Technical Manual

GoTaq® 1-Step RT-qPCR System

INSTRUCTIONS FOR USE OF PRODUCT A6020.

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1. Description

GoTaq® 1-Step RT-qPCR System[a,b,c,d] is a reagent system for quantitative analysis of RNA using a one-step reverse transcription-quantitative PCR (RT-qPCR) protocol. The GoTaq® 1-Step RT-qPCR System combines the benefits of GoScript™ Reverse Transcriptase and GoTaq® qPCR Master Mix for efficient, sensitive and linear one-step RT-qPCR quantification over a wide range of RNA template inputs.

The GoTaq® 1-Step RT-qPCR System contains the proprietary fluorescent DNA-binding dye, BRYT Green® dye that exhibits greater fluorescence enhancement, upon binding to double-stranded DNA (dsDNA), than SYBR® Green I.

1. Description (continued)

The GoTaq® 1-Step RT-qPCR System offers:

- Linear quantification over a wide range of RNA input amounts.
- Robust activity in the presence of inhibitors.
- Premixed GoTaq® qPCR Master Mix, 2X, with optimized formulation for both RT and PCR activities.
- A hot-start DNA polymerase
- Can be used with any real-time instrument capable of detecting SYBR® Green I or FAM dye.
- Compatible with fast and standard instrument programs
- Flexible, universal qPCR premix formulation with a "low" (33nM at 1X) concentration of carboxy-X-rhodamine (CXR) reference dye. A tube of 30µM CXR Reference Dye is provided for use with real-time PCR instruments that require additional reference dye (see Section 3.B).
- GoScript™ RT Mix for 1-Step RT-qPCR, 50X: Combines optimized amounts of GoScript™ Reverse Transcriptase, RNasin® Plus RNase Inhibitor and additives to enhance single-step reactions.
- Mg^{2+}, 25mM, supplied separately. Allows optimization of reactions that require additional Mg^{2+}.

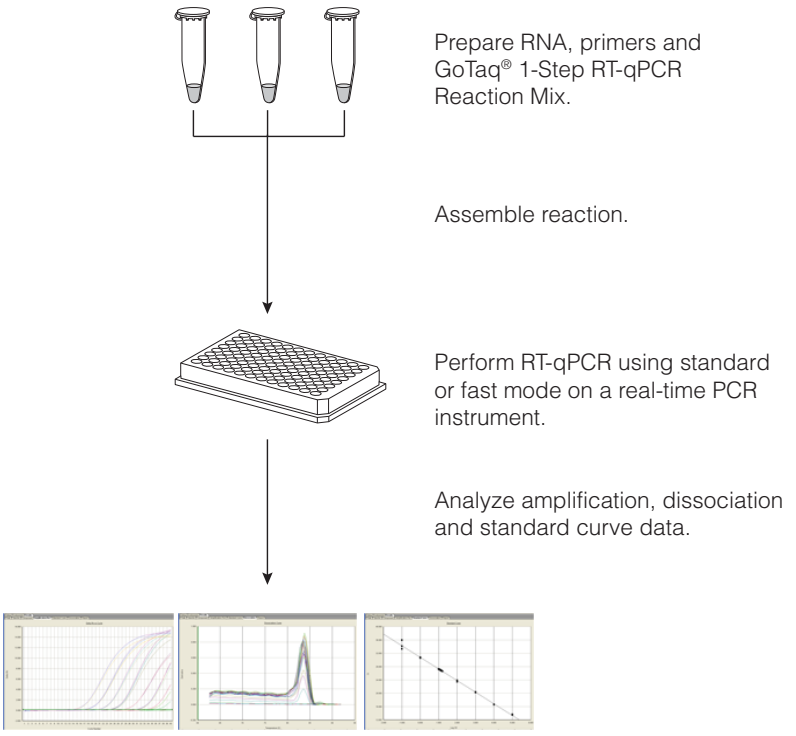

Figure 1. Overview of the GoTaq® 1-Step RT-qPCR protocol.

2. Product Components and Storage Conditions

Product	Size	Cat.#
GoTaq® 1-Step RT-qPCR System	200 reactions	A6020

For in vitro research use only. Not for use in diagnostic procedures. Each system contains sufficient reagents for 200 × 50µl GoTaq® RT-qPCR assays. Includes:

- 5 × 1ml GoTaq® qPCR Master Mix, 2X
- 1 × 200µl GoScript™ RT Mix for 1-Step RT-qPCR, 50X
- 200µl CXR Reference Dye, 30µM
- 750µl $MgCl_2$, 25mM
- 2 × 13ml Nuclease-Free Water

Storage Conditions: Store all components at –20°C in a nonfrost-free freezer. Protect components from light at all times.

For best results, mix solutions **gently** yet thoroughly, immediately after thawing, being careful to minimize aeration and foaming. Vortexing and overmixing will result in poor product performance due to loss of fluorescence. Keep solutions on ice.

Note: 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. Reagents can withstand up to three freeze-thaw cycles.

Available Separately

Product	Size	Cat.#
CXR Reference Dye, 30µM*	100µl	C5411
Nuclease-Free Water	50ml	P1193

*For in vitro research use only. Not for use in diagnostic procedures.

3. General Considerations

3.A. Spectral Properties

The BRYT Green® dye 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 for GoTaq® 1-Step RT-qPCR reactions.

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 for GoTaq® 1-Step RT-qPCR reactions.

3.B. Instrument Compatibility

GoTaq® 1-Step RT-qPCR System can be used with any real-time PCR instrument designed for excitation and detection of SYBR® Green I or FAM™ dyes. Standard SYBR® Green I or FAM™ calibration prepares the instrument for analysis of the BRYT Green® dye.

At 1X concentration, GoTaq® qPCR Master Mix contains 33nM CXR Reference Dye. Standard ROX™ calibration prepares the instrument for analysis of the CXR Reference Dye. This concentration of CXR Reference Dye is appropriate for instruments that recommend qPCR reagents with a "low" level of ROX™ dye or no-ROX™ formulation.

Note: Some instrumentation is designed to excite/detect using CXR/ROX at a concentration of 30–50nM, some instruments specifically need 500nM, and others do not normalize CXR/ROX at all.

Examples of instruments that require additional CXR Reference Dye are listed below.

Instruments that Require Supplemental CXR:

- Applied Biosystems 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 Do Not Require 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/MJ Research Chromo4™ Real-Time Detector
- 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

Instruments that are designed for "Fluorescein Dynamic Well Factor" (no Fluorescein addition is needed):

- 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

3.C. General Laboratory Precautions

Aeration is detrimental to the stability and detection of DNA-binding dyes. However, it is essential to gently and thoroughly mix all GoTaq® qPCR Master Mix solutions and reaction mixes after each freeze-thaw cycle. Mix **gently** by swirling, inversion or pipetting. Avoid vigorous vortexing to minimize bubbles. If a vortex is used, mix at minimum speed for the most gentle agitation. Loss of dye fluorescence can result from oxidation due to vigorous mixing.

Protect all GoTaq® qPCR Master Mix solutions from light. Protect plates and tubes during storage. Store all reagents, reaction mixes and assembled reactions on ice.

3.D. Important Assay-Specific Considerations

Reference Dye

Use the same concentration of reference dye in all the GoTaq® 1-Step RT-qPCR runs as recommended for your instrument platform. This is important for proper instrument performance and run-to-run data correlation.

Use GoTaq® qPCR Master Mix with its premixed "Low" CXR (33nM at 1X concentration) with an instrument platform that requires a "Low" level of reference dye.

Use GoTaq® qPCR Master Mix with its premixed "Low" CXR with an instrument platform that does not require any reference dye.

For instruments requiring addition of CXR dye, adjust the concentration of CXR in GoTaq® 1-Step RT-qPCR reaction mixes using the separate stock tube of CXR Reference Dye, 30µM, as a supplement.

Prepare reaction mixes in batch to achieve a consistent concentration of CXR Reference Dye in across the plate. This is important for well-to-well and run-to-run data correlation.

Do **not** add high levels of ROX™/CXR to reactions run on instruments with low ROX™ optical specifications. High ROX/CXR levels cause noise in run data in "low" ROX instruments.

RNA Amount

RNA sample input may range from 500fg to 100ng, depending on target abundance and RNA quality.

Prepare dilutions of RNA using the Nuclease-Free Water provided or a low ionic strength qPCR-compatible buffer (e.g., sterile 10mM Tris, 0.1mM EDTA [pH 8.0]). Use sterile, nuclease-free, nonstick microcentrifuge tubes whenever possible. Keep on ice.

Primers

Target-specific primers should be designed and prepared following generally published guidelines for dye-based, one-step RT-qPCR. The guidelines recommend 1X assay concentrations of 50–300nM for each primer.

It is important to determine the functional quality of each primer synthesis and optimal concentration of each forward and reverse primer sequence in the pair, at the $MgCl_2$ assay concentration. Evaluate the primer pair sequence and concentration of forward and reverse primer for specific, linear quantification over the desired range of sample input. To improve specificity and qPCR efficiency, $MgCl_2$ concentration or annealing/extension conditions can be optimized. Prepare a stock of the primer pair in PCR-compatible buffer (e.g., sterile 10mM Tris, 0.1mM EDTA [pH 8.0]) in single-use aliquots. Store at –20°C).

Reverse Transcription

GoScript™ Reverse Transcriptase has an optimal activity at 37°C. The general protocol outline includes primer extension at ≥37°C for 15 minutes. These recommendations provide efficient quantification of most templates using the GoTaq® 1-Step RT-qPCR System. Alternative annealing and extension incubation times and temperatures may be experimentally determined for each assay model.

$MgCl_2$ Concentration

GoTaq® qPCR Master Mix, 2X, provides a final (1X) concentration of 2mM $MgCl_2$, which is optimal for quantification of most targets. For challenging quantifications or to modify the stringency of the conditions, $MgCl_2$ concentration may be adjusted using the 25mM stock provided.

Assay Optimization

GoTaq® 1-Step RT-qPCR System performs under a wide range of cycling parameters. The general protocol may be modified to achieve the most sensitive, broad range and specific results.

Protocols

Develop a protocol for efficient quantification of your experimental target and primer pair. Use a 5- to 7-point standard curve method that encompasses the quantities of target in your unknown samples to identify the optimal cDNA synthesis, denaturation, annealing and extension temperatures and times, and the cycle number required for 100% efficiency.

Verify the quantitative function of your RT-qPCR assay by including no template controls (NTC), minus- RT controls, and positive target control.

3.D. Important Assay-Specific Considerations (continued)

Instrument Programming

Program the real-time instrument before setting up the reaction plate to provide for immediate start of the assembled reactions.

Use the following program outline as a guide for coupled incubations for Reverse Transcription, RT inactivation/Hot-start activation, three-step qPCR, and dissociation. Cycling conditions can be optimized as appropriate for your experimental system.

Table 1. General Thermocycler Program.

Stage	# of Cycles	Program in Standard or Fast Mode
1. Reverse Transcription	1	≥37°C for 15 minutes
2. RT inactivation/Hot-start activation	1	95°C for 10 minutes
3. 3-Step qPCR:		
a. Denature	40	95°C for 10 seconds
b. Anneal/Collect Data		60°C for 30 seconds
c. Extend		72°C for 30 seconds
4. Dissociation	1	60-95°C

4. GoTaq® 1-Step RT-qPCR Protocol

Materials to Be Supplied by the User

- RNA template (e.g., experimental and standard RNA of highest quality, nuclease-free, inhibitor-free, intact; can be total RNA, mRNA, viral RNA, in vitro transcript RNA)
- target-specific forward and reverse primers designed for use in one-step RT-qPCR
- sterile, DNA-free, nuclease-free, nonstick microcentrifuge tubes, multiwell plates and low-retention barrier tips
- nuclease-free pipettors designated for pre-amplification
- optical multiwell reaction plates and seals compatible with the real-time PCR instrument
- real-time PCR instrument
- microcentrifuge
- centrifuge with adapter for multiwell plates
- real-time analysis software
- chilled block or ice bucket with wet ice
- vortex mixer
- disposable gloves and other personal protective wear
- **optional:** positive-control DNA standards for qPCR

Note: Use nonstick tubes and multiwell plates for the entire protocol.

4.A. Protocol

The following instructions describe the preparation of 20µl or 50µl reactions .

Prepare GoTaq® 1-Step RT-qPCR Reaction Mix as a single batch that includes the common components, such as GoTaq® 1-Step RT-qPCR Master Mix, CXR dye, nuclease-free water and GoScript™ RT Mix. Divide the batch into individual volumes then add the remaining components (Table 2).

Determine the number of control and experimental reactions in the assay. Make a sufficient volume of reaction mix to provide for ≥3 replicates of each reaction, plus a 10% volume excess.

Prepare new reaction mix for each experiment. Do not save or reuse reaction mix.

4.A. Protocol (continued)

1. Thaw the components of the GoTaq® 1-Step RT-qPCR System, the RNA templates and the primer pair on ice, at room temperature or at 37°C.
2. Visually inspect to ensure that thawing is complete. Immediately mix each thawed component solution thoroughly. Swirl using a vortex mixer at **low speed** to minimize aeration. Keep the thawed reagents on ice.

   Note: Protect all reagents that include GoTaq® qPCR Master Mix from light.
3. Combine the component volumes in a nonstick sterile tube, on ice (Table 2). After each addition, mix the combinations gently and thoroughly, by drawing up and down in the pipette tip. If using a vortex mixer, use low speed to minimize aeration.

Table 2. GoTaq® 1-Step RT-qPCR Reaction Mix.

Component	Volume per 20µl Reaction	Volume per 50µl Reaction	Final Concentration in Reaction
GoTaq® qPCR Master Mix, 2X	10µl	25µl	1X
Forward Primer, 10X	2µl	5µl	50–300nM
Reverse Primer, 10X	2µl	5µl	50–300nM
GoScript™ RT Mix for 1-Step RT-qPCR, 50X **or** Nuclease-Free Water for Minus-RT control	0.4µl	1.0µl	1X
RNA Template (500fg–100ng) (or Nuclease-free Water for No-Template Control)	4µl	10µl	variable
Optional: Supplemental $MgCl_2$, 25mM*	__µl	__µl	≥2mM
Optional: Supplemental CXR Reference Dye, 30µM**	__µl	__µl	≥33nM**
Nuclease-Free Water	**to 20µl**	**to 50µl**	–

*Add to supplement the $MgCl_2$ provided in Master Mix

**__Guidelines__ for addition of CXR Reference Dye (30µM) to the reaction mix to achieve a final concentration of 0.5µM:
31µl per 100-reaction batch for 20µl reactions, **or**
78µl per 100-reaction batch for 50µl reactions.

4.A. Protocol (continued)

4. Carefully distribute the reaction volumes to the wells of the reaction plate on ice, using caution to avoid cross contamination.
5. Cover the wells with an optical plate seal.
6. Centrifuge the plates for up to 1 minute at room temperature to collect the reaction volumes and eliminate residual air bubbles from the well contents.
7. Keep the reactions chilled on ice or in a cold block whenever possible. Protect the reactions from light. Keep the plate on ice during transfer to the instrument.
8. Place the reaction plate into the prepared instrument (prewarmed and programmed). Start the run immediately.

4B. GoTaq® 1-Step RT-qPCR Run

Preprogram the real-time instrument to avoid run start delays.

1. Select SYBR® (or FAM™) as the detection dye.
2. Select ROX™ detection for normalization using CXR as the passive reference dye.
3. Select to run in Standard or Fast Mode.
4. Designate at which step the data will be collected in each qPCR cycle (e.g., during the annealing step, or at an alternate temperature step in each cycle).
5. For each stage program temperature, duration and number of repeats for optimal results. As a guide, Table 1 outlines general incubation parameters that are appropriate for most assays. The program may be modified as necessary for optimal results, based upon assay time requirements, the required level of sensitivity, and the specificity of the target/primer sequences. Experimental optimization might include variation of the following:
   a. Reverse transcription temperature (>37°C).
   b. Reverse transcription time (greater or less than 15 minutes).
   c. Reverse transcriptase inactivation/hot-start activation time and temperature.
   d. Denaturation, annealing and extension times and temperatures of each qPCR cycle.
   e. Number of steps in each qPCR cycle (e.g., two-step or other)
   f. Increased or reduced number of cycles.

5. 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.
2. Dorak, M.T (2009) Glossary of real-time PCR terms. This can be viewed online at **www.dorak.info/genetics/glosrt.html**.
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.

6. Related Products

Real-Time PCR

Product	Size	Cat.#
GoTaq® qPCR Master Mix*	200 reactions	A6001
	1,000 reactions	A6002
GoTaq® 2-Step RT-qPCR System	50 RT reactions + 200 qPCR reactions	A6010

*For in vitro research use only. Not for use in diagnostic procedures.

RNA Purification, Manual Systems

Product	Size	Cat. #
SV Total RNA Isolation System	10 preps	Z3101
	50 preps	Z3100
	250 preps	Z3105
PureYield™ RNA Midiprep System	50 prep	Z3741
	10 prep	Z3740

Manual or Automated RNA Purification

Product	Size	Cat.#
SV 96 Total RNA Isolation System	1 x 96 preps	Z3500
	5 x 96 preps	Z3505
Vac-Man® 96 Vacuum Manifold	1 each	A2291

Automated RNA Purification

Product	Size	Cat.#
Maxwell® 16 LEV simplyRNA Cells Kit	48 preps	AS1270
Maxwell® 16 LEV simplyRNA Tissue Kit	48 preps	AS1280
Maxwell® 16 Total RNA Purification Kit	48 preps	AS1050
MagneSil® Total RNA mini-Isolation System	4 plate	Z3351

Accessories

Product	Size	Cat.#
GoScript™ Reverse Transcription System	50 reactions	A5000
	100 reactions	A5001
GoScript™ Reverse Transcriptase	100 reactions	A5003
	500 reactions	A5004
RNasin® Plus RNase Inhibitor	2,500u	N2611
	10,000u	N2615
Recombinant RNasin® Ribonuclease Inhibitor	2,500u	N2511
Nuclease-Free Water	50ml	P1193

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[a]U.S. Pat. No. 5,552,302.

[b]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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Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
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