TECHNICAL MANUAL

ImProm-II™ Reverse Transcription System

Instructions for use of Product
A3800

Revised 1/14
TM236

ImProm-II™ Reverse Transcription System

All technical literature is available on the Internet at: www.promega.com/protocols/ Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

1. Description

The ImProm-II™ Reverse Transcription System[a] is a convenient kit that includes a reverse transcriptase and an optimized set of reagents designed for efficient synthesis of first-strand cDNA in preparation for PCR amplification. The components of the ImProm-II™ Reverse Transcription System can be used to reverse transcribe RNA templates starting with either total RNA, poly(A)+ mRNA or synthetic transcript RNA. Figure 1 provides an overview of the

reverse transcription procedure. The optimized reaction buffer and powerful reverse transcriptase provided in the ImProm-II™ Reverse Transcription System enable robust, full-length cDNA synthesis for the reproducible analysis of rare or long messages (Figures 2 and 3). These conditions have been developed for cDNA synthesis or for easy transition to gene-specific target amplification. Volumes from 1µl to 20µl of the reverse transcription reaction can be directly amplified using *Taq* DNA polymerase in PCR. cDNA synthesis using the ImProm-II™ Reverse Transcription System can also be coupled with amplification using the Plexor® qPCR System for two-step real-time quantitative RT-PCR (Section 4.D).

For ImProm-II™ Reverse Transcription System citations visit:
www.promega.com/citations/

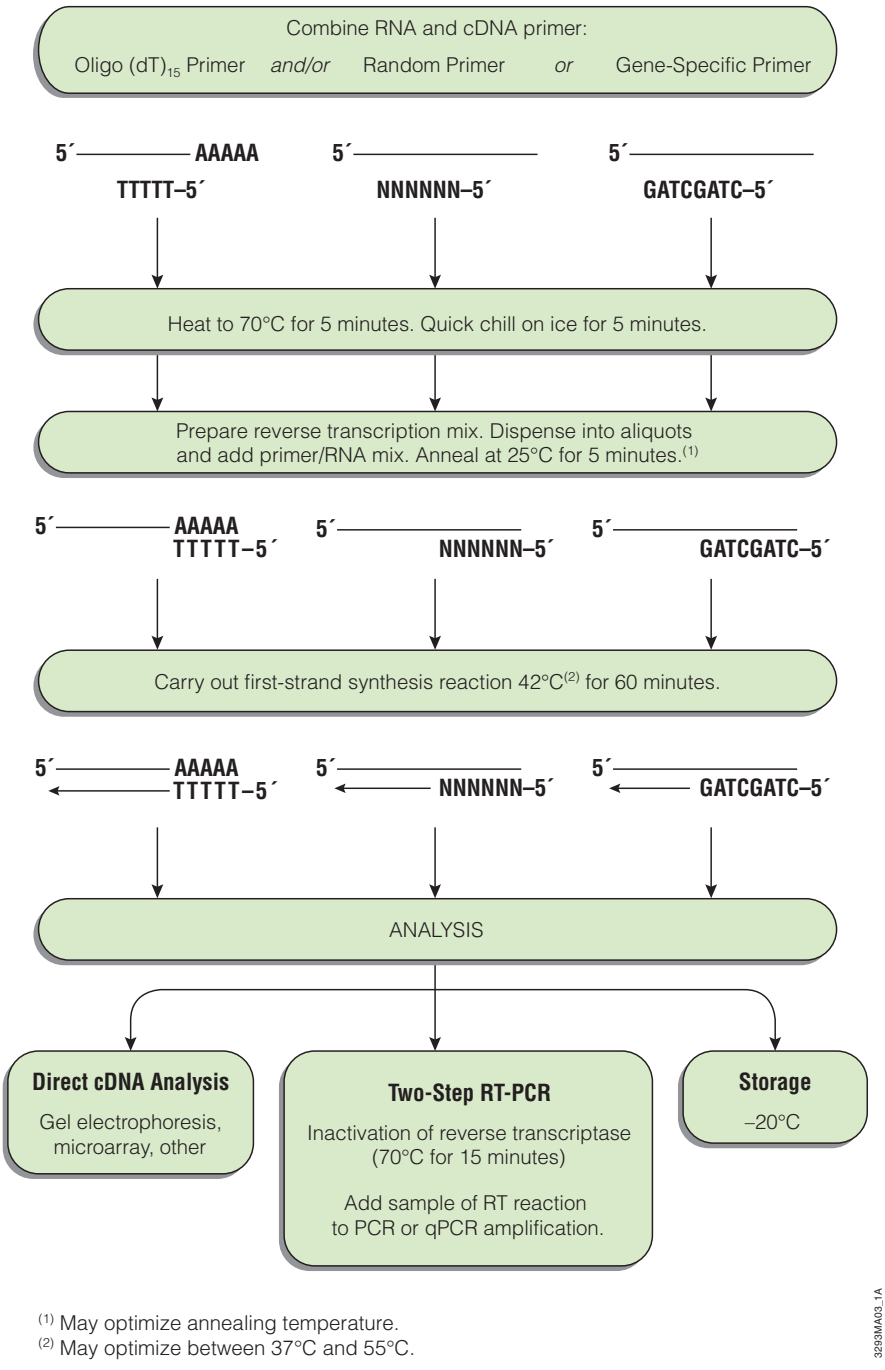
2. Product Components and Storage Conditions

Product	Size	Cat.#
ImProm-II™ Reverse Transcription System	100 reactions	A3800

Each system contains sufficient reagents for 100 first-strand cDNA synthesis reactions of 20µl each. Additionally, each system includes sufficient 1.2kb positive control RNA transcript and gene-specific primers for use in 5 control RT-PCR amplifications.

- 100µl ImProm-II™ Reverse Transcriptase
- 600µl ImProm-II™ 5X Reaction Buffer
- 1.2ml $MgCl_2$
- 320µl dNTP Mix
- 50µg Oligo$(dT)_{15}$ Primer
- 50µg Random Primers
- 5µg 1.2kb Kanamycin Positive Control RNA
- 100µl Upstream Control Primer
- 100µl Downstream Control Primer
- 2.5ml Nuclease-Free Water
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor
- 1 Protocol

Storage Conditions: Store all system components at –20°C. Thaw and maintain the ImProm-II™ 5X Reaction Buffer, the ImProm-II™ Reverse Transcriptase, dNTP Mix and the 1.2kb Kanamycin Positive Control RNA on ice during use. For long-term storage, the 1.2kb Kanamycin Positive Control RNA may be stored at –70°C in single-use aliquots. See the expiration date on the system label.



(1) May optimize annealing temperature.
(2) May optimize between 37°C and 55°C.

Figure 1. Schematic overview of cDNA synthesis and downstream analysis options using the ImProm-II™ Reverse Transcription System.

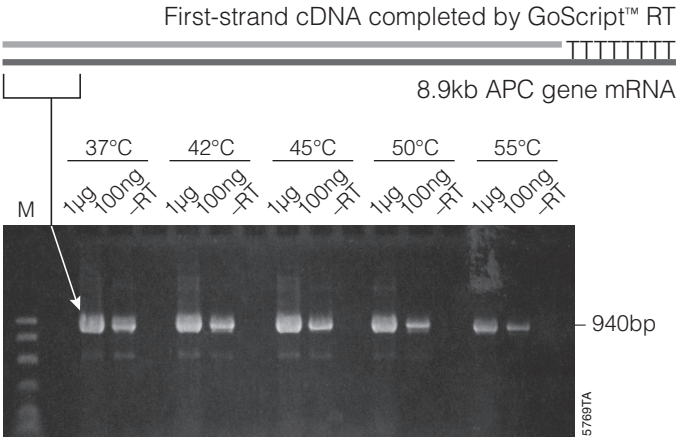


Figure 2. Full-length 8.9kb adenomatous polyposis coli (APC) cDNA synthesized over a range of temperatures (37–55°C) using the ImProm-II™ Reverse Transcription System as demonstrated by selective amplification of terminal 3´ sequences in two-step RT-PCR. The indicated amounts of RNA and 0.5µg Oligo(dT)$_{15}$ Primer were used in an ImProm-II™ Reverse Transcription System first-strand synthesis reaction in the presence of 1u/µl Recombinant RNasin® Ribonuclease Inhibitor and 3mM $MgCl_2$. The reverse transcription reactions were first annealed at 25°C and then incubated at temperatures ranging from 37–55°C for 60 minutes. After thermal inactivation of the reverse transcriptase, each 20µl reverse transcription reaction was used in a 100µl PCR using APC gene-specific primers and 5 units of GoTaq® DNA Polymerase. The APC primers, (5´–ATGGCTGCAGCTTCATATGATC–3´) and (5´–CCACCTTGGTTCCCAGATGAC–3´), were designed to anneal with the 3´ end of any APC cDNA that has been extended to the 5´ end of the 8.9kb message, selectively amplifying only the terminal 940 bases of the full-length 8.9kb cDNA sequence. A "hot-start" method was used to initiate the PCR by adding 5 units of GoTaq® DNA Polymerase directly to the reactions after they had been equilibrated at 95°C in a Perkin Elmer Model 480 thermal cycler. After a 2-minute denaturation, PCR proceeded through 38 cycles (94°C for 1 minute; 60° for 1 minute; 72°C for 3 minutes), followed by a final extension at 72°C for 5 minutes. Samples of each RT-PCR were analyzed for the presence of the 940bp amplicon by electrophoresis on a 2% GTG agarose, TAE ethidium bromide gel. The marker (M) is PCR Markers (Cat.# G3161), and the marker bands shown are 700, 800, 900 and 1,000bp.

Note: The characteristics of the products of the reverse transcriptase reaction run in the 37–55°C temperature range may vary, depending on the RNA template and on the method of analysis of the products.

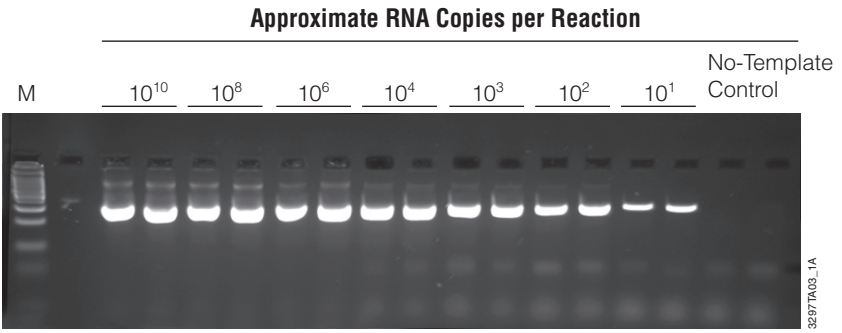


Figure 3. Full-length 1.2kb cDNA synthesized over a range of poly(A)+ transcript RNA template concentrations using the ImProm-II™ Reverse Transcription System as demonstrated by selective amplification of terminal 3´ cDNA sequences in two-step RT-PCR. Titrated amounts of the 1.2kb Kanamycin Positive Control RNA (dilutions representing approximately 10^{10} copies (0.01µg) to 10^1 copies) were combined with 0.5µg Oligo(dT)$_{15}$ Primer and used in an ImProm-II™ Reverse Transcription System first-strand synthesis reaction in the presence of 1u/µl Recombinant RNasin® Ribonuclease Inhibitor and 6mM $MgCl_2$. The reverse transcription reactions were incubated at 42°C for 60 minutes. After thermal inactivation of the reverse transcriptase, each 20µl reaction was used in a 100µl PCR that included the Upstream and Downstream Control Primers provided with this system following the cycling program described in Section 4.C except that 38 cycles were run. Samples of each RT-PCR were analyzed for the presence of the 323bp amplicon by electrophoresis on a 4% GTG agarose, TBE ethidium bromide gel. The marker (M) is 100bp DNA Ladder (Cat.# G2101).

3. General Considerations

3.A. General Laboratory Precautions

- Use designated work areas and pipettors for pre- and post-amplification steps. This precaution is intended to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid (DNA and RNA) from one experiment to the next.
- Wear gloves and change them often.
- Prevent contamination by using barrier or positive displacement pipette tips.
- Use sterile, nuclease-free thin-walled reaction tubes.
- The ImProm-II™ Reverse Transcriptase, ImProm-II™ 5X Reaction Buffer, dNTP Mix and Control RNA should be kept chilled before use. Thaw on ice; do not heat in a warming block to thaw.

3.B. ImProm-II™ Reverse Transcription System Reaction Characteristics

(See Section 5 for additional information on optimizing reaction conditions.)

- **RNA Template:** Total RNA, poly(A)+ mRNA or synthetic RNA transcript may be used. For optimal results, the RNA should be free of DNA contamination. See Section 5.A for considerations on RNA preparation and concentration per reaction.
- **RNase Inhibition:** Though not required for ImProm-II™ Reverse Transcription reaction efficiency under RNase-free conditions, the addition of Recombinant RNasin® Ribonuclease Inhibitor is recommended for experimental systems.
- **Magnesium:** Magnesium chloride is added to the reactions as a separate component. It is not included in the ImProm-II™ 5X Reaction Buffer. The final magnesium concentration of the reaction should be optimized between 1.5 and 8mM. See Section 5.B for details regarding optimization of magnesium concentration.
- **Primer Options:** The ImProm-II™ Reverse Transcription System is qualified using three methods of priming first-strand cDNA synthesis: gene-specific primers, oligo$(dT)_{15}$ primers or random hexamer primers. Section 5.C describes these options in more detail.
- **Enzyme Concentration:** The ImProm-II™ Reverse Transcriptase is designed to work most efficiently when 1µl of enzyme is added per 20µl reverse transcription reaction.
- **Reaction Temperature:** The ImProm-II™ Reverse Transcriptase is active across a range of 37–55°C, with greatest activity between 37–42°C. See Section 5.F and Figure 2 for more detailed information.
- **Controls:** A positive control template and control PCR primers for cDNA amplification are included in this system. To facilitate optimization and troubleshooting, perform both positive and negative control reactions as described in Sections 4 and 5.E.
  - + The positive control reaction tests the reverse transcriptase activity.
  - – The negative (no-template) control reaction reveals the presence of contaminating templates.
  - – The negative (no-reverse transcriptase) control reaction verifies the absence of DNA template contamination.

4. Detailed Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.A.)

- commercially autoclaved, nuclease-free, thin-walled reaction tubes, 0.5ml
- sterile, aerosol-resistant tips and pipettors
- high-quality, experimental target RNA diluted in nuclease-free water
- ice-water bath
- 25°C, 42°C and 70°C controlled-temperature water baths or heat blocks

Options for subsequent amplification in RT-PCR include:

- gene-specific primers for PCR priming
- *Taq* DNA polymerase and appropriate reaction buffer
- PCR Nucleotide Mix and magnesium chloride, 25mM, or alternative amplification system such as PCR Master Mix (Cat.# M7501)

This procedure outlines the synthesis of cDNA for subsequent amplification using PCR. Reverse transcription reactions of up to 1µg of total RNA, poly(A)+ mRNA or synthetic transcript RNA are performed in 20µl reactions comprised of components of the ImProm-II™ Reverse Transcription System. Experimental RNA is combined with the experimental primer, or an aliquot of the positive control RNA is combined with oligo$(dT)_{15}$. The primer/template mix is thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix is assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. In experimental systems, the addition of 1u/µl of Recombinant RNasin® Ribonuclease Inhibitor is recommended but optional. As a final step, the template-primer combination is added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction is incubated at 42°C for up to one hour. Because no cleanup or dilution is necessary following the cDNA synthesis, the product may be directly added to amplification reactions. This procedure outlines the method proposed to amplify the entire 20µl reaction or a 1µl aliquot of the cDNA synthesis reaction product in 100µl PCR amplifications.

4.A. Target RNA and Primer Combination and Denaturation

Note: This section describes 2 negative controls. See Section 5.E for details.

1. Place sterile, thin-walled dilution tubes and reaction tubes on ice. Thaw the experimental RNA or the 1.2kb Kanamycin Positive Control RNA on ice, and return any unused portion to the freezer as soon as aliquots are taken. If dilutions of the RNA target are made, dispense the dilution volumes of Nuclease-Free Water into aliquots and chill on ice.

2. On ice, combine the experimental RNA (up to 1µg) and the cDNA primer in Nuclease-Free Water for a final volume of 5µl per RT reaction. Multiply the volumes to accommodate multiple reactions if more than one reaction is planned using a single RNA:primer combination.

Positive Control	
1.2kb Kanamycin Positive Control RNA (1µg)	2µl
Oligo(dT)$_{15}$ Primer (0.5µg/reaction)	1µl
Nuclease-Free Water	2µl
Final volume	**5µl**

Negative (No-Template) Control	
Primer [Oligo(dT)$_{15}$, (0.5µg/reaction) or Random Primer (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)][1]	Xµl
Nuclease-Free Water	Xµl
Final volume	**5µl**

[1]10–20pmol of primer in a 20µl reaction is equal to 0.5–1µM. A general formula for calculating nanograms of primer equivalent to 10pmol is 3.3 × b, where b is the number of bases in the primer.

Experimental Reaction	
Experimental RNA (up to 1µg/reaction)[1]	Xµl
Primer [Oligo(dT)$_{15}$ (0.5µg/reaction) or Random Primer (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)]	Xµl
Nuclease-Free Water	Xµl
Final volume	**5µl**

[1]10^2–10^{10} copies of a specific target RNA template or 1pg–1µg total RNA or poly(A)+ mRNA.

3. Close each tube of RNA tightly. Place the tubes into a preheated 70°C heat block for 5 minutes. Immediately chill in ice-water for at least 5 minutes. Spin each tube for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. Keep the tubes closed and on ice until the reverse transcription reaction mix is added.

4.B. Reverse Transcription

1. Prepare the reverse transcription reaction mix by combining the following components of the ImProm-II™ Reverse Transcription System in a sterile 1.5ml microcentrifuge tube on ice. Prepare sufficient mix to allow 15µl for each cDNA synthesis reaction to be performed. Determine the volumes needed for each component, and combine them in the order listed. Vortex gently to mix, and keep on ice prior to dispensing into the reaction tubes.

Experimental Reaction	
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$ (final concentration 1.5–8.0mM)[1]	1.2–6.4µl
dNTP Mix (final concentration 0.5mM each dNTP)[2]	1.0µl
Recombinant RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final volume	**15.0µl**

[1]Mg^{2+} concentration should be optimized between 1.5–8.0mM.

[2]If isotopic or nonisotopic incorporation is desired to monitor this first-strand cDNA synthesis, α[^{32}P]-dCTP or other modified nucleotides may be supplemented into the dNTP Mix. See Section 4.D for analysis suggestions.

Negative (No-Reverse Transcriptase) Control	
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$ (final concentration 1.5–8.0mM)	1.2–6.4µl
dNTP Mix (final concentration 0.5mM each dNTP)	1.0µl
Recombinant RNasin® Ribonuclease Inhibitor (optional)	20u
Final volume	**15.0µl**

Positive-Control	
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$ (final concentration 6mM)	4.8µl
dNTP Mix (final concentration 0.5mM each dNTP)	1.0µl
Recombinant RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final volume	**15.0µl**

2. Add 15µl aliquots of the reverse transcription reaction mix to each reaction tube on ice. Be careful to prevent cross-contamination. Add 5µl of RNA and primer mix to each reaction for a final reaction volume of 20µl per tube. If there is a concern about evaporation in subsequent steps, overlay the reaction with a drop of nuclease-free mineral oil to prevent evaporation and condensation.

3. **Anneal:** Place the tubes in a controlled-temperature heat block equilibrated at 25°C, and incubate for 5 minutes.

4.B. Reverse Transcription (continued)

4. **Extend:** Incubate the tubes in a controlled-temperature heat block at 42°C for up to one hour. The extension temperature may be optimized between 37°C and 55°C.

5. The reactions may be stopped at this point for analysis of the cDNA as outlined in Section 4.D. The reactions may be maintained frozen for long-term storage.

6. **Inactivate Reverse Transcriptase:** If the experimental goal is to proceed with PCR, the reverse transcriptase must be thermally inactivated prior to amplification. Incubate the reaction tubes in a controlled-temperature heat block at 70°C for 15 minutes.

4.C. PCR Amplification

Note: ImProm-II™ Reverse Transcription reaction conditions support PCR amplification. No dilution of the cDNA is necessary. Add heat-inactivated reverse transcription reaction products directly to the PCR mix.

1. The cDNA may be directly amplified by adding the products of the heat-inactivated reverse transcription reaction to the PCR mix and proceeding with thermal cycling. As a general example, reaction volumes outlined in this procedure represent the addition of 20µl or 1µl fraction of the reverse transcription reaction into 100µl PCR amplifications. The volumes may be scaled for reactions less than 100µl. Carryover concentrations of magnesium chloride, dNTPs, buffer and primers must be considered when combining the PCR mix components.

2. Prepare the PCR mix, minus the cDNA sample, by combining the components in a sterile, 1.5ml microcentrifuge tube on ice. Combine the components in the order listed, vortex gently to mix, and keep on ice prior to dispensing to the reaction tubes. In this example, the final volume of PCR mix should be sufficient for a final reaction volume of 100µl once the cDNA volume is added. Scale the volumes to accommodate the total number of PCR amplifications being performed.

   Because of the ionic conditions, magnesium concentration and dNTP concentration of the reverse transcription reaction, the amount of magnesium and dNTP added to the PCR varies, depending on how much RT reaction is used as template. For example, for a 100µl PCR that contains 20µl of RT product, 8µl of 10X thermophilic polymerase reaction buffer is added to support the 80µl PCR mix addition. If 5µl of RT reaction were added to 95µl of PCR mix, 9.5µl of 10X thermophilic polymerase reaction buffer would be needed. Similar considerations must be given to the magnesium and dNTP additions.

   Note: In the table of PCR component additions shown, the amounts of 10X reaction buffer, 25mM $MgCl_2$ and dNTP mix combined in the PCR amplifications correspond to the specified volume of reverse transcription reaction that is added.

The example shown in the table below details the amplification conditions recommended for the amplification of either 1µl or 20µl of the cDNA synthesized in the positive control reverse transcriptase reactions containing the 1.2kb Kanamycin Positive Control RNA template in a 100µl PCR.

3. Dispense the appropriate volume of PCR mix to each reaction tube sitting on ice. Aliquots of the first-strand cDNA from the reverse transcription reaction are added last to give a final reaction volume of 100µl per tube. The amount of reverse transcription reaction used in the PCR may be modified after experimental optimization.

   Overlay the reaction with two drops of nuclease-free mineral oil to prevent evaporation and condensation.

   Note: PCR volumes may be varied from 20µl to 100µl for your experimental system.

Component	Volume per 100µl reaction (1µl RT reaction)	Volume per 100µl reaction (20µl RT reaction)
Nuclease-Free Water	65.1µl	53.6µl
10X thermophilic polymerase reaction buffer (without $MgCl_2$)	9.9µl	8.0µl
$MgCl_2$, 25mM (2mM final concentration)[1]	7.8µl	3.2µl
PCR Nucleotide Mix, 10mM (0.2mM final)	2.0µl	1.0µl
Upstream Control Primer, (1µM final concentration)	6.6µl	6.6µl
Downstream Control Primer (1µM final concentration)	6.6µl	6.6µl
Taq DNA polymerase (5.0 units)	1.0µl	1.0µl
PCR mix	99µl	80µl
volume of RT reaction	1.0µl	20.0µl
Total PCR volume	**100.0µl**	**100.0µl**

[1]For experimental systems, Mg^{2+} should be optimized between 1.5mM and 2.5mM.

4. Place the reactions in a thermal cycler that has been preheated to 94°C. An optimized program for amplification using the Upstream and Downstream Control Primers provided with this system is given below.

Denaturation	94°C for 2 minutes
25 cycles:	
Denaturation	94°C for 1 minute
Annealing	60°C for 1 minute
Extension	72°C for 2 minutes
Final extension	72°C for 5 minutes
Hold	4°C

5. After the cycle is complete, analyze the products or store the amplifications at –20°C.

4.D. cDNA Quantitation Using the Plexor® qPCR System

cDNA synthesized using ImProm-II™ Reverse Transcriptase can be amplified and quantified using the Plexor® qPCR System. cDNA samples may be used directly or diluted in MOPS/ EDTA Buffer prior to amplification. As a starting point for dilution, dilute sample and reference standard cDNA reactions 1:10 in MOPS/EDTA Buffer, then add 5µl of these diluted reactions to the reaction mix. For additional information, refer to the *Plexor® Two-Step qRT-PCR System Technical Manual* #TM264.

4.E. Analysis

1. The products of the first-strand cDNA synthesis reaction may be analyzed or detected using a number of methods. Alkaline agarose gel electrophoresis is recommended for visualization of isotopically labeled cDNA (1). For a description of this method, refer to the PCR Applications Chapter of the *Protocols and Applications Guide* (2).

2. Analyze the PCR products by agarose gel electrophoresis using approximately 5–10% of the total reaction. The products should be readily visible on an ethidium bromide-stained gel. The amplification product obtained using the positive control RNA with the Upstream and Downstream Control Primers is 323bp long.

5. Protocol Optimization

The ImProm-II™ Reverse Transcriptase System can be used for efficient first-strand cDNA synthesis from a variety of RNA templates: total RNA, poly(A)+ mRNA or synthetic RNA transcripts. The ultimate yield of full-length cDNA obtained from this system is influenced by many factors including the quality of the starting RNA, abundance of the specific target in the RNA population, magnesium concentration, choice of cDNA primer and reaction temperature.

5.A. RNA Template

Successful reverse transcription is dependent on the integrity and purity of the RNA used as the template. Procedures for creating and maintaining an RNase-free environment are described in Section 3 and in Blumberg, 1987 (3). Use sterile tubes, pipette tips, gloves and nuclease-free reagents for all preparation steps. When using RNA isolated from samples high in ribonuclease activity, we recommend adding 20 units of RNasin® Ribonuclease Inhibitor to each 20µl reverse transcription reaction.

Total RNA, poly(A)+ mRNA or synthetic RNA transcripts over a wide range of concentrations can be used as a template in reverse transcription reactions. The optimal amount of starting RNA will be specific to the experimental design, but these recommendations are offered as a starting point. For reverse transcription reactions where direct analysis of the cDNA will proceed without target amplification, we recommend that 1µg of poly(A)+ mRNA or synthetic

transcript RNA be used per reaction. In reverse transcription reactions where the cDNA sequences will be amplified by PCR, excellent results can be obtained using total RNA template levels of approximately 1pg for highly abundant targets to 1µg for rare or long targets (>8kb). The amount of starting RNA must be optimized for each experimental system.

5.B. Magnesium Concentration Optimization

The ImProm-II™ Reverse Transcription System supplies magnesium chloride as a separate component to allow for optimization. The ImProm-II™ 5X Reaction Buffer **does not** contain magnesium chloride. The ImProm-II™ Reverse Transcriptase requires magnesium for activity and tolerates a wide range of magnesium concentrations. We recommend using between 1.5mM and 8mM. Reverse transcription reactions designed to make full-length cDNA from long messages (>7kb) are more efficient at lower magnesium concentrations. Short messages are more efficiently produced at higher magnesium concentrations. As illustrated in Section 4.B, 6mM $MgCl_2$ is suggested for the positive control reverse transcription reactions.

5.C. Primer Options and Design

There are three ways to prime the synthesis of first-strand cDNA using an RNA template. Reverse transcription reactions may be primed using oligo(dT) primer, random primers or gene-specific primers.

Sequence-specific primers that anneal to the 3´ end of the RNA of interest are used to generate specific cDNAs from an RNA target.

Priming using random hexamers is the most general method of initiating cDNA synthesis from a variety of RNA templates. Random hexamers can be used to prime first-strand cDNA synthesis from all RNA molecules, including those that do not possess a poly(A)+ tail and RNA isolated from prokaryotic sources. Use of random primers will yield a population of cDNA products primed internally along the entire RNA sequence.

Oligo$(dT)_{15}$ priming initiates first-strand synthesis by annealing to the 3´ end of any polyadenylated RNA molecule. Reverse transcription of total RNA or mRNA will yield oligo$(dT)_{15}$-primed products from any poly(A)+ RNA. The synthesis does not require knowledge of the experimental gene sequence or annealing characteristics. The population of cDNAs produced using this method allow many different downstream analyses from a single reverse transcription reaction. Thus priming with oligo$(dT)_{15}$ is the method of choice in most cases and is described in the procedure outlined for the control reactions in Section 4.

Regardless of primer choice, the final concentration of primer in the reaction must be optimized. In the protocol outlined in Section 4.A, we recommend 0.5µg of oligo$(dT)_{15}$ or random primers per 20µl reverse transcription reaction or 0.5–1µM final concentration of a gene-specific primer as a starting point for optimization.

5.D. Reverse Transcriptase Enzyme Concentration

The ImProm-II™ Reverse Transcriptase supplied with this system is formulated for efficient first-strand cDNA synthesis or RT-PCR applications. For robust activity in any of these applications, use 1µl of the enzyme in a 20µl reaction.

5.E. Control Reactions

To facilitate optimization and troubleshooting of the reverse transcription reaction or associated RT-PCR, perform both positive and negative control reactions. RNA template and gene-specific primers for amplification are supplied with the ImProm-II™ Reverse Transcription System for this use. The recommended protocols for reverse transcription and subsequent PCR amplification using *Taq* DNA polymerase and reaction components are described in Section 4.C.

Positive Control: First-strand cDNA synthesis is performed using 1µg or less of the 1.2kb Kanamycin Positive Control RNA as a template and 0.5µg of Oligo(dT)$_{15}$ Primer. The resulting cDNA can be amplified by PCR using the kanamycin gene-specific Upstream and Downstream Control Primers. The optimized design of this ImProm-II™ Reverse Transcription System allows direct amplification of 1–20µl of the reverse transcription reaction by PCR.

Negative (No-Template) Control: No-template control reactions are performed by omitting the target RNA from the reactions while including the Oligo(dT)$_{15}$ Primer. Gene-specific control primers are included in the subsequent PCR amplification. No-template control reactions are outlined in Section 4.A.

Negative (No-Reverse Transcriptase) Control: To test for the presence of contaminating genomic DNA or plasmid DNA in the RNA template, a no-reverse transcriptase reaction may be performed. When the no-reverse transcriptase reaction is amplified by PCR using *Taq* DNA polymerase and all experimental gene-specific primers, DNA sequences that are introduced with the RNA target and not synthesized by the reverse transcriptase can be detected. The recommended protocol for such a control is described in Section 4.B.

5.F. Temperature

In many experimental situations, the RNA template and the cDNA primer require thermal denaturation prior to reverse transcription; therefore, we include a heat-denaturation step in this protocol. Combine the RNA and primer in a separate tube as described in Section 4.A. Incubate in a controlled-temperature heat block at 70°C for 5 minutes, then quick-chill in ice-water prior to adding to the reverse transcription reaction mix. Do not incubate the reverse transcription reaction mix including the ImProm-II™ Reverse Transcriptase enzyme at this elevated temperature, as the enzyme will be inactivated. The denatured template and primer mixture can be added to the reverse transcription reaction mix for standard reverse transcription at 42°C.

In many cases, cDNA synthesis through a region containing a high degree of secondary structure proceeds more efficiently at higher temperatures. The ImProm-II™ Reverse Transcriptase enzyme is active over a range of 37–55°C (Figure 2). The characteristics of the reverse transcriptase reaction products generated in this temperature range may vary, depending on the RNA template and the method of analysis of the products. We recommend performing the reverse transcription reaction for 60 minutes at 42°C as a starting point for experimental optimization.

Following cDNA synthesis, the reaction products may be manipulated without steps to remove the enzyme or other reaction components. The cDNA can be stored overnight in the reverse transcription reaction mix at –20°C or –70°C. If PCR amplification is to proceed in a two-step RT-PCR format, the reverse transcriptase must be inactivated to obtain efficient amplification because of unspecified interactions between many reverse transcriptase enzymes, cDNAs and thermophilic DNA polymerases. We recommend an intermediate incubation at 70°C for 15 minutes to thermally inactivate the enzyme. The procedure outlined in Section 4.B includes a 15-minute incubation at 70°C as an appropriate treatment that maintains the integrity of the cDNA but heat-denatures the reverse transcriptase.

5.G. Subsequent cDNA Amplification

The synthesized cDNA may be added directly to PCR amplifications. Unlike other first-strand systems, there will be no inhibitory effects encountered when the entire 20µl reaction is added to a PCR amplification as long as the final $MgCl_2$ concentration is kept at an optimal level. When dividing one first-strand cDNA synthesis reaction into multiple PCRs, aliquots of the reverse transcription reaction as small as 1µl can be used in each PCR. The robust reaction conditions of the ImProm-II™ Reverse Transcription System make many flexible applications possible. The method outlined in Section 4.C describes two-step RT-PCR of the 1.2kb Kanamycin Positive Control RNA using either 20µl or 1µl of the reverse transcription reaction in a 100µl PCR. The volumes of PCR components assembled take into account the carryover of buffer, magnesium and dNTP from the reverse transcription reaction to achieve the final concentration of each component.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low yield of full-length first-strand cDNA	cDNA degradation. Residual DNase from the RNA preparation method may be digesting the cDNA. Use the SV Total RNA Isolation System (Cat.# Z3100) to prepare RNA.
	RNA degradation. • Always use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol-resistant tips and gloves. Ensure that reagents, tubes and tips are kept RNase-free by using sterile technique. • Isolate RNA in the presence of Promega RNasin® Ribonuclease Inhibitor. • RNA storage conditions are very important. Store at –70°C. Keep RNA target in single-use aliquots to minimize freeze-thaw cycles. Once thawed, keep RNA on ice. • Add 1µg of the positive control RNA to the reverse transcription with the experimental RNA and amplify the resulting cDNA using the Upstream and Downstream Control Primers to determine if RNase contamination of the RNA template is a problem. • Use Promega RNasin® Ribonuclease Inhibitor to inhibit degradation of target during cDNA synthesis (20u/20µl reaction).
	Inhibitors present in RNA preparation. Inhibitors such as SDS, EDTA, polysaccharides, heparin, guanidine thiocyanate or other salts may carry over from some RNA preparations and interfere with first-strand cDNA synthesis. Use the SV Total RNA Isolation System (Cat.# Z3100) to prepare RNA. To determine if the experimental RNA preparation contains an inhibitor, add the positive control RNA to the reverse transcription with the experimental RNA and amplify the resulting cDNA using the Upstream and Downstream Control Primers to assess the degree of inhibition of cDNA synthesis.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low yield of full-length first-strand cDNA (continued)	Primer-annealing temperature too high. Verify that the initial annealing incubation was carried out at an appropriate temperature to encourage primer-target hybridization. A primer melting temperature (T_m) calculator is available at: **www.promega.com/biomath/#melt/**
	RNA has a high degree of GC-rich secondary structure. • Heat RNA target and primer at 70°C for 5 minutes, then place immediately on ice to ensure denaturation of template and primer and minimize refolding. • Add agents that facilitate strand separation to melt out RNA secondary structure. Try supplementing the RT reaction with 1M betaine, 2% DMSO or 5% deionized formamide. • If the cDNA primer is a gene-specific sequence that has a T_m >50°C, try adding the target and primer directly from the 70°C denaturation into a prewarmed reaction mixture (45–55°C) to minimize formation of secondary structure.
	RNA target sequence contains strong transcriptional pauses. In cases where the entire gene sequence is not required in the cDNA, choose random primers or primers specific for proximal regions of the target rather than oligo$(dT)_{15}$ to initiate cDNA synthesis.
	Primer specificity. Verify that the "downstream" cDNA primer was designed to be complementary to the distal 3´ sequences of the RNA. Verify that the primer sequence is equivalent to the "antisense" sequence.
	Incorrect primer:RNA ratio. Use 0.5µg oligo$(dT)_{15}$ or random hexamers per 20µl cDNA synthesis reaction. Use 0.5–1.0µM gene-specific primer per reaction.
	dNTP concentration too high. Do not use more than 0.5mM final concentration of dNTP in the cDNA synthesis.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low yield of full-length first-strand cDNA (continued)	Nonoptimal magnesium concentration. Magnesium salts occasionally cause degradation of long cDNA. If denaturing gel analysis of ^{32}P-labeled cDNA shows evidence of degradation, try reducing the concentration of the $MgCl_2$. Optimal concentrations are between 1.5mM and 8.0mM.
	Reverse transcriptase inactivation prior to cDNA synthesis. Be certain to combine the RT reaction mix with the target after the thermal denaturation of RNA and primer. Thermal denaturation will inactivate the ImProm-II™ Reverse Transcriptase.
	"Hot start" needed. Occasionally, the yield of full-length cDNA that is synthesized can be increased by using a "hot-start" reverse transcription method. To use this method, add the ImProm-II™ Reverse Transcriptase to the cDNA synthesis reaction after the initial annealing incubation at 25°C and subsequent equilibration at 42°C. (**Note:** This will not work for random hexamer priming.)
RT-PCR amplification product has a molecular weight higher than expected	Genomic DNA sequences related to the RNA template contaminate the RNA preparation. Use the SV Total RNA Isolation System (Cat.# Z3100) protocol, which includes steps to remove genomic DNA.
RT-PCR amplification products have multiple or unexpected sizes	Contamination by another target RNA or DNA. Use positive displacement pipets or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and post-amplification. Wear gloves and change them often. Use UNG (4) or another sterilization technique to prevent DNA carryover to subsequent reactions.
	Multiple target sequences exist in the target RNA. Design new, more specific primers for the experiment.
	Low-molecular-weight products due to nonspecific primer annealing. • Optimize the annealing temperature used during PCR. A T_m calculator is available at: **www.promega.com/biomath/#melt/** • Design PCR primers with no complementary sequences at their 3´ ends.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
RT-PCR amplification products have multiple or unexpected sizes (continued)	Suboptimal magnesium concentration. Optimize magnesium concentration for each target/primer set.
	Vortex thawed magnesium solution prior to use.
	Reverse transcriptase effect on primer-dimer artifact synthesis in RT-PCR. Make sure to thoroughly heat-inactivate the reverse transcription reactions prior to use (5,6).
Low yield of RT-PCR amplification product	Insufficient cDNA added to PCR amplification. If 1µl of cDNA is not detected following PCR amplification, try amplifying the entire 20µl reverse transcription in a 100µl PCR.
	RNA:cDNA hybrid prevents efficient amplification. cDNA yield and amplification sensitivity can be increased by removing or degrading the RNA prior to PCR. Add an RNase H digestion step to optimize the results.
	Poor primer design. • If the reaction products appear to be entirely primer artifacts, the reaction may not have amplified the desired RT-PCR product because of primer-primer interactions. Make sure the primers are not self-complementary. • Check the length and T_m of the PCR primers.
	Too little thermophilic DNA polymerase added to PCR. Add up to 5 units of *Taq* DNA polymerase to the amplification reaction to minimize any inhibitory effects of the reverse transcriptase.
	Suboptimal magnesium concentration for PCR. Optimize the final magnesium concentration in the amplification reaction. Be sure to consider the amount of magnesium that is introduced with the cDNA synthesis reaction when setting up the PCR amplification.
	Nucleotides degraded. Keep nucleotide solutions frozen in aliquots, thaw quickly, and keep on ice once thawed. Avoid multiple freeze-thaw cycles.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low yield of RT-PCR amplification product (continued)	Suboptimal annealing temperature. Optimize annealing temperature of each cycle by increasing or decreasing the temperature in increments of 1°C.
	Extension time too brief for amplicon length. To minimize the interactive effects of the reverse transcriptase and thermophilic DNA polymerase, design the thermal cycling program with a longer extension time in each cycle. Begin with 1 minute per kilobase per cycle, and increase to 2 minutes or more if necessary.
	Too few cycles in PCR. To detect rare or difficult RNA targets by RT-PCR, increase the cycle number to 40 to maximize sensitivity.
	Wrong reaction tubes used. Make sure to use thin-walled reaction tubes for optimal heat transfer during PCR.
	Use only sterile, nuclease-free commercially autoclaved tubes for PCR. Autoclaving eliminates volatile contaminants that inhibit amplification.
	Mineral oil problem. Use only high-quality, nuclease-free light mineral oil. Do not use autoclaved mineral oil.
	Target sequence not present in target RNA. Redesign experiment or try other sources of target RNA.

7. References

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8. Appendix

8.A. Composition of Buffers and Solutions

Positive Control RNA

1.2kb kanamycin resistance gene mRNA prepared by in vitro transcription and supplied at a concentration of 0.5µg/µl in TE buffer

Control Primer Sequences

Upstream Control Primer:
5´–GCC ATT CTC ACC GGA TTC AGT CGT C–3´

Downstream Control Primer:
5´–AGC CGC CGT CCC GTC AAG TCA G–3´

8.B. Related Products

Reverse Transcription

Product	Concentration	Size	Cat.#
ImProm-II™ Reverse Transcriptase		10 reactions	A3801
		100 reactions	A3802
		500 reactions	A3803
Access RT-PCR System		100 reactions	A1250
		500 reactions	A1280
Access RT-PCR Introductory System		20 reactions	A1260
Reverse Transcription System		100 reactions	A3500
AMV Reverse Transcriptase	10u/µl	300u	M5101
	10u/µl	1,000u	M5108
	20–25u/µl	600u	M9004
M-MLV Reverse Transcriptase	200u/µl	10,000u	M1701
	200u/µl	50,000u	M1705
Ribonuclease H	0.5–2u/µl	50u	M4281
	0.5–2u/µl	250u	M4285

Cat.# A3801, A3802 and A3803 contain ImProm-II™ Reverse Transcriptase, 5X ImProm-II™ Reaction Buffer and $MgCl_2$ only.

Real-Time Quantitative Amplification

Product	Size	Cat.#
Plexor® qPCR System	200 reactions	A4031[1], A4011[2]
Plexor® One-Step qRT-PCR System	200 reactions	A4041[1], A4021[2]
Plexor® Two-Step qRT-PCR System	200 reactions	A4061[1], A4051[2]

[1]Cat.# A4031, A4041, A4061 are available in Europe or through Distributors supported by Promega European Branch Offices.

[2]Cat.# A4011, A4021, A4051 are available in all other countries, including the United States.

8.B. Related Products (continued)

Thermostable DNA Polymerases and PCR Master Mixes

Product	Size	Cat.#
GoTaq® Green Master Mix	100 reactions	M7112[1], M7122[2]
	1,000 reactions	M7113[1], M7123[2]
GoTaq® Colorless Master Mix	100 reactions	M7142[1], M7132[2]
	1,000 reactions	M7143[1], M7133[2]

GoTaq® Master Mixes are premixed solutions containing GoTaq® DNA Polymerase, GoTaq® Reaction Buffer (Green or Colorless), dNTPs and Mg^{2+}.
[1]Cat.# M7112, M7113, M7142, M7143 are available in Europe or through Distributors supported by Promega European Branch Offices.
[2]Cat.# M7122, M7123, M7132, M7133 are available in all other countries, including the United States.

Product	Size	Cat.#
GoTaq® Flexi DNA Polymerase	100u	M8301[1], M8291[2]

Available in additional sizes. GoTaq® Flexi DNA Polymerase includes 5X Green GoTaq® Flexi Reaction Buffer, 5X Colorless GoTaq® Flexi Reaction Buffer and Magnesium Chloride Solution, 25mM. Reaction buffers are magnesium-free.
[1]Cat.# M8301 is available in Europe or through Distributors supported by Promega European Branch Offices.
[2]Cat.# M8291 is available in all other countries, including the United States.

Product	Size	Cat.#
GoTaq® DNA Polymerase	100u	M3171[1], M3001[2]

Available in additional sizes.
[1]Cat.# M3171 is available in Europe or through Distributors supported by Promega European Branch Offices.
[2]Cat.# M3001 is available in all other countries, including the United States.

Product	Concentration	Size	Cat.#
PCR Master Mix	2X	10 reactions	M7501
	2X	100 reactions	M7502
	2X	1,000 reactions	M7505

May not be available in Europe. Premixed solution of GoTaq® DNA polymerase, reaction buffer, dNTPs and Mg^{2+}. One reaction refers to a 50µl reaction.

Product	Concentration	Size	Cat.#
Pfu DNA Polymerase	2–3u/µl	100u	M7741
	2–3u/µl	500u	M7745
Tfl DNA Polymerase*	5u/µl	100u	M1941
	5u/µl	1,000u	M1945
Tth DNA Polymerase	5u/µl	100u	M2101
	5u/µl	500u	M2105

*Includes magnesium-free 10X reaction buffer and a tube of 25mM $MgCl_2$.

Reagents and dNTPs

Product	Concentration	Size	Cat.#
RNasin® Plus RNase Inhibitor	40u/µl	2,500u	N2611
	40u/µl	10,000u	N2615
Recombinant RNasin® Ribonuclease Inhibitor	20–40u/µl	2,500u	N2511
	20–40u/µl	10,000u	N2515
RNasin® Ribonuclease Inhibitor	20–40u/µl	2,500u	N2111
	20–40u/µl	10,000u	N2115
RQ1 RNase-Free DNase	1u/µl	1,000u	M6101

Product	Size	Cat.#
PCR Nucleotide Mix, 10mM	200µl	C1141
	1,000µl	C1145
dATP, 100mM	40µmol	U1201
dCTP, 100mM	40µmol	U1221
dGTP, 100mM	40µmol	U1211
dTTP, 100mM	40µmol	U1231
dATP, dCTP, dGTP, dTTP, 100mM each	40µmol each	U1240
	25µmol each	U1420
	10µmol each	U1330
Lambda DNA/HindIII Markers	100µg (200 lanes)	G1711
100bp DNA Ladder	250µl (50 lanes)	G2101

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[a]U.S. Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

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