification and subsequent characterization of unknown coding sequences.

2.2 Summary of the 5' RACE System

The 5' RACE System is a set of prequalified reagents intended for synthesis of first strand cDNA, purification of first strand products, homopolymeric tailing, and preparation of target cDNA for subsequent amplification by PCR. Control RNA, DNA, and primers are provided for monitoring system performance. The procedure is summarized in figure 1.

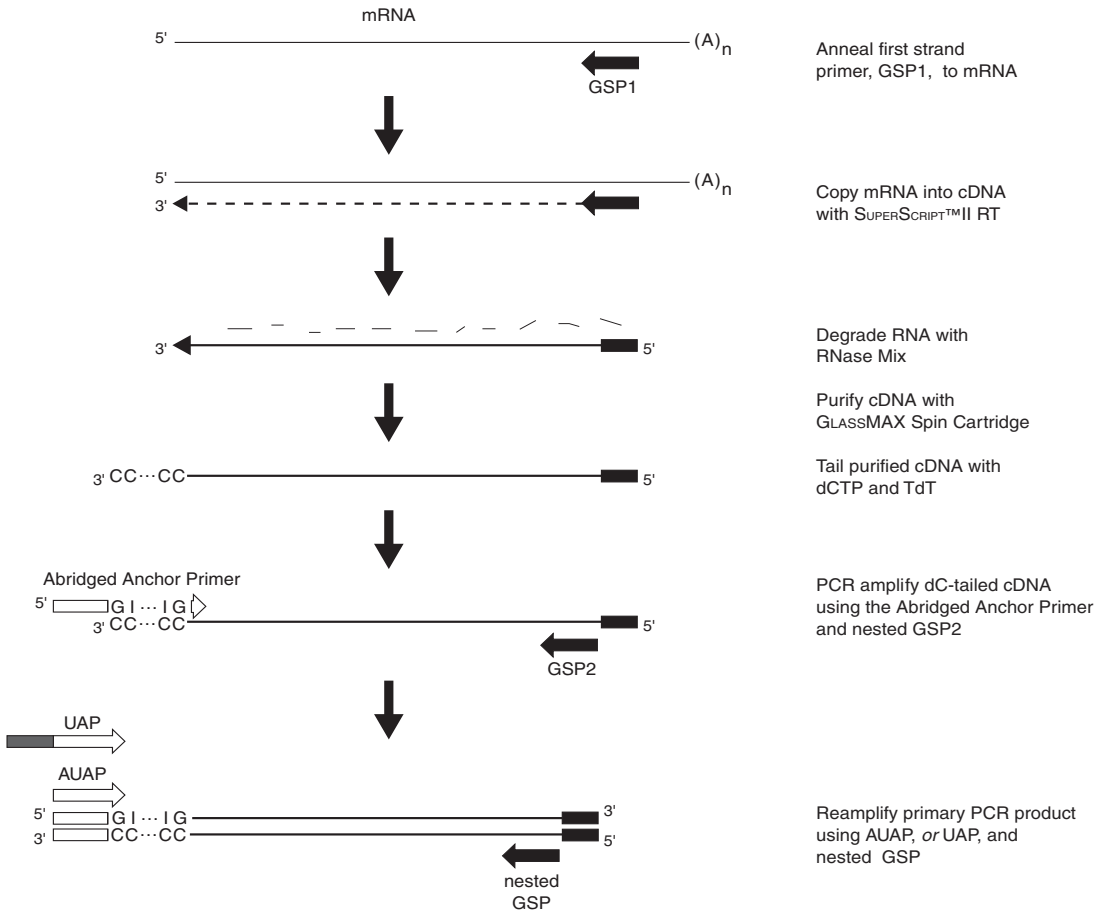

Figure 1. Overview of the 5' RACE Procedure.

First strand cDNA is synthesized from total or poly(A)$^+$ RNA using a gene-specific primer (GSP1) that the user provides and SuperScript™ II, a derivative of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) with reduced RNase H activity. After first strand cDNA synthesis, the original mRNA template is removed by treatment with the RNase Mix (mixture of RNase H, which is specific for RNA:DNA heteroduplex molecules, and RNase T1). Unincorporated dNTPs, GSP1, and proteins are separated from cDNA using a S.N.A.P. Column. A homopolymeric tail is then added to the 3'-end of the cDNA using TdT and dCTP. Since the tailing reaction is performed in a PCR-compatible buffer, the entire contents of the reaction may be directly amplified by PCR without intermediate organic extractions, ethanol precipitations, or dilutions. PCR amplification is accomplished using *Taq* DNA polymerase, a nested, gene-specific primer (GSP2, designed by the user) that anneals to a site located within the cDNA molecule, and a novel deoxyinosine-containing anchor primer (patent pending) provided with the system.

Following amplification, 5' RACE products can be cloned into an appropriate vector for subsequent characterization procedures, which may include sequencing, restriction mapping, preparation of probes to detect the genomic elements associated with the cDNA of interest, or *in vitro* RNA synthesis. The Abridged Anchor Primer (AAP), Abridged Universal Amplification Primer (AUAP), Anchor Primer (AP) [available separately], and Universal Amplification Primer (UAP) include recognition sequences for *Mlu* I, *Sal* I, and *Spe* I to facilitate restriction endonuclease cloning of RACE products (figure 2).

Overview

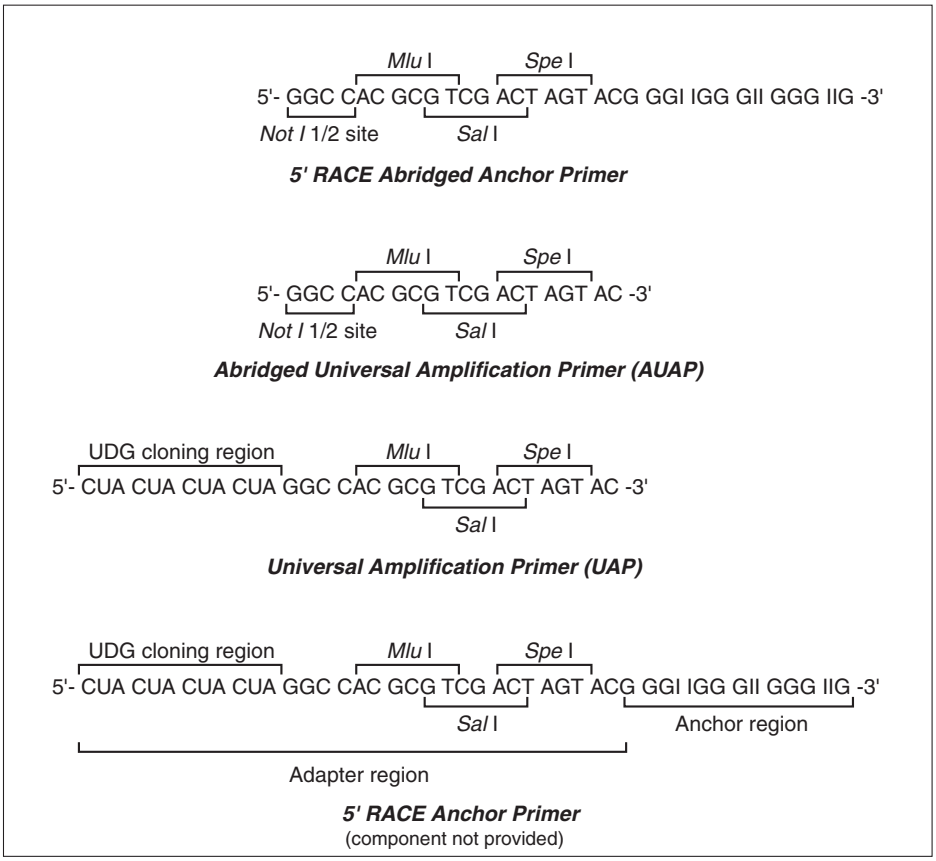

Figure 2. Sequence of the 5' RACE Primers.

5' RACE products may also be used for procedures that do not require an intermittent cloning step such as dsDNA cycle sequencing (12,18) or probe preparation (13). However, additional rounds of PCR using the AUAP, or UAP, in conjunction with either progressively nested GSPs or size-selected products from the initial PCR, may be required to confer an adequate level of specificity to the process to permit direct characterization of RACE products.

Details of the individual steps of 5' RACE are discussed below. Review this information carefully before beginning. The RNA isolation, design of primers, and the amplification protocols are most important for optimal results.

2.2.1 Isolation of 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 (19), and to prevent introduction of RNases and inhibitors of RT (20, 52). The guanidine isothiocyanate/acid-phenol method, originally described by Chomzynski and Sacchi (21), is the recommended method for RNA isolation. See section 3.4.1.

2.2.2 Design of 5' RACE Primers

The sensitivity and the specificity of the first strand synthesis and the subsequent PCR depend upon good primer design. A minimum of two antisense gene-specific primers (GSP) are **required** for 5' RACE and must be supplied by the user.

1. GSP1 primes first-strand cDNA synthesis (figure 1). Design this primer to anneal at least 300 bp from the mRNA 5'-end so that the cDNA can be easily purified using a S.N.A.P. column.
2. A second nested primer, GSP2, that anneals to sequences located 3' (with respect to cDNA not mRNA) of GSP1 is required for PCR. GSP2 can anneal immediately adjacent to GSP1 or at sequences located further upstream of GSP1 within the cDNA product. Other sequentially nested GSPs may be required depending on the efficiency and specificity of the primary PCR.

In general, these primers should be highly specific for their target sequences, able to form stable duplexes with their target sequences, and free of secondary structure. The key rules for primer design are discussed in section 3.4.2 (as well as in references 22-26). The primers provided in this system were carefully designed for successful 5' RACE.

The anchor primers contain 3' sequence complementary to the homopolymeric tail and additional 5' sequence that encodes an adapter region, comprised of restriction endonuclease sites and other functional sequences which facilitate cloning and characterization of 5' RACE products. Normally, homopolymer primers create melting temperatures that are either higher [poly (dG)•poly (dC)] or lower [poly (dA)•poly (dT)] than a typical GSP. They also can have poor specificity that can lead to mispriming at internal sequences. To minimize these problems, our anchor primers (figure 2, patent pending) were designed with the selective placement of deoxyinosine residues in the poly (dG) portion. This design eliminates the need to use the mixtures of anchor and adapter primers described in the original method (6,7).

Note: While the anchor primers enable efficient amplification of many target sequences, they may not be an idealized solution for all 5' RACE applications. It is possible that the anchor primer may anneal at certain gene-specific sequences. Therefore, as in any RACE procedure, specificity of the anchor primer for the oligo-dC tail should be tested by performing amplification reactions with cDNA subjected to dC-tailing both in the presence of and absence of TdT.

Deoxyinosine has the capacity to base-pair with all four bases; however, it does so with varying affinities. The order of stabilities for the different combinations, from greatest to least stable, reported by Martin *et al*. are as follows: I:C, I:A, I:T, and I:G. I:C pairs were found to be slightly less stable than A:T pairs (27). The selective placement of deoxyinosine residues in the 3' region of the anchor primer maintains low stability on the primer's 3'-end (ΔG = -8.2 kCal/mol) and creates a melting temperature (T_m) for the 16-base anchor region (66.6°C) which is comparable to that of a typical 20-mer primer with 50% GC content (22,23). This maximizes specific priming from the oligo-dC tail, minimizes priming at internal C-rich regions of the cDNA, and establishes a relationship of a "balanced" T_m for the anchor region to that of GSP2, which is required for efficient PCR (6,7).

The Abridged Universal Amplification Primer (AUAP) and Universal Amplification Primer (UAP) are used to reamplify primary 5' RACE PCR products in applications such as nested PCR or enrichment of RACE products for cloning. The AUAP contains a restriction endonuclease site sequence (adapter region) homologous to the adapter region of the anchor primer.

The UAP is composed of the same adapter region as the AUAP plus a dUMP-containing sequence at the 5'-end of the primer required for uracil DNA glycosylase (UDG)-mediated cloning of 5' RACE products. The original 5' RACE Anchor Primer is available separately for applications that require UDG cloning of 5' RACE products directly from the primary PCR. The UAP, the 5' RACE Anchor Primer or any dUMP-containing primer should not be used to prime DNA synthesis with any archaeobacterial polymerase (*Pfu* DNA Polymerase, *Pwo* DNA Polymerase, etc.), including long PCR enzyme mixtures (28,29), because dUMP inhibits these polymerase activities.

2.2.3 First Strand cDNA Synthesis from Total RNA

The capture of mRNA 5'-ends is dependent on complete cDNA synthesis. The use of RNase H^- RT for first strand synthesis results in greater full-length cDNA synthesis and higher yields of first strand cDNA than obtained with other RTs (20,30). SuperScript™ II RT has been engineered to retain the full DNA polymerase activity found in M-MLV RT (31). The enzyme exhibits increased thermal stability and may be used at temperatures up to 50°C. Because SuperScript™ II RT is not inhibited significantly by ribosomal and transfer RNA, it may be used effectively to synthesize first strand cDNA from a total RNA preparation. The RNA template is removed from the first strand cDNA product as described below.

2.2.4 Removal of RNA Template by RNase Mix

After cDNA synthesis, RNase Mix, a mixture of RNase H and RNase T1, is used to degrade the RNA. The digestion is performed following thermal inactivation of the RT in order to reduce the potential for hairpin-primed second-strand synthesis (catalyzed by RT) which can obscure the accessibility of the cDNA ends to TdT. Template RNA in the cDNA:RNA hybrid is degraded by RNase H and the single-stranded RNAs are degraded by RNase T1. This eliminates possible renaturation of template RNA to cDNA. TdT does not use RNA as a substrate (32); however, RNA may inhibit tailing and subsequent PCR of the cDNA (33). Use of the RNase Mix upon completion of first strand synthesis is cru-

cial to the efficiency of the tailing reaction because TdT exhibits a marked preference for single-stranded substrates (34,35).

2.2.5 Purification of First Strand Product

Excess nucleotides and GSP1 must be removed from the first strand product. Otherwise, residual GSP1 will be tailed by TdT and will compete for Abridged Anchor Primer during PCR (figure 3). Because of the large amounts of GSP1 relative to cDNA product, a stringent purification procedure is required (6,7,36). The S.N.A.P. column procedure, adapted from a method described by Vogelstein and Gillespie (37), provides a rapid and efficient means to purify first strand product. In the presence of the chaotropic agent, sodium iodide, cDNA >200 bases are bound to the silica-based membrane. Buffer components, dNTPs, enzymes, and oligonucleotides remain in solution and are removed by centrifugation with the effluent. Residual impurities and sodium iodide are removed by passing several volumes of 1X wash buffer followed by a 70% ethanol rinse through the S.N.A.P. column. Purified cDNA is recovered in distilled water and may be used directly in the TdT tailing reaction.

2.2.6 Homopolymeric Tailing of cDNA

TdT tailing creates the abridged anchor primer binding site on the 3'-end of the cDNA. Efficient tailing is necessary to provide:

1. A high proportion of tailed cDNA molecules for efficient amplification of first strand products.
2. Homopolymeric tails of sufficient length to allow the primer to anneal.
3. Homopolymeric tails of uniform length to produce a homogeneous amplification product.
4. A buffer compatible with the PCR buffer system.

The 5' RACE System tailing reaction has been optimized to meet these criteria. The 5' RACE System uses a tailing buffer [10 mM Tris-HCl (pH 8.4), 25 mM KCl and 1.5 mM $MgCl_2$] supplemented with 200 µM dCTP for homopolymeric tailing of first strand cDNA. The tailing reaction is highly sensitive to the concentration of each buffer component.

Concentrations of $MgCl_2$ in excess of 1.5 mM may significantly inhibit both the length of the tail and the percentage of molecules tailed. In general, components such as Tris buffers and salts have been reported to be inhibitory to TdT (32), and $CoCl_2$ has classically been chosen over $MgCl_2$ as the optimal divalent cation for tailing reactions (35). However, careful manipulation of buffers containing these components has been shown to produce results that are highly effective for 5' RACE (36).

Double-stranded 3' termini and hairpin structures may significantly impair homopolymeric tailing of cDNA; therefore, a brief denaturation procedure prior to tailing is used to disrupt any secondary structure in the cDNA.

The choice of nucleotide for homopolymeric tailing has been a subject of debate. Each nucleotide offers unique advantages and disadvantages. The 5' RACE System uses dC-tailing to complement our unique Abridged Anchor Primer (see section 2.2.2). dA-tailing permits the use of the same oligo-dT anchor primer for both 5' and 3' RACE procedures. However, because A:T base pairs are less stable than G:C base pairs, longer stretches of dAs or dTs are required for priming as compared to dGs or dCs.

2.2.7 Amplification of Target cDNA

Successful 5' RACE is extremely dependent on the efficiency and specificity of the PCR. 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 thermal cycling protocol, may be required. The optimal **free** magnesium concentration for efficient amplification is reported to be between 0.7 and 0.8 mM (38). Since magnesium can bind deoxynucleoside 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

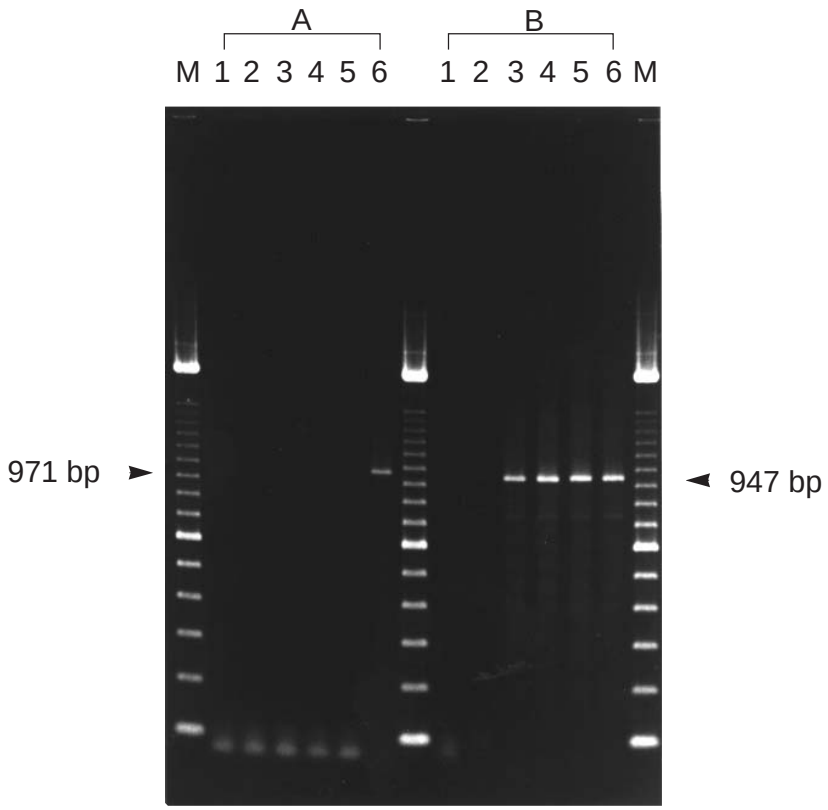

Figure 3. Effect of GSP1 on 5' RACE. A large-scale preparation of control cDNA was synthesized using 100 μg of an *in vitro* transcribed RNA essentially as described in *Methods* with the amount of components scaled appropriately. RNA template was degraded using RNase Mix. The cDNA product was purified by phenol:chloroform extraction followed by Sephadex® G-150 chromatography. Purified product was quantified by A_{260} and diluted in TE buffer. 4 x 10^5 copies of cDNA were tailed as described in the presence of 1, 0.1, 0.01, 0.001, or 0 pmoles of GSP1, lanes 1-6 respectively. One-fourth (1 x 10^5 copies of cDNA) of each reaction was amplified by PCR using Anchor Primer in combination with either (A) the control GSP1 or (B) the control GSP2 (see figure 4). One tenth (5 μl) of each PCR was analyzed by 1.5% agarose gel in 1X TBE stained with 0.5 μg/ml ethidium bromide. Lane M, GIBCO BRL 100 bp DNA Ladder.

specificity (39). Higher nucleotide concentrations, however, can be used to improve product yield as well as to promote 3'-terminal T-mismatches (40). This factor may warrant consideration if a degenerate oligonucleotide is used as GSP2. Additionally, since a degenerate primer represents a composite of many different priming sequences, higher primer concentrations (≥1 µM) are generally required.

Typical thermal cycling parameters are provided in the protocols. However, optimal conditions depend not only on the primers and template, but also the type of PCR tube as well as the thermal cycler.

There are four times and temperatures that must be considered:

1) Preamplification denaturation of the template DNA (PAD)

2) Denaturation of product DNA at the beginning of each cycle

3) Annealing of the primers to the denatured DNA

4) Extension of the primers by the polymerase

Steps 2-4 constitute a cycle and are repeated usually 30-35 times followed by a final extension time of 5-10 min and then a holding temperature of 5°C.

Many PCR protocols use a PAD step of 3 to 5 min. However, an extended PAD is not usually necessary and may impair the ability to amplify longer sequences (41). The denaturation temperature and time should be sufficient to completely separate target strands, yet minimized to reduce deamination and depurination of target DNA. For thin-walled tubes in thermal cyclers which use the sample temperature (or calculated sample temperature) to control temperature cycling, a denaturation time of 10 s to 15 s at 94°C is adequate. Likewise, an annealing time of 20 s to 30 s is usually ample. In contrast, PCR in conventional 0.5-ml microcentrifuge tubes may require 1 min for complete denaturation and 30 s to 1 min for annealing. Optimal annealing temperature is dependent upon the thermodynamic properties of the primers (see section 3.4.2). However, well-designed primers, *i.e.* primers with unstable 3'-ends ($\Delta G > -9$ kCal/mol), can function effectively in PCR over a broad range of annealing temperatures (41). A general rule for extension time is to allow 1 min for every 1 kb of target sequence. If primer T_ms are ≥68°C, a two step PCR, which cycles between denaturation at 94°C and combined annealing and extension at 68°C can be used. For a detailed discussion of parameters affecting PCR, please refer to Innis and Gelfand (42) or Saiki (38, 43).

Nonspecific annealing and extension of primers prior to the initial denaturation step of the PCR process may adversely affect the efficiency and specificity of amplification. These artifacts can be minimized by using the “hot start” technique (44,45) which requires the addition of either *Taq* DNA polymerase, dNTPs, or $MgCl_2$ after reactions have been equilibrated at 75°C to 80°C. For many applications it is sufficient to assemble the reactions on ice, in thin-walled PCR tubes and directly transfer the tubes to a thermal cycler equilibrated to the initial denaturation temperature, 94°C. This is the procedure used in section 3.9.

Amplification of a target cDNA synthesized with the 5' RACE System requires priming with two oligonucleotides. The Abridged Anchor Primer, which is specific for the oligo-dC tail added by TdT, serves as the sense primer. The antisense primer (GSP2), provided by the user, should anneal to an internal (nested) site within the cDNA sequence (with respect to the primer used for first strand synthesis, GSP1) and may include sequence elements that facilitate subsequent cloning steps (section 4.2).

Use of a nested GSP2 is essential for effective PCR (6,7,36). This not only adds a level of specificity to the process, but it prevents “primer-dimer” amplification of residual GSP1 that may carry through the cDNA purification procedure. This effect is illustrated in figure 3. Residual GSP1, which is subsequently tailed by TdT, is copied by extension of the anchor primer during PCR. This results in amplification of the tailed GSP1 sequence and blocks amplification of cDNA.

2.2.8 Cloning 5' RACE Amplification Products

Conventional cloning methods that typically involve end-repair and blunt-end cloning can be problematic for amplified products (18,46,47). An alternative is a rapid and efficient cloning method involving the use of UDG (48-50). This method requires that the user design a nested GSP2 containing a 5'-$(CAU)_4$ sequence. Incorporation of dUMP into the nested GSP2 may be accomplished with minimal expense on most automated synthesizers or by ordering through Invitrogen's Custom Primers (see section 3.4.2).

An 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 (51). In this procedure, PCR products from the primary PCR with the Abridged Anchor Primer, or nested amplification reaction primed with the AUAP, are treated with T4 DNA polymerase to generate a *Not* I 5' overhang.

Another approach to cloning is to digest the 5' RACE product using one of the restriction endonuclease sites designed into the AUAP (see figure 2) (1). The user may also design unique restriction sites into the GSP, exploit a site present in the cDNA sequence or end-repair the 5' RACE product prior to restriction-endonuclease digestion (46).

3 Methods

3.1 Components and Storage

Note: The 5' RACE system does not include *Taq* DNA polymerase or the reagents required for cloning.

The components of the 5' RACE System are as follows. Sufficient material is provided for 10 reactions. One reaction prepares specific cDNA from 1-5 µg of a total RNA or 50-500 ng poly $(A)^+$ RNA preparation for amplification by anchored PCR. The amount of RNA will vary depending on the application. Control RNA, DNA, and primers are included to verify the performance of the system and may be added to experimental RNA preparations to monitor the efficiency of each step or to troubleshoot potential problems.

Note: The 10X PCR buffer does not contain $MgCl_2$. Therefore, $MgCl_2$ must be added to the first strand reaction mix.

Component	Volume	Storage
Reagents:		
10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	500 µl	–20°C
25 mM $MgCl_2$	500 µl	–20°C
10 mM dNTP mix [10 mM each dATP, dCTP, dGTP, dTTP]	100 µl	–20°C
0.1 M DTT	100 µl	–20°C
SuperScript™ II Reverse Transcriptase (200 units/µl)	10 µl	–20°C
RNase mix	10 µl	–20°C
5X tailing buffer [50 mM Tris-HCl (pH 8.4), 125 mM KCl, 7.5 mM $MgCl_2$]	500 µl	–20°C
2 mM dCTP	50 µl	–20°C
terminal deoxynucleotidyl transferase	15 µl	–20°C
5' RACE abridged anchor primer (AAP, 10 µM)	80 µl	–20°C
universal amplification primer (UAP, 10 µM)	40 µl	–20°C
abridged universal amplification primer (AUAP, 10 µM)	40 µl	–20°C
DEPC-treated water	1.25 ml	–20°C
control gene-specific primer 1 (GSP1, 1 µM)	25 µl	–20°C
control nested gene-specific primer 2 (GSP2, 10 µM)	80 µl	–20°C
control PCR primer, gene-specific primer 3 (GSP3, 10 µM)	20 µl	–20°C
control DNA (2 x 10^4 copies/µl; ~0.1 pg/µl)	100 µl	–20°C
control RNA (50 ng/µl)	10 µl	–70°C
DNA Purification System:		
S.N.A.P. Columns	10 columns	4°C
Collection tubes	10 tubes	4°C
binding solution (6 M sodium iodide)	30 ml	4°C
Wash buffer concentrate	1 ml	4°C

Note: Do not freeze the DNA Purification System.

3.2 Additional Materials Required

The following items are required for use with the 5' RACE System, but are not included.

- sterilized, RNase-free thin-walled 0.2 or 0.5-ml PCR tubes;
- automatic pipettes capable of dispensing 1 to 20 µl and 20 to 200 µl;
- sterilized, RNase-free disposable tips for automatic pipettes;
- disposable latex gloves;
- sterilized, distilled water;
- absolute ethanol;
- GSP1 (cDNA primer, user-defined);
- GSP2 (nested primer for PCR amplification of dC-tailed cDNA, user-defined, appropriately engineered);
- microcentrifuge capable of generating a relative centrifugal force of 13,000 x *g*;
- 37°C, 42°C, 65°C, and 70°C water baths or heat blocks (or use thermal cycler);
- *Taq* DNA Polymerase;
- programmable thermal cycler; and
- mineral oil (if necessary for your thermal cycler)

Note: All temperature incubations may be performed in an appropriately programmed thermal cycler. This eliminates the need for multiple fixed temperature baths or heat blocks.

3.3 Performance and Limitations of Procedures

The 5' RACE System has been functionally tested using the control RNA according to the protocols described in this manual using GIBCO BRL *Taq* DNA Polymerase. Following PCR, a distinct 711-bp band was visible by agarose gel electrophoresis and ethidium bromide staining. While the 5' RACE system provides a direct and reliable solution for the preparation of tailed cDNA, PCR with single-sided specificity remains highly challenging. Success with the system is extremely dependent on the efficiency and specificity of the PCR used to amplify your tailed cDNA. *Taq* DNA polymerase from other suppliers may not function as well in the buffers provided in this system. The PCR protocol, section 3.8, is intended as a starting point. Optimal amplification parameters for your target gene may vary.

Obtaining longer 5' RACE products, *i.e.* greater than 1 kb, adds an additional challenge to the procedure. The 5' RACE System has been used successfully with eLONGase®, Enzyme Mix, an enzyme system designed for amplification of long templates, to obtain an increased yield of amplification product as well as substantially increased length of 5' RACE products. The principle barrier to long(er) 5' RACE lies in the specificity and efficiency of PCR. Critical success factors include primer design, PCR optimization, and a systematic experimental strategy that includes amplification of primary PCR using nested, gene-specific primers. Truncated products can yield informative sequence data that can be applied in additional 5' RACE experiments as one walks toward the 5'-end.

3.4 Advance Preparations

Please review the advance preparation guidelines discussed in this section prior to starting to work with the 5' RACE System. To achieve optimal results, it is also recommended that you review Chapter 2 before using this system.

3.4.1 Isolation of Total RNA

One of the most important factors affecting the synthesis of substantially full-length cDNA is the isolation of intact RNA. Therefore, it is important to optimize the isolation of RNA and to prevent introduction of RNases (19) and inhibitors of RT such as guanidinium salts, SDS and EDTA (20,52). The recommended method of RNA isolation is the guanidine isothiocyanate/acid-phenol method originally described by Chomzynski and Sacchi (21).The TRIzol® Reagent method (53) is an improvement of the original single-step method of Chomczynski and Sacchi and can be used for the preparation of RNA from as little as 10^3 cells (54). Total RNA isolated with TRIzol® Reagent is undegraded and essentially free of protein and DNA contamination. To maintain intact RNA, an RNase-free environment (see section 5.7) is critical.

Total RNA may contain small amounts of genomic DNA that may be amplified along with the target cDNA. The presence of this double-stranded DNA is not likely to cause problems in 5' RACE, since it is inefficiently tailed prior to amplification. As a precaution, however, perform a control experiment without RT. 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 to section 6.3. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary although incorporating this step may facilitate the detection of rare mRNA transcripts.

3.4.2 Design of the Gene-Specific Primers

Efficient and specific PCR is highly dependent on effective primer design. This is especially true for RACE applications since the PCR is carried out with only a single GSP. No method of primer design can guarantee successful amplification, so all primers must be tested in PCR before they can be pronounced “good”.

Primers for PCR (GSP2 AND GSP3): Effective primers form stable, highly specific duplexes with their target sequences, and are free of secondary structure such as hairpin loops and dimers (22,23,25,55). High stability, *i.e.,* G:C clamps, in the 5'- and central regions of the primer confer hybridization stability with the target sequence. Primers with unstable 3'-ends (ΔG > -9 kCal/mol) often result in higher specificity, since the potential to misprime at nontarget sites is reduced. Generally, this condition can be met by including no more than two G or C residues in the last five 3'-bases (22,23). Additionally, 3'-terminal complementarity should be minimized since primer-dimer artifacts may significantly reduce PCR efficiency. Therefore the nested amplification primer should be examined for dimer formation with the anchor primer, as well as itself. Computer algorithms that have been developed (22,56-58) often facilitate this analysis as well as secondary structure analysis.

The next important parameter for primers is the T_m (the temperature at which 50% of the primer and its complementary sequence are present in a duplex DNA molecule.) The T_m is necessary to establish an annealing temperature for PCR. The annealing temperature should be low enough to guarantee efficient annealing of the primer to the target, but high enough to minimize nonspecific binding. Since a single GSP is used in RACE, use as stringent an annealing temperature as possible to minimize amplification of nonspecific products. As a good starting point, choose an annealing temperature a few degrees below the estimated T_ms of the primer pair (see section 5.8 for 5' RACE primer T_ms). In practice, primers with T_ms between 60°C and 75°C usually can function effectively in 5' RACE. Ideally, the T_m of primers used for PCR should be closely matched (6).

Several methods are available to estimate the T_m of primers. These provide only estimates and the optimal annealing temperature must be established experimentally. When designing UDG cloning primers, consider dU residues as if they were dT residues for the calculations (see section 5.8.).

Primer for First Strand cDNA Synthesis (GSP1): For GSP1, follow the same rules used for PCR primer selection. The T_m should be appropriate for the relatively low temperature (42°C) of the cDNA synthesis reaction. A short primer (16 to 20 bases) facilitates efficient separation of GSP1 from cDNA product. Efficient recovery of cDNA from the S.N.A.P. column requires a product of at least 200 bases in length. Therefore, we recommend that GSP1 anneal to sequences located at least 300 bases from the mRNA 5'-end. Again, even well-designed primers may not function efficiently in priming first strand synthesis. For example, secondary structure of the mRNA at the annealing site may prevent efficient annealling or extension of the primer.

Primers for Subsequent Cloning: The user defined GSPs need to be compatible with the cloning method. Add the following to the 5'-end of the nested GSP:

for T4 DNA polymerase cloning 5'–CGA–3' (use with AUAP)

for restriction endonuclease cloning, an appropriate adapter region (59) is required.

It should be noted that in cases where only limited peptide sequence information is available, degenerate GSPs may be prepared. An alternative to the synthesis of degenerate primers is the substitution of dI residues at wobble-base positions (60-62).

3.4.3 1X Wash Buffer for S.N.A.P. Procedure

Prior to using the system for the first time, a 1X wash buffer must be prepared from the wash buffer concentrate.

1. Pipette 1 ml of the wash buffer concentrate into a 50-ml graduated cylinder.
2. Add 18 ml of distilled water and 21 ml of absolute ethanol. Mix thoroughly.
3. Transfer to an appropriate-sized glass bottle. Cap and store at 4°C.

3.4.4 70% Ethanol Wash for S.N.A.P. Procedure

1. Add 35 ml of absolute ethanol and 15 ml of distilled water to a 50-ml graduated cylinder.
2. Transfer to an appropriate-sized glass bottle. Cap and store at 4°C.

3.5 First Strand cDNA Synthesis

A detailed protocol for using the control RNA in 5' RACE is provided in Chapter 5. You may wish to use this protocol to familiarize yourself with the procedure before attempting 5' RACE with your sample.

This procedure is designed to convert specific RNA sequence(s) from a background of 1-5 µg of total RNA into first strand cDNA. In general, 100 to 500 ng of total RNA should provide sufficient material for the amplification of low copy messages by 5' RACE (6). Although poly(A)$^+$ RNA may be used in this protocol to enrich for very rare messages, this level of purity is typically not necessary. If you wish to use the control RNA, refer to Sections 5.2 and 5.5 for proper procedure.

Note: Extreme care should be taken to avoid contamination of samples by RNase. (See section 5.7)

1. Add the following to a 0.5-ml microcentrifuge tube (or thin-walled PCR tube if using a thermal cycler):

Component	Amount
GSP1	2.5 pmoles (~10 to 25 ng)
sample RNA	1-5 µg
DEPC-treated water (or sterile, distilled water)	sufficient for a final volume of 15.5 µl

Note: If you choose to use placental RNase inhibitor in the reaction, it should be added *after* the addition of DTT. Appropriate volume adjustments should be made in step 1. See precautionary notes regarding the use of RNase inhibitor proteins in Chapter 5.

2. Incubate the mixture 10 min at 70°C to denature RNA. Chill 1 min on ice. Collect the contents of the tube by brief centrifugation and add the following in the order given:

Component	Volume (µl)
10X PCR buffer	2.5
25 mM $MgCl_2$	2.5
10 mM dNTP mix	1
0.1 M DTT	2.5
final volume	8.5

Note: For high GC content mRNA, use the Alternate Protocol given in section 6.1 to help reduce interference by secondary structure of mRNA.

The final volume of step 1 and 2 is 24 µl.

3. Mix gently, and collect the reaction by brief centrifugation. Incubate for 1 min at 42°C.

Note: Keep enzymes on ice during the procedure. Mix, and quickly centrifuge each component before use.

4. Add 1 µl of SuperScript™ II RT. Mix gently and incubate for 50 min at 42°C. **Note**: 30 min incubation is usually sufficient for short (<4 kb) mRNAs. Longer transcripts require at least 50 min to synthesize enough cDNA for a consistent signal in long PCR.

   Final composition of the reaction:

   20 mM Tris-HCl (pH 8.4)
   50 mM KCl
   2.5 mM $MgCl_2$
   10 mM DTT
   100 nM cDNA primer (GSP1)
   400 µM each dATP, dCTP, dGTP, dTTP
   1-5 µg (~40 ng/µl) RNA
   200 units SuperScript™ II RT

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.

5. Incubate at 70°C for 15 min to terminate the reaction.
6. Centrifuge 10 to 20 s and place the reaction at 37°C.
7. Add 1 µl of RNase mix, mix gently but thoroughly, and incubate for 30 min at 37°C.
8. Collect the reaction by brief centrifugation and place on ice.

Note: Possible stopping points are noted in the protocol with the STOP icon.

The procedure may be stopped at this point and the reactions stored at -20°C. STOP

3.6 S.N.A.P. Column Purification of cDNA

1. Equilibrate the binding solution to room temperature.
2. For each sample to be purified, equilibrate ~100 µl of sterilized, distilled water at 65°C for use in step 9.
3. Add 120 µl of binding solution (6 M NaI) to the first strand reaction.
4. Transfer the cDNA/NaI solution to a S.N.A.P. column. Centrifuge at 13,000 *x g* for 20 s.
5. Remove the cartridge insert from the tube and transfer the flowthrough to a microcentrifuge tube. Save the solution until recovery of the cDNA is confirmed. Place the cartridge insert back into the tube.
6. Add 0.4 ml of **COLD** (4°C) 1X wash buffer to the spin cartridge. Centrifuge at 13,000 x *g* for 20 s. Discard the flowthrough. Repeat this wash step **three** additional times.
7. Wash the cartridge two times with 400 µl of **COLD** (4°C) 70% ethanol as described in step 6.
8. After removing the final 70% ethanol wash from the tube, centrifuge at 13,000 x *g* for 1 min.
9. Transfer the spin cartridge insert into a fresh sample recovery tube. Add 50 µl of sterilized, distilled, water (**preheated to 65°C**) to the spin cartridge. Centrifuge at 13,000 x *g* for 20 s to elute the cDNA. STOP

Note: 1X wash buffer and 70% ethanol must be prepared prior to use and used cold (see section 3.4.3).

Note: The binding solution must be at room temperature for efficient binding of the DNA.

Note: Failure to remove all the ethanol can result in poor recovery of the DNA.

Note: It is very important that the distilled water be at 65°C in order to maximize recovery of DNA.

3.7 TdT Tailing of cDNA

Variable amounts of purified cDNA from the S.N.A.P. column purification may be used in the TdT-tailing reaction. Factors, such as the amount of RNA used in the first strand reaction and relative abundance of the desired mRNA, should be considered. If desired, the cDNA pool may be concentrated by lyophilization and the entire contents used in the tailing reaction. To evaluate the specificity of the subsequent amplification reaction from the oligo-dC tail, inclusion of a control reaction that omits TdT is recommended.

1. Add the following components to each tube and mix gently:

Component	Volume (µl)
DEPC-treated water	6.5
5X tailing buffer	5.0
2 mM dCTP	2.5
S.N.A.P.-purified cDNA sample	10.0
final volume	24.0

Note: The 5X tailing buffer already contains $MgCl_2$. DO NOT add it separately.

2. Incubate for 2 to 3 min at 94°C. Chill 1 min on ice. Collect the contents of the tube by brief centrifugation and place on ice.
3. Add 1 µl TdT, mix gently, and incubate for 10 min at 37°C.

Note: The TdT has been optimized for 5' RACE. Increased amounts of TdT in the tailing reaction may inhibit PCR.

Final composition of the reaction:

10 mM Tris-HCl (pH 8.4)
25 mM KCl
1.5 mM $MgCl_2$
200 µM dCTP
cDNA
TdT

4. Heat inactivate the TdT for 10 min at 65°C. Collect the contents of the reaction by brief centrifugation and place on ice. STOP

3.8 PCR of dC-tailed cDNA

Tailed cDNA obtained from the preceding protocol may be amplified directly by PCR. Amplification of > 5 µl volumes of the tailing reaction requires appropriate adjustments for buffer, $MgCl_2$, and dNTP concentrations in the PCR.

1. Equilibrate the thermal cycler block to 94°C. In most cases, the "good start" procedure gives specific amplification products. For some target and primer sets, "hot start" has been reported to improve the specificity of the reaction (44,45).
2. Add the following to a 0.2 or 0.5-ml thin-wall PCR tube sitting on ice:

Note: It is very important to have the reaction mixture ice-cold to avoid nonspecific binding and extension of primers.

Component	Volume (µl)
sterilized, distilled water	31.5
10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	5.0
25 mM $MgCl_2$	3.0
10 mM dNTP mix	1.0
nested GSP2 (prepared as 10 µM solution)	2.0
Abridged Anchor Primer (10 µM)	2.0
dC-tailed cDNA	5.0
final volume	49.5

3. Add 0.5 µl of *Taq* DNA polymerase (5 units/µl) immediately before mixing.
4. Mix the contents of the tube (*Taq* DNA polymerase is added immediately before going into the thermal cycler) and overlay with 50 to 100 µl of mineral oil (if necessary).

   Final composition of the reaction:

   20 mM Tris-HCl (pH 8.4)
   50 mM KCl
   1.5 mM $MgCl_2$
   400 nM GSP2
   400 nM Abridged Anchor Primer
   200 µM each dATP, dCTP, dGTP, dTTP
   tailed cDNA
   2.5 units *Taq* DNA polymerase

5. Transfer tubes directly from ice to the thermal cycler preequilibrated to the initial denaturation temperature (94°C).
6. Perform 30 to 35 cycles of PCR. A typical cycling protocol for cDNA with ≤1 kb amplified region is:

   PAD: 94°C for 1-2 min
   Cycle:
   Denaturation: 94°C for 0.5-1 min
   Annealing of primers: 55°C for 0.5-1 min
   Primer extension: 72°C for 1-2 min
   Followed by:
   Final extension: 72°C, 5-7 min
   Indefinite hold: 5°C, until samples are removed.

7. Analyze 5-20 µl of 5' RACE products by agarose gel electrophoresis according to standard protocols, using appropriate size standards (19). Either TAE or TBE electrophoresis buffer may be used for the procedure. The volume of the sample used for analysis will depend on the volume and thickness of the sample well. If products will be extracted for reamplification, ultraviolet (UV) visualization of ethidium bromide-stained products should be performed using either a long wavelength (356-nm) UV or 302-nm wavelength source to minimize DNA nicking.

Note: The cycling protocol and annealing/extension temperatures may need to be optimized for each target sequence and/or thermal cycler. See section 2.2.7.

3.9 Nested Amplification

Often a single PCR of 25 to 35 cycles will not generate enough specific product to be detectable by ethidium bromide staining. Increasing the number of cycles performed during the PCR beyond 35 cycles may generate numerous nonspecific products and is not recommended. Similarly, high target levels may exacerbate amplification of nonspecific products during PCR and contribute to the production of a heterologous smear of PCR products. Instead, a dilution of the original PCR (0.1 to 0.05%) can be re-amplified (7) using the AUAP or UAP and a nested GSP (see figures 1 and 2, see Chapter 4 for more information). If there is insufficient sequence information to design a nested GSP, re-amplification of gel purified, size-selected PCR products using the UAP, or AUAP, and original GSP is useful for enriching specific 5' RACE products or installation of dUMP-cloning sequences for UDG cloning. Nested PCR may also be conveniently conducted using a plug of agarose from the gel analysis of the initial 5' RACE reaction (see section 6.5) (63).

1. Dilute a 5 µl aliquot of the primary PCR into 495 µl TE buffer [10 mM Tris-HCl, (pH 8.0), 1 mM EDTA].
2. Equilibrate the thermal cycler block to 94°C.
3. Add the following to a 0.2 or 0.5-ml thin-wall PCR tube sitting on ice.

Component	Volume (µl)
sterilized, distilled water	33.5
10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	5.0
25 mM $MgCl_2$	3.0
10 mM dNTP mix	1.0
nested GSP (prepared as 10 µM solution)	1.0
AUAP or UAP (10 µM)	1.0
dilution of primary PCR product	5.0
final volume	49.5

Note: It is very important to have the reaction mixture ice cold to avoid nonspecific binding and extension of primers.

Note: UDG cloning requires amplification with the UAP and appropriately designed nested GSP. Use the AUAP if using an archaeobacterial DNA polymerase or long PCR enzyme mixture.

4. Add 0.5 µl of *Taq* DNA polymerase (5 units/µl) immediately before mixing.
5. Mix the contents of the tube (*Taq* DNA polymerase is added immediately before going into the thermal cycler) and overlay with 50 to 100 µl of mineral oil (if necessary).

   Final composition of the reaction:

   20 mM Tris-HCl (pH 8.4)
   50 mM KCl
   1.5 mM $MgCl_2$
   200 nM nested GSP
   200 nM UAP or AUAP
   200 µM each dATP, dCTP, dGTP, dTTP
   diluted primary PCR product
   2.5 units *Taq* DNA polymerase

6. Transfer tubes directly from ice to the thermal cycler preequilibrated to the initial denaturation temperature.
7. Perform 30 to 35 cycles of PCR.
8. Analyze 5 to 20 µl of the amplified sample, using agarose gel electrophoresis, ethidium bromide staining, and the appropriate molecular size standards.

Note: Cycle number will depend on the amount and complexity of the target. As few as 5 cycles can be used to install dUMP-sequences for UDG cloning.

Interpretation of Results

4

Analysis of 5' RACE Results: Following PCR, products may be analyzed by agarose gel electrophoresis (1% to 2%) and ethidium bromide staining. Band intensity and size distribution of resulting products depends on the specificity of GSPs used for cDNA synthesis and PCR, the complexity and relative abundance of target cDNA, and the PCR conditions used. Amplification products may vary from a single specific band to multiple discrete products to a broad diffuse smear. Incomplete cDNA synthesis, aberrant priming of GSPs during first strand synthesis or PCR, mispriming by the anchor primer, as well as primer-dimer and other PCR artifacts may contribute to the complexity of products obtained by 5' RACE. Identification of specific product bands may be complicated by the presence of nonspecific products that are dependent on both reverse transcription and dC-tailing (36). If sequences are available for use as internal probes, it is strongly recommended that Southern blot analysis be used to identify specific product bands. Specific products can also be identified using a diagnostic restriction endonuclease digestion if the amplified cDNA sequence contains a known restriction site.

5' RACE Controls: Several controls may facilitate interpretation of results. Products that result from amplification of contaminating genomic DNA can be identified from control reactions that omit RT. An alternative approach is to include control reactions that use genomic DNA as target (6). Specificity of the anchor primer for the oligo-dC tail should be examined by performing amplification reactions with cDNA subjected to dC-tailing both in the presence and absence of TdT. Additional controls that amplify dC-tailed cDNA using each primer individually (either the Abridged Anchor Primer or GSP2) may be useful in identifying nonspecific products that result from mispriming.

Nested Amplification: 5' RACE of rare messages may require additional PCR using a nested GSP and either the UAP or AUAP (see section 3.9). Generally, a dilution of the original PCR is used as target. A nested primer is composed of sequences located 3' to the original primer (GSP2). For 5' RACE, this would be an antisense primer that anneals closer to the mRNA 5'-end. Purification of the original PCR product from primers and primer-dimer products may significantly improve the specificity and efficiency of nested amplification procedures. Ultimately, the 5' RACE procedure should produce a single prominent band on an agarose gel. This may require additional rounds of PCR using successively nested GSPs.

Decisions regarding the design of a nested primer will depend on the amount of sequence information available for the target of interest and on the results of the original amplification reaction. When performing 5' RACE with a nested primer, sequences specific for downstream cloning manipulations (see section 3.4.2) must be designed into the nested GSP.

5 Troubleshooting Guide

5.1 Testing the 5' RACE System Using the Control RNA and DNA

When using the 5' RACE System for the first time, we suggest performing an experiment using the control RNA to become familiar with the 5' RACE System procedure and to verify proper functioning of all components in the protocol, including your reagents and equipment for PCRs. The control RNA provided with the 5' RACE System is an 891-bp, *in vitro* transcribed RNA from the chloramphenicol acetyltransferase (CAT) gene that has been engineered to contain a 3' poly(A) tail. It may be used alone or added to your RNA preparation to test system performance in a background of heterologous nucleic acid. This is useful to test for the presence of contaminating nucleases. If desired, dilutions of the control RNA may be used to determine the sensitivity of the system or to model the abundance of the desired mRNA.

Note: Extreme care should be exercised when working with the control DNA so the 5' RACE reagents do not become contaminated. Use of aerosol barrier pipette tips significantly reduces the potential for the introduction of aerosols.

The control DNA was constructed by cloning the control 5' RACE product into pAMP1. Tailed cDNA was amplified using the 5' RACE Anchor Primer and control GSP2 containing additional UDG cloning sequences. The 4.8-kb pAMP1 5'RACE recombinant contains the oligo-dC tail sequence and may be used as a PCR positive control to verify the performance of the Abridged Anchor Primer, 5' RACE Anchor Primer, AUAP, UAP, or the control GSP3, in conjunction with the control GSP2. Alternately, it may be used to optimize PCR parameters with the Abridged Anchor Primer for your reaction conditions or thermal cycling device.

Two different PCRs are used to verify system performance. Conversion of first strand cDNA and recovery of cDNA after S.N.A.P. purification are assayed by a CAT cDNA-specific PCR using the control GSP3 and GSP2. Addition of the oligo-dC tail to purified control cDNA is assayed by PCR using the Abridged Anchor Primer and control GSP2. This strategy is depicted schematically in figure 6. Sequences for the control primers are presented in figure 4. The annealing sites for the control primers and resulting amplification products are shown in figure 5, panel 1. Note: The user may find it advantageous to adopt a similar RT-PCR strategy for their message and design an appropriate sense gene-specific primer (GSP3) to facilitate troubleshooting problems that may arise during 5' RACE with their message.

control GSP1	5'-TTG TAA TTC ATT AAG CAT TCT GCC-3'
control GSP2	5'-GAC ATG GAA GCC ATC ACA GAC-3'
control GSP3	5'-CGA CCG TTC AGC TGG ATA TTA C-3'

Figure 4. Sequences of the Control Primers.

Typical results for the procedure using the control RNA, both alone and in a background of 1 µg HeLa total RNA, are shown in figure 5, panel 2. A distinct 711-bp 5' RACE PCR product (solid arrow) should be visible by ethidium bromide staining. Other products, generally visible as faint bands or a diffuse smear, can result from spurious priming by GSP2 or the anchor primer, incomplete cDNA synthesis, and primer-dimer artifacts. If the control RNA is used in a background of heterologous RNA, nonspecific 5' RACE products, that are dependent on both RT and TdT, may be observed. This effect is illustrated by the 310-bp HeLa-derived 5' RACE product (open arrow). The presence of these nonspecific but genuine 5' RACE products is primarily a function of the specificity of the GSPs and emphasizes the need for characterization and enrichment of specific amplification products prior to cloning or sequencing.

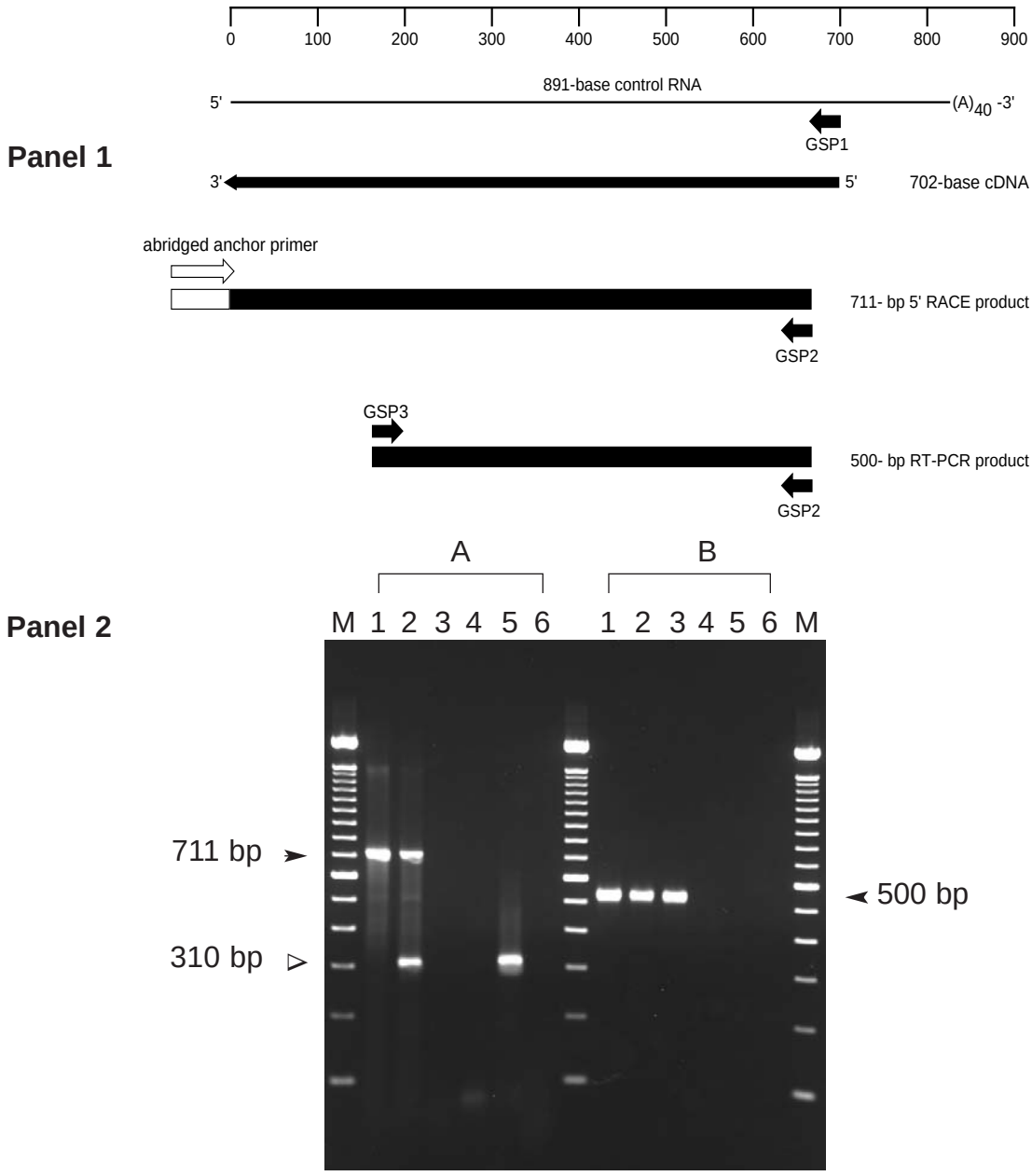

Figure 5. Agarose Gel Analysis of 5' RACE Products Using Control RNA. Panel 1. Schematic of the CAT RNA control system. Arrows designate 3' termini. The oligo-dC anchor is depicted as an open box. Panel 2. Control RNA (1 x 10^7 copies) was added to 1-µg aliquots of total RNA isolated from HeLa cells and subjected to 5' RACE amplified through 35 cycles with either (A) the anchor primer and control GSP2 or (B) control GSP2 and control GSP3 as described in this section. Reaction products were analyzed by gel electrophoresis in a 1.5% agarose gel (1X TBE) and stained with 0.5 µg/ml ethidium bromide. Lane 1, 1 x 10^7 copies of control RNA. Lanes 2 to 5 contain 1 µg of HeLa total RNA. Lanes 2-4 contain 1 x 10^7 copies of control RNA. Lane 3, no TdT control. Lane 4, no RT control. Lane 5, HeLa RNA only. Lane 6, water control. Target level is expressed as input values of RNA. Analyzed products represent 0.5% of target input given above. Lane M, GIBCO BRL 100 bp DNA Ladder. Open arrow, HeLa-derived 5' RACE product.

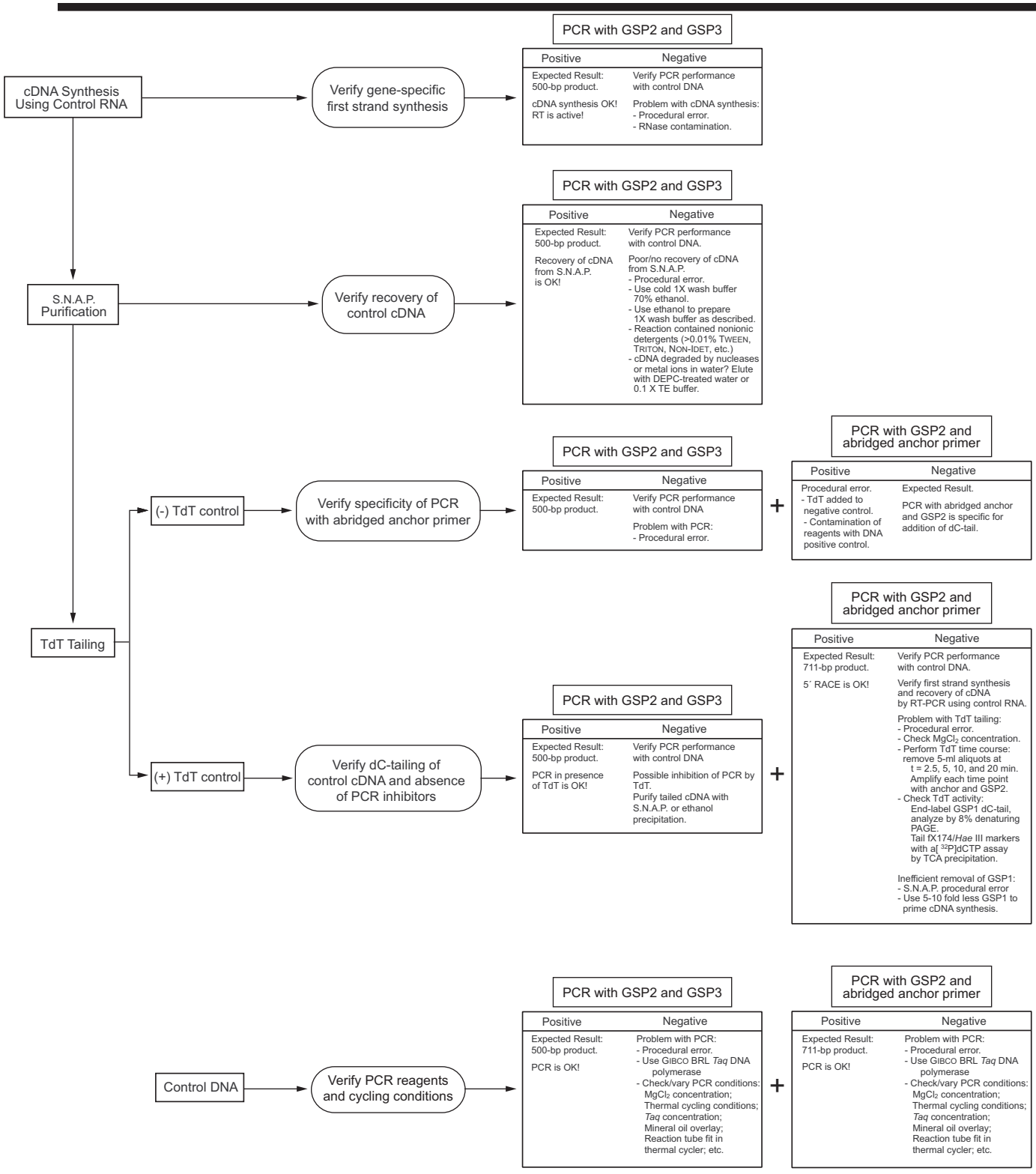

Figure 6: Flow Diagram for Troubleshooting the 5' RACE System Using the Control RNA and DNA.

5.2 Control First Strand cDNA Synthesis

The RNA template for this step is control RNA and the primer is control GSP 1. If you wish to test performance in a background of your RNA we suggest doing two first strand reactions: (Control RNA + Control GSP 1) and (Control RNA +Control GSP 1 in the presence of your sample RNA). Adjust the volume of DEPC-treated water appropriately so that the final volume in step 1 is still 15.5 µl**.**

Note: Mix and quickly centrifuge each component before use.

1. Add the following to a 0.5-ml microcentrifuge tube:

Component	Volume (µl)
Control GSP1 (1 µM)	2.5
control RNA	1.0
DEPC-treated water	12.0
final volume	15.5

2. Incubate the mixture 10 min at 70°C to denature RNA. Chill 1 min on ice. Collect the contents of the tube by brief centrifugation and add the following in the order given:

Component	Volume (µl)
10X PCR buffer	2.5
25 mM $MgCl_2$	2.5
10 mM dNTP mix	1.0
0.1 M DTT	2.5
final volume	8.5

   The combined total volume of steps 1 and 2 is 24 µl.

3. Mix gently, and collect the reaction by brief centrifugation. Incubate for 1 min at 42°C.
4. Add 1 µl of SuperScript™ II RT. Mix gently and incubate for 50 min at 42°C.
5. Incubate at 70°C for 15 min to terminate the reaction.
6. Centrifuge 10 to 20 s and place the reaction at 37°C.
7. Add 1 µl of RNase Mix, mix gently, and incubate for 30 min at 37°C.
8. Collect the reaction by brief centrifugation and place on ice.
9. Transfer a 2-µl aliquot of the control first strand cDNA to a 1.5-ml microcentrifuge tube containing 998 µl of TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]. Label the tube “A” and retain for later PCR.
10. Purify the remainder of the control DNA using the S.N.A.P. column. STOP

5.3 S.N.A.P. Column Purification of the Control cDNA

1. Equilibrate the binding solution to room temperature.

Note: The binding solution must be at room temperature for efficient binding of the DNA.

2. Equilibrate ~100 µl of distilled water at 65°C (for each sample to be purified) for use in step 9.
3. Add 120 µl of binding solution (6 M NaI) to the remainder of the control first strand reaction.
4. Transfer the cDNA/NaI solution to a S.N.A.P. column. Centrifuge at 13,000 x *g* for 20 s.
5. Remove the cartridge insert from the tube and transfer the flowthrough to a microcentrifuge tube. Save the solution until recovery of the cDNA is confirmed. Place the cartridge insert back into the tube.
6. Add 0.4 ml of **COLD** (4°C) 1X wash buffer to the spin cartridge. Centrifuge at 13,000 x *g* for 20 s. Discard the flowthrough. Repeat this wash step **three** additional times.

Note: 1X wash buffer and 70% ethanol must be prepared prior to use and used cold (see section 3.4.3 and 3.4.4).

7. Wash the cartridge two times with 400 µl of **COLD** (4°C) 70% ethanol as described in step 6.

Note: Failure to remove all the ethanol can result in poor recovery of the DNA.

Note: It is very important that the distilled water be at 65°C in order to maximize recovery.

8. After removing the final 70% ethanol wash from the tube, centrifuge at 13,000 x *g* for 1 min.
9. Transfer the spin cartridge insert into a fresh sample recovery tube. Add 50 µl of distilled water (**preheated to 65°C**) to the spin cartridge. Centrifuge at 13,000 x *g* for 20 s to elute the cDNA.
10. Transfer a 5-µl aliquot of the purified control first strand cDNA to a 0.5-ml microcentrifuge tube containing 495 µl of TE buffer. Label the tube "B" and retain for later PCR. STOP

5.4 TdT Tailing of the Control First Strand cDNA

A control reaction that omits TdT is included because amplification of cDNA from this control reaction can provide important data for troubleshooting RACE. For example, inclusion of a control reaction that omits TdT will help evaluate the specificity of the amplification reaction from the oligo-dC tail. Label two 0.5-ml microcentrifuge tubes "C" and "D", respectively.

1. In order to more sensitively test the efficiency of the tailing reaction, prepare a 100-fold dilution of the purified control cDNA. Add 1 µl of the cDNA to a 0.5-ml tube containing 99 µl of sterilized, distilled water.
2. Add the following components to each tube:

Component	Volume (µl)
DEPC-treated water (or sterile, distilled water)	6.5
5X Tailing Buffer	5.0
2 mM dCTP	2.5
S.N.A.P.-purified control cDNA (1:100 dilution)	10.0
final volume	24

Note: The 5X tailing buffer has $MgCl_2$ already present. DO NOT add it separately.

3. Incubate for 2 to 3 min at 94°C. Chill 1 min on ice. Collect the contents of the tube by brief centrifugation and place on ice.
4. Add 1 µl TdT to tube(s) "C".
5. Add 1 µl DEPC-treated water to tube(s) "D".
6. Gently mix the contents of each reaction and incubate for 10 min at 37°C.
7. Heat inactivate the TdT for 10 min at 65°C. Collect the contents of the reaction by brief centrifugation and place on ice. Retain each reaction for later PCR. STOP

5.5 PCR of cDNA, Tailed cDNA and Control DNA

The PCR of the various aliquots from each step and the tailed cDNA product(s) is done using two sets of primers in two separate PCR mixes. An additional reaction that contains 5 µl of the control DNA as target is used as a positive control to verify PCR. One set of primers will be control GSP2 and control GSP3. Specific amplification of the control first strand cDNA product, or control DNA, results in a prominent 500-bp band when analyzing products by agarose gel electrophoresis and ethidium bromide staining. The other set of primers will be control GSP2 and Abridged Anchor Primer and should result in a prominent 711-bp product (and no product from the no TdT control) for both the tailed cDNA and the control DNA. The sample templates to be amplified are:

Note: Extreme care should be exercised when working with the control DNA so the 5' RACE reagents do not become contaminated. Use of aerosol barrier pipet tips significantly reduces the potential for the introduction of aerosols.

"A" cDNA from the first strand reaction (checks the efficiency of the cDNA synthesis reaction)

"B" S.N.A.P. eluate sample: (checks the recovery of cDNA from S.N.A.P. column)

"C" Tailed cDNA: (actual 5' RACE product; checks the efficiency of tailing)

"D" No TdT control: (checks the specificity of amplification of the tailed product, and presence of inhibitors of PCR)

Control DNA: (checks PCR with both primer sets)

You may have more than one tube for each set, depending on the number of first

strand reactions you have.

1. Make two PCR mixes, one for each primer set:
   Mix I = Control GSP2 and GSP3
   Mix II = Control GSP2 and Abridged Anchor Primer

Add the following components on ice to an appropriately sized sterile tube, *e.g.* 1.5-ml microcentrifuge tube. Make enough mix for n+2 reactions where n = number of template samples to be amplified. (Minimum value for n is 5: A + B +C + D + Control DNA). Volumes given in the table are for 50 µl PCRs.

Mix I (GSP2 and GSP3)

Component	Volume (µl) per reaction	Volume (µl) 7X mix n=5	Volume (µl) 12X mix n=10
DEPC-treated water	33.5	234.5	402.0
10X reaction buffer	5.0	35.0	60.0
25 mM $MgCl_2$	3.0	21.0	36.0
10 mM dNTP mix	1.0	7.0.	12.0
control GSP2 (10 µM)	1.0	7.0	12.0
control GSP3 (10 µM)	1.0	7.0	12.0
Taq DNA Polymerase (5 units/µl)	0.5	3.5	6.0
final volume	45.0	315.0	540.0

Note: Add the *Taq* DNA polymerase just before you are ready to pipet the mix into the PCR tubes containing template.

Mix II (GSP2 and Abridged Anchor Primer)

Component	Volume (µl) per reaction	Volume (µl) 7X mix n=5	Volume (µl) 12X mix n=10
DEPC-treated water	31.5	220.5	378.0
10X reaction buffer	5.0	35.0	60.0
25 mM $MgCl_2$	3.0	21.0	36.0
10 mM dNTP mix	1.0	7.0	12.0
control GSP2 (10 µM)	2.0	14.0	24.0
Abridged Anchor Primer	2.0	14.0	24.0
Taq DNA Polymerase 5 units/(µl)	0.5	3.5	6.0
final volume	45.0	315.0	540.0

2. Pipet 5 µl of template samples (A,B,C,D, and control DNA) into two sets appropriately labeled thin-walled 0.2 or 0.5 ml PCR tubes. There will be one set for each PCR mix.
3. Add 45 µl of Mix I to one set of tubes and add 45 µl of Mix II to the second set of tubes.
4. Mix the contents of the tubes and overlay with 50 to 100 µl of mineral oil (if necessary).
5. Equilibrate the thermal cycler block to 94°C.
6. Transfer tubes directly from ice to the hot thermal cycler.
7. Perform 35 cycles of PCR:
   Denature 94°C for 1 min
   Anneal 63°C for 30 s
   Extend 72°C for 2 min

Note: Temperatures may vary slightly among thermal cyclers. The annealing temperature can be adjusted (61° - 65°C) to compensate for this variation.

8. Incubate the reaction for 10 min at 72°C following the last cycle of PCR, then maintain reactions at 4°C.
9. Analyze 5-20 µl of 5' RACE products by agarose gel electrophoresis according to standard protocols, using appropriate size standards (19). Either TAE or TBE electrophoresis buffer may be used for the procedure. The volume of the sample used for analysis will dependent on the volume and thickness of the sample well. If products will be extracted for reamplification, ultraviolet (UV) visualization of ethidium bromide-stained products should be performed using either a long wavelength (356-nm) UV or 302-nm wavelength source to minimize DNA nicking.

Troubleshooting Guide

5.6. General Troubleshooting Guidelines for the 5' RACE System

Problem	Possible Cause	Suggested Remedy
No bands after electrophoretic analysis of amplified products	5' RACE amplification product may be present but in too low a concentration for detection by ethidium bromide staining	Perform Southern blot analysis of amplifiction products using internal sequence as probe.
		Amplify agarose gel purified material of expected size range using the AUAP or UAP, and user's GSP2.
		Perform nested primer amplification from either purified amplification products, dilution of original PCR (0.05%), or agarose gel plug using the UAP,or AUAP, and user's nested GSP.
		For further discussion, see Chapter 4, Interpretation of Results and section 6.5. Refer to Chapter 6 for specific protocols.
	Procedural error in first strand cDNA synthesis, purification of cDNA product, TdT tailing, or PCR	Use the control RNA to verify conversion of first strand product, recovery following purification and dC tailing.
		Design sense GSP from available 5'-mRNA sequence data. Verify first strand conversion of desired message by PCR using two gene-specific primers.
	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.
		Note: Inhibitors of RT include sodium dodecyl sulfate (SDS), EDTA, guanidinium salts, and glycerol. Inhibitors of M-MLV-RT include sodium pyrophosphate and spermidine. SuperScript™ RT is inhibited 50% by 0.0025% SDS, 1 mM EDTA , 15 mM guanidine isothiocyanate, 17% DMSO, 50% glycerol, 5% formamide, 4 µg/ml heparin and 4 mg/ml glycogen (52).
		Test for the presence of inhibitors by mixing 1 µg of control RNA with 1 µg of sample RNA and comparing yields of first strand cDNA or by PCR of control band.
	Target mRNA has secondary structure that interferes with annealing of GSP1	Redesign GSP1 and/or use section 6.1.1.
	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 6.1.1.

Problem	Possible Cause	Suggested Remedy
No bands after electrophoretic analysis of amplified products	RNase contamination	Perform first strand synthesis with the control RNA both alone and added to sample RNA to determine if RNase is present. Assay for control cDNA by PCR using control GSP2 and GSP3. See section 5.1.
		Maintain aseptic conditions to prevent RNase contamination. See section 5.7.
		Use placental RNase inhibitor during first strand cDNA synthesis.
		Note: Placental RNase inhibitor requires sulfhydryl reagents for maximal RNase binding activity (19). Always add RNase inhibitor to reactions after the addition of dithiothreitol. Treatments which denature the protein, such as high temperature incubation, exposure to oxidizing conditions, or repeated freezing and thawing, can release RNases initially bound by the inhibitor. These RNases may subsequently degrade RNA preparations in downstream procedures.
	Inefficient tailing of cDNA	Perform TdT time course: remove 5-µl aliquots at t = 2.5, 5, 10, and 20 min. Amplify each time point using abridged anchor primer and GSP2.
	cDNA did not tail due to strong secondary structure of 3'-end	Denature cDNA prior to tailing by incubation at 94°C for 2 to 3 min, then quick chill on ice. Perform the tailing reaction on ice for 1 h to keep 3'-end denatured and available for TdT.
		Alternatively, use of cosolvents *e.g.* DMSO (≤ 20%) has been been found to increase efficiency of tailing.
	Inhibition of PCR by TdT	Purify tailed cDNA by ethanol precipitation in the presence of inert carrier, *i.e.* glycogen, and 2.5 M NH_4OAc.
	Polysaccharides and small RNAs coprecipitate with mRNA	Ethanol precipitate the RNA preparation; treat the pellet as described in section 6.4.
	Polymerase used in PCR was from an archaeobacterium and dUMP primers were used	Use abridged anchor primer, or AUAP, and non-dUMP-containing GSP in PCR using eLONGase® reagents or an archaeobacterial polymerase.
		Use *Taq* DNA polymerase for PCR with the UAP and dUMP-containing primers.

Troubleshooting Guide

Problem	Possible Cause	Suggested Remedy
Agarose gel analysis shows strong primer-dimer product, but no visible gene-specific product	Inefficient removal of GSP1 during S.N.A.P. procedure	Design GSP1 according to the guidelines discussed in section 3.4.2. Reduce the concentration of GSP1 used in first strand synthesis. Remove dC-tailed GSP1 with an additional S.N.A.P. purification after TdT tailing and second strand synthesis as described in section 6.2. **Note:** dCTP and the 5' RACE anchor primer may be substituted for dATP and the 3' RACE system adapter primer described in this protocol. Substitute dU for dT in GSP1. Degrade dU-GSP1 following cDNA synthesis with UDG. Purify cDNA by ethanol precipitation in the presence of inert carrier, *i.e.* glycogen, and NH_4OAc. **Note:** This technique may be useful for 5' RACE of short cDNAs that bind poorly to the S.N.A.P. column.
	Primer-dimer product between the anchor primer and GSP2	Minimize nonspecific annealing of primers by initiation of PCR at an elevated temperature (75°C to 80°C). See section 3.9.
Unexpected bands after electrophoretic analysis of "nested" amplification products	Contamination by genomic DNA Spurious priming in the PCR	Pretreat RNA as described in section 6.3. Vary the parameters of the PCR: increase annealing temperature, decrease annealing time, reduce $MgCl_2$ concentration, etc. (24-26, 38, 42, 43). Minimize nonspecific annealing of primers by initiation of PCR at an elevated temperature (75°C to 80°C). See section 3.9. Perform PCR control amplifications as discussed in Chapter 4.
Gene-specific 5' RACE products appear as a smear. Unable to isolate full length 5' RACE product	RNA preparation is degraded or of poor quality	Isolate new preparation of total RNA.
Absence of discrete product bands. 5' RACE products appear as a heterogeneous smear	Potentially, a normal result	Identfiy gene-specific products by Southern blot hybridization. Enrich for specific products by reamplification of gel-purified material or nested primer amplification.

Problem	Possible Cause	Suggested Remedy
Absence of discrete product bands. 5' RACE products appear as a heterogeneous smear	High levels of poly $(A)^+$ RNA (≥1 µg) used for first strand synthesis may contribute to nonspecific products. RNA-primed, (GSP1 independent) cDNA may compete for TdT and also for anchor primer during the PCR.	Reduce target level or optimize tailing conditions. Perform TdT time course: remove 5-µl aliquots at t = 2.5, 5, 10, and 20 min. Amplify each time point using abridged anchor primer and GSP2.
5' RACE product does not correspond to known mRNA sequence. Product is dependent on TdT addition of dC-tail	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 6.1.1. Use cosolvents *e.g.*, 5-10% DMSO or 10-20% glycerol compatible with the RT in first strand reaction (52) to help eliminate secondary structure while maintaining RT activity.
5' RACE product does not correspond to full length mRNA sequence. Product is not dependent on TdT addition of dC-tail	Internal mispriming by the anchor primer at C-rich sequence	Deoxyinosine-containing anchor primer may not be suitable for amplification of C-rich cDNA. Use alternate 5' RACE strategy. See section 6.2.
5' RACE product does not correspond to known mRNA sequence.	Aberrant priming of gene-specific primers	Perform control amplification reactions as discussed in Chapter 4, 5' RACE Controls. Verify identity of 5' RACE product by hybridization or diagnostic restriction endonuclease cleavage prior to cloning or sequencing. Increase the specificity of GSP1 by increasing the temperature of first strand synthesis (up to 50°C). Optimize PCR conditions to maximize specificity of GSP2 and anchor primer. Increase annealing temperature, decrease annealing time, decrease $MgCl_2$ concentration, *etc*. Design new GSP1 and/or GSP2. See section 3.4.2.

Troubleshooting Guide

Problem	Possible Cause	Suggested Remedy
Poor cloning efficiency	Inefficient ligation	Increase the incubation time in the ligation reaction; decrease the temperature.
		Remove dNTPs prior to ligation.
		Use a ligation-free cloning method such as the CloneAmp system.
	Poor restriction endonuclease digestion due to residual bound *Taq* DNA Polymerase	Treat PCR products with proteinase K (64). See section 6.6.
	Fill-in of overhangs by residual *Taq* DNA polymerase	Extract PCR products with phenol:chloroform and purify by ethanol precipitation or treat with proteinase K (see above) before restriction endonuclease digestion.
	Clone is unstable in host cells	Try different strain of bacterial cells such as Stbl2™ competent cells.

5.7 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, except sterilized pipettes, centrifuge tubes, culture tubes, or any similar labware that is explicitly stated to be sterile. Wear latex gloves for all manipulations involving RNA.
2. Dedicate a separate set of automatic pipettes 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 them with sterilized, distilled water, and autoclave them.
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 filter them to 0.2 µm using sterilized, disposable filterware.

Note: Buffers containing primary amines (such as Tris) cannot be effectively treated with DEPC.

6. If all else fails, most aqueous buffer solutions can be treated with 0.01% (v/v) DEPC and autoclaved.
7. Use aerosol-resistant pipet tips.

5.8 T_m Values for 5' RACE and Control Primers

Oligonucleotide primer T_ms can vary widely depending on the method of calculation. A comparison of T_m determinations (°C) for the 5' RACE Anchor Primer, Abridged Anchor Primer, UAP, AUAP, control GSP2, and control GSP3 using the 2(AT) + 4(GC), %GC, and nearest neighbor methods are summarized in table 1. See figure 2 for sequences of the 5' RACE primers, and figure 4 for sequences of the control primers. The nearest neighbor analysis method (65) relies not only on the quantity of each base, but on the primary sequence of the DNA. Comparison of experimentally derived T_ms to that predicted by the available estimates for a number of oligonucleotides has found the nearest neighbor method to be the most accurate (22,66). However, the precise reaction conditions should be considered in the calculation. Other methods are either limited by the size of the oligonucleotide for which they are valid or derived for salt and temperature conditions that are different from those for PCR (see legend, table 1, and 19,67-70).

Troubleshooting Guide

Table 1. Comparison of T_m Values for 5' RACE and Control Primers calculated by different methods

Primer	2(AT) + 4(GC)[1]	%GC[2]	NN[3]	NN_{PCR}[4]
GI Anchor Region	52	72	60.5	66.6
Anchor Primer	150	90.3	93.9	90.1
Abridged Anchor Primer	118	90.0	94.4	92.5
UAP	98	83.8	75.8	75.0
AUAP	66	79.3	67.8	71.5
GSP2	64	75.3	64.5	69.0
GSP3	66	75.4	66.5	69.2

T_m values (°C) for 5' RACE and control primers were calculated using OLIGO™ 5.0 according to the formulae given below. The GI Anchor Region comprises the 16-base deoxyinosine-containing sequence at the 3'-end of the Abridged Anchor or Anchor Primer. The efficiency and specificity of annealing of this sequence to the dC-tail in the early PCR cycles is critical to the overall efficiency of 5' RACE. The disparity of T_m values obtained using different formulae highlights the importance of empirical determination of optimal annealing temperature for any given primer pair.

1) The T_m of many oligonucleotides can be estimated by assigning 2°C to each A and T and 4°C to each G and C (63,64).

   2(AT) + 4(GC): 2 x (numbers of A's + number of T's) + 4 x (number of C's + number of G's).

   This estimate was derived for hybridizations in high salt concentration (1M) and is only valid for oligonucleotides less than 18 bases in length (35).

2) Another method for estimating the T_m is based on the percent GC in the oligonucleotide (35,65,66). The formula was developed for hybridization to DNA immobilized on a solid support using molecules under 100 bases in length in conditions of high salt.

   %GC: $Tm = 81.5 + 16.6 \log[salt^+] + 0.41[\%GC] - (675 / \text{number of bases in oligonucleotide})$

3) NN: nearest neighbor T_m (19) calculated using second order hybridization kinetics, 100 pM primer 1 M Na^+.

4) NN_{PCR}: nearest neighbor T_m (19) calculated for standard PCR conditions using first order hybridization kinetics, 200 nM primer, 50 mM Na^+, 0.7 mM free Mg^{++} (effective $[Na^+]$ = 155.6 mM).

Additional Protocols

6

6.1 5' RACE of G:C Rich cDNA

The 5' RACE System protocol provides a rapid and reliable solution for capturing 5'-end mRNA sequences. The physical properties of each message, however, will influence the efficacy of the 5' RACE procedure. Some messages may require alternative strategies. Poly(dC) tracts which may be present in G:C rich cDNA can serve as effective priming sites for the deoxyinosine-containing anchor primer. PCR of such a template results in truncated products. In this case, a modification of the dATP-tailing method described by Frohman *et al*. (1, 6) is recommended.

Classical 5' RACE methodologies use the PCR process to install adapter priming sequences and amplify the cDNA from the homopolymer tail. However, PCR from homopolymer sequences, and especially oligo(dT), can be highly problematic. Because of these difficulties, a second-strand synthesis procedure is used to install the adapter priming site prior to PCR. An oligo(dT)-containing primer such as the 3' RACE Adapter Primer (AP) is used to prime second-strand synthesis (figure 7). Following purification, 5'-end sequences are amplified using the AUAP, or UAP, and a user-defined, nested gene-specific primer. This single-cycle installation, followed by a purification step to remove excess oligo(dT) primer, removes the potential for internal mispriming by the primer during each PCR cycle, and results in a higher proportion of “full-length” amplification products.

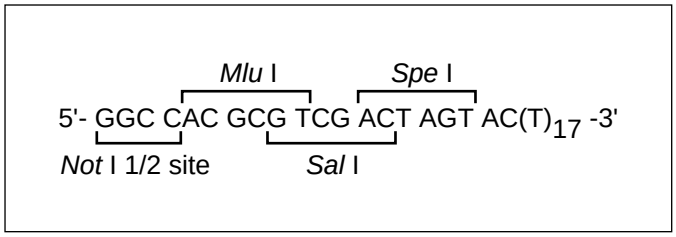

Figure 7. Sequence of the 3' RACE Adapter Primer.

6.1.1 Alternative Protocol for First Strand cDNA Synthesis of Transcripts with High GC Content

Problems with 5' RACE due to secondary structure of the target mRNA often can 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 50°C before adding it to the primer and RNA. Use of a thermal cycler 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
0.5 mM each dATP, dCTP, dGTP, and dTTP
100 nM GSP1
1-5 µg total RNA
200 units SuperScript™ II RT
Reaction volume: 50 µl

Additional Protocols

1. Mix and briefly centrifuge each component before use.
2. Combine 1-5 µg of total or 50-500 ng of poly(A)$^+$ - RNA and DEPC-treated water to a final volume of 20 µl in a 0.2 or 0.5-ml microcentrifuge tube (or thin-walled PCR tube if using a thermal cycler).
3. Add 5 µl of the 1 µM GSP1 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. For n samples make n+1 reaction mix. The table below shows volumes for n=1.

Component	Volume (µl) 1 reaction	Volume (µl) n +1
DEPC-treated water	6.5	13.0
10X PCR buffer	5.0	10.0
25 mM $MgCl_2$	5.0	10.0
10 mM dNTP mix	2.5	5.0
0.1 M DTT	5.0	10.0
final volume	24.0	48.0

6. Prewarm the reaction mix to 50°C immediately before use.
7. Add 24 µl of the prewarmed reaction mixture and 1µl (200 units) of SuperScript™ II to the RNA/primer mixture to each sample tube, mix gently and continue to incubate at 50°C for 50 min.
8. Terminate the reactions by incubating the tubes at 70°C for 15 min. Chill on ice.
9. Collect the reactions by brief centrifugation. Add 1 µl of RNase mix to each tube and incubate for 30 min at 37°C.
10. Add 225 µl of binding solution (6 M NaI) and proceed to section 3.6.

6.2 dA-Tailing of cDNA

Variable amounts of purified cDNA may be used in the TdT-tailing reaction depending on the application. Factors such as the amount of RNA used in the first strand reaction and relative abundance of the desired mRNA should be considered. If desired, the cDNA pool may be concentrated by lyophilization and the entire contents used in the tailing reaction. Inclusion of a control reaction that omits TdT is recommended.

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

Component	Volume (µl)
DEPC-treated water	6.5
5X Tailing Buffer	5.0
2 mM dATP	2.5
S.N.A.P.-purified cDNA sample	10.0
final volume	24.0

2. Incubate at 94°C for 2 to 3 min. Chill for 1 min on ice. Collect the contents of the tube by brief centrifugation and place on ice.
3. Add 1 µl of TdT, mix gently, and incubate at 37°C for 10 min.

   Final composition of the reaction:

   10 mM Tris-HCl (pH 8.4)
   25 mM KCl
   1.5 mM $MgCl_2$
   200 µM dATP
   TdT
4. Heat-inactivate the TdT for 10 min at 65°C. Collect the contents of the reaction by brief centrifugation and place on ice.

6.2.1 Second-Strand Synthesis

This protocol has been adapted for enzymes supplied with the 5' RACE system. In general, SuperScript™ will utilize a DNA template with equivalent efficacy to that of an RNA template. However, the user may want to consider the use of other DNA-directed DNA polymerases and appropriately adjust reaction conditions (49).

1. Add the following components to the TdT reaction (25 µl):

Component	Volume (µl)
sterile, distilled water	8.0
10X PCR buffer[200 mM Tris-HCl (pH 8.4), 500 mM KCl]	4.0
25 mM $MgCl_2$	3.5
0.1 M DTT	5.0
10 mM dNTP mix	2.5
3' RACE AP (10 µM)	1.0
final volume	24.0

2. Equilibrate the reaction at 50°C for 2 min.
3. Add 1 µl of SuperScript™ II RT.
4. Incubate the reaction for 50 min at 50°C.
5. Add 225 µl of binding solution (6 M NaI) to the second strand reaction. Purify second-strand product using a S.N.A.P. column as described in section 3.6. Elute second-strand product using warm (65°C) TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA].

6.2.2 PCR of cDNA

1. Equilibrate the thermal cycler block to 94°C. In most cases, the “good start” procedure gives specific amplification products. For some target and primer sets, “hot start” has been reported to improve the specificity of the reaction (44,45).

Note: Do not use dUMP-containing primers with any archaeobacterial polymerase (*Pfu* DNA Polymerase, *Pwo* DNA Polymerase, etc.) or long PCR enzyme mixture.

2. Add the following to a 0.2 or 0.5-ml thin-wall PCR tube sitting on ice:

Component	Volume (µl)
sterilized, distilled water	28.5
10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	5.0
25 mM $MgCl_2$	3.0
10 mM dNTP mix	1.0
nested GSP2 (prepared as 10 µM solution)	1.0
AUAP (10 µM) or UAP (10 µM)	1.0
purified second-strand cDNA	10.0
final volume	49.5

Note: It is very important to have the reaction mixture ice-cold to avoid non-specific binding and extension of primers.

3. Add 0.5 µl of *Taq* DNA polymerase (5 units/µl) immediately before mixing.
4. Mix the contents of the tube and overlay with 50 to 100 µl of mineral oil (if necessary).

   Final composition of the reaction:

   20 mM Tris-HCl (pH 8.4)
   50 mM KCl
   1.5 mM $MgCl_2$
   200 nM GSP2
   200 nM UAP/AUAP
   200 µM each dATP, dCTP, dGTP, dTTP
   2.5 units *Taq* DNA polymerase

Note: The cycling protocol and annealing/extension temperatures may need to be optimized for each target sequence and/or thermal cycler. See section 2.2.7.

5. Transfer tubes directly from ice to the thermal cycler preequilibrated to the initial denaturation temperature (94°C).
6. Perform 30 to 35 cycles of PCR.

Additional Protocols

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.

7. Analyze 5 to 20 µl of the amplified sample, using agarose gel electrophoresis, ethidium bromide staining, and the appropriate molecular size standards. If the positive control RNA was used, a prominent 711-bp band will be visible.

6.3 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. Amplification Grade DNase I has been extensively purified to remove trace ribonuclease activities.

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

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

2. Incubate at room temperature for 15 min.
3. Terminate the reaction by addition of 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.

6.4 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 5S RNA) during the RNA isolation procedure. You may be able to recover highly purified mRNA using the following protocol adapted from Sambrook *et al*. (19).

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 x *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 NaOAc (pH 5.2) and 2 volumes of absolute (100%) ethanol (–20°C). Place the tube at –20°C for 30 min. Centrifuge at 14,000 x *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 x *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 section 3.5

6.5 Amplification from an Agarose Plug

This procedure is intended to provide added specificity to the amplification procedure prior to cloning or sequencing (1,18) 5' RACE products. The following protocol, adapted from Buck and Axel (63), should be performed after completion of nested amplifcation (section 3.9), but prior to performing the cloning procedures described below.

1. Analyze 5-20 µl of 5' RACE products by agarose gel electrophoresis according to standard protocols, using appropriate size standards (19). Either TAE or TBE electrophoresis buffer may be used for the procedure. The volume of the sample used for analysis will depend on the volume and thickness of the sample well. Ultraviolet (UV) visualization of ethidium bromide-stained products should be performed using either a long wave UV or 302-nm wavelength source to minimize DNA nicking if products will be extracted for reamplification.
2. Using a capillary pipette, remove a small (1-5 µl) agarose plug from the area of the gel containing the band of interest.
3. Transfer the agarose plug to a 0.5-ml microcentrifuge tube.
4. Amplify as described in section 3.9. Added specificity may be gained from the procedure by performing a nested PCR using a second nested GSP (composed of cDNA sequences 3' of the original GSP2) and the UAP. If sequence data for a nested GSP is unavailable, re-amplification of the agarose plug using GSP2 and the UAP should still provide sufficient enrichment of specific 5' RACE products for cloning.

Note: Do not carry over any mineral oil from the PCR.

6.6 Proteinase K Treatment of 5' RACE Product

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 (42).

1. Extract the sample once with phenol:chloroform (1:1) and once with chloroform.
2. Add 0.1 volume of proteinase K solution [500 µg/ml proteinase K, 10 mM Tris-HCI (pH 7.5), 1 mM EDTA].
3. Incubate for 30 min at 37°C, and then for 10 min at 65°C.
4. Extract once with phenol:chloroform (1:1) and once with chloroform.
5. Transfer the aqueous layer to a fresh 1.5-ml microcentrifuge tube.
6. Ethanol-precipitate by addition of 0.1 volume of 3M NaOAc (pH 5.2) and 2 volumes of absolute ethanol (–20°C). Place tube at –20°C for 30 min.
7. Centrifuge at 13,000 x *g* for 30 min. at 4°C. Remove supernatant, rinse pellet with 70% ethanol and air-dry the pellet at room temperature.
8. Resuspend the sample in 10 µl of TE buffer before advancing to cloning procedures.

6.7 Cloning Using T4 DNA Polymerase

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 (51). In this procedure, PCR products from the primary PCR with the Abridged Anchor Primer, or nested amplification reaction primed with the AUAP, are treated with T4 DNA polymerase to generate a *Not* I 5' overhang. Similarly, the user may design a site into their GSP2 (see section 3.4.2.). Figure 9 depicts this procedure using an *Acc* I-compatible end-derived from the GSP2. Alternative restriction endonucleases that yield ends compatible with either *Not* I or *Acc* I are shown in table 2.

Additional Protocols

Table 2. Protruding Ends Compatible with *Acc* I or *Not* I.

Protruding End	Unambiguous			Ambiguous
5´-CG	*Aci* I	*Hin*P1 I	*Nsp*V	*Acc* I
		Hpa II	*Psp*1406 I	
		Mae II	*Taq* I	
	*Bsa*H I	*Msp* I		
	Cla I	*Nar* I		
5´-GGCC	*Eae* I	*Not* I	*Xma* III	

1. Following amplification with the Abridged Anchor Primer, or nested PCR with the AUAP, 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]	5.0
dTTP (1 mM)	5.0
dATP (1 mM)	5.0
T4 DNA polymerase (1 unit/µl)	1.0

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 (19) and resuspend in 5 µl of TE Buffer.
4. Prepare the vector for ligation by digestion with *Acc* I and *Not* I (19). 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 using 1 to 2 µl of the vector-ligated DNA.

Note: For additional information about ligation reactions using T4 DNA ligase, see reference 74.

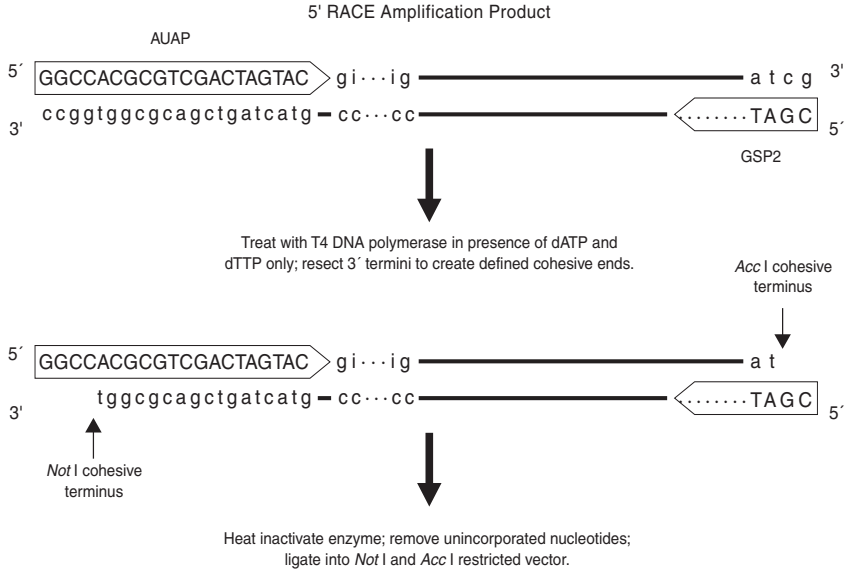

Figure 9: Cloning of 5' RACE Products using T4 DNA Polymerase.

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7

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