Technical Manual

GoScript™ Reverse Transcription System

INSTRUCTIONS FOR USE OF PRODUCTS A5000 AND A5001.

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GoScript™ Reverse Transcription System

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1. Description

The GoScript™ 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 optimized in preparation for PCR amplification. The components of the GoScript™ Reverse Transcription System can be used to reverse transcribe RNA templates starting with total RNA, poly(A)+ mRNA or synthetic transcript RNA. Figure 1 provides an overview of the reverse transcription procedure. The optimized reaction buffer and reverse transcriptase provided in the GoScript™ Reverse Transcription System enable robust, full-length cDNA synthesis for reproducible analysis of rare or long messages.

1. Description (continued)

These conditions were developed for cDNA synthesis or for easy transition to gene-specific target amplification. Up to 5µl of the reverse transcription reaction can be directly amplified using *Taq* DNA polymerase in a 25µl PCR. GoScript™ Reverse Transcriptase is qualified for use in two-step RT-qPCR using GoTaq® qPCR and Plexor® qPCR Systems. In two-step RT-qPCR using GoTaq® qPCR Master Mix, samples of the GoScript™ Reverse Transcription System can be added directly, up to 20% v/v.

2. Product Components and Storage Conditions

Product	Size	Cat.#
GoScript™ Reverse Transcription System	50 reactions	A5000

Each system contains sufficient reagents for 50 first-strand cDNA synthesis reactions of 20µl each.

- 50µl GoScript™ Reverse Transcriptase
- 300µl GoScript™ 5X Reaction Buffer
- 750µl $MgCl_2$ (25mM)
- 200µl PCR Nucleotide Mix
- 50µg $Oligo(dT)_{15}$ Primer
- 50µg Random Primers
- 1.25ml Nuclease-Free Water
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor

Product	Size	Cat.#
GoScript™ Reverse Transcription System	100 reactions	A5001

Each system contains sufficient reagents for 100 first-strand cDNA synthesis reactions of 20µl each.

- 100µl GoScript™ Reverse Transcriptase
- 600µl GoScript™ 5X Reaction Buffer
- 1.2ml $MgCl_2$ (25mM)
- 200µl PCR Nucleotide Mix
- 50µg $Oligo(dT)_{15}$ Primer
- 50µg Random Primers
- 1.25ml Nuclease-Free Water
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor

Available Separately

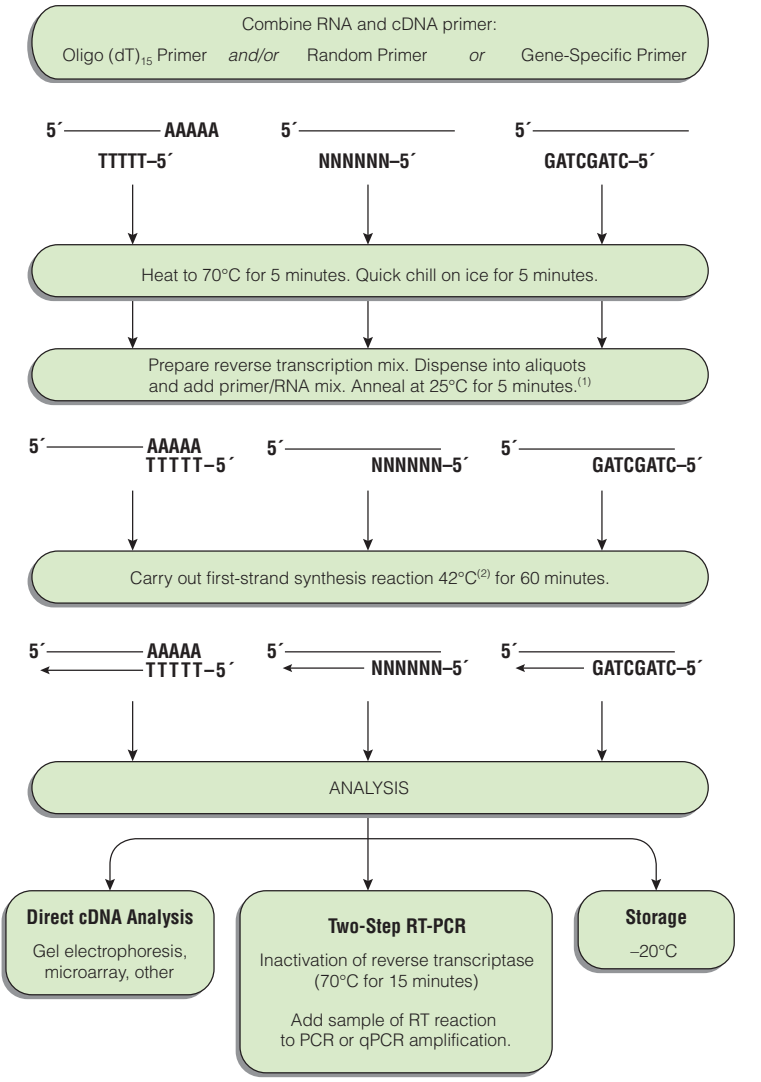
Product	Size	Cat.#
GoScript™ Reverse Transcriptase	10 reactions	A5002
GoScript™ Reverse Transcriptase	100 reactions	A5003
GoScript™ Reverse Transcriptase	500 reactions	A5004

Storage Conditions: Store all system components at –20°C. Thaw and maintain the GoScript™ 5X Reaction Buffer, the GoScript™ Reverse Transcriptase and PCR Nucleotide Mix on ice during use. Mix the GoScript™ 5X Reaction Buffer until no visible precipitate is present. See the expiration date on the system label.

3. 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 GoScript™ Reverse Transcriptase, GoScript™ 5X Reaction Buffer and PCR Nucleotide Mix should be kept chilled before use. Thaw on ice; do not thaw by heating in a warming block.

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 the method of analysis of the products.



(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 GoScript™ Reverse Transcription System.

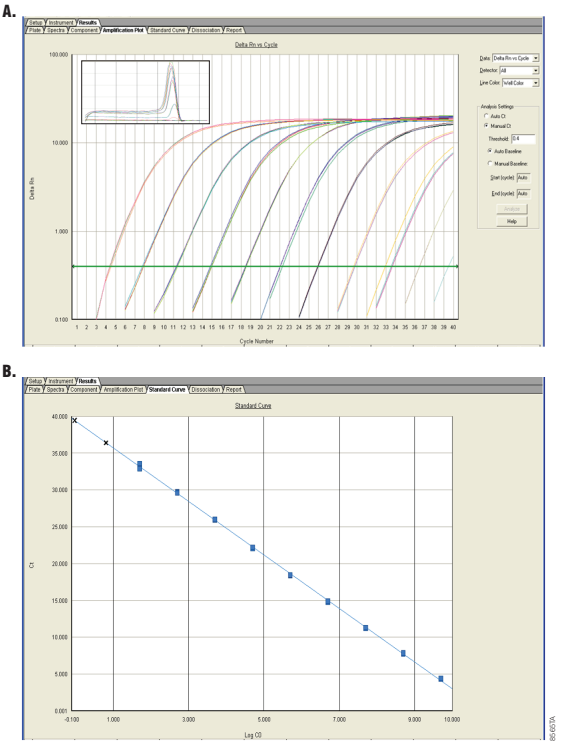


Figure 2. GoScript™ Reverse Transcriptase cDNA synthesis coupled with GoTaq® qPCR Master Mix can detect input template over a 9-log order dynamic range. **Panel A.** Ten RNA template mixes were assembled to model a series of tenfold differences of a specific RNA abundance (1×10^2 to 1×10^{11} copies of MS2 bacteriophage RNA or 0.2fg to 200ng) in a constant mass of 10ng of human total RNA. cDNAs for each RNA sample were prepared using GoScript™ Reverse Transcriptase plus oligo(dT) and random primer in parallel with minus-RT control reactions. Each reverse transcription reaction was diluted (1:10) for use as template in cDNA-specific qPCR. Replicate (n = 3) GoTaq® qPCR at each level of total-cDNA template, minus-RT control and no-template control (NTC) was performed using primers specific to MS2 cDNA. Semilog-scale amplification plots are shown on the left, including the results of the standard, "unknown", minus-RT controls and NTC (undetected) reactions. Inset (**Panel A**) shows dissociation profile for the 50-, 500- and 5,000-copy standard and 5-copy detected levels of MS2 + cDNA and NTC reactions, demonstrating specificity. Standard values are defined as the number of copies of input MS2 bacteriophage RNA in each sample of diluted reverse transcription reaction: 5×10^9 down to 5×10^1 copies in 500pg of human total RNA. Standard curve shown in **Panel B** illustrates the quantitation and detection of the "unknown" samples. • = 10-fold standard dilutions; xx = detection of 5-copy samples.

4. Detailed Protocol

Materials to Be Supplied by the User

- 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 or GoTaq® Hot Start Polymerase (Cat.# M5001)
- PCR Nucleotide Mix and magnesium chloride, 25mM, or alternative amplification system such as GoTaq® Green Master Mix (Cat.# M7122) or GoTaq® Colorless Master Mix (Cat.# M5133)
- qPCR system, i.e., GoTaq® qPCR Master Mix (Cat.# A6001) or Plexor® qPCR System (Cat.# A4011)

This procedure outlines the synthesis of cDNA for subsequent amplification using PCR or qPCR. Reverse transcription reactions of up to 5µg of total RNA, poly(A)+ mRNA or synthetic transcript RNA are performed in 20µl reactions comprised of components of the GoScript™ Reverse Transcription System. Experimental RNA is combined with the experimental primer. 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 (PCR Nucleotide Mix) and ribonuclease inhibitor. In experimental systems, 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 cDNA synthesis, the product may be directly added to amplification reactions. This procedure outlines the method proposed to amplify up to a 5µl aliquot of the cDNA synthesis reaction product in 25µl PCR amplifications.

4.A. First-Strand cDNA Synthesis

The following procedure is designed to convert up to 5µg of total RNA or up to 500ng of poly(A) RNA into first-strand cDNA.

1. Mix and briefly centrifuge each component before use. Combine the following:

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

2. Close each tube of RNA tightly. Place tubes into a preheated 70°C heat block for 5 minutes. Immediately chill in ice-water for at least 5 minutes. Centrifuge 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.

3. Prepare the reverse transcription reaction mix by combining the following components of the GoScript™ Reverse Transcription System in a sterile 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.

Component	Amount
Nuclease-Free Water (to a final volume of 15µl)	Xµl
GoScript™ 5X Reaction Buffer	4.0µl
$MgCl_2$ (final concentration 1.5–5.0mM)[1]	1.2–4.0µl
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)[2]	1.0µl
Recombinant RNasin® Ribonuclease Inhibitor (optional)	20u
GoScript™ Reverse Transcriptase	1.0µl
Final volume	**15.0µl**

[1]Mg^{2+} concentration should be optimized. We recommend 1.5–5.0mM. ($MgCl_2$ is provided at 25mM.)

[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 in the PCR Nucleotide Mix. See Section 4.D for analysis suggestions.

4.A. First-Strand cDNA Synthesis (continued)

4. 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.

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

6. **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.

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

7. **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.B. cDNA Quantification Using qPCR

cDNA synthesized using GoScript™ Reverse Transcriptase can be amplified and quantified using the GoTaq® qPCR Master Mix or Plexor® qPCR Systems. cDNA samples may be used directly or diluted prior to amplification. As a starting point for dilution, dilute sample and reference standard cDNA reactions 1:10, then add 5µl of these diluted reactions to the reaction mix. For additional information, refer to the *GoTaq® qPCR Master Mix Technical Manual,* #TM318, or the *Plexor® qPCR System Technical Manual*, #TM262.

The synthesized cDNA may be added directly to PCR amplifications. Unlike other first-strand systems, there will be no inhibitory effects encountered when up to 20% of the reaction is added to a PCR amplification as long as the final $MgCl_2$ concentration is kept at an optimal level. The robust reaction conditions of the GoScript™ Reverse Transcription System make many flexible applications possible. The method outlined in Section 4.C describes two-step RT-PCR using either 1µl or 5µl of the reverse transcription reaction in a 25µl 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.

1. Heat inactivate the reverse transcription reaction.
2. Proceed directly to GoTaq® or Plexor® qPCR target-specific quantitative analysis of the cDNA.
3. Alternatively, heat-inactivated reactions may be stored frozen for future use.
4. GoTaq® and Plexor® qPCR accommodate addition of up to 20% of the total reaction volume as template GoScript™ Reverse Transcriptase reaction volume.
5. The cDNA may be added directly to the qPCR as undiluted reverse transcription reaction product, or it may be diluted.
6. The dilution factor must be experimentally determined to be appropriate for the amount of RNA template mass and proportional reverse-transcript representation in the cDNA sample.
7. Generally, for cDNA quantitation, using the default analysis settings of many real-time instruments, the amount of cDNA used as template in GoTaq® qPCR should not exceed that correlating with a proportional 100ng of input total RNA. The cDNA generated from highly abundant transcripts can be detected in less than 1pg of total RNA.

4.C. PCR Amplification for Endpoint Analysis

Note: GoScript™ 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 amplified directly 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 5µl or 1µl fraction of the reverse transcription reaction to 25µl PCR amplifications. The volumes may be scaled for reactions less than 25µl. Carryover concentrations of magnesium chloride, PCR Nucleotide Mix, 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 25µl once the cDNA volume is added. Scale the volumes to accommodate the total number of PCR amplifications being performed.

4.C. PCR Amplification for Endpoint Analysis (continued)

Due to 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 PCR Nucleotide mix combined in the PCR amplifications correspond to the specified volume of reverse transcription reaction that is added.

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 25µl per tube. The amount of reverse transcription reaction used in the PCR may be modified after experimental optimization.

   Note: PCR volumes may be varied: Volumes up to 50µl are commonly used.

Component	Volume per 25µl reaction (1µl RT reaction)	Volume per 25µl reaction (5µl RT reaction)
Nuclease-Free Water (to be determined)	__µl	__µl
5X GoTaq® Flexi Reaction Buffer	5.0µl	4.0µl
$MgCl_2$, 25mM (2mM final concentration)	2.0µl	0.8µl
PCR Nucleotide Mix, 10mM (0.2mM final)	0.5µl	0.25µl
upstream primer (1µM final concentration)	__	__
downstream primer (1µM final concentration)	__	__
GoTaq® Flexi DNA polymerase (5.0 units)	.25µl	.25µl
PCR mix volume	**24µl**	**20µl**
+		
cDNA volume	1.0µl	5.0µl
Total PCR volume	**25µl**	**25µl**

4. Place the reactions in a thermal cycler that has been preheated to 94°C.
5. After thermal cycling is complete, analyze the products, or store the amplifications at –20°C.

4.D. Analysis

1. Products of the first-strand cDNA synthesis reaction may be analyzed or detected using a number of methods. Alkaline agarose gel electrophoresis is recommended to visualize 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.

5. Protocol Optimization

The GoScript™ Reverse Transcription 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 using 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.

For information on optimizing qPCR reactions see the *GoTaq® qPCR Master Mix Technical Manual, #TM318* or PCR Applications chapter of the Protocols and Applications guide, online: **www.promega.com/paguide/chap1.htm**

5.A. GoScript™ Reverse Transcription System Reaction Characteristics

- **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 GoScript™ Reverse Transcription reaction efficiency under RNase-free conditions, 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 GoScript™ 5X Reaction Buffer. The final magnesium concentration should be optimized between 1.5mM and 5mM. See Section 5.C for details regarding optimization of magnesium concentration.
- **Primer Options:** The GoScript™ 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.D describes these options in more detail.
- **Enzyme Concentration:** The GoScript™ Reverse Transcriptase works most efficiently when 1µl of enzyme is added per 20µl reverse transcription reaction.

5.A. GoScript™ Reverse Transcription System Reaction Characteristics (continued)

- **Reaction Temperature:** The GoScript™ Reverse Transcriptase is active across a range of 37–55°C, with greatest activity at 37–42°C. See Section 5.G for more detailed information.
- **Controls:** To facilitate optimization and troubleshooting, perform negative control reactions as described in Section 5.F.
  - 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.

5.B. RNA Template

Successful reverse transcription depends on the integrity and purity of the RNA 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 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.C. Magnesium Concentration Optimization

The GoScript™ Reverse Transcription System supplies magnesium chloride as a separate component to allow optimization. The GoScript™ 5X Reaction Buffer **does not** contain magnesium chloride. The GoScript™ Reverse Transcriptase requires magnesium for activity and tolerates a wide range of magnesium concentrations. We recommend using 1.5mM–5mM. Reverse transcription reactions designed to make full-length cDNA from long messages are more efficient at lower magnesium concentrations. Short messages are more efficiently reverse transcribed at higher magnesium concentrations.

5.D. Primer Options and Design

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

Regardless of primer choice, the final primer concentration in the reaction must be optimized. In the protocol outlined in Section 4.A, we recommend 0.5µg of oligo$(dT)_{15}$ and/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.E. Reverse Transcriptase Enzyme Concentration

The GoScript™ 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.F. Control Reactions

To facilitate optimization and troubleshooting of the reverse transcription reaction or associated RT-PCR, perform negative control reactions.

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.

5.F. Control Reactions (continued)

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.

5.G. Temperature

In many experimental situations, the RNA template and 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 GoScript™ 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 GoScript™ Reverse Transcriptase enzyme is active over a range of 37–55°C. 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, 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 due to 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.A includes a 15-minute incubation at 70°C as an appropriate treatment that maintains cDNA integrity but heat-denatures the reverse transcriptase.

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 RNasin® Ribonuclease Inhibitor. • RNA storage conditions are very important. Store at –70°C. Keep RNA target in concentrated, single-use aliquots to minimize freeze-thaw cycles. Once thawed, keep RNA on ice. • Use RNasin® Ribonuclease Inhibitor to inhibit degradation of target during cDNA synthesis (20u/20µl reaction).
	Storage of dilute samples. RNA template adhered to walls of low-quality reaction tubes. This is especially a problem with low-concentration samples or serially diluted samples. Possible solutions are: 1) dilute the RNA template with an inert carrier, such as background RNA, as exemplified in MS2 model, diluted in background of human total RNA, or 2) make fresh dilutions of RNA, and use immediately. Do not store low-concentration RNA dilutions for later use.
	Inhibitors were 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.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low yield of full-length first-strand cDNA (continued)	Primer-annealing temperature was 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 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 of cDNA synthesis reaction. Use 0.5–1.0µM gene-specific primer per reaction.
	dNTP concentration was too high. Do not use more than 0.5mM final concentration of PCR Nucleotide Mix in the cDNA synthesis.

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, reduce the $MgCl_2$ concentration. Optimal concentrations are between 1.5mM and 5.0mM.
	Reverse transcriptase inactivation prior to cDNA synthesis. Be certain to combine the RT reaction mix with the target after thermal denaturation of RNA and primer. Thermal denaturation will inactivate the GoScript™ Reverse Transcriptase.
	"Hot start" needed. Occasionally, the yield of full-length cDNA can be increased by using a "hot-start" reverse transcription method. To use this method, add the GoScript™ 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 product has a molecular weight higher than expected	Genomic DNA sequences related to the RNA template contaminated the RNA preparation. Use the SV Total RNA Isolation System (Cat.# Z3100) protocol, which includes steps to remove genomic DNA.
	Design primers to span introns, covering the exon-exon junction, to eliminate any amplification from genomic DNA.
RT-PCR 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.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
RT-PCR products have multiple or unexpected sizes (continued)	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.
	Magnesium concentration was suboptimal. 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 product	Insufficient cDNA was 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 was 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.

Symptoms	Causes and Comments
Low yield of RT-PCR product (continued)	Magnesium concentration was suboptimal 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 were degraded. Store nucleotide solutions frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles.
	Annealing temperature was suboptimal. Optimize annealing temperature of each cycle by increasing or decreasing the temperature in increments of 1°C.
	Extension time was 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 PCR cycles. To detect rare or difficult RNA targets by RT-PCR, increase the cycle number to 40 to maximize sensitivity.
	Wrong reaction tubes were 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 was not present in target RNA. Redesign experiment or try other sources of target RNA.

7. References

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2. *Protocols and Applications Guide*, Online Edition (2004) Promega Corporation. (**www.promega.com/paguide/**)
3. Blumberg, D.D. (1987) Creating a ribonuclease-free environment. *Meth. Enzymol.* **152**, 20–4.
4. Longo, M.C., Berninger, M.S. and Hartley, J.L. (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93**, 125–8.
5. Chumakov, K.M. (1994) Reverse transcriptase can inhibit PCR and stimulate primer-dimer formation. *PCR Methods Appl.* **4**, 62–4.
6. Sellner, L.N., Coelen, R.J. and Mackenzie, J.S. (1992) Reverse transcriptase inhibits *Taq* polymerase activity. *Nucl. Acids Res.* **20**, 1487–90.

8. Related Products

Reverse Transcription

Product	Concentration	Size	Cat.#
GoScript™ Reverse Transcriptase		10 reactions	A5002
		100 reactions	A5003
		500 reactions	A5004
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.# A5002, A5003 and A5004 contain GoScript™ Reverse Transcriptase, 5X GoScript™ Reaction Buffer and $MgCl_2$ only.

Real-Time Quantitative Amplification

Product	Size	Cat.#
GoTaq® qPCR Master Mix	5ml	A6001
	25ml	A6002

DNA Polymerases and PCR Master Mixes

Product	Size	Cat.#
GoTaq® G2 Hot Start Polymerase	100u	M7401
	500u	M7405
	2,500u	M7406
	10,000u	M7408
GoTaq® G2 Hot Start Green Master Mix	100 reactions	M7422
	1,000 reactions	M7423
GoTaq® G2 Hot Start Colorless Master Mix	100 reactions	M7432
	1,000 reactions	M7433
GoTaq® G2 Green Master Mix	100 reactions	M7822
	1,000 reactions	M7823
GoTaq® G2 Colorless Master Mix	100 reactions	M7832
	1,000 reactions	M7833
GoTaq® G2 Flexi DNA Polymerase	100u	M7801
	500u	M7805
	2,500u	M7806
	10,000u	M7808
GoTaq® G2 DNA Polymerase	100u	M7841
	500u	M7845
	2,500u	M7848

8. Related Products (continued)

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

9. Summary of Change

The Storage Conditions were updated in the 9/18 revision of this document.

[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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