TECHNICAL MANUAL

GoTaq® Probe 2-Step RT-qPCR System

Instructions for Use of Product
A6110

Revised 9/18
TM380

GoTaq® Probe 2-Step RT-qPCR System

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

1. Description

The GoTaq® Probe 2-Step RT-qPCR System[a] is optimized for quantitative PCR assays in the hydrolysis probe detection format. The system protocol facilitates detection and relative quantification of RNA expression levels via a two-step RT-qPCR method using integrated components:

- GoScript™ Reverse Transcription System
- GoTaq® Probe qPCR Master Mix

The GoScript™ Reverse Transcription System includes an optimized reaction buffer and reverse transcriptase designed to enable efficient synthesis of first-strand cDNA in preparation for PCR amplification. The cDNA product can be added directly to downstream qPCR amplification reactions.

An overview of the protocol is shown in Figure 1.

The GoTaq® Probe qPCR Master Mix is provided as a ready-to-use, stabilized 2X formulation that includes all components for qPCR, including GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs and a proprietary reaction buffer, but not template, primers and probe. This master mix does not contain a reference dye; a separate tube of carboxy-X-rhodamine (CXR) reference dye is included with this system, allowing you to add reference dye to amplification reactions if desired.

1. Description (continued)

The GoTaq® Probe qPCR Master Mix provides resistance to a wide range of PCR inhibitors. This formulation uses antibody-mediated hot-start chemistry, allowing reaction setup to be performed at room temperature. The master mix also employs rapid hot-start activation and processive enzymes, making it compatible with both standard and fast instrument cycling programs.

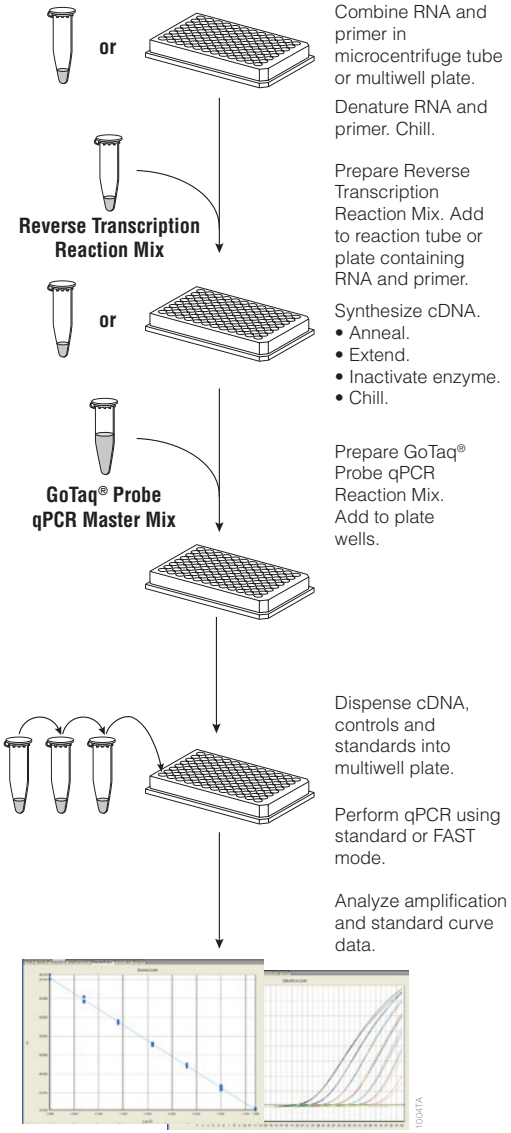

Figure 1. An overview of the GoTaq® Probe 2-Step RT-qPCR protocol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GoTaq® Probe 2-Step RT-qPCR System	**2ml**	**A6110**

For Research Use Only. Not for use in diagnostic procedures. Each system contains sufficient reagents for 200 × 20µl GoTaq® Probe 2-Step RT-qPCR assays. Includes:

- 2 × 1ml GoTaq® Probe qPCR Master Mix, 2X
- 750µl $MgCl_2$, 25mM
- 100µl CXR Reference Dye
- 300µl GoScript™ 5X Reaction Buffer
- 50µl GoScript™ Reverse Transcriptase
- 50µg Oligo$(dT)_{15}$ Primer
- 200µl PCR Nucleotide Mix, 10mM
- 50µg Random Primers
- 2,500u Recombinant RNasin® Ribonuclease Inhibitor
- 3 × 1.25ml Nuclease-Free Water

Storage Conditions: Store all components between –30°C and –10°C. Protect components from light at all times. Thaw the GoScript™ 5X Reaction Buffer on ice and mix until no visible precipitate is present. Store the buffer on ice after thawing. For best results, mix thawed solution gently to minimize aeration and foaming, and store on ice. For short-term storage and frequent use, the GoTaq® Probe qPCR Master Mix, 2X, can be stored at 2–10°C for up to 3 months if protected from light.

Available Separately

PRODUCT	SIZE	CAT.#
GoTaq® Probe qPCR Master Mix*	**2ml**	**A6101**
	10ml	**A6102**
GoTaq Probe 1-Step RT-qPCR System*	**2ml**	**A6120**
	12.5ml	**A6121**
Nuclease-Free Water	**50ml**	**P1193**

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

3. General Considerations

3.A. Prevention of Contamination

We recommend the following precautions to prevent contamination:

- Use designated work areas and pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acids from one experiment to the next.
- Wear gloves and change them often.
- Do not open the reaction plate or strip wells after amplification is complete. Opening the reaction plate or strip wells increases the risk of contaminating subsequent reactions with the amplified product.
- Use aerosol-resistant pipette tips.

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3.B. qPCR Primers and Probes

The concentrations of primers and probes should be optimized for each primer/probe combination. For gene expression assays, primer and probe concentrations may need to be adjusted based on target abundance. As a general rule, a concentration of 900nM for PCR primers and 250nM for the hydrolysis probe is a recommended starting point. Concentrations of PCR primers can range from 200nM to 1µM, while probe concentration can range from 100nM to 300nM; titrations should be performed to ensure optimal results.

We recommend preparing and storing PCR primers and hydrolysis probes as 20X solutions.

3.C. CXR Reference Dye

The GoTaq® Probe qPCR Master Mix does not contain a reference dye; however, a separate tube of carboxy-X-rhodamine (CXR) reference dye is included with this system, allowing you to add reference dye if desired. Adding the reference dye will help maximize effectiveness of the GoTaq® Probe qPCR Master Mix when used with real-time PCR instruments that allow normalization. The CXR reference dye has the same spectral properties as ROX™ dye. The dye is provided at a concentration of 30µM.

Some instrumentation is designed to normalize with a low concentration of ROX™ reference dye. We recommend that the CXR reference dye be added to a final concentration of 30nM for instruments that recommend a low level of ROX™ dye. Other instruments require ROX™ at a high concentration for normalization. We recommend that the CXR Reference Dye be added to a final concentration of 500nM for instruments that recommend a high level of ROX™ dye.

The recommended dye levels for various instruments are listed below. Directions for supplementing the GoTaq® Probe qPCR Master Mix with CXR Reference Dye are included in Section 5.A.

Instruments That Do Not Require Supplemental Reference Dye

- 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
- Bio-Rad iCycler iQ® and iQ®5 Real-Time PCR Detection Systems
- Bio-Rad MyiQ™ Real-Time PCR Detection System
- Roche LightCycler® 480 Real-Time PCR System
- Eppendorf Mastercycler® ep realplex Real-Time PCR System

Instruments That Require Low Levels (30nM) of Reference Dye

- Applied Biosystems 7500 and 7500 FAST Real-Time PCR System
- Applied Biosystems QuantStudio® Real Time PCR Systems
- Applied Biosystems ViiA® 7 Real-Time PCR System
- Stratagene/Agilent Mx3000P® and Mx3005P® Real-Time PCR Systems
- Stratagene/Agilent Mx4000® Multiplex Quantitative PCR System

Instruments That Require High Levels (500nM) of Reference Dye

- Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems
- Applied Biosystems 7300 and 7900HT Real-Time PCR System

4. Preparing cDNA using the GoScript™ Reverse Transcription System

Materials to Be Supplied By the User

- nuclease-free reaction tubes
- sterile, aerosol-resistant tips and pipettors
- RNA template
- 25°C, 45°C and 70°C controlled-temperature heat blocks
- ice-water bath

The GoScript™ Reverse Transcription System includes a reverse transcriptase and optimized reagents for efficient synthesis of first-strand cDNA in preparation for PCR amplification. Because no cleanup or dilution is necessary following the cDNA synthesis, the product can be added directly to amplification reactions.

The final reaction volume in this protocol is 20µl. The volumes given here may be scaled for larger or smaller reaction volumes.

RNA Template

The amount of RNA required to detect the target of interest depends on the abundance of that RNA target in each sample. As a starting point to detect RNA at unknown expression levels, we recommend using 100ng of total RNA template per cDNA synthesis reaction. A high-copy-number RNA transcript may be detected in as little as 10pg, while a low-copy-number RNA transcript may require more than 100ng. Up to 1µg of RNA may be used in each reaction.

For optimal results, the RNA template should be free of genomic DNA contamination. This is of particular importance when amplifying targets within a single exon to avoid amplifying any contaminating genomic DNA.

cDNA Synthesis Negative Control

To test for the presence of contaminating genomic DNA in the RNA templates, we recommend performing a no-reverse transcriptase reaction.

Note: RQ1 RNase-Free DNase (Cat.# M6101) can be used to remove DNA from RNA samples prior to RT-qPCR.

1. Denature RNA and primers.
   a. On ice, combine the RNA template and primers as described below.

Component	Volume
RNA template (DNA-free)	1–5µl
Oligo $(dT)_{15}$ Primer (0.5µg/µl)	1µl
Random Primers (0.5µg/µl)	1µl
Nuclease-Free Water	to a total volume of 7µl

   b. Close each tube tightly. Place tubes in a 70°C heat block for 5 minutes. Immediately chill in an ice-water bath for at least 5 minutes. Centrifuge each tube for 10 seconds in a microcentrifuge to collect the contents at the bottom and maintain the original volume. Keep the tubes closed and on ice until the reverse transcription reaction mix is added in Step 4.

4. Preparing cDNA using the GoScript™ Reverse Transcription System (continued)

2. Determine the number of reactions to be set up, including negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does require using a small amount of extra reagent, it ensures that you have enough reaction mix for all samples.

3. Prepare the Reverse Transcription Reaction Mix by combining the following components of the GoScript™ Reverse Transcription System in the order listed below in a sterile 1.5ml microcentrifuge tube on ice. Vortex gently to mix, and store on ice.

Reaction Mix Component	Reverse Transcription Reaction Mix (Volume per Reaction)	Minus-Reverse Transcriptase Reaction Mix (Volume per Reaction)
Nuclease-Free Water	4.9µl	5.9µl
GoScript™ 5X Reaction Buffer	4µl	4µl
$MgCl_2$	1.6µl	1.6µl
PCR Nucleotide Mix	1µl	1µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl	0.5µl
GoScript™ Reverse Transcriptase	1µl	0µl
Final volume	13µl	13µl

4. Add 13µl of the Reverse Transcription Reaction Mix to each RNA/primer tube for a final reaction volume of 20µl.

5. Place tubes in a 25°C heat block, and incubate for 5 minutes to allow the primers and RNA to anneal.

6. Incubate tubes in a 42°C heat block for 45 minutes.

7. Inactivate the reverse transcriptase by incubating tubes in a 70°C heat block for 15 minutes.

   Note: The reverse transcriptase must be thermally inactivated prior to amplification.

8. Proceed to qPCR amplification, or store cDNA at –20°C.

5. qPCR Protocol

Materials to Be Supplied by the User

- real-time PCR instrument and related equipment (i.e., optical-grade PCR plates and appropriate plate covers)
- sterile, aerosol-resistant pipette tips
- nuclease-free pipettors dedicated to pre-amplification work
- cDNA template
- qPCR primers and probe

5.A. Adding CXR Reference Dye to the GoTaq® Probe qPCR Master Mix (Optional)

Some real-time PCR instruments require addition of the CXR Reference Dye; see Section 3.C. If you wish to add CXR Reference Dye to your amplification reactions, we recommend adding an aliquot of concentrated CXR Reference Dye to the 1ml tube of the GoTaq® Probe qPCR Master Mix. Depending on your instrument, the CXR Reference Dye should be added to either the low dye (30nM) concentration or high dye (500nM) concentration (see Section 3.C).

1. Thaw the CXR Reference Dye and GoTaq® Probe qPCR Master Mix. Do not thaw at elevated temperatures (i.e., above room temperature).
2. Vortex the CXR Reference Dye and GoTaq® Probe qPCR Master Mix for 3–5 seconds to mix.
3. Add CXR Reference Dye to the 1ml tube of GoTaq® Probe qPCR Master Mix as follows:

   When using an instrument designated as a high-dye instrument, add 33.4µl of CXR Reference Dye, which is supplied at a concentration of 30µM, to the 1ml tube of GoTaq® Probe qPCR Master Mix.

   When using an instrument designated as a low-dye instrument, add 2µl of CXR Reference Dye, which is supplied at a concentration of 30µM, to the 1ml tube of GoTaq® Probe qPCR Master Mix.
4. Vortex for 3–5 seconds to mix.
5. Mark the tube to indicate that you have performed this step. Store the GoTaq® Probe qPCR Master Mix with CXR at –20°C.

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5.B. Assembling the GoTaq® Probe qPCR Reaction Mix

The GoTaq® Probe qPCR Master Mix uses a hot-start chemistry, allowing reaction setup to be performed at room temperature.

We recommend assembling the following reactions for each set of qPCRs:

Sample cDNA Template. Add 2–5µl of the cDNA prepared in Section 4 directly to each amplification reaction. Alternatively, dilute the sample cDNA prior to amplification. As a starting point for dilution, the sample cDNA can be diluted 1:10 in nuclease-free water; add 2–5µl of diluted cDNA to the amplification reaction.

No-Template Control. Include a no-template control (NTC) reaction for each set of reactions. Add 2–5µl of nuclease-free water to these reactions in place of the cDNA template. There should be no amplification product detected in the NTC reaction.

Negative (No Reverse Transcriptase) cDNA Control. The no-reverse transcriptase cDNA control serves as a negative amplification control. If reverse transcriptase is absent during cDNA synthesis, then no real-time amplification should be observed.

The final reaction volume in this protocol is 20µl. The volumes given here may be scaled for larger or smaller reaction volumes.

1. Thaw the GoTaq® Probe qPCR Master Mix and Nuclease-Free Water. Do not thaw the GoTaq® Probe qPCR Master Mix at elevated temperatures (i.e., above room temperature).
2. Vortex the GoTaq® Probe qPCR Master Mix for 3–5 seconds to mix.
3. Determine the number of reactions to be set up, including negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach requires the use of a small amount of extra reagent, it ensures that you will have enough reaction mix for all samples.
4. Prepare the reaction mix (minus the cDNA template) by combining the GoTaq® Probe qPCR Master Mix, PCR primers, hydrolysis probe and Nuclease-Free Water. The cDNA template is added in Step 6. Vortex briefly to mix.

Component	Volume	Final Concentration
GoTaq® Probe qPCR Master Mix, 2X	10µl	1X
Forward primer (20X)	1µl	200nM–1µM
Reverse primer (20X)	1µl	200nM–1µM
Hydrolysis probe (20X)	1µl	100–300nM
cDNA Template	2–5µl	≤250ng
Nuclease-Free Water	to a final volume of 20µl	

Note: The concentrations of primers and hydrolysis probe should be optimized for each primer combination.

5. Add the appropriate volume of reaction mix to each PCR tube or well of an optical-grade PCR plate.
6. Add the cDNA template (or water for the no-template control reactions) to the appropriate wells of the reaction plate.
7. Seal the tubes or optical plate; centrifuge briefly to collect the contents of the wells at the bottom. Protect from extended light exposure or elevated temperatures before cycling. The samples are ready for thermal cycling.

6. Thermal Cycling

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® DNA Polymerase activation	1	95°C	2 minutes
Denaturation	40	95°C	15 seconds
Annealing and extension		60°C	1 minute

FAST Cycling Conditions

Step	Cycles	Temperature	Time
GoTaq® DNA Polymerase activation	1	95°C	2 minutes
Denaturation	40	95°C	3 seconds
Annealing and extension		60°C	30 seconds

7. 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.

8. Related Products

Real-Time PCR

Product	Size	Cat.#
GoTaq® qPCR Master Mix	5ml	A6001
	25ml	A6002
GoTaq® 1-Step RT-qPCR System	5ml	A6020
GoTaq® 2-Step-RT-qPCR System	5ml	A6010

RNA Purification, Manual Systems

Product	Size	Cat. #
ReliaPrep™ RNA Cell Miniprep System	10 preps	Z6010
ReliaPrep™ RNA Tissue Miniprep System	10 preps	Z6110
ReliaPrep™ FFPE Total RNA Miniprep System	10 reactions	Z1001
SV Total RNA Isolation System	10 preps	Z3101
PureYield™ RNA Midiprep System	10 preps	Z3740

Additional sizes are available.

Manual or Automated RNA Purification

Product	Size	Cat.#
SV 96 Total RNA Isolation System	1 × 96 each	Z3500
	5 × 96 each	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
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

9. Summary of Changes

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