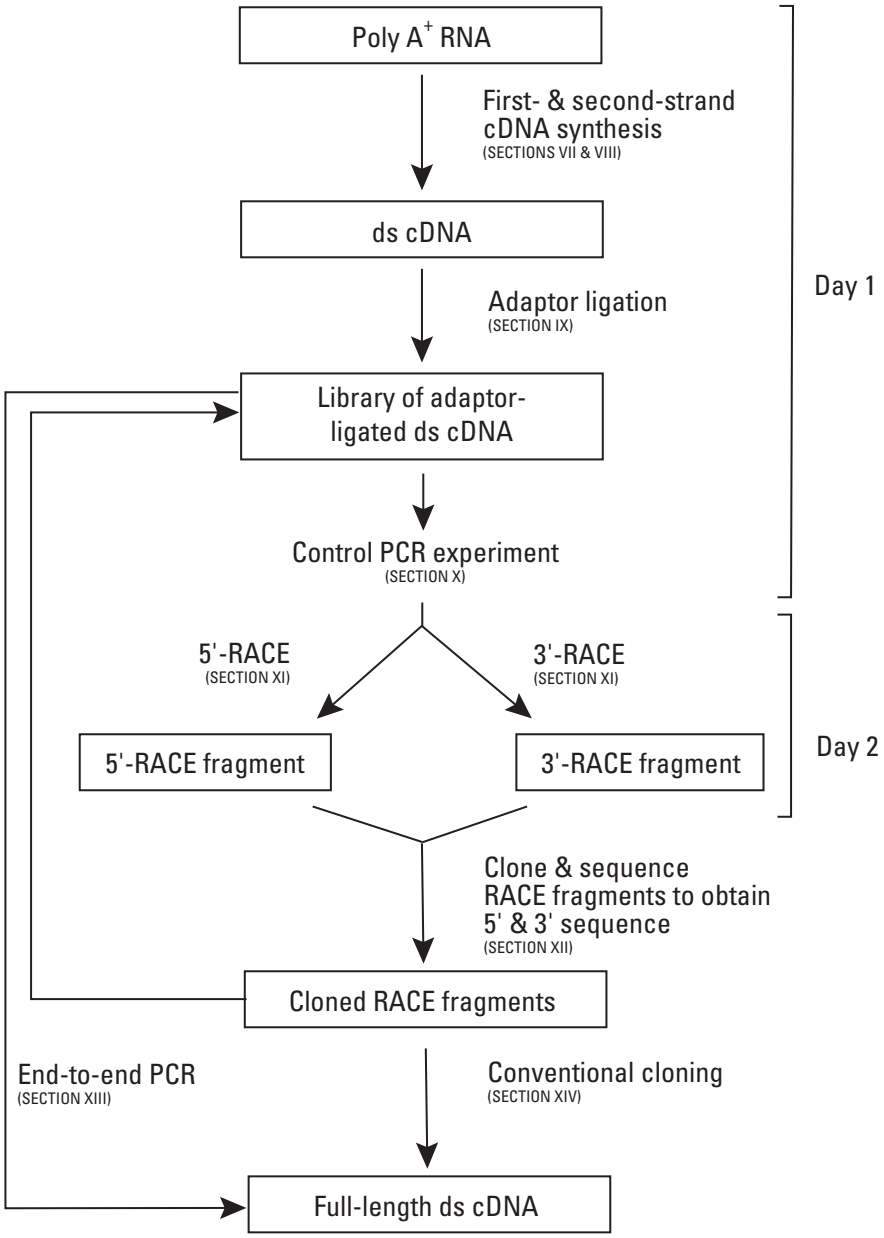
Protocol-at-a-Glance

Marathon® cDNA Amplification Kit
Protocol-at-a-Glance
(PT1115-2)

Marathon cDNA amplification is a fairly complex, multiday procedure. Please read the *User Manual* before using this abbreviated protocol, and refer to it often for interpretation of results during the course of your experiments. The Protocol-at-a-Glance is provided for your convenience, but is not intended for first-time users.



Overview of the Marathon procedure. This figure depicts the order in which different reaction products are generated. Note that with the cloned RACE fragments you can use a restriction site in an overlapping region and a site in the cloning vector to obtain both parts of the complete cDNA and subclone them together to obtain a full-length cDNA transcript, or you can sequence the 5' end of the 5' product and the 3' end of the 3' product to obtain additional sequence information. Using this additional sequence information, you can design 5' and 3' gene-specific primers to use in LD PCR with the adaptor-ligated ds cDNA to obtain the full-length cDNA.

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A. First-Strand cDNA Synthesis

1. For each reaction (including the Control Human Placental Poly A$^+$ RNA), combine the following in a sterile 0.5 ml microcentrifuge tube:

1 µg (1–4 µl)	RNA sample (poly A$^+$)
1 µl	cDNA Synthesis Primer (10 µM)

2. Add sterile H_2O to a final volume of 5 µl.
3. Mix contents and spin the tube briefly in a microcentrifuge.
4. Incubate the tube at 70°C for 2 min.
5. Cool the tube on ice for 2 min.
6. Spin the tube briefly to collect the contents at the bottom.
7. Add the following to each reaction tube:

2 µl	5X First-Strand Buffer
1 µl	dNTP Mix (10 mM)
1 µl	[α-^{32}P]dCTP (1 µCi/µl) *
1 µl	AMV Reverse Transcriptase (20 units/µl)
10 µl	Total volume

* [α-^{32}P]dCTP is optional. If you choose not to add [α-^{32}P]dCTP, add 1µl of H_2O. If using [α-^{32}P]dCTP with a specific activity of 10 µCi/µl, be sure to dilute tenfold with H_2O.

8. Mix the contents of the tube by gently pipetting.
9. Spin the tube briefly to collect the contents at the bottom.
10. Incubate the tube at 42°C for 1 hr in an air incubator.
    Note: Using a water bath or thermocycler for this incubation may reduce the volume of the reaction mixture (due to evaporation) and therefore reduce the efficiency of first-strand synthesis.
11. Place the tube on ice to terminate first-strand synthesis.
12. Proceed directly to second-strand synthesis.

B. Second-Strand cDNA Synthesis

Note: All components and reaction vessels should be prechilled on ice.

1. For each reaction (including the positive control), combine the following components in the reaction tube from Step A.11:

(10 µl	First-strand reaction)
48.4 µl	Sterile H_2O
16 µl	5X Second-Strand Buffer
1.6 µl	dNTP Mix (10 mM)
4 µl	20X Second-Strand Enzyme Cocktail
80 µl	Total volume

2. Mix contents thoroughly with gentle pipetting.
3. Spin the tube briefly to collect the contents at the bottom.
4. Incubate the tube at 16°C for 1.5 hr.
5. Add 2 µl (6 units) of T4 DNA Polymerase and mix thoroughly with gentle pipetting.
6. Incubate the tube at 16°C for 45 min.
7. Add 4 µl of the EDTA/glycogen Mix to terminate second-strand synthesis.
8. Add 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
9. Vortex thoroughly.
10. Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate phases.
11. Carefully transfer the top aqueous layer to a clean 0.5 ml microcentrifuge tube. Discard the interface and lower phase.
12. Add 100 µl of chloroform:isoamyl alcohol (24:1) to the aqueous layer and vortex thoroughly.
13. Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate phases.
14. Remove the top aqueous layer and place in a clean 0.5 ml microcentrifuge tube.
15. Add one-half volume of 4 M ammonium acetate (e.g., if you recovered ≈70 µl at Step 14, add 35 µl of 4 M ammonium acetate).
16. Add 2.5 volumes of room temperature 95% ethanol. (e.g., if your volume at Step 15 was ≈105 µl, add 263 µl of 95% ethanol.)
17. Vortex the mixture thoroughly.

18. Spin the tube immediately in a microcentrifuge at 14,000 rpm at room temperature for 20 min.
    Note: Do not chill the ethanol precipitate prior to centrifugation. Incubation at low temperatures does not improve the yield of ethanol precipitation with ammonium acetate and may precipitate impurities that will inhibit subsequent steps.
19. Remove the supernatant carefully.
    Note: If you included [α-^{32}P]dCTP in the reaction, check the efficiency of the precipitation. The pellet should contain 1–10% of the total radioactivity in the sample.
20. Gently overlay the pellet with 300 µl of 80% ethanol.
21. Spin in a microcentrifuge at 14,000 rpm for 10 min.
22. Carefully remove the supernatant.
    Note: If you included [α-^{32}P]dCTP, check that the pellet still contains counts to be sure that you did not lose the sample.
23. Air dry the pellet for approximately 10 min to evaporate residual ethanol.
24. Dissolve the precipitate in 10 µl of H_2O and store at –20°C.
25. **[Optional]** We recommend that you estimate the yield of your experimental ds cDNA products and compare the yield and size range to the ds cDNA made from the positive control Human Placental RNA. This information will help you dilute your sample in Step C.5.
    a. Analyze 2 µl of your experimental ds cDNA and 2 µl of the positive control ds cDNA on a 1.2% agarose/EtBr gel with suitable DNA size markers.
    b. If you cannot see your experimental ds cDNA via EtBr staining and you included [α-^{32}P]dCTP, dry the agarose gel using a vacuum gel drying system and expose x-ray film to the gel overnight at –70°C.

C. Adaptor Ligation

Note: Allow 5X DNA Ligation Buffer to completely thaw at room temperature and keep it at room temperature for 30 min before use. **Do not put the 5X DNA Ligation Buffer on ice.**

1. For each reaction (including the positive control), combine the following reagents in a 0.5 ml microcentrifuge test tube at room temperature and in the order shown:

5	µl	ds cDNA
2	µl	Marathon cDNA Adaptor (10 µM)
2	µl	5X DNA Ligation Buffer
1	µl	T4 DNA Ligase (1 unit/µl)
10	µl	Total volume

2. Mix by vortexing and spin briefly in a microcentrifuge.
3. Incubate at either:
   • 16°C overnight; or
   • room temperature (19–23°C) for 3–4 hr.
4. Heat at 70°C for 5 min to inactivate the ligase.
5. Using the following guidelines, dilute your adaptor-ligated ds cDNA to a concentration which is suitable for subsequent RACE PCR procedures (≈0.1 µg/ml).
   a. If you compared your yield of ds cDNA to that obtained with the positive control RNA:
      - If the yield of your experimental sample is equal to or greater than the yield of the positive control, dilute 1 µl of the reaction mixture with 250 µl of Tricine-EDTA Buffer.
      - If the yield of your experimental sample is less than the yield of the positive control, dilute the reaction mixture with proportionately less Tricine-EDTA Buffer. For example, if your sample contained fivefold less cDNA than the positive control, dilute 1 µl in 50 µl of Tricine-EDTA Buffer.

        (If you cannot see your ds cDNA with EtBr staining, you will probably need to repeat Sections A–C using fresh, or more, poly A$^+$ RNA.)

   b. If you did not compare your yield of ds cDNA to that obtained with the positive control RNA, prepare separate 1/50 and 1/250 dilutions of adaptor-ligated ds-cDNA in Tricine-EDTA Buffer. Perform the subsequent RACE PCR reactions using the specified amount of both dilutions until you determine which dilution gives you the best results.
6. Dilute 1 µl of the positive control reaction mixture with 250 µl of Tricine-EDTA Buffer.
7. Store the undiluted adaptor-ligated cDNA at –20°C for future use.
8. Heat the diluted ds cDNA at 94°C for 2 min to denature the ds cDNA.
9. Cool the tube on ice for 2 min.
10. Briefly spin the tube in a microcentrifuge to collect the contents in the bottom of the tube. Store at –20°C until ready for RACE PCR. At this stage, you essentially have a library of adaptor-ligated ds cDNA. The RACE reactions in Section E use only a fraction of this material for each RNA of interest.

D. Control PCR Experiment

Prior to performing 5'- and 3' -RACE reactions with your adaptor-ligated ds cDNA, we *strongly* recommend that you perform the positive control RACE PCR experiment in Section X of the User Manual. *This control is essential if you are not using Advantage® 2 Polymerase Mix and a hot-lid or non-hot-lid thermal cycler.*

E. Rapid Amplification of cDNA Ends (RACE)

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 III.
   For 3'-RACE: prepare PCR reactions as shown in Table IV.
   Add the components in the order shown in PCR tubes.

TABLE III: SETTING UP 5'-RACE PCR REACTIONS

Test Tube No. / Description: / Component	1 Experimental Sample	2 TFR Pos. Ctrl *	3 GSP 1 + 2 Pos. Ctrl	4 AP1 only Neg. Ctrl	5 GSP1 only Neg. Ctrl
Diluted adaptor-ligated expt'l cDNA	5 µl	---	5 µl	5 µl	5 µl
Diluted adaptor-ligated pos. ctrl cDNA	---	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 TFR 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

* The TFR Positive Control should generate a 2.6 kb product.

TABLE IV: SETTING UP 3'-RACE PCR REACTIONS

Test Tube No. / Description: / Component	1 Experimental Sample	2 TFR Pos. Ctrl †	3 GSP 1 + 2 Pos. Ctrl	4 AP1 only Neg. Ctrl	5 GSP2 only Neg. Ctrl
Diluted adaptor-ligated expt'l cDNA	5 µl	---	5 µl	5 µl	5 µl
Diluted adaptor-ligated pos. ctrl cDNA	---	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 TFR 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

† The TFR Positive Control should generate a 2.9 kb product.

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

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

Non-hot-lid thermal cycler:

- 94°C for 1 min
- 5 cycles:
  94°C 30 sec
  72°C 4 min◊
- 5 cycles:
  94°C 30 sec
  70°C 4 min◊
- 20–25 cycles:
  94°C 20 sec
  68°C 4 min◊

Hot-lid thermal cycler:

- 94°C for 30 sec
- 5 cycles:
  94°C 5 sec
  72°C 4 min◊
- 5 cycles:
  94°C 5 sec
  70°C 4 min◊
- 20–25 cycles:
  94°C 5 sec
  68°C 4 min◊

Program 2 (if GSP T_m = 60–65°C):

Non-hot-lid thermal cycler:

- 94°C for 1 min
- 25–30 cycles:
  94°C 30 sec
  68°C 4 min◊

Hot-lid thermal cycler:

- 94°C for 30 sec
- 25–30 cycles:
  94°C 5 sec
  68°C 4 min◊

Notes on cycling:

You may need to determine the optimal cycling parameters for your gene empirically.

◊ 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.
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 of the User Manual.)
   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).

F. 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 be done by: (1) Comparison of RACE products obtained with GSPs and NGSPs; (2) Southern blotting; and (3) Cloning and sequencing. Characterization of your RACE products at this point can prevent confusion and wasted effort in your subsequent experiments, even when both RACE reactions produce a single major product.

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 XIII of the User Manual).
2) Generation of Full-Length cDNA by Cloning (Section XIV of the User Manual).