Marathon-Ready™ cDNA User Manual

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I. Introduction & Protocol Overview

Marathon-Ready™ cDNAs are premade "libraries" of adaptor-ligated ds cDNA ready for use as templates in Marathon® cDNA amplification—a method for performing both 5'- and 3'-RACE from the same template (Chenchik et al., 1995; 1996; Figure 1). Marathon technology has been cited in more than 140 research articles. (For a complete list of citations, please visit **www.clontech.com**.) The method is made possible by Clontech's patented suppression PCR technology (Siebert et al., 1995) and other innovations in the design of the Marathon Adaptor (see Appendix B). When compared to conventional kits used for 5'-RACE, Marathon RACE reactions are more efficient and reproducible with considerably less smearing and fewer false bands. Because the protocol uses enzyme mixes designed for long-distance PCR (LD PCR; Barnes, 1994; Cheng et al., 1994), Marathon RACE reactions are capable of amplifying much larger templates than can be amplified with conventional RACE methods. Furthermore, given the lower rate of misincorporation observed with LD PCR, Marathon RACE products should have higher fidelity to the sequence of the original RNA.

Marathon cDNA amplification is a flexible tool. Many researchers use this method—and Marathon-Ready cDNAs—in place of conventional RACE kits to amplify just the 5' or 3' end of a particular cDNA. Others perform both 5'- and 3'-RACE, and many then go on to clone full-length cDNAs using one of the two methods described in the latter part of the protocol. In many cases, researchers obtain full-length cDNAs without ever constructing or screening a cDNA library.

Examples of 5'- and 3'-RACE using the Control G3PDH Primers with different Marathon-Ready cDNAs are given in Figure 2A. G3PDH Primers are provided with each Marathon-Ready cDNA and are an important positive control in Marathon RACE experiments. Figure 2B gives 5' and 3' Marathon RACE results for human actin and human transferrin receptor (TFR) genes, while Figure 4 gives several examples of large (5–9 kb), full-length cDNAs generated by Marathon cDNA amplification. Table I at the end of this introduction gives several other examples of Marathon-amplified cDNAs.

The only requirement for Marathon cDNA amplification is that you use an LD PCR-compatible polymerase mix and know at least 23–28 nucleotides (nt) of sequence information in order to design gene-specific primers (GSPs) for the 5'- and 3'-RACE reactions. (Additional sequence information will facilitate analysis of your RACE products.) This minimal requirement for sequence information means that Marathon cDNA amplification is well suited for characterizing RNAs identified as expressed sequence tags (ESTs; Sikela & Auffray, 1993) or by methods such as differential display (Liang & Pardee, 1992) or RNA fingerprinting (Welsh et al., 1992; Welsh et al., 1994). In particular, Marathon cDNA amplification is an excellent tool for cloning full-length cDNAs corresponding to differentially expressed mRNAs identified with the Clontech PCR-Select™ cDNA Subtraction Kit (Cat. No. 637401) or the Delta® Differential Display Kit (Cat. No. 937405). Marathon-Ready cDNAs can also be used to obtain full-length clones of partial cDNAs obtained through library screening.

I. Introduction & Protocol Overview continued

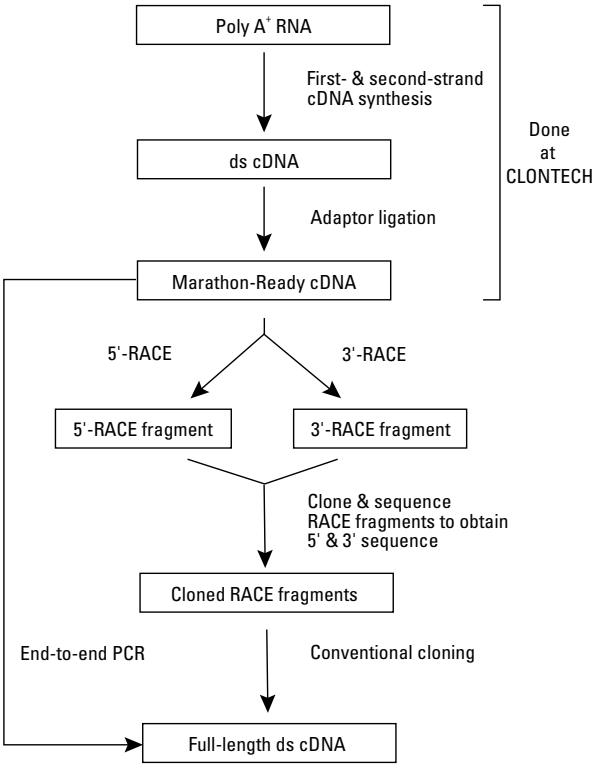

Figure 1. Overview of Marathon procedure. A more detailed flow chart of the Marathon procedure can be found in Appendix A. The cloned 5'- & 3'-RACE fragments can be subcloned using a restriction site in the overlapping region and a site in the cloning vector to obtain both parts of the complete cDNA to obtain a full-length cDNA. Or you can sequence the 5' end of the 5' product and the 3' end of the 3' product to obtain additional sequence information. This additional sequence information can be used to design 5' and 3' gene-specific primers to use in LD PCR with the Marathon-Ready cDNA to obtain the full-length cDNA.

Marathon-Ready cDNAs also have several nonRACE applications. For example, you can use them to obtain full-length copies of published cDNAs—simply design flanking 5' and 3' GSPs from the published sequence and amplify the cDNA directly from an appropriate Marathon-Ready cDNA. Once you make the necessary primers, you will have the cDNA you need in just a day and be ready for your next experiment. Marathon-Ready cDNAs can also be used instead of poly A^+ RNAs (and cDNA synthesis) in many RT-PCR experiments, including characterization of tissue-specific patterns of gene expression and characterization of polymorphic mRNAs and multigene families (Chenchik & Siebert, 1996).

I. Introduction & Protocol Overview continued

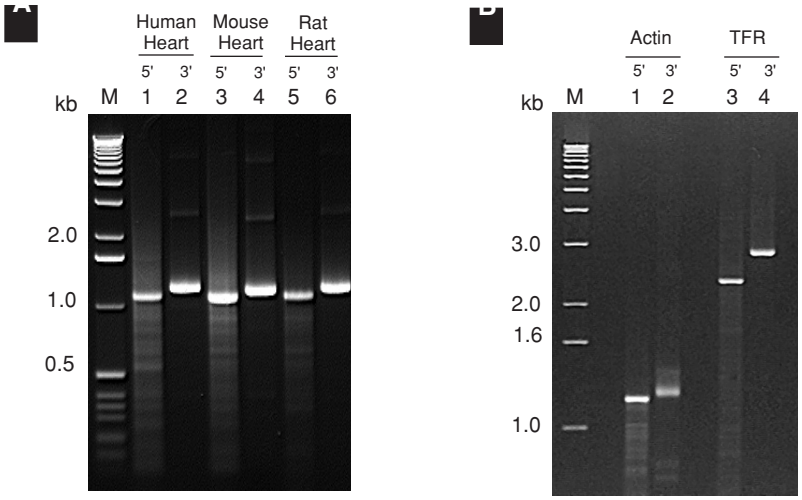

Figure 2. Typical Marathon 5'- & 3'-RACE results. Panel A: Results using the Control G3PDH Primers. The G3PDH Primers anneal to a portion of the G3PDH gene that is conserved among human, rat, and mouse. Lanes 1 & 2: 5'- & 3'-RACE from Human Heart Marathon-Ready cDNA. Lanes 3 & 4: 5'- & 3'-RACE from Mouse Heart Marathon-Ready cDNA. Lanes 5 & 6: 5'- & 3'-RACE from Rat Heart Marathon-Ready cDNA. Lanes M: DNA size markers. **Panel B: Results using Actin and TFR primers.** Lane 1: Negative control reaction primed with AP1 alone. Lane 1: 1.2-kb 5'-RACE product generated with actin primers. Lane 2: 1.3-kb 3'-RACE product generated with actin primers. Lane 3: 2.6-kb 5'-RACE product generated with TFR primers. Lane 4: 2.9-kb 3'-RACE product generated with TFR primers. Lane M: DNA size markers.

Overview of the Marathon cDNA amplification protocol

An overview of Marathon cDNA amplification is presented in Figure 1. For Marathon-Ready cDNAs, cDNA synthesis and adaptor ligation are performed at Clontech 5'- and/or 3'-RACE can be completed in one day. The time required to characterize the RACE products and to generate the full-length cDNA can vary greatly depending on the particular target. As you read the following description and set up your experiments, you may find it useful to refer to the detailed flow chart (Figure 6; Appendix A) and the diagram of the Marathon cDNA template and primers (Figure 4; Section V).

- **Primer Design** (Section V)

  Gene-Specific Primers (GSPs) should be:
  - 23–28 nt
  - 50–70% GC
  - $T_m \geq 65°C$; best results are obtained if $T_m \geq 70°C$ (enables the use of touchdown PCR)

I. Introduction & Protocol Overview continued

You will need to design gene-specific primers for the 5'- and/or 3'-RACE reactions (GSP1 and GSP2, respectively). Nested primers (NGSP1 and NGSP2) will facilitate analysis of your RACE products, as described in Section VIII, and can be used for nested RACE PCR if necessary. Primer design is discussed in detail in Section V, and Figure 4 shows the relationship of primers and template used in Marathon RACE reactions.

- **Construction of Marathon-Ready cDNAs**

  Construction of Marathon-Ready cDNAs at Clontech begins with cDNA synthesis from high-quality poly A^+ RNA. First-strand synthesis uses a modified lock-docking oligo(dT) primer with two degenerate nucleotide positions at the 3' end. These nucleotides position the primer at the start of the poly-A tail and thus eliminate the 3' heterogeneity inherent with conventional oligo(dT) priming (Chenchik et al., 1994; Borson et al., 1994). The carefully optimized Marathon reaction conditions give consistently high yields and size distributions of first-strand cDNA synthesis.

  Second-strand synthesis is performed according to the method of Gubler & Hoffmann (1983) with a cocktail of E. coli DNA polymerase I, RNase H, and E. coli DNA ligase. The conditions and enzyme concentrations for second-strand cDNA synthesis have been optimized to produce high yields of ds cDNA. Typically less than 15% of the second-strand syntheses are primed by hairpin-loop formation.

  Following creation of blunt ends with T4 DNA polymerase, the ds cDNA is ligated to the Marathon cDNA Adaptor. (See Appendix B for information on the design and the sequence of the Marathon cDNA Adaptor.) This adaptor is partially double-stranded and is phosphorylated at the 5' end to facilitate blunt-end ligation of the adaptor to both ends of the ds cDNA by T4 DNA ligase. Blunt-end ligation is more efficient than homopolymeric tailing or ligation of an adaptor to single-stranded cDNA by T4 RNA ligase, so a higher percentage of the resulting cDNA molecules contain the terminal structure required for RACE. This is a primary reason why Marathon 5'-RACE reactions are more efficient and reproducible than 5'-RACE methods based on tailing or ss ligation (Frohman et al., 1988; Dumas et al., 1991; Harvey & Darlison, 1991). Finally, the adaptor-ligated cDNA is diluted to the appropriate concentration and is packaged as Marathon-Ready cDNA.

- **Marathon RACE Reactions** (Section VI)

  Each tube of Marathon-Ready cDNA is essentially an uncloned library of adaptor-ligated ds cDNA. Enough material is provided for you to perform 5' and 3' Marathon RACE for many different genes simply by using different gene-specific primers. Marathon RACE reactions should be performed with a 50X polymerase mix containing a combination of DNA polymerases suitable for long-distance PCR. We recommend Clontech's Advantage® 2 Polymerase Mix (Cat. Nos. 639201, 639202), which was specifically developed for LD

I. Introduction & Protocol Overview continued

PCR using cDNA templates and is the polymerase mix used to optimize the protocols in this User Manual. This 50X mix contains TITANIUM™ Taq DNA Polymerase—a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR (Kellogg et al., 1994)—and a minor amount of a proofreading polymerase. The Advantage® 2 Polymerase Mix is also available in the Advantage 2 PCR Kit (Cat. Nos. 639206, 639207).

We recommend that you first perform Marathon RACE reactions using a LD PCR polymerase mix as stated above. If your cDNA of interest has high GC content, you can use the Advantage-GC 2 Polymerase Mix (Cat. No. 639114) or PCR Kit (Cat. Nos. 639119, 639120) for subsequent analysis. For applications in which the highest fidelity product (< 3.5 kb) is desired, use the Advantage-HF 2 PCR Kit (Cat. Nos. 639123, 639124). For more information, see Section X (Troubleshooting RACE Reactions).

Performing 5'- and 3'-RACE from the same template is made possible by the design of the Marathon® cDNA Adaptor, which is described in detail in Appendix B. Both 5'- and 3'-RACE reactions are primed with an internal gene-specific primer (GSP) and the Marathon Adaptor Primer (AP1). The adaptor-ligated cDNA does not contain a binding site for AP1. During the first round of thermal cycling, the GSP is extended to the end of the adaptor, creating an AP1 binding site at the 5' (or 3') terminus of the cDNA. In subsequent cycles, both AP1 and the GSP can bind, allowing exponential amplification of the cDNA of interest. Nonspecific products are greatly reduced because the AP1 binding cannot be created on the general population of cDNA molecules, which also lack binding sites for the GSPs.

- **Characterization of RACE Products** (Section VII)

  Before generating the full-length cDNA, we strongly recommend that you characterize your RACE products to confirm that you have amplified the desired target. This can be done by one or more of the following: (1) comparing PCR products obtained using GSP1 and AP1 to product generated with NGSP1 and AP1; (2) probing a Southern blot of your PCR products with an internal gene-specific probe (e.g., labeled NGSP1); and (3) cloning and sequencing your RACE products. In general, we recommend that you obtain at least some sequence information.

  Careful characterization of your RACE products at this point can prevent confusion and wasted effort in your subsequent experiments, even when both RACE reactions produce single major products. For example, when we cloned and sequenced the “single” RACE product observed with actin primers in Figure 2, we found that this single band actually contained cDNAs from three different actin genes. Characterization is essential at this point if you have multiple RACE products or suspect that you are working with a member of a multigene family.

I. Introduction & Protocol Overview continued

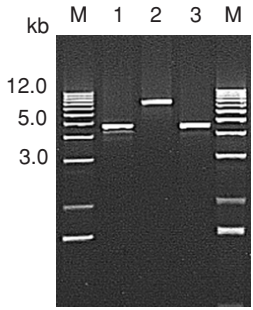

Figure 3. Examples of large full-length cDNAs generated by end-to-end PCR of Marathon-Ready cDNAs. Full-length cDNAs were generated using an Advantage Polymerase Mix and 5' and 3' GSPs obtained by partial sequencing of 5' and 3' Marathon RACE products. The template for Lanes 1 & 2 was Human Skeletal Muscle Marathon-Ready cDNA (Cat. No. 639313); the template for Lane 3 was Human Placenta Marathon-Ready cDNA (Cat. No. 639311). Lane 1: Full-length ILGFR1 cDNA (5.0 kb; 32 cycles; 7-min extension). Lane 2: Full-length ILGFR2 (8.9 kb; 28 cycles; 10-min extension). Lane 3: Full-length TFR cDNA (5.0 kb; 25 cycles; 7-min extension). M: 1-kb DNA ladder.

- Options for Generating Full-Length cDNA

After RACE products have been characterized by partial or complete sequencing, the full-length cDNA can be generated by one of two methods:

(1) Generation of Full-Length cDNA by PCR (Section VIII)
A standard LD-PCR reaction with GSPs from the 5' and 3' ends of your gene can be used to amplify the full-length cDNA from the Marathon-Ready cDNA. The sequence of the 5' and 3' GSPs is usually obtained by sequencing the 5' end of the 5'-RACE product and the 3' end of the 3'-RACE product. Figure 3 shows three examples of full-length cDNAs generated by end-to-end PCR.

(2) Generation of Full-Length cDNA by Cloning (Section IX)
Cloned, overlapping 5'- and 3'- RACE fragments can be used to generate the full-length cDNA using a restriction site in the overlapping region (if one exists) and sites in the Marathon Adaptor and/or cDNA Synthesis Primer.

In general, PCR using flanking GSPs is more direct and less subject to complications or artifacts. With cloning, there is a slight chance of joining 5' and 3' cDNA fragments derived from two different transcripts; this could occur with two different forms of a polymorphic RNA or with transcripts from a multigene family. In contrast, with end-to-end PCR, the 5' and 3' GSPs will amplify the full length of a single cDNA, so there is no chance of generating a hybrid cDNA. Virtually all cDNAs are within the range of LD PCR.

No method of cDNA synthesis can guarantee a full-length cDNA, particularly at the 5' end. Determining the true 5' end requires some combination of RNase protection assays, primer extension assays, and cDNA or genomic sequence information. Many Marathon cDNAs include the complete 5' end of the cDNA; however, the action of T4 DNA polymerase may remove some nucleotides (typically 1–20) from the 5' end of the cDNA. Severe secondary structure may also block the action of RT and/or Taq DNA polymerase in some instances. In our experience, Marathon RACE products and full-length cDNAs compare favorably in this regard with cDNAs obtained by conventional RACE

I. Introduction & Protocol Overview continued

or from libraries. To obtain the maximum possible amount of 5' sequence, we recommend that you sequence the 5' ends of 5–10 separate clones of the 5'-RACE product.

TABLE I: EXAMPLES OF cDNAs AMPLIFIED BY MARATHON RACE

Gene [a]	Poly A+ RNA	Abundance of mRNA	Size of amplified cDNA (kb)
Transferrin receptor	Placental	Low–med.	5.1
Actin	Placental	High	1.9
Rat lung-specific protein	Lung	High	2.1
Inducible nitric oxide synthase	Placental	Low–med.	4.1
GCSF receptor	Thymus	Low–med.	2.9
Insulin-like growth factor receptor type 1	Thymus	Low–med.	5.1
Insulin-like growth factor receptor type 2	Thymus	Low–med.	8.9
HIV-induced genes 1 & 2 identified by differential display	HIV-infected macrophage	Med.	0.7, 1.7[b]
Interferon-α	Placental	Low	2.7
G3PDH	Placental	High	1.5
β_2-microglobulin	Placental	High	0.6

[a] Human unless otherwise indicated.

[b] When conventional RACE failed to produce the 5' ends of these two genes, Marathon RACE was used.

II. List of Components

Store all components at –20°C.

The following reagents are sufficient for 30 Marathon® RACE reactions:

- 150 µl Marathon-Ready cDNA (~ 0.1 ng/µl in Tricine-EDTA buffer)
- 50 µl Adaptor Primer 1 (AP1; 10 µM)
- 50 µl Nested Adaptor Primer 2 (AP2; 10 µM)
- 20 µl Control 5'-RACE G3PDH Primer (10 µM)
- 20 µl Control 3'-RACE G3PDH Primer (10 µM)

Note: See Figure 8 (Appendix B) for the sequences of the Marathon cDNA Adaptor and Primers. The T_m's of AP1 and AP2 are 71°C and 77°C, respectively. However, only 22 of the 27 nt in AP1 bind the adaptor during PCR, so the effective T_m of AP1 is actually several degrees lower. The lower effective T_m of AP1 is one reason touchdown PCR works well with Marathon RACE reactions.

III. Additional Materials Required

The following reagents are required but not supplied:

- **Advantage 2 Polymerase Mix (50X)**

  You will need a Taq-based 50X polymerase mix suitable for LD PCR. A single polymerase will not give satisfactory results in most experiments. The Marathon cDNA amplification protocol has been optimized with Clontech's Advantage 2 Polymerase Mix (Cat. Nos. 639201, 639202). This LD PCR enzyme mix has been specifically developed for amplification of cDNA templates of all sizes. The Advantage 2 polymerase mix contains TITANIUM™ Taq DNA Polymerase—a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus TaqStart™ Antibody to provide automatic hot-start PCR (Kellogg et al., 1994)—and a minor amount of a proofreading polymerase. Advantage 2 Polymerase Mix is also available in the Advantage 2 PCR Kit (Cat. Nos. 639206, 639207).

- **10X PCR reaction buffer** (Included with Advantage 2 Polymerase Mix and in the Advantage 2 PCR Kit)

  Use the 10X reaction buffer supplied with your source of native or truncated Taq DNA polymerase in all reactions that call for 10X PCR buffer.

- **50X dNTP Mix** (10 mM each of dATP, dCTP, dGTP, and dTTP; 1X concentration: 0.2 mM. Included in the Advantage 2 PCR Kit)

- **0.5-ml PCR reaction tubes** We recommend Applied Biosystems GeneAmp 0.5-ml reaction tubes (Cat. Nos. N801-0737 or N801-0180).

- **Tricine-EDTA buffer** (recommended instead of TE for dissolving and/or diluting PCR templates.) (10 mM Tricine KOH [pH 9.2] + 0.1 mM EDTA)

IV. General Considerations of Marathon® cDNA Amplification

- The cycling parameters throughout this protocol have been optimized using a Applied Biosystems DNA Thermal Cycler 480 or GeneAmp PCR Systems 2400/9600, the Advantage 2 PCR Kit and Control G3PDH Primers. The optimal cycling parameters may vary with different 50X polymerase mixes, templates, gene-specific primers, and thermal cyclers. For example, the efficiency of RACE PCR depends on the abundance of the mRNA of interest in the poly A^+ RNA sample and different primers will have different optimal annealing/extension temperatures.
- **You must use some form of hot start in the 5'-RACE and 3'-RACE PCR.** The following protocols have been optimized using TaqStart Antibody (Kellogg et al., 1994) in the 50X polymerase mix. Hot start can also be performed manually (D'Aquila et al., 1991) or using wax beads (Chou et al., 1992).
- We recommend the Tricine-EDTA buffer for resuspending and diluting your DNA samples throughout this protocol. Tricine buffers maintain their pH at high temperature better than Tris-based buffers. Tris-based buffers can lead to low pH conditions that can degrade DNA.
- When resuspending pellets or mixing reactions, gently pipet the solution up and down or tap the bottom of the tube, and then spin briefly to bring all contents to the bottom of the tube.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last. Make sure that the enzyme is thoroughly mixed with the reaction mixture by gently pipetting the mixture up and down.
- Use the recommended amounts of enzyme. These amounts have been carefully optimized for the Marathon amplification protocol and reagents.
- Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing of this reagent. For more information, see Molecular Cloning: A Laboratory Manual by Sambrook and Russell, (2001).

V. Primer Design

A. Primer Sequence

Gene-Specific Primers (GSPs) should be:

- 23–28 nt
- 50–70% GC
- $T_m \geq 65°C$; best results are obtained if $T_m \geq 70°C$ (enables the use of touchdown PCR)

The relationship of the primers used in the Marathon RACE reactions to the template and resulting RACE products is shown in detail in Figure 4. For the complete Marathon protocol, you will need at least two GSPs: an antisense primer for the 5'-RACE PCR and a sense primer for the 3'-RACE PCR. If you are doing only 5'- or 3'-RACE, you will only need one GSP. All primers should be 23–28 nt long; there is generally no advantage to using primers longer than 30 nt. The primers shown in Figure 4 will create overlapping 5'- and 3'-RACE products, which, if a suitable restriction site is located in the region of overlap, can subsequently be joined by restriction digestion and ligation to create the full-length cDNA. Using primers designed to give overlapping RACE products also means that these primers can be used together to generate the overlapping fragment if the overlap is at least 100–200 bp. This provides a useful control for PCR. However, it is not absolutely necessary to use primers that give overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. Additionally, the primers themselves can overlap (i.e., be complementary).

GSPs should have GC content of 50–70% and a T_m of at least 65°C; whenever possible the T_m should be 70°C or higher as determined by nearest neighbor analysis (Freier et al., 1986). In our experience, longer primers with annealing temperatures of at least 70°C give more robust amplification in RACE, particularly from difficult samples. T_m's of 70°C or higher allow you to use touchdown PCR (Section C below). T_m's of GSP1 and GSP2 can be calculated or determined experimentally by doing PCR at different temperatures. Avoid using self-complementary primer sequences which can fold back and form intramolecular hydrogen bonds. Similarly, avoid using primers that have complementarity to the Marathon AP1 Primer, particularly in the 3' ends.

For further assistance with primer design and T_m calculation, consult the world wide web at: **http://alces.med.umn.edu/VGC.html**

Note: Do not incorporate restriction sites into the 5' ends of the 5' and 3' GSPs. In our experience, the presence of these extra sequences can lead to increased background.

V. Primer Design continued

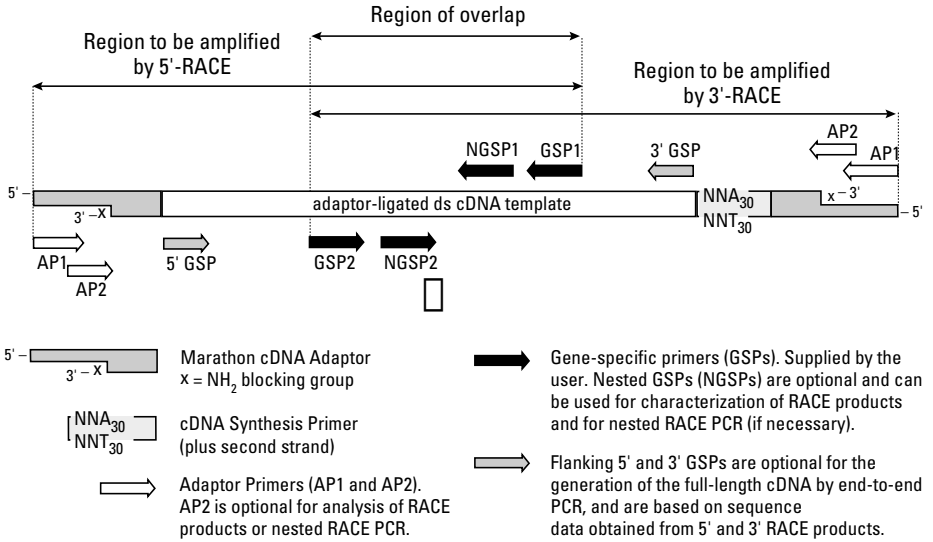

Figure 4. The template and primers used in Marathon RACE reactions. cDNA synthesis and adaptor ligation create a population of cDNAs with the structure depicted above. This population is the Marathon-Ready cDNA which is essentially a library of uncloned ds cDNA from which you can amplify many different cDNAs using different sets of GSPs. If the GSPs create overlapping 5'- and 3'-RACE products, the products can subsequently be joined by restriction digestion and ligation to create the full-length cDNA (provided there are suitable restriction sites in the region of overlap). Also, GSPs designed to give overlapping RACE products can be used together to generate a useful control for PCR. However, it is not absolutely necessary to use primers that give overlapping fragments. The specificity of Marathon RACE reactions is greatly enhanced by the absence of an AP1 binding site on the Marathon-Ready cDNAs. This site is created on the cDNA of interest by extension from the GSP during the first RACE cycle. The amine group on the Marathon cDNA Adaptor blocks extension of the 3' end of the adaptor-ligated ds cDNAs, and thus prevents formation of an AP1 binding site on the general population of cDNAs.

B. Location of Primer Sequences within a Gene

We have had good success using the Marathon-Ready cDNA to amplify 5' and 3' cDNA fragments that extend up to 6.5 kb from the GSP sites. If possible, choose your primers so that the 5'- and 3'-RACE products will be 3 kb or less.

If designing primers that produce overlapping 5'- and 3'-RACE products, it is helpful to design the gene-specific PCR primers so that the overlap between GSP1 and GSP2 is at least 100–200 bases. In this way, a stretch of known sequence will be incorporated into the amplified 5' and 3' fragments and can be used to verify that the correct gene was amplified.

V. Primer Design continued

C. Touchdown PCR

We have found that touchdown PCR (Don et al., 1991; Roux, 1995) significantly improves the specificity of Marathon RACE PCR. Touchdown PCR involves using an annealing/extension temperature, during the initial PCR cycles, that is several degrees higher than the T_m of the AP1 Primer. Although primer annealing (and amplification) is less efficient at this higher temperature, it is also much more specific. The higher temperature also enhances the suppression PCR effect with AP1 (see Appendix B). If the T_m of your GSP >70°C, only gene-specific synthesis occurs during these initial cycles, and this allows a critical amount of gene-specific product to accumulate. The annealing/extension temperature is then reduced to the AP1 Primer T_m for the remaining PCR cycles, permitting efficient, exponential amplification of the gene-specific template.

As noted above, we recommend using primers with T_m's > 70°C to allow you to use the touchdown cycling programs in the protocol. (Nontouchdown cycling programs are also included for use with primers with T_m's < 70°C.)

D. Nested Primers

Do not use nested PCR in your initial experiments. The AP1 Primer and a GSP will usually generate a good RACE product with a low level of nonspecific background. However, a Southern blot or nested GSPs (NGSP1 and NGFP2; see Figure 4) are very useful for characterizing your RACE products. Furthermore, nested PCR may be necessary in some cases where the level of background or nonspecific amplification in the 5'- or 3'-RACE reaction is too high with a single GSP. In nested PCR, a primary amplification is performed with the outer primers and, if a smear appears, then an aliquot of the primary PCR product is reamplified using the inner primers. The Marathon protocols include optional steps indicating where nested primers can be used. The nested AP2 Primer provided with the kit can be used for both 5'- and 3'-RACE.

Nested primers should be designed according to the guidelines discussed above. If possible, nested primers should not overlap (like AP1 and AP2); if they must overlap (due to limited sequence information), the 3' end of the inner primer should have as much unique sequence as possible. Be sure that nested primers do not contain sequences that can hybridize to the outer gene-specific primer, particularly at their 3' ends.

V. Primer Design continued

E. Controls to Test Gene-Specific Primers (GSPs)

When performing the RACE reactions, we recommend that you perform the following controls to test your GSPs:

1. Negative control with single primers

   Include a negative control containing only the appropriate GSP (antisense primer for 5'-RACE; sense primer for 3'-RACE) and the adaptor-ligated ds cDNA. The GSPs should not give any bands in the absence of the AP1 Primer. If significant amounts of product are seen with this control, it may be necessary to alter the cycling parameters, use nested primers, or redesign your original primer.

2. Positive control with both GSPs (only possible if using primers that produce overlapping 5' and 3' fragments)

   To confirm that your gene is expressed in the Marathon-Ready cDNA that you have chosen and that your GSPs work as intended, set up a positive control containing both GSPs. This should produce a band corresponding to the combined length of your GSPs and the overlap between the primers (i.e., the region of overlap between the 5'- and 3'-RACE products). If this band is missing, then 1) your gene may not be expressed in the tissue from which the Marathon-Ready cDNA was prepared; 2) you made to optimize the cycling parameters for your GSPs; or 3) you may to design new GSPs.

VI. Rapid Amplification of cDNA Ends (RACE)

The procedure below describes how to set up the RACE PCR reactions. We recommend that you also perform a positive control 5'-RACE using the positive control primer and AP1. Although the Nested Adaptor Primer (AP2) is provided, nested PCR is generally not necessary in Marathon RACE reactions.

All Marathon RACE reactions have been optimized with Clontech's Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR. If you choose not to use Advantage 2 Polymerase Mix, you must use your polymerase mix with some form of hot start PCR (i.e., TaqStart Antibody, wax beads, or manual hot start) to minimize background in your RACE reactions.

1. Prepare enough PCR master mix for all of the PCR reactions plus one additional tube. The same master mix can be used for both 5'- and 3'-RACE reactions. For each 50-µl reaction, mix the following reagents:

36 µl	H_2O
5 µl	10X cDNA PCR Reaction Buffer
1 µl	dNTP mix (10 mM)
1 µl	Advantage 2 Polymerase Mix (50X)
43 µl	Final volume

   Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

2. **For 5'-RACE:** prepare PCR reactions as shown in Table II.

   For 3'-RACE: prepare PCR reactions as shown in Table III.

   Add the components in the order shown in 0.5-ml PCR tubes.

3. Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

   Note: This is not necessary if you are using a hot-lid thermal cycler.

(Protocol continues on page 20.)

TABLE II: SETTING UP 5'-RACE REACTIONS

Test Tube No.: / Description: / Component	1 Experimental Sample	2 G3PDH Pos. Ctrl [a]	3 GSP 1 + 2 Pos. Ctrl [b]	4 AP1 only Neg. Ctrl [c]	5 GSP1 only Neg. Ctrl [c]
Marathon-Ready cDNA	5 µl	5 µl	5 µl	5 µl	5 µl
AP1 Primer (10 µM)	1 µl	1 µl	---	1 µl	---
GSP1 (antisense primer; 10 µM)	1 µl	---	1 µl	---	1 µl
GSP2 (sense primer; 10 µM)	---	---	1 µl	---	---
Control 5'-RACE G3PDH Primer (10 µM)	---	1 µl	---	---	---
H_2O	---	---	---	1 µl	1 µl
Master Mix	43 µl	43 µl	43 µl	43 µl	43 µl
Final volume	50 µl	50 µl	50 µl	50 µl	50 µl

Notes

Controls 3–5 provide useful information if your initial RACE reactions do not give the expected results. For a complete discussion of controls, see Section X (Troubleshooting RACE Reactions).

[a] The G3PDH Positive Control should generate a 1.09-kb product.

[b] If performing both 5'- and 3'-RACE with overlapping products, the "GSP 1+2" Positive Control is useful to confirm that the gene of interest is expressed in the Marathon-Ready cDNA. This control can only be performed if your GSP1 & 2 primers are designed to give overlapping 5'- and 3'-RACE products. An alternative is to use the control 5'- and 3'-RACE G3PDH Primers. This will generate a 900-bp fragment.

[c] The "AP1 only" and "GSP1 only" negative controls are particularly useful if your 5'-RACE reactions produce a smear or extra bands. If one of these controls also produces a smear or extra bands similar to what was observed in the experimental RACE reaction, you may need to design new primary primers or perform a secondary RACE amplification with the appropriate nested primer(s).

TABLE III: SETTING UP 3'-RACE REACTIONS

Component / Test Tube No.: / Description:	1 Experimental Sample	2 G3PDH Pos. Ctrl [a]	3 GSP 1 + 2 Pos. Ctrl [b]	4 AP1 only Neg. Ctrl [c]	5 GSP2 only Neg. Ctrl [c]
Marathon-Ready cDNA	5 µl	5 µl	5 µl	5 µl	5 µl
AP1 Primer (10 µM)	1 µl	1 µl	---	1 µl	---
GSP2 (sense primer; 10 µM)	1 µl	---	1 µl	---	1 µl
GSP1 (antisense primer; 10 µM)	---	---	1 µl	---	---
Control 3'-RACE G3PDH Primer (10 µM)	---	1 µl	---	---	---
H_2O	---	---	---	1 µl	1 µl
Master Mix	43 µl	43 µl	43 µl	43 µl	43 µl
Final volume	50 µl	50 µl	50 µl	50 µl	50 µl

Notes

Controls 3–5 provide useful information if your initial RACE reactions do not give the expected results. For a complete discussion of controls, see Section X (Troubleshooting RACE Reactions).

[a] The G3PDH Positive Control should generate a 1.2-kb product.

[b] If performing both 5'- and 3'-RACE with overlapping products, the "GSP 1+2" positive control is a useful control to confirm that the gene of interest is expressed in the Marathon-Ready cDNA. This control can only be performed if your GSP1 & 2 primers are designed to give overlapping products. An alternative is to use the control 5'- and 3'-RACE G3PDH Primers. This will generate a 900-bp fragment.

[c] The "AP1 only" and "GSP2 only" negative controls are particularly useful if your 3'-RACE reactions produce a smear or extra bands. If one of these controls also produces a smear or extra bands similar to what was observed in the experimental RACE reaction, you may need to design new primary primers or perform a secondary RACE amplification with the appropriate nested primer(s).

VI. Rapid Amplification of cDNA Ends (RACE) continued

4. Commence thermal cycling using one of the following programs (programs 1 and 2 work with the Control 5'-RACE G3PDH Primers and AP1):

Program 1 (preferred; use if GSP T_m > 70°C):

DNA Thermal Cycler 480:	GeneAmp Systems 2400/9600 (or hot-lid thermal cycler):
• 94°C for 1 min	• 94°C for 30 sec
• 5 cycles:	• 5 cycles:
94°C 30 sec	94°C 5 sec
72°C 4 min*	72°C 4 min*
• 5 cycles:	• 5 cycles:
94°C 30 sec	94°C 5 sec
70°C 4 min*	70°C 4 min*
• 20–25 cycles:	• 20–25 cycles:
94°C 20 sec	94°C 5 sec
68°C 4 min*	68°C 4 min*

Program 2 (if GSP T_m = 60–65°C):

DNA Thermal Cycler 480:	GeneAmp Systems 2400/9600 (or hot-lid thermal cycler):
• 94°C for 1 min	• 94°C for 30 sec
• 25–30 cycles:	• 25–30 cycles:
94°C 30 sec	94°C 5 sec
68°C 4 min*	68°C 4 min*

Notes on cycling:

You may need to determine the optimal cycling parameters for your gene empirically. If you see weak bands or no bands, perform five additional cycles at 68°C. For more suggestions on optimizing RACE PCR conditions, refer to Section X.

* The optimal extension time depends on the length of the fragment being amplified. We typically use 4 min for cDNA fragments of 2–5 kb. For 0.2–2-kb targets, we reduce the extension time to 2–3 min. For 5–10-kb targets, we increase the extension time up to 10 min.

5. When cycling is completed, analyze 5 µl from each tube, along with appropriate DNA size markers, on a 1.2% agarose/EtBr gel.

VI. Rapid Amplification of cDNA Ends (RACE) continued

6. [Optional] If the primary PCR reaction fails to give the distinct band(s) of interest or produces a smear, you may wish to perform a Southern blot using:
    a. a cDNA probe
    b. a nested primer as a probe

    Or, you may wish to perform a secondary, or "nested," PCR reaction using the AP2 primer supplied with Marathon-Ready cDNA and a NGSP. (See the discussion in Section V. Primer Design.)
    a. Dilute 5 µl of the primary PCR product into 245 µl of Tricine-EDTA buffer.
    b. Repeat steps 1–5 above, using:
        - 5 µl of the diluted primary PCR product in place of the Marathon-Ready cDNA.
        - 1 µl of the AP2 primer and 1 µl of your nested antisense GSP.
        - Fewer cycles (15–20 instead of 25–30).

At Clontech, we have successfully used Marathon-Ready cDNAs to amplify the 5'- and 3'-RACE fragments of several different genes from poly A^+ RNA. Typically, only one major band is generated, although in some cases minor bands are also visible. Although a nested AP2 Primer is provided, nested primers generally are not needed for successful Marathon amplification, particularly if you use primers with T_m's > 70°C and the preferred cycling programs for touchdown PCR. If you do not know the complete structure of your gene, you may be able to predict the size of the correctly amplified product via Northern blot analysis. Certain genes will give multiple bands due to the presence of a multigene family or multiple RNAs. If there are multiple products, you may need to determine which are real (e.g., the products of alternative transcription start sites, alternative splicing sites, or related genes) and which are artifacts (e.g., the result of pausing by RT, high GC content, nonspecific priming during RACE PCR, etc.).

VII. Characterization of RACE Products

At this point, we recommend that you characterize your RACE fragments and confirm that you have amplified the desired product. This can prevent confusion and wasted effort when you try to generate the full-length cDNA, even if you have single major products from both the 5'- and 3'-RACE reactions. Characterization is especially important if you have multiple bands or if you suspect that you are working with a member of a multigene family. For example, the "single" RACE products generated using actin primers in Figure 2 (Introduction) contain cDNAs from three different members of the actin gene family.

Below we describe three methods for characterizing RACE products: (1) Comparison of RACE products obtained with GSPs and NGSPs; (2) Southern blotting; and (3) Cloning and sequencing. We recommend that you obtain at least some sequence confirmation before attempting to generate the full-length cDNA. For options 1 and 2, you will need nested GSPs for analyzing your 5'- and 3'-RACE products. Section X also contains information that may help you interpret your results. For more detailed protocols for blotting and cloning, see Sambrook and Russell (2001) or other appropriate laboratory manuals.

A. Comparison of RACE Products Obtained with GSPs & NGSPs

For the 5'-RACE products, compare the products of primary amplifications performed with AP1 and GSP1 to the products obtained using AP1 and NGSP1 and the same cycling conditions and Marathon-Ready cDNA as a template. (For 3'-RACE, compare the products obtained from amplifications performed with AP1 and GSP2 to those obtained with AP1 and NGSP2.) This is a good test of whether multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If bands are real (i.e., the result of correct priming), they should be slightly smaller in the reaction using the NGSP. The difference in mobility of the products should correspond to the positions of the GSPs and NGSPs in the cDNA structure. (**Note**: Do not use AP2 in these reactions, because it will cause a size decrease in **all** of the PCR products.) If you have multiple bands with AP1 and GSP1 (or GSP2), some of these may disappear upon amplification with AP1 and NGSP1 (or NGSP2).

B. Southern Blot Analysis

Much stronger confirmation can be obtained by probing a Southern blot of your RACE products using an internal gene-specific probe (usually one of your other GSPs). This method can be particularly useful for determining which bands are real when RACE produces multiple bands. (Multiple bands are more common with 5'-RACE than with 3'-RACE.) Figure 5 shows Southern analysis of the 5'-RACE products from the insulin-like growth factor receptor type 2 cDNA (ILGFR2). The ILGFR2 mRNA, which is large (~9 kb) and relatively rare, is one of the most difficult targets we have analyzed using the Marathon cDNA amplification.

VII. Characterization of RACE Products continued

1. Repeat the RACE reactions and examine the products on an agarose/EtBr gel.
2. Photograph the gel, then transfer the DNA to a nylon membrane using standard blotting procedures.
3. Prepare a hybridization probe that does not have sequences in common with GSP1 (or 2). The probe can be end-labeled NGSP1 (or 2). Alternatively, if your GSPs define overlapping 5' and 3' fragments, GSP2 can be used as a probe to characterize your 5'-RACE products, and GSP1 can be used as a probe to characterize your 3'-RACE products. Nick-translated or random-primed internal restriction fragments (from a previously cloned partial cDNA) can also be used.
4. Hybridize the probe to the Southern blot, wash under moderate-to-high stringency conditions, and expose x-ray film.
5. Compare the hybridization pattern to the photograph of the agarose/EtBr gel.

   Generally, you will want to isolate the RACE product(s) that correspond(s) to the largest band(s) on the Southern blot. As seen in Figure 5, there may be larger RACE products that appear on the agarose gel but do not hybridize to the gene-specific probe. These bands are generally due to nonspecific priming. Smaller bands that hybridize to your probe may be the result of incomplete reverse transcription; however, you cannot exclude the possibility that some of these shorter bands are real and correspond to alternatively spliced transcripts, transcripts derived from multiple promoters, or other members of a multigene family.

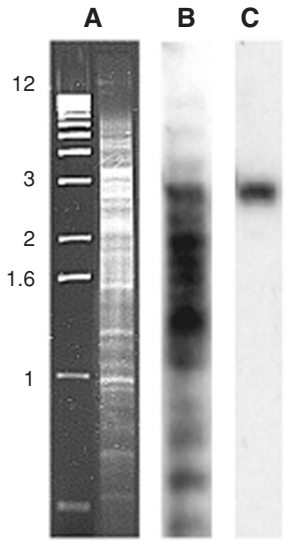

Figure 5. Identifying the correct RACE products by Southern blotting. Panel A: Agarose/EtBr gel showing the products of 5' Marathon RACE using a GSP derived from the ILGFR2 cDNA (Lane 2; expected product ~3 kb). Lane 1: 1-kb DNA ladder. **Panel B:** Southern blot of the gel seen in Panel A probed with a NGSP for ILGFR2. Note that the hybridization signal at the top of the blot is considerably lower than the top of the DNA smear seen in Panel A. To obtain the full-length cDNA, a second gel was run and the portion of the gel corresponding to just below the 3-kb size marker was excised. The DNA was eluted and cloned, and multiple independent clones were tested as described in the protocol to identify the largest insert derived from the ILGFR2 gene. **Panel C**. The same blot was reprobed with an internal gene-specific probe derived from the 5'-end of the cDNA. This confirms that the band at the top of Panel B is the correct 5'-RACE product. (Most researchers will not have the necessary probe to confirm their 5'-RACE product in this manner.)

VII. Characterization of RACE Products continued

6. Once you have determined the band(s) of interest, repeat the RACE reaction, gel-purify the DNA in the band(s) of interest using your preferred method of DNA recovery from agarose gels, resuspend in 30 µl of Tricine-EDTA buffer, and proceed with your experiments (after confirming that you have cloned the correct RACE product).

C. Cloning & Sequencing RACE Products

1. Gel-purify the RACE product(s) of interest using your preferred method of DNA recovery from agarose gels. In our experience, silica bead-based methods work well for RACE products up to about 2.5 kb. With longer fragments, we have obtained the best results using electroelution or DNA purification cartridges. (If you choose another method of DNA purification, resuspend your DNA in 30 µl of Tricine-EDTA Buffer.)
2. Verify recovery of the desired DNA fragment by examining 5 µl on an agarose/EtBr gel.
3. Clone the purified PCR product directly into a T/A-type PCR cloning vector. Alternatively, you may be able to clone into conventional vectors using the Not I, Srf I, Xma I, and EcoR I restriction sites in the Marathon Adaptor and/or cDNA Synthesis Primer, and restriction sites introduced in your GSP.
4. Identify clones containing gene-specific inserts by colony hybridization using a ^{32}P-end-labeled, nested GSP as a probe or by sequencing from your GSP. For 5'-RACE products, we recommend picking at least 8 –10 different independent clones in order to obtain the maximal possible amount of sequence at the 5' end. (Reverse transcription does not always proceed all the way to the 5' end of the mRNA template [especially for long templates]. Furthermore, the action of T4 DNA polymerase removes 0–20 bases from the 5'-end.)

   Once you have identified the clones containing the largest gene-specific inserts, obtain as much sequence data as you can. Ideally, you will be able to sequence the entire open reading frame, as well as 5' and 3' untranslated regions.

After RACE products have been characterized by partial or complete sequencing, you have two options for generating the full-length cDNA:

1) Generation of Full-Length cDNA by PCR (Section VIII).
2) Generation of Full-Length cDNA by Cloning (Section IX).

VIII. Generation of Full-Length cDNA by PCR

We have used this method successfully with several transcripts in the 5–10-kb range and with several other smaller transcripts. We have had success with both abundant and relatively rare mRNAs. Please note that amplification of large cDNAs requires significantly longer extension times, as described in Step 6; however, if the extension time is too long, some smearing may be observed. Careful primer design is critical.

All Marathon PCR reactions have been optimized with Clontech's Advantage® 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR. If you choose not to use the Advantage 2 Polymerase Mix, you must use some form of hot start with your polymerase mix.

1. Design 5' GSP and 3' GSP primers based on the sequence obtained from your 5'- and 3'-RACE products. These primers should be derived from the 5' and 3' ends of the cDNA (as shown in Figure 4) and should be 23–28 nt long. We do not recommend adding restriction sites to the ends of your primers, as we have observed higher background in some cases. Consult the guidelines in Section V for more information on the design of the primers. In some cases it may be necessary to design nested 5' and 3' primers; however, we recommend you first try to amplify the full-length cDNA with a single pair of primers.
2. Prepare enough master mix for all PCR reactions and one extra reaction to ensure sufficient volume. For each 50-µl PCR reaction, mix the following reagents:

36	µl	H_2O
5	µl	10X cDNA PCR Reaction Buffer
1	µl	dNTP Mix (10 mM)
1	µl	Advantage 2 Polymerase Mix (50X)
43	µl	Final volume

3. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
4. Prepare PCR reactions as shown in Table IV. Add the components in the order shown in 0.5-ml PCR tubes and mix gently.

VIII. Generation of Full-Length cDNA by PCR continued

TABLE IV: SETTING UP PCR TO AMPLIFY FULL-LENGTH cDNA

Component / Test Tube No. :	1 Full-length	2 * 5'-RACE	3 * 3'-RACE
Marathon-Ready cDNA	5 µl	5 µl	5 µl
5' GSP primer (10 µM)	1 µl	1 µl	---
3' GSP primer (10 µM)	1 µl	---	1 µl
GSP1 primer (10 µM)	---	1 µl	---
GSP2 primer (10 µM)	---	---	1 µl
Master Mix	43 µl	43 µl	43 µl
Total volume	50 µl	50 µl	50 µl

* The 5'- and 3'-RACE reactions are optional controls. The 5' and 3' GSPs are critical for the success of full-length amplification. If the full-length amplification does not work, the most likely reason is the design of these primers. These controls can help determine whether you have a problem with one of the primers.

5. Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

   Note: This is not necessary if you are using a hot-lid thermal cycler.

6. Commence thermal cycling using the following program:

DNA Thermal Cycler 480:	GeneAmp Systems 2400/9600 (or "hot-lid" thermal cycler):
• 94°C for 1 min	• 94°C for 30 sec
• 25 cycles: 94°C 30 sec 72°C 2–15 min*	• 25 cycles: 94°C 5 sec 72°C 2–15 min*

* The extension time in min should equal the expected length of the cDNA (to the nearest kb) plus 2 min (e.g., if your expected product is 6 kb, use 8 min [6 + 2]).

Note: you may need to determine the optimal cycling parameters for your gene empirically. If you see weak bands or no bands, perform an additional five cycles. For additional suggestions on optimizing RACE PCR conditions, refer to Section XVI.

7. Analyze 5 µl of each sample on a 1.2 % agarose/EtBr gel.

   Expected results: In most cases, you should see a single major product. If so, proceed to steps 8–12 for gel purification of the full-length cDNA. Gel purification is recommended (instead of cloning the PCR product directly) even though the product of this reaction is often a single strong band.

   If you do not see a single major product, refer to Section X.

VIII. Generation of Full-Length cDNA by PCR continued

8. Pour a preparative 1.2% agarose gel in TAE buffer with EtBr (0.3 µg/ml).

   Note: Do not use TBE buffer, as we have had difficulty cloning full-length cDNAs purified from TBE gels.

9. Load the remaining 45 µl of the PCR amplification mixture on the gel, along with appropriate DNA size markers.
10. Using a medium- to long-wave length UV light (≥ 300 nm) to visualize the DNA, cut out the band corresponding to the fused, full-length cDNA.

    Note: Be careful to minimize exposure of your DNA to UV.

11. Purify the DNA fragment.

    Purify the band of interest using your preferred method of DNA recovery from agarose gels. In our experience, silica bead-based methods work well for PCR products up to about 2.5 kb. With longer fragments, we have obtained the best results using electroelution or DNA purification cartridges. (If you choose another method of DNA purification, resuspend your DNA in 30 µl of Tricine-EDTA Buffer.)
12. Clone the full-sized cDNA into a T/A-type PCR cloning vector.

In our experience, large cDNAs can be damaged during purification by exposure to UV in the presence of EtBr. If your full-length cDNA is longer than 3 kb, we suggest that you test the quality of the purified primary PCR product by repeating the reaction in Steps 4–6 using 5 µl of a 1/50 dilution of your PCR product as a template. If the DNA is damaged, reamplification will not give a single, strong band. If you cannot amplify a full-length cDNA that can be readily reamplified, we recommend that you clone the PCR product directly (i.e., without gel purification) and screen (by hybridization with a gene-specific probe) for colonies that contain full-length, gene-specific inserts.

To be certain of obtaining the correct product, we recommend that you always pick several transformants and confirm the insertion of the full-length cDNA of interest. Again, this is especially important with cDNAs larger than about 3 kb.

IX. Generation of Full-Length cDNA by Cloning

If you have cloned overlapping 5'- and 3'-RACE products, and if there is a restriction site in the overlapping portion of the cDNA sequence, it is fairly easy to generate the full-length cDNA by standard cloning techniques. (Note that restriction sites introduced with your GSP are not suitable for this purpose, since using such a site to fuse your 5' and 3' fragments would, in most cases, introduce foreign sequence into the middle of your cDNA. For the same reason, do not fuse your 5' and 3' cDNA fragments using restriction sites in polylinkers adjacent to your cloned RACE products.) Simply digest each fragment with the enzyme, and join them using T4 DNA ligase. Clone the resulting full-length cDNA into the vector of your choice using the restriction sites introduced by the Marathon Adaptor (which is on both ends of full-length cDNAs created in this manner) and the Marathon cDNA synthesis primer (on the 5' end). The Marathon Adaptor contains sites for Not I and Xma I (sticky ends) and Srf I (blunt ends), while the cDNA Synthesis Primer contains Not I and EcoR I sites (See Appendix B). This facilitates easy directional cloning of the Srf I/Not I, Srf I/EcoR I, Xma I/Not I or Xma I/EcoR I fragments, or non-directional cloning of Not I/Not I or Srf I/Srf I fragments into suitable vectors. Srf I and Not I are extremely rare in mammalian genomes, occurring approximately once in 10^6 bp, and hence are unlikely to be present in most cDNAs. Alternatively, if you are working directly with the products of your 5'- and 3'-RACE reactions, you can clone the full-sized cDNA directly into any T/A-type PCR cloning vector.

X. Troubleshooting RACE Reactions

Tables II and III in the User Manual include several controls that will help you troubleshoot the reaction if yields are suboptimal. These include:

- **Tube No. 2:** 5'- or 3'-RACE PCR using the Control G3PDH Primer and the AP1 Primer to amplify your Marathon-Ready cDNA.

  A smear of large molecular weight material may appear at the top of some lanes. As discussed in Appendix B, the upper limit of the suppression PCR effect is about 6 kb. The large, nonspecific amplification products that do appear in some Marathon experiments generally do not interfere with interpretation of RACE results; however, care must be taken to avoid these products when cloning and otherwise using your RACE products for subsequent experiments.

- **Tube No. 3:** An additional positive control using both GSPs to amplify your Marathon-Ready cDNA. If you do not have suitable 5'- and 3'-GSPs (i.e. GSPs that create overlapping 5'- and 3'-RACE products), use the control 5'- and 3'-RACE G3PDH Primers. This should give a single band corresponding to the overlap between the primers. This result confirms that your cDNA, or the G3PDH cDNA, is present in (and can be amplified from) your Marathon-Ready cDNA.

- **Tube No. 4:** A negative control using AP1 by itself to amplify your Marathon-Ready cDNA. With less than 30 cycles, this should produce no product. (AP1 may produce some large [5–8 kb] smear product with higher cycle numbers; RACE products can generally still be seen in the presence of these bands.) If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters or perform a secondary amplification using the AP2 Primer.

- **Tube No. 5:** A negative control using each GSP by itself. This control should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters, perform a secondary amplification using nested primers, or redesign your original primer.

A. General Considerations.

- **Troubleshooting GC-rich templates:** If your PCR product is not the expected size, especially your 5'-RACE product, it may be due to difficulty amplifying a GC-rich template. Clontech offers the Advantage-GC 2 Polymerase Mix and PCR Kit for efficient amplification of GC-rich templates. However, when using this polymerase mix or kit, the master mix recipes will need to be modified to include GC-Melt™ and for the 5X PCR Reaction Buffer, instead of a 10X buffer supplied with most polymerases. Additionally, the PCR parameters may need to be optimized for these templates. We recommend that you perform the initial RACE reactions with Advantage 2 Polymerase Mix, then perform the RACE reactions using the Advantage-GC 2 Polymerase Mix to confirm the product is the same size in both reactions.

X. Troubleshooting RACE Reactions continued

- **High-fidelity PCR:** If you are going to sequence or clone your RACE products for further analysis, we recommend performing your RACE reactions using the Advantage-HF 2 PCR Kit. The Advantage-HF 2 PCR Kit is designed to yield products of less than 3.5 kb with exceptionally high fidelity. Based on sequence data derived from PCR products amplified for 25 cycles, the Advantage-HF 2 PCR Kit provides an accuracy rate that is more than 25X higher than Taq polymerase (January 1999 Clontechniques). This kit may not be ideal for cDNA templates that are greater than 3.5 kb, but it is especially well suited for applications in which the RACE product will be sequenced or cloned for use in additional experiments. Again, the initial RACE reactions should be performed using the Advantage 2 Polymerase Mix to confirm the product is present and that the GSPs work well.
- **Troubleshooting touchdown PCR:** When troubleshooting touchdown PCR, we recommend that you begin by modifying the final (third) set of cycle parameters (i.e., the 20–25 cycles performed at 68°C in Program 1). If you do not observe an amplified product after 20 cycles at 68°C, run five additional cycles. If the product still does not appear, add an additional 3–5 cycles at 68°C or try amplifying preforming a new PCR experiment with 25 cycles of: 65°C, 30 sec; 68°C, 4 min. The last program is especially useful if you suspect that your GSP has a T_m close to or less than 70°C.
- **Adapting the Marathon protocol for thermal cyclers other than the Applied Biosystems DNA Thermal Cycler 480 or GeneAmp Systems 2400/9600:** As noted elsewhere in this manual, cycling parameters in this protocol must be optimized if you are using a thermal cycler other than the Applied Biosystems DNA Thermal Cycler 480 or GeneAmp Systems 2400/9600. If you have access to one of these thermal cycler, we strongly recommend that you use it.

B. No bands are observed in your positive control (GSP1 + GSP2).

The control PCR reaction using your sense and antisense GSPs and your Marathon-Ready cDNA to amplify the internal fragment of your gene is very important. If this reaction fails to produce the expected internal cDNA fragment, there are at least four possible explanations:

- There may be a problem with your 50X polymerase mix. If you are not using Advantage 2 Polymerase Mix, consider switching. The Marathon protocol was optimized with Advantage 2 Polymerase Mix.
- Your gene may be expressed weakly or not at all in your Marathon-Ready cDNA. It may be necessary to try another Marathon-Ready cDNA or make your own from the optimal tissue source using the Marathon cDNA Amplification Kit (Cat. No. 634913).

X. Troubleshooting RACE Reactions continued

- There is a problem with your primers. This could be due either to poor primer design or poor primer preparation. First try lowering your annealing/extension temperature. If this does not work, you may need to design new primers or repurify your GSPs to remove impurities.
- The AMV-RT cannot effectively copy the full-length cDNA due to strong secondary structure and/or high GC content. This is indicated if the 3'-RACE works, but the 5'-RACE does not, and the positive control (GSP1 + GSP2) does not produce the expected fragment.

You may be able to obtain more information by amplifying the internal fragment (using GSP1 and GSP2) using genomic DNA as the template. If this produces the expected band, this indicates that your primers are usable and the problem is either (a) the target RNA is a poor template for AMV RT; or (b) the RNA is not expressed in the tissue source you have chosen. Note, however, that this is not a conclusive test, since your primers may be separated by an intron in the genomic DNA. If this is the case, amplification of genomic DNA will give a larger fragment than expected or no fragment at all.

C. No bands are observed with the experimental cDNA sample, but the G3PDH positive control gives the expected product.

1. Increase the cycle number.
2. Lower the annealing temperature by increments of 2–5°C.
3. Generate different GSPs.
4. Try another Marathon-Ready cDNA or make your own from the optimal tissue source using the Marathon cDNA Amplification Kit, (Cat. No. 634913).

D. RACE cDNA product consists of multiple bands.

In many cases, your initial experiments will produce multiple 5'- and/or 3'-RACE products. As mentioned above, you will have to determine which are real and which are artifacts. While the following guidelines will help you eliminate artifacts, confirmation of real and complete bands will require additional studies such as mapping of transcription start sites, intron/exon structure and polyadenylation sites, and genomic sequencing.

Multiple fragments do not mean you cannot proceed with generating the full-length cDNA, however, you may save time in the long run if you try to eliminate nonspecific fragments by troubleshooting the reactions. If multiple fragments persist and you want to proceed, you generally will want to start with the largest fragment from each RACE reaction, since it is most likely to be a true, complete RACE product.

X. Troubleshooting RACE Reactions continued

Sources of "real" multiple RACE products

Individual genes can give rise to multiple sizes of transcripts—and hence to multiple RACE fragments—via at least three mechanisms:

- Alternative splicing can cause multiple products in either 5'- or 3'-RACE.
- Use of different transcription initiation sites causes multiple 5'-RACE products.
- Use of different polyadenylation sites causes multiple 3'-RACE products.

Alternatively, the gene may be a member of a multigene family, in which case your "gene-specific" primers may simultaneously amplify several highly homologous cDNAs.

Distinguishing true polymorphic forms of an RNA is a matter for scientific investigation. Different RNA forms of the same gene may be most abundant in different Marathon-Ready cDNAs.

Sources of artifacts

Multiple bands often do not correspond to actual and complete transcripts. These artifact RACE products can be divided into two classes—incomplete and nonspecific.

There are several possible sources of incomplete fragments, which are generated from correctly primed sites.

- Premature termination of first-strand cDNA synthesis caused by pausing of RT generally causes multiple 5'-RACE products. This is a common problem with larger RNAs, and it is a difficult problem to overcome, since it is due to an intrinsic limitation of RT.
- Difficulty in amplifying certain "difficult" genes can cause multiple products in either 5'- or 3'-RACE; often a result of high GC content.

Nonspecific RACE products arise from nonspecific binding of the primer to multiple sites in the ds cDNA or primer-dimer artifacts.

Suggestions:

- If you have not already done so, repeat your RACE reactions with all of the recommended controls. In particular, be sure that your GSPs do not give bands when used alone, and that they give a single band when used together. If either GSP alone gives persistent bands, we recommend altering the cycling parameters or designing nested primers as discussed below.
- If you have not already done so, repeat your RACE reactions using some form of hot start PCR (antibody-mediated, wax beads, or manual).

X. Troubleshooting RACE Reactions continued

- If multiple bands persist, try altering the PCR cycling parameters:
  a. Increase the stringency of your PCR by raising the annealing temperature in increments of 2–5°C. In many cases, bands arising from nonspecific priming will disappear while real or incomplete products will persist.
  b. Reduce the cycle number. Again, bands arising from nonspecific priming may disappear, while real or incomplete products will persist.
  c. Reduce the extension time.
  d. In the case of large RACE products, increasing the extension time may help eliminate extra bands.
- If multiple bands persist, try designing a new set of primers:
  a. Redesign your primers so that they have a T_m > 70°C and use the cycling parameters for touchdown PCR.
  b. We recommend that you design new primers that will give RACE products that are slightly different in size than those expected with the original primers. These new primers can either be used by themselves or in combination with the original primers in "nested PCR". In nested PCR the product of a PCR reaction is reamplified using a second set of primers that is internal to the original primers. This often greatly reduces the background and nonspecific amplification seen with either set of primers alone. The design of nested primers is discussed in Section V.
  c. Prior to performing nested RACE PCR, we recommend that you perform separate primary amplifications with AP1 and either the GSP or NGSP. This is a good test of whether multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If the multiple bands are real (i.e., the result of correct priming), they should be present in both reactions, but slightly smaller in the reaction using the nested primers. The difference in mobility of the products should correspond to the positions of the GSP and NGSP in the cDNA structure.

E. RACE cDNA product is smeared.

Note: Some Marathon reactions produce very complex patterns of bands that appear almost as smears. See Figure 5 for an example. Section VIII gives some guidelines for interpreting these complex patterns and isolating the band(s) of interest.

You can try optimizing your RACE reactions using the troubleshooting tips described above for multiple bands.

XI. Troubleshooting Generation of Full-Length cDNA by PCR

In most cases, you should see a single major product of the size predicted from Northern analysis or analysis of your 5'- and 3'-RACE products. If you do not observe a single, major band and if you cannot resolve your full-length cDNA by optimizing the extension time and cycle number, we suggest that you design additional (nested) 5' and 3' GSPs. Most problems with the full-length PCR reaction are due to poor primers and can be corrected simply by using better primers. If one of the controls in Table V also does not work, try that nested primer first. Try additional primary PCR reactions with different combinations of flanking primers (i.e., 5' GSP and 3' NGSP; 5' NGSP and 3' GSP; 5' NGSP and 3' NGSP). If that doesn't work, then try nested PCR.

See Section XI for additional suggestions on interpreting your results and optimizing your PCR reactions.

XII. References

Marathon® cDNA amplification has been cited in more than 200 research articles. For a complete list of citations, see Clontech's web site (http://www.clontech.com).

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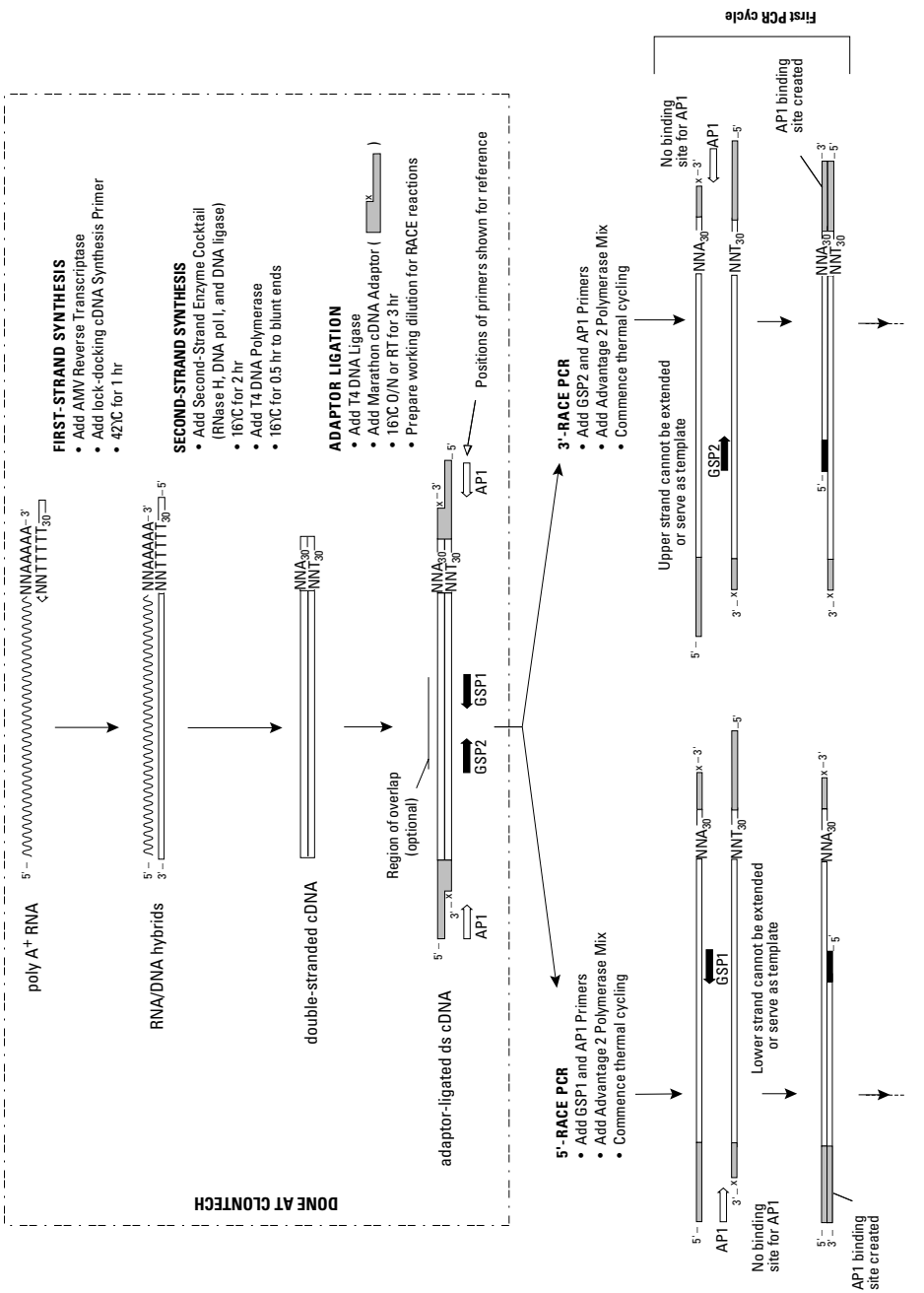
XIII. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

	Cat. No.
• Marathon® cDNA Amplification Kit	634913
• Advantage® 2 PCR Kit	639206 639207
• Advantage® 2 Polymerase Mix	639201 639202
• Advantage®-GC 2 PCR Kit	639119 639120
• Advantage®-GC 2 Polymerase Mix	639114
• Advantage®-HF 2 PCR Kit	639123 639124
• QUICK-Clone™ cDNAs	many
• TaqStart® Antibody	639250 639251
• Atlas® cDNA Expression Arrays	many
• SMART™ cDNA Library Construction Kit	634901
• SMART™ PCR cDNA Synthesis Kit	634902
• SMART™ RACE cDNA Amplification Kit	634914
• Clontech PCR-Select™ cDNA Subtraction Kit	637401
• Multiple Tissue cDNA (MTC™) Panels	many
• Delta® Differential Display Kit	637405
• GenomeWalker™ Kits	many
• Total RNA Panels	many
• Poly A^+ RNAs	many
• Genomic and cDNA Libraries	many
• Multiple Tissue Northern (MTN®) Blots	many

Appendix A: Detailed Flow Chart of Marathon® Procedure



Appendix A: Flow Chart of Marathon® Procedure continued

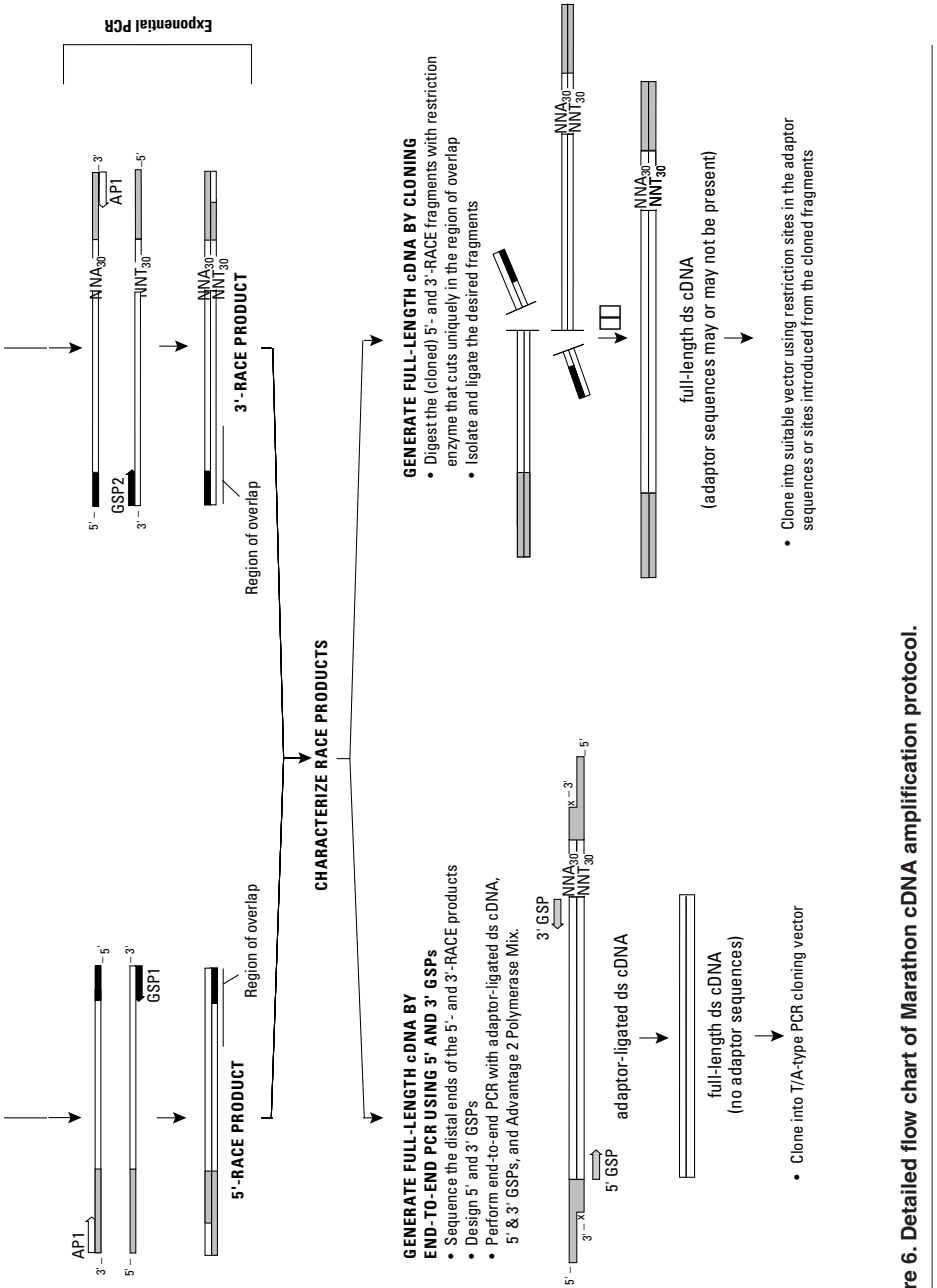


Figure 6. Detailed flow chart of Marathon cDNA amplification protocol.

Appendix B: Marathon® cDNA Adaptor & Primers

The Marathon cDNA Adaptor has three design features that are critical to the success of Marathon cDNA amplification:

- The use of a 5'-extended adaptor that has no binding site for the AP1 Primer used in primary PCR. An AP1 binding site can only be generated by extension of the gene-specific primer (GSP).
- Blocking of the exposed 3' end of the adaptor with an amine group to prevent extension of the 3' end (which would create an AP1 binding site and allow nonspecific amplification).
- The use of an adaptor primer that is shorter than the adaptor itself (suppression PCR). As shown in Figure 7, the suppression PCR effect prevents amplification of templates where the 3' end has been extended to create an AP1 binding site. Though rare, such extension does occur, presumably due to incomplete amine modification or incomplete adaptor ligation. Given the exponential nature of PCR amplification, such events would lead to nonspecific amplification and unacceptable backgrounds in the absence of suppression PCR.

Each of these features helps eliminate nonspecific amplification among the general population of cDNA fragments. Together, they allow amplification of a specific target from a very complex mixture of DNA fragments—all of which have the same terminal structure—using a single set of GSPs.

Appendix B: Marathon® cDNA Adaptor & Primers continued

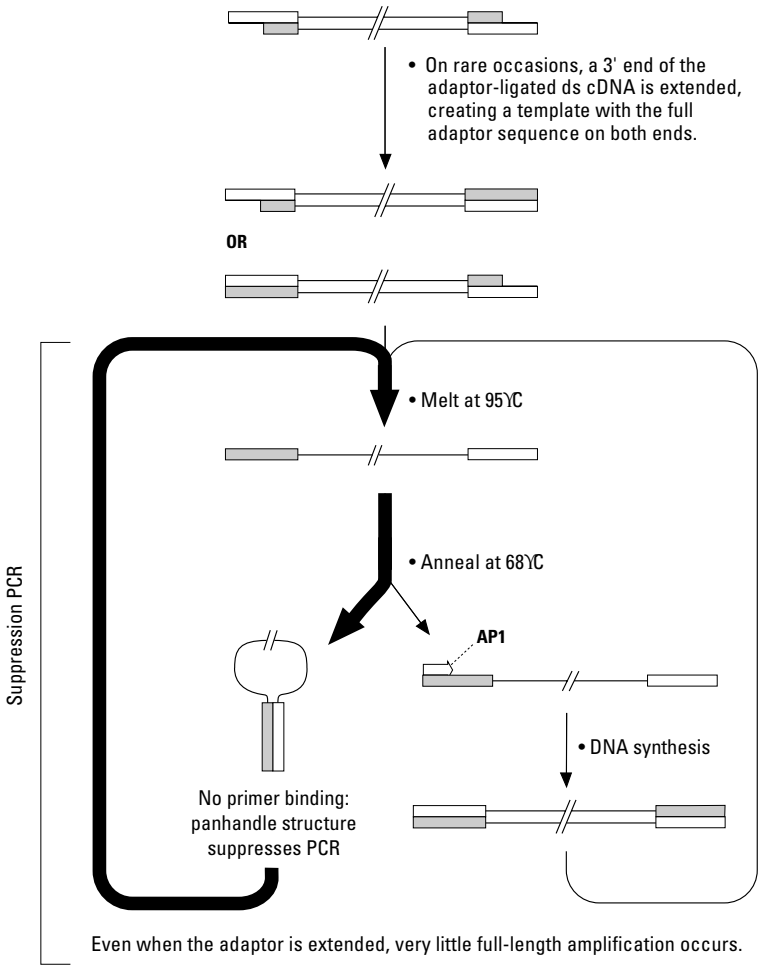
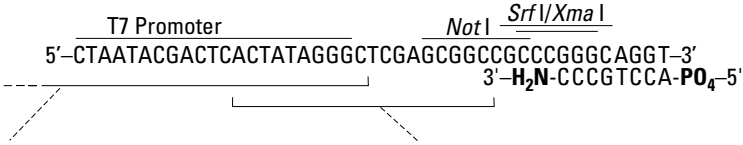


Figure 7. The suppression PCR effect. In rare cases, the 3' end of the Marathon Adaptor gets extended. (Though rare, such extension does occur, presumably due to incomplete amine modification during oligonucleotide synthesis or incomplete adaptor ligation.) This creates a molecule that has the full-length adaptor sequence on both ends and can serve as a template for end-to-end amplification. Without suppression PCR, these rare events would lead to unacceptable backgrounds in RACE reactions due to the exponential nature of PCR amplification. However, in suppression PCR, the adaptor primer is much shorter than the adaptor itself. Thus, during subsequent thermal cycling, nearly all the DNA strands will form the "panhandle" structure shown above, which cannot be extended. At the appropriate annealing/extension temperature, this intramolecular annealing event is strongly favored over (and more stable than) the intermolecular annealing of the much shorter adaptor primer to the adaptor.

Appendix B: Marathon® cDNA Adaptor & Primers continued

Marathon cDNA Adaptor:



Adaptor Primer 1 (AP1; 27-mer):
5′–CCATCCTAATACGACTCACTATAGGGC–3′

Nested Adaptor Primer 2 (AP2; 23-mer):
5′–ACTCACTATAGGGCTCGAGCGGC–3′

Marathon cDNA Synthesis Primer (52-mer): 5′–TTCTAGAATTCAGCGGCCGC(T)$_{30}$N$_{-1}$N–3′

*Eco*R I *Not* I

N$_{-1}$ = G, A, or C; N = G, A, C, or T
Degenerate nucleotides anchor primer at base of poly-A tail

5'-RACE G3PDH Primer (24-mer): 5′–GGTCTTACTCCTTGGAGGCCATGT–3′

3'-RACE G3PDH Primer (25-mer): 5′–GACCCCTTCATTGACCTCAACTACA–3′

Figure 8: Sequences of the Marathon® cDNA Adaptor & Primers. The T_m's of AP1 and AP2 are 71°C and 77°C, as determined by nearest neighbor analysis (Freier et al., 1986). Note, however, that only 22 of the 27 nt in AP1 bind the Adaptor during the first cycle of PCR, so the effective T_m of AP1 may be actually several degrees lower. The lower effective T_m of AP1 is the reason touchdown PCR works well with Marathon RACE reactions.

Notes

Notes

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Suppression PCR is covered by U.S. Patent No. 5,565,340. Foreign patents pending.

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