TECHNICAL MANUAL

GoTaq® 2-Step RT-qPCR System

Instructions for use of Product
A6010

Revised 1/14
TM337

Promega

GoTaq® 2-Step RT-qPCR System

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1. Description

GoTaq® 2-Step RT-qPCR System[a,b] is a reagent system for quantitative analysis of RNA using a two-step reverse transcription-quantitative PCR (RT-qPCR) protocol. The components and protocol allow cDNA synthesis using the GoScript™ Reverse Transcription System and quantification using the GoTaq® qPCR Master Mix. The GoTaq® 2-Step RT-qPCR System contains a new fluorescent DNA-binding dye that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA (dsDNA) than SYBR® Green I. GoTaq® qPCR Master Mix can be used with any real-time instrument capable of detecting SYBR® Green I or FAM™ dye. GoTaq® qPCR Master Mix contains a low level of CXR reference dye. A separate tube of CXR Reference Dye is included for use with instruments that require a higher level of reference dye than that in the GoTaq® qPCR Master Mix.

1. Description (continued)

The GoTaq® 2-Step RT-qPCR System combines the benefits of GoScript™ Reverse Transcriptase and dye-based GoTaq® qPCR Master Mix for efficient, sensitive and linear two-step RT-qPCR quantification over a wide range of RNA template inputs. Furthermore, the system has a straightforward protocol to analyze RNA with several formats for flexible use in routine or challenging RNA quantifications.

For additional information on component systems, refer to the *GoScript™ Reverse Transcription System Technical Manual* #TM316 and *GoTaq® qPCR Technical Manual* #TM318.

The GoTaq® 2-Step RT-qPCR System offers:

- High-efficiency, high-sensitivity, full-length cDNA synthesis
- Linear quantification over a wide range of RNA input amounts
- Robust activity in the presence of inhibitors
- GoScript™ Reverse Transcriptase for flexible options in cDNA synthesis
- Direct addition of cDNA or diluted cDNA to qPCR
- GoTaq® qPCR Master Mix formulated with a hot-start DNA polymerase optimized for specific, sensitive qPCR
- Rapid hot-start activation and robust enzyme processivity compatible with fast and standard instrument programs
- Flexible, universal qPCR premix formulation with a low concentration of carboxy-X-rhodamine (CXR) reference dye. A tube of concentrated CXR reference dye is provided for use with real-time PCR instruments that require additional reference dye. (See Section 3.B.)

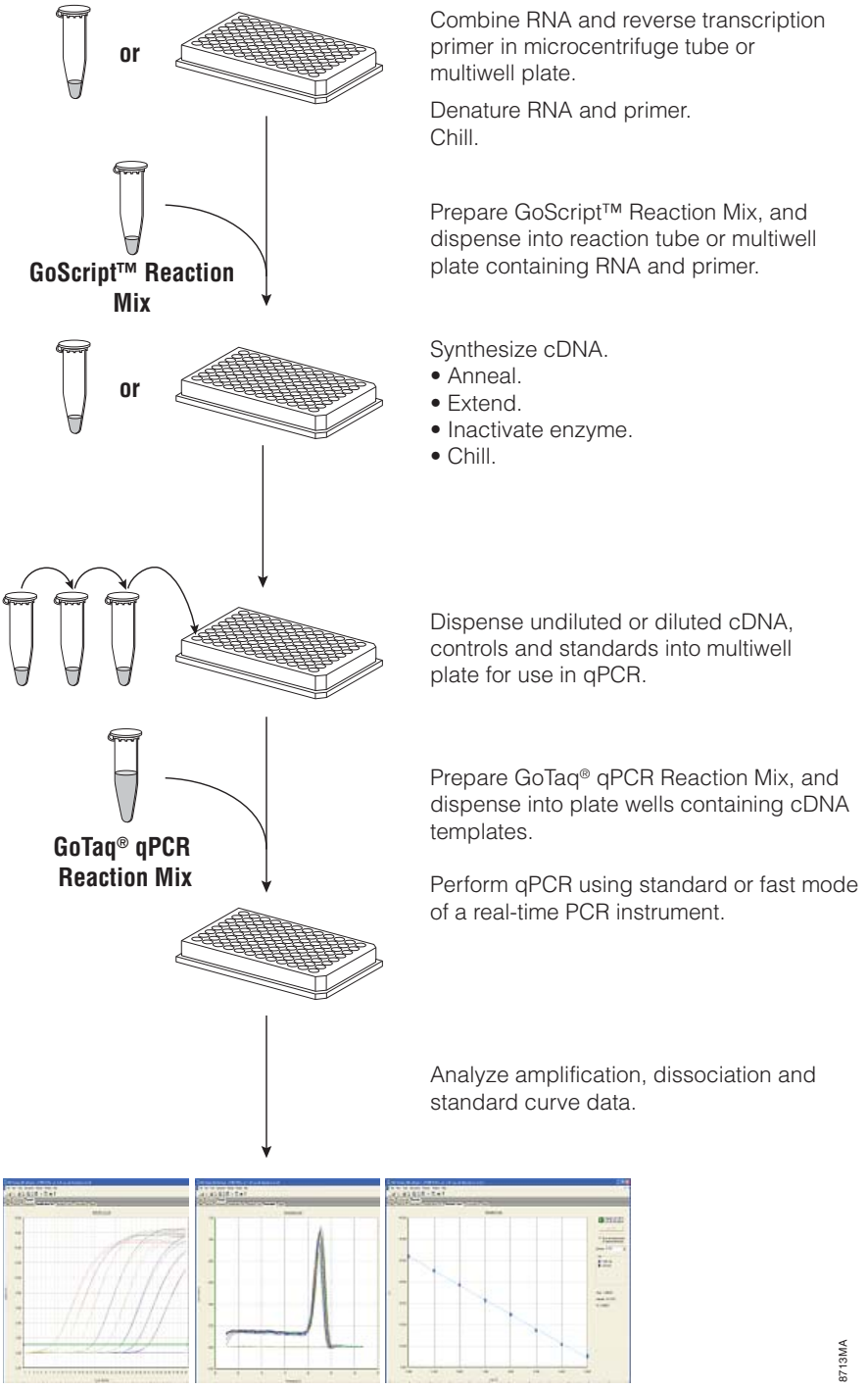

Figure 1. An overview of the GoTaq® 2-Step RT-qPCR protocol.

2. Product Components and Storage Conditions

Product	Size	Cat.#
GoTaq® 2-Step RT-qPCR System	200 reactions	A6010

For Research Use Only. Not for use in diagnostic procedures. Each system contains sufficient reagents for 50 × 20µl GoScript™ Reverse Transcriptase reactions and 200 × 50µl GoTaq® qPCR assays. Includes:

- 50µl GoScript™ Reverse Transcriptase
- 300µl GoScript™ 5X Reaction Buffer
- 750µl $MgCl_2$, 25mM
- 200µl PCR Nucleotide Mix, 10mM
- 50µg Oligo$(dT)_{15}$ Primer, 500µg/ml
- 50µg Random Primers, 500µg/ml
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor
- 5 × 1ml GoTaq® qPCR Master Mix, 2X
- 100µl CXR Reference Dye
- 2 × 13ml Nuclease-Free Water

Storage Conditions: Store all components at –20°C. Protect components from light at all times. For best results, mix thawed solutions gently to minimize aeration and foaming, and keep on ice. For short-term storage and frequent use, the GoTaq® qPCR Master Mix, 2X, may be kept at 2–8°C for up to 3 months if protected from light.

Available Separately

Product	Size	Cat.#
GoTaq® qPCR Master Mix*	200 reactions	A6001
	1,000 reactions	A6002
GoScript™ Reverse Transcription System*	50 reactions	A5000
	100 reactions	A5001
GoScript™ Reverse Transcriptase**	100 reactions	A5003
	500 reactions	A5004
Nuclease-Free Water**	50ml	P1193

*For Research Use Only. Not for use in diagnostic procedures.
**For Laboratory Use.

3. General Considerations

3.A. Spectral Properties

The proprietary dye in the GoTaq® qPCR Master Mix has spectral properties similar to those of SYBR® Green I: Excitation at 493nm and emission at 530nm. Use the instrument optical settings for SYBR® Green I assays with reactions containing GoTaq® qPCR Master Mix.

The CXR reference dye has the same spectral properties as ROX™ dye: Excitation at 580nm and emission at 602nm. Use the instrument optical settings for ROX™ dye with reactions containing GoTaq® qPCR Master Mix.

3.B. Instrument Compatibility

GoTaq® qPCR Master Mix can be used with any real-time PCR instrument capable of detecting SYBR® Green I or FAM™ dyes. Standard SYBR® Green I or FAM™ calibration prepares the instrument for analysis of the Promega proprietary green dye.

GoTaq® qPCR Master Mix contains a low level of CXR reference dye. Standard ROX™ calibration prepares the instrument for analysis of the CXR reference dye.

This low level of CXR reference dye is appropriate for instruments that recommend qPCR reagents with a low level of ROX™ dye or no-ROX™ formulation.

Some real-time PCR instruments recommend use of a high level of reference dye (e.g., 500nM ROX™ dye) as the normalization dye during qPCR. The GoTaq® 2-Step RT-qPCR System provides a separate tube of CXR Reference Dye to supplement these reactions. If you are using an instrument that requires a high concentration of reference dye, supplement the GoTaq® qPCR Reaction Mix with 0.5µl of CXR Reference Dye per 50µl reaction. Examples of instruments that require additional CXR Reference Dye are listed below.

Instruments that Require Supplemental CXR

- ABI PRISM® 7000 and 7700 Sequence Detection System
- Applied Biosystems 7300 and 7900HT Real-Time PCR System
- Applied Biosystems GeneAmp® 5700 Thermal Cycler
- Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems

Instruments that may Require Fluorescein for Well Factor Detection*

- Bio-Rad CFX96 Real-Time PCR Detection System
- Bio-Rad iCycler® iQ™ and iQ™5 Real-Time PCR Detection System
- Bio-Rad MyiQ™ Real-Time PCR Detection System

*Add 10–20nM fluorescein to each well prior to PCR.

3.B. Instrument Compatibility (continued)

Instruments that Require No Supplemental CXR

- Applied Biosystems 7500 and 7500 FAST Real-Time PCR System
- Bio-Rad CFX96 Real-Time PCR Detection System
- Bio-Rad DNA Engine Opticon® and Opticon® 2 Real-Time PCR Detection Systems
- Bio-Rad iCycler® iQ™ and iQ™5 Real-Time PCR Detection Systems
- Bio-Rad/MJ Research Chromo4™ Real-Time Detector
- Bio-Rad MyiQ™ Real-Time PCR Detection System
- Cepheid SmartCycler® System
- Corbett Rotor-Gene™ 3000 and 6000 Real-Time Rotary Analyzer
- Eppendorf Mastercycler® ep *realplex* Real-Time PCR System
- Roche LightCycler® 480 Real-Time PCR System
- Stratagene MX3000P® and Mx3005P® Real-Time PCR Systems
- Stratagene Mx4000® Multiplex Quantitative PCR System

3.C. General Laboratory Precautions

Use sterile technique in designated, RNase-free workspace for pre-amplification activities. Periodically clean work surfaces and equipment with 10% bleach to eliminate RNase, amplicon and fluorescent-dye contamination.

Use separate designated work areas, reagents and equipment for post-amplification steps. This will minimize the potential for cross-contamination among samples and prevent carryover of nucleic acid from one experiment to the next. Wear clean gloves and disposable lab coats, and change them often.

Prevent contamination by using barrier, aerosol-resistant or positive-displacement pipette tips. Prevent fluorescent contamination of reagents, tubes and instruments. Use plasticware that is vendor-certified to be sterile, nuclease-free, DNA-free and nonstick.

Aeration is detrimental to DNA-binding dyes. Air bubbles interfere with fluorescence detection in real-time PCR assays. Mix all GoTaq® qPCR Master Mix solutions gently by inversion or pipetting rather than vigorous vortexing to minimize bubbles.

Protect all GoTaq® qPCR Master Mix solutions from light. Protect plates and tubes during storage.

4. GoTaq® 2-Step RT-qPCR Protocol

Materials to Be Supplied by the User

- experimental RNA
- DNA-free reference RNA
- positive-control DNA standards for qPCR, if desired
- gene-specific reverse transcription primer, if desired
- qPCR primers
- sterile, DNA-free, nuclease-free, nonstick microcentrifuge tubes
- sterile, DNA-free, nuclease-free, nonstick multiwell plates
- sterile, DNA-free, nuclease-free, low-retention barrier tips
- nuclease-free pipettors designated for pre-amplification
- optical multiwell reaction plates and seals compatible with the real-time PCR instrument
- alternative normalization dye, if required (e.g., fluorescein for Bio-Rad instruments)
- ice bath
- controlled-temperature blocks or thermal cycler for cDNA synthesis
- real-time PCR instrument
- microcentrifuge
- centrifuge with adapter for multiwell plates
- vortex mixer

Note: We recommend using nonstick tubes and multiwell plates for the entire protocol.

4.A. Prepare RNA and Reverse Transcription Primer

Perform denaturation using controlled-temperature blocks at 70°C and 4°C. Alternatively, program a thermal cycler to heat and cool the samples.

1. Combine RNA and reverse transcription primer in individual microcentrifuge tubes or wells of a multiwell plate on ice. Close the tubes tightly, or seal plates with an adhesive cover.

   Note: The total volume of RNA and reverse transcription primer per reaction should not exceed 50% (v/v) of the GoScript™ reverse transcription reaction (e.g., no more than 10µl for each 20µl cDNA synthesis).

   RNA and Reverse Transcription Primer

Component	Volume	Final Concentration for 20µl Reaction
RNA (up to 5µg/reaction)	__µl	
Primer [Oligo(dT)$_{15}$ Primer and/or	1µl	0.025µg/µl
Random Primer or	1µl	0.025µg/µl
gene-specific primer]	__µl	1µM
Nuclease-Free Water (up to a final volume of 10µl)	__µl	
Final Volume	**10µl**	

   Note: Component volumes may be scaled as appropriate.

2. **Optional:** Place the sealed tubes or reaction plate into a preheated 70°C heat block. Incubate at 70°C for 5 minutes to denature. Chill the reactions at 4°C for 5 minutes.

3. Centrifuge the tubes or plate briefly to collect any condensation. Store RNA and reverse transcription primer on ice prior to adding the GoScript™ Reaction Mix.

4.B. Synthesize cDNA with GoScript™ Reverse Transcriptase

Perform cDNA synthesis using controlled-temperature blocks at the temperatures given below. Alternatively, program a thermal cycler to heat and cool the cDNA synthesis reactions.

1. Thaw the components, mix thoroughly by inversion or gentle vortexing, and combine in the order specified below. Prepare 10µl of GoScript™ Reaction Mix for each sample in a sterile microcentrifuge tube on ice. Mix gently, and use immediately. Ensure there is sufficient volume of reaction mix for all experimental samples, reference samples and control reactions.

   Optional: Prepare a minus-reverse transcriptase control reaction by substituting Nuclease-Free Water for the GoScript™ Reverse Transcriptase as described in the table below.

Component	GoScript™ Reaction Mix	Minus-Reverse Transcriptase Reaction Mix	Final Concentration for 20µl Reaction
Nuclease-Free Water (to a final volume of 10µl)	1.5µl	2.5µl	
GoScript™ 5X Reaction Buffer	4µl	4µl	1X
$MgCl_2$, 25mM	2µl	2µl	2.5mM
PCR Nucleotide Mix, 10mM	1µl	1µl	0.5mM
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl	0.5µl	20 units
GoScript™ Reverse Transcriptase	1µl	0µl	
Final Volume	**10µl**	**10µl**	

2. In reaction tubes or wells of a multiwell reaction plate, combine the following reaction components on ice:

Component	Reverse Transcriptase Reaction
GoScript™ Reaction Mix[1]	10µl
RNA and reverse transcription primer[2] prepared in Section 4.A	10µl
Final Volume	**20µl**

[1]For the minus-reverse transcriptase control reaction, use the minus-reverse transcriptase reaction mix prepared in Step 1.

[2]The volume of RNA and reverse transcription primer should not exceed 50% (v/v) of the final reaction volume.

4.B. Synthesize cDNA with GoScript™ Reverse Transcriptase (continued)

3. Incubate the reactions as indicated below in temperature-controlled blocks or a thermal cycler.

Step	Temperature	Time
Anneal (Optional)	25°C	5 minutes
Extend	42°C	1 hour
Inactivate	70°C	15 minutes
Chill	4°C	Hold

Note: The annealing, extension and inactivation conditions can be modified. See Section 5.E.

4. Store cDNA at 4°C or on ice for immediate analysis. Alternatively, store cDNA at –20°C until use.

4.C. Quantify cDNA with GoTaq® qPCR Master Mix

Quantify diluted or undiluted GoScript™ reverse transcription products synthesized in Section 4.B and DNA standards using the GoTaq® qPCR Master Mix. When diluting the cDNA products, the optimal dilution must be determined empirically (see Section 5.F). We also recommend performing no-template (negative) control and minus-reverse transcriptase control reactions.

1. Prepare the standard DNA dilution series and experimental samples in nuclease-free water. Store on ice until use. Carefully add 10µl of each GoScript™ cDNA template or reference standard to the appropriate wells of the reaction plate. For no-template (negative) control reactions, add nuclease-free water instead of cDNA template. Temporarily cover the plate, and store on ice.

   Notes:

   1. Template volume should not exceed 20% of the final reaction volume (e.g., 10µl of cDNA + 40µl of GoTaq® qPCR Reaction Mix = 50µl qPCR).
   2. For best results, the experimental cDNA templates and reference standards should have similar ionic conditions.
   3. Add a volume of cDNA template that corresponds to approximately ≤100ng of input RNA. For reproducible results and to avoid degradation of cDNA template, prepare all dilutions of cDNA template and reference standards using tubes certified to be nuclease-free and made of nonstick PCR-grade material.

2. Thaw the GoTaq® qPCR Master Mix at room temperature or on ice. Mix by gentle inversion to ensure that it is thoroughly mixed. To avoid foaming, do **not** vortex or shake vigorously because bubbles interfere with detection. Store GoTaq® qPCR Master Mix on ice until use. Shield the tube to minimize exposure to light during reaction setup.

3. Determine the number of reactions to be set up. This should include reference standards and no-template (negative control) reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does waste a small amount of each reagent, it ensures that you will have enough GoTaq® qPCR Reaction Mix for all samples. It also ensures that each reaction contains the same master mix. If you are using multiple sets of PCR primers, omit the primers from the GoTaq® qPCR Reaction Mix, take an aliquot for each set of primers used, and then add the primers separately to each reaction mix subset. This ensures consistency for each primer set.

   GoTaq® qPCR Reaction Mix

Component	Volume	Final Concentration in 50µl Reaction
GoTaq® qPCR Master Mix, 2X	25µl	1X
Nuclease-Free Water (to a final volume of 40µl)	__µl	
Forward and reverse qPCR primers[1]	__µl	200nM each
Final Volume	**40µl**	

[1]A range of primer concentrations can be used. See Section 5.B.

Notes:

1. Some real-time PCR instruments require an additional 0.5µl of CXR Reference Dye in the 50µl reactions. See Section 3.B.
2. Some real-time PCR instruments, such as Bio-Rad instruments, require a normalization dye (e.g., fluorescein) for well factor calculation. See Section 3.B.
3. The standard reaction volume is 50µl. Components may be scaled as appropriate.

4. In reaction tubes or wells of a multiwell reaction plate, combine the following reaction components on ice or at room temperature. Seal the reaction plate.

Component	Volume
cDNA template or reference standard[1,2]	10µl
GoTaq® qPCR Reaction Mix	40µl
Final qPCR Volume	**50µl**

[1]Use 10µl of water for no-template control.
[2]The volume of template or reference standard should not exceed 20% of the final reaction volume.

Note: Protect the plate from light, and avoid cross-contamination as reactions are assembled.

4.C. Quantify cDNA with GoTaq® qPCR Master Mix (continued)

5. Centrifuge the reaction plate using a swinging-bucket centrifuge at low speed for 1 minute to collect all reaction components and eliminate residual air bubbles.

6. Program the real-time PCR instrument for standard or fast qPCR following the instrument manufacturer's instructions and these guidelines:

   - Select SYBR® or FAM™ as the detection dye for the entire plate.
   - Select the ROX™ channel to detect CXR as the reference dye for the entire plate.
   - Select a standard or fast, two-step, 40-cycle qPCR and dissociation program.
   - Designate that data will be collected during the annealing/extension step of each cycle.

   Note: The cycling parameters below are offered as a guideline and may be modified as necessary for optimal results.

Standard Cycling Conditions

Step	Cycles	Temperature	Time
GoTaq® Hot Start Polymerase activation	1	95°C	2 minutes
Denaturation	40	95°C	15 seconds
Annealing/Extension		60°C	1 minute
Dissociation	1	60–95°C	

Fast Cycling Conditions

Step	Cycles	Temperature	Time
GoTaq® Hot Start Polymerase activation	1	95°C	2 minutes
Denaturation	40	95°C	3 seconds
Annealing/Extension		60°C	30 seconds
Dissociation	1	60–95°C	

7. Place the plate in the preprogrammed real-time PCR instrument, and press "Start".

8. When the run is complete, collect and analyze the amplification and dissociation data.

5. Optimization of the GoTaq® 2-Step RT-qPCR System

The protocol in this Technical Manual outlines the use of the GoTaq® 2-Step RT-qPCR System. Please refer to the *GoScript™ Reverse Transcription System Technical Manual* #TM316 and *GoTaq® qPCR Technical Manual* #TM318 for detailed optimization suggestions.

Note: We recommend using nonstick tubes and multiwell plates for the entire protocol.

5.A. RNA Template Amount

The amount of RNA that yields linear quantification results may vary considerably. We recommend the following guidelines for RNA amounts per 20µl reverse transcription reaction:

- Total RNA: 5µg–1pg
- Poly (A+) RNA: 500ng–100fg
- Viral or in vitro transcript RNA molecules: 1×10^{10}–10 copies

5.B. Reverse Transcription Primer Sequence and Concentration

It is essential to use the same primers in all experiments. Increased primer concentration can improve cDNA synthesis. We recommend the following primer concentrations:

Primer	Standard Protocol	Optional Range
Oligo$(dT)_{15}$	0.025µg/µl	0.01–0.125µg/µl
Random Primer	0.025µg/µl	0.01–0.1µg/µl
Oligo$(dT)_{15}$ + Random Primer	0.025µg/µl each primer	Each 0.01–0.1µg/µl
Gene-specific primer	1µM	0.5–1µM

5.C. Magnesium Chloride Concentration

The GoScript™ 5X Reaction Buffer **does not** contain magnesium chloride. Magnesium chloride must be added to the GoScript™ Reaction Mix, as described in Section 4.B, Step 1. We recommend a final concentration of 2.5mM magnesium chloride for best results in most GoScript™ cDNA syntheses for GoTaq® 2-Step RT-qPCR assays. The concentration of $MgCl_2$ can be optimized, if desired. We recommend a range of 1.5–5.0mM.

The GoTaq® qPCR Master Mix **does** include magnesium chloride at a concentration of 2mM, which is optimal for quantifying diluted or undiluted cDNA that has been synthesized in the presence of 2.5mM $MgCl_2$. Any cDNA dilutions should be performed in nuclease-free water.

To optimize magnesium concentration in GoScript™ Reverse Transcriptase reactions or GoTaq® qPCR analyses of challenging targets, titrate the magnesium chloride in 0.25mM increments by adding the 25mM $MgCl_2$ provided.

5.D. RNA and Reverse Transcription Primer Denaturation Conditions

For optimal cDNA synthesis, the RNA and reverse transcription primer must be heat-denatured to melt RNA secondary structure such as hairpins or loops that interfere with full-length cDNA synthesis. The benefits of heat denaturation should be determined empirically. We recommend the following:

Denaturation Options	Recommendations
Vary denaturation temperature	Incubate RNA and reverse transcription primer at 65–75°C. The standard protocol recommends a denaturation temperature of 70°C, but the optimal denaturation temperature might be higher or lower. For example, increase the denaturation temperature to maximize denaturation of complex RNA secondary structures or reduce the temperature to maintain RNA quality for synthesizing longer cDNAs.
Perform warm-start cDNA synthesis	Add the 70°C RNA and reverse transcription primer directly, without chilling, to GoScript™ Reaction Mix warmed to reaction temperature (e.g., 42°C) to enhance primer specificity or for full-length reverse transcription of RNAs with challenging template secondary structure.
Eliminate denaturation	Combine RNA and reverse transcription primer directly with ice-cold GoScript™ Reaction Mix to minimize process time or for a simplified protocol when lower sensitivity is acceptable.

5.E. GoScript™ cDNA Synthesis

For optimal cDNA synthesis using GoScript™ Reverse Transcriptase, annealing, extension or inactivation conditions and the volume of GoScript™ reverse transcription reaction added to the GoTaq® qPCR Reaction Mix may be varied. The benefits of these modifications should be empirically determined. We recommend the following:

Reverse Transcription Parameter	Comments
Annealing	To enhance specificity of gene-specific primers, increase annealing temperature to >25°C.
	To enhance sensitivity with maximized annealing of primer to RNA template, increase annealing time to >5 minutes.
Extension	To save time, reduce extension time from 60 minutes to as little as 5 minutes. Most of the cDNA is synthesized within the first 15 minutes of the extension incubation.
	To maximize yield of full-length cDNA ≥12kb, reduce the extension temperature to <42°C.
	To enhance reverse transcription of RNAs with challenging secondary structure, increase extension temperature >42°C.
	To maximize cDNA yields, increase extension time to >1 hour.
	To enhance efficiency, vary the extension conditions (e.g., by performing 40 cycles consisting of 1 minute at 37°C and 1 second at 50°C).
Inactivation	To minimize time requirements, increase temperature to >70°C, and reduce time to <15 minutes.

5.F. Reaction Assembly

The GoTaq® 2-Step RT-qPCR System allows direct quantification of GoScript™ reaction products by GoTaq® qPCR Master Mix. cDNA template volumes should not exceed 20% of the total qPCR volume (e.g., 10µl of cDNA + 40µl of GoTaq® qPCR Reaction Mix = 50µl qPCR volume).

For best results, quantify undiluted cDNA products and standards in similar ionic conditions. Prepare dilutions of cDNA and reference standards using nuclease-free water. Use this same stock of nuclease-free water for the qPCR negative control.

The optimal dilution factor must be determined experimentally for each target and depends on the cDNA concentration, the amount of RNA in the reverse transcription reaction and the specific target sequence abundance.

5.G. GoTaq® qPCR Primers and Target

Design primers for minimal amplification of genomic DNA sequences, pseudogenes or other genes. We recommend:

qPCR primers	15–25 nucleotides long Span exon-exon junction containing an intron to differentiate between RNA versus genomic DNA amplification 50% GC content (20% to 80%) Similar T_m for each primer
Amplicon	50–250bp long

For most GoTaq® qPCR assays, 200nM of each primer is sufficient. Optimal primer concentrations range from 50–500nM each, depending on target-specific requirements, and should be determined experimentally.

6. General References for qPCR

1. Bustin, S.A. *et al.* (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–22.
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3. Fleige, S. and Pfaffl, M.W. (2006) RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Aspects Med.* **27**, 126–39.
4. Lefever, S. *et al.* (2009) RDML: Structured language and reporting guidelines for real-time quantitative PCR data. *Nucleic Acids Res.* **37**, 2065–9.
5. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–8.

[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.

[b]**NOTICE TO PURCHASER: LIMITED LICENSE**
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