Takara Bio USA

Quant-X™ One-Step qRT-PCR TB Green® Kit User Manual

Cat. No. 638317
(050919)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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I. Introduction

Quantitative reverse transcription PCR (qRT-PCR) is a highly sensitive and versatile technique that is widely used for measuring gene expression in tissues and cultured cells. Traditionally, RT-PCR is performed in two reaction steps. In the initial reaction, first-strand cDNA is reverse-transcribed from total or poly A+ RNA using a reverse transcriptase. Then, in a separate reaction, the cDNA is typically diluted and then amplified by PCR using a thermostable DNA polymerase. Such two-step procedures require multiple handling steps and either multiple tubes or the sequential addition of enzymes and other reagents to a single tube. In contrast, the Quant-X One-Step qRT-PCR TB Green Kit allows first-strand cDNA synthesis and qPCR to be performed in one step—in a single tube, with a single optimized buffer, and a single enzyme mix. No additional reagents are required after the reaction is initiated (Figure 1). This significantly reduces the risk of cross-contamination and provides a very convenient technique for detecting and quantitating gene expression.

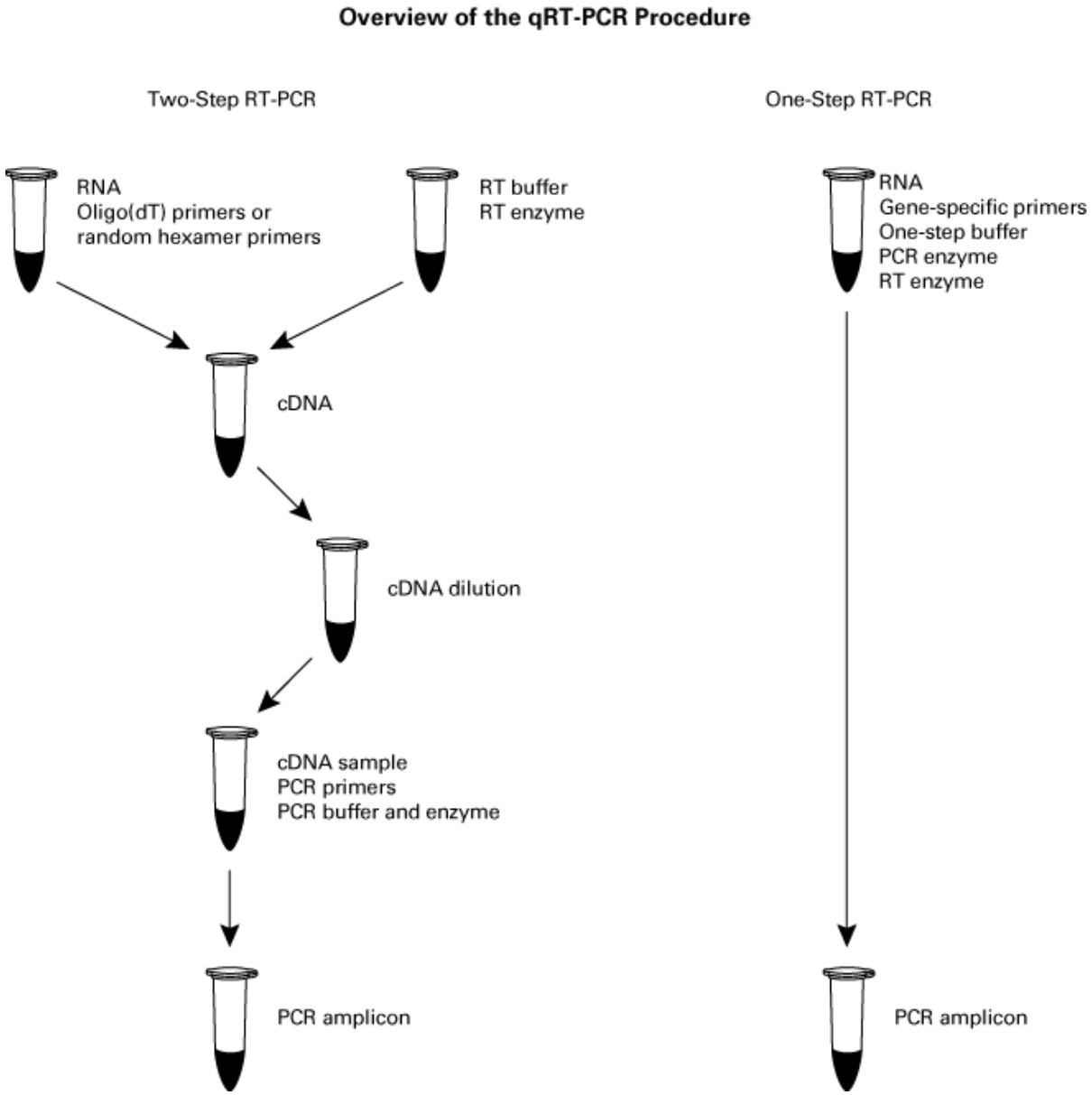

Figure 1. Comparison of two-step vs. one-step qRT-PCR procedures.

II. List of Components

Store all components at –20°C. **Shield the 2X Quant-X Buffer from light.**

This kit contains sufficient reagents for 200 qRT-PCR reactions of 25 µl each.

Quant-X One-Step qRT-PCR TB Green Kit (Cat. No. 638317):

- 3 tubes 2X Quant-X Buffer (0.84 ml per tube)
- 100 µl Quant-X Enzyme (5 U/µl)
- 100 µl RT Enzyme Mix
- 2 tubes RNase-Free Water (1.25 ml per tube)
- 100 µl 50X ROX Reference Dye LSR
- 100 µl 50X ROX Reference Dye LMP

III. Additional Materials Required

- The following materials are required but not supplied:
- **Recombinant DNase I (RNase-free)** (Cat. No. 2270A) [Optional]
- **EASY Dilution (for Real Time PCR)** (Cat. No. 9160) [Optional]—prevents RNA from sticking to plastic, facilitating the quantitation of low concentrations of RNA.
- **Gene-specific PCR primers**—refer to Section IV. B. Primer Design
- **PCR-grade water (RNase free)**
  Avoid using autoclaved H_2O; the recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- Work areas and pipettors free of contaminating RNA and RNases.
- **PCR reaction tubes or plate (reaction specific)**
- **Aerosol-free PCR pipette tips** suitable for use with the above pipettors and preferably equipped with hydrophobic filters
- **Pipettors** dedicated for pre-PCR
- **Pipettors** dedicated for RNA template
- **Real-time thermal cycler**
- **RNA template for standard curve generation:** We recommend using our qPCR Human Reference Total RNA (Cat. No. 636690)

IV. General Considerations

A. RNA Preparation

The use of pure, non-degraded total RNA is critical for synthesizing high-quality cDNA for PCR. RNA should have an A_{260}/A_{280} ratio of 1.8 or higher and should be evaluated on a denaturing agarose gel (containing formaldehyde) to verify its integrity prior to cDNA synthesis.

1. Total RNA can be prepared by any standard method. See Sambrook et al. 2001 as a reference. Be sure that your protocol includes a DNase I digestion step to ensure that your RNA is free of contaminating DNA.
2. To avoid contamination and degradation of RNA, follow these precautions:
   - Wear gloves to avoid RNase contamination from hands.
   - Wipe all pipettes with 70% ethanol or isopropanol before RNA work.
   - Use sterile pipette tips.
3. Store pure RNA at –70°C or as an ethanol precipitate at –20°C. Avoid multiple freeze-thaw cycles.

B. Primer Design

Primer design is the single largest variable in PCR applications, and the single most important factor in determining the success or failure of PCR reactions. Always check (and recheck) your primer design before synthesizing (or ordering) your primers. When designing primers, consider the following:

1. Make sure your primer sequences are gene-specific. Do not use oligo(dT) or random primers.
2. To reduce the likelihood of amplifying genomic DNA, design at least one primer to span an exon-exon junction. Such primers prevent the amplification of genomic DNA because the presence of the intron interferes with primer binding. However, make sure that most of the primer binds to the 5' exon, otherwise, even in the presence of the intron, enough of the 3' end of the primer could bind to allow replication of the target sequence.

   Alternatively, primer pairs that are separated by at least one intron will help distinguish between products amplified from cDNA and those amplified from genomic DNA. Such primers will amplify much larger products from genomic DNA than from cDNA, allowing you to easily distinguish between the two products by melting curve analysis and/or agarose gel electrophoresis. If intron-separated primer sites cannot be used, a negative control reaction should be performed as described in Section IV.D.4. (below).
3. Primers should be between 17–25 bases in length and have a GC content between 40–60%.
4. Each primer should have a melting temperature (Tm) between 58–65°C. The Tm difference between the two primers should be ≤ 4 ºC.
5. Avoid runs of identical nucleotides. The last five nucleotides at the 3' end of each primer should contain no more than two guanines (G) and/or cytosines (C). In addition, we recommend that you make the last nucleotide at the 3' end of each primer a G or a C.
6. Avoid complementarity within each primer to prevent hairpin structures; avoid complementarity between primer pairs to prevent primer dimers. There should be no more than 3 bases of complementary sequence within a primer or between primer pairs. In addition, primer pairs should have no more than 2 bases of complementary sequence.
7. synthesizing your primers, perform a BLAST search to determine if the primers you designed are unique and specific (**www.ncbi.nlm.nih.gov/BLAST/**).

C. Amplicon Design

1. Amplicons should be between 80–150 bp long. Shorter amplicons will give higher PCR efficiencies; longer amplicons will give a higher overall fluorescence intensity (ΔRn).
2. Select amplicons with a GC content between 40–60%.
3. Avoid sequences with extensive secondary structure. Use a computer program such as Mfold, available on the Mfold web server (**www.bioinfo.rpi.edu/applications/mfold/**), to predict secondary structure in your proposed amplicon (Mathews et al. 1999; Zuker 2003).

D. Good PCR Practices

1. Prepare reactions with dedicated pipettors in a dedicated work space.
2. Because of the small volumes used in experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be certain not to carry over extra solution on the outside of the pipette tip during transfer. When adding liquid to a tube, immerse the tip into the reaction mixture, deliver the contents from the pipette tip into the mixture, and then rinse the tip by pipetting up and down several times.
3. Prepare a Master Mix containing the appropriate volumes of all of the reagents required for multiple RT-PCR reactions. This eliminates the need for repeated pipetting of individual reaction components into each reaction tube. We highly recommend using a Master Mix when multiple reactions are required because it greatly reduces tube-to-tube variation. If, for example, multiple templates are being tested with the same primer pair, include the primers in the Master Mix. If one template is being tested with multiple primer sets, include the template in the Master Mix. If you are setting up several sets of parallel samples, assemble multiple Master Mixes (e.g., each with a different set of primers). Make sure the Master Mix has enough volume to allow for additional 'No Reaction Controls' for each template, and 'No Template Controls' (NTC) for each Master Mix, as well as an extra 25 µl to accommodate pipetting errors. The Master Mix should be thoroughly mixed before use.
4. Always include positive and negative controls (e.g., H_2O instead of RNA template for the negative control).

   NOTE: An optional negative control can be performed to help distinguish between fragments amplified from cDNA and those derived from genomic DNA contaminants in your RNA sample. To prepare this control, simply omit the RT Enzyme Mix from a reaction containing your RNA sample.
5. At a minimum, each reaction should be performed in duplicate; however, triplicate reactions will allow more accurate statistical analysis.

V. Quant-X One-Step qRT-PCR TB Green Kit Protocols

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

A. Protocol: Preparing for Quant-X One-Step qRT-PCR TB Green assays

1. General considerations:
    a. Thaw all of the reaction components—except the Quant-X Enzyme and the RT Enzyme Mix—at room temperature. Gently vortex each component and centrifuge briefly to collect the contents in the bottom of the tube. Store on ice until ready to use.
    b. Keep the Quant-X Enzyme and the RT Enzyme Mix at –20°C until just before use. Gently mix the enzymes before use and centrifuge briefly prior to pipetting—avoid generating air bubbles. The enzymes contain 50% glycerol and are very viscous, so pipet slowly. Return the enzymes to the freezer promptly after use.
    c. Use 0.1–1.0 µM of each primer per reaction. For most targets, 0.2 µM of each primer is adequate.
    d. Depending on the expression level of your target gene, use between 10 pg and 100 ng of total RNA per reaction. For most genes, a total of 50 ng of template RNA is sufficient.
    e. If you are using ROX™ as a reference dye on a real-time PCR thermal cycler not mentioned in this user manual, use the amount recommended by the manufacturer of your thermal cycler.
2. Dilute the RNA standard:
    a. Dilute the RNA standard with PCR-grade dH_2O, on ice, in an RNase-free area. If you suspect the RNA is sticking to your plastic ware, we recommend diluting with EASY Dilution Buffer (Cat. No. 9160) instead of water.
    b. Prepare 5-fold serial dilutions of your RNA standard. Dilute the RNA to the concentrations indicated in Table I.

NOTES—IMPORTANT!

i. To generate the standard curve, you will need to use 2–4 µl of RNA per reaction, depending on your real-time instrument and your final reaction volume (see Table II). If your final reaction volume is 20–25 µl, you will need 2 µl RNA per well; if your final reaction volume is 50 µl, you will need 4 µl RNA per well.

ii. We recommend using our qPCR Human Reference Total RNA (Stock conc. 1 mg/ml; Cat. No. 636690)

iii. Use a new pair of gloves when diluting the RNA template to help prevent degradation by RNases.

Table I. Concentrations of RNA Template for Standard Curve

Tube #	RNA conc. (if using 2 µl* RNA/rxn)	RNA conc. (if using 4 µl* RNA/rxn)	Quantity of RNA per Reaction
1	50 ng/µl	25 ng/µl	100 ng
2	10 ng/µl	5 ng/µl	20 ng
3	2 ng/µl	1 ng/µl	4 ng
4	0.40 ng/µl	0.20 ng/µl	0.8 ng
5	0.08 ng/µl	0.04 ng/µl	0 .16 ng
6	0.016 ng/µl	0.008ng/µl	0.032 ng
7	0.0032 ng/µl	0.0016 ng/µl	0.0064 ng

*See Note 2.b.i., above.

3. Dilute your experimental RNA sample(s).

    a. Dilute all RNA samples with PCR-grade dH_2O, on ice, in an RNase-free area. If you suspect the RNA is sticking to your plastic ware, we recommend diluting with EASY Dilution Buffer (Cat. No. 9160) instead of water.

    b. We recommend using 10 pg–100 ng of total RNA per reaction.

    NOTE: You will need to add 2–4 µl of RNA per reaction, depending on your real-time instrument and your final reaction volume (see Table II). If your final reaction volume is 20–25 µl, add 2 µl RNA per well; if your final reaction volume is 50 µl, add 4 µl RNA per well.

B. Protocol: Setting up Quant-X One-Step RT-PCR TB Green assays

1. Prepare the Master Mix:

   a. In a pre-PCR area or a template-free PCR workstation, prepare a Master Mix on ice by adding each component as indicated in Table II. Add all reagents in the order shown.

   b. Make sure you use enough of each reagent for all of your reactions, plus an additional reaction to accommodate pipetting errors. Ideally, each reaction should be performed in triplicate; however, duplicate reactions will suffice:

Table II. Master Reaction Mixes Recommended for Different Real-Time PCR Instruments

	qPCR Instrument			
	Roche LightCycler	Cepheid SMARTCycler II/ Stratagene MX3000P QPCR System/ Takara Thermal Cycler Dice Real Time System	ABI PRISM 7000, 7700, & 7900HT/ ABI 7300, 7500, & 7500 Fast Real-Time PCR Systems	
Reagent	**Reagent volume (µl per well) for each instrument**			
RNase-Free Water	6.4	8.5	6.0	16.0
Quant-X Buffer (2X)	10.0	12.5	10.0	25.0
Forward Primer (10 µM)[a]	0.4	0.5	0.4	1.0
Reverse Primer (10 µM)[a]	0.4	0.5	0.4	1.0
ROX Reference Dye LSR or LMP (50X)[b]	—	—	0.4	1.0
Quant-X Enzyme	0.4	0.5	0.4	1.0
RT Enzyme Mix	0.4	0.5	0.4	1.0
Total volume per well	**18.0**	**23.0**	**18.0[c]**	**46.0[d]**

[a] We recommend using 0.1–1.0 µM of each primer.

[b] The Kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments that are equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488 nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. **Be certain to use the formulation that is appropriate for your real-time instrument!**

[c] This 18 µl reaction is for ABI instruments using 384-well plates and 96-well Fast Thermal Cycling plates.

[d] This 46 µl reaction is for ABI instruments using 96-well plates, single tubes and 8-tube strips.

2. Mix the Master Mix well by pulse-vortexing at least 5 times, then centrifuge briefly. Transfer the amount of Mix indicated for your real-time instrument (see the last row of Table II) into each well of a PCR plate or 8-well strip, or individual PCR capillary tubes. Centrifuge briefly.

3. Add the diluted RNA standard, total RNA test sample(s), and controls to the appropriate wells. If you transferred 18 µl or 23 µl of Master Mix into each well in Step 2, add 2 µl of RNA per well; if you transferred 46 µl of Master Mix into each well in Step 2, add 4.0 µl of RNA per well. To the No Template Control, add PCR-grade water instead of RNA.

4. Seal the wells according to the procedure recommended for the real time instrument being used.

5. Program the thermal cycler with the following conditions:

Table III. Recommended Thermal Cycling Conditions for Different Real-Time PCR Instruments

	qPCR Instrument									
	Roche LightCycler		Cepheid SMARTCycler II		Stratagene MX3000P QPCR System		ABI PRISM 7000, 7700, & 7900HT/ ABI 7300 & 7500 Real-Time PCR Systems/Takara Thermal Cycler Dice Real Time System		ABI 7500 Fast Real-Time PCR System	
Reaction Cycles	**Thermal cycling conditions for each instrument**									
Reverse Transcription (1 Cycle)	42°C 95°C	5 min[a] 10 sec[a]	42°C 95°C	5 min 10 sec	42°C 95°C	5 min 10 sec	42°C 95°C	5 min 10 sec	42°C 95°C	5 min 10 sec
qPCR (40 Cycles):	95°C 60°C	5 sec[a] 20 sec[a]	95°C 60°C	5 sec 20 sec	95°C 54°C	5 sec 30 sec	95°C 60°C	5 sec 30–34 sec[c]	95°C 60°C	3 sec 25 sec
Melting/Dissociation Curve (1 Cycle):	95°C 65°C 95°C	0 sec[a] 15 sec[a] 0 sec[b]	Melting Curve		Dissociation Curve		Dissociation Curve		Dissociation Curve	

[a] 20°C/sec

[b] 0.1°C/sec

[c] Program 30 sec on the ABI PRISM 7700 & 7900HT and the Takara Thermal Cycler Dice; 31 sec on the ABI 7000 & 7300; and 34 sec on the ABI 7500.

NOTE: Although Table III shows the optimized cycling conditions for a selection of commonly used real-time PCR instruments, the Quant-X One-Step qRT-PCR TB Green Kit can be used with a variety of real-time instruments and is not limited to those listed in the table. If your instrument is not listed in the table, please refer to the user manual supplied with the instrument to determine optimal cycling conditions.

6. Spin the reactions briefly, then place them into the real-time PCR instrument and begin thermal cycling.
7. After the reaction is complete, verify the amplification and melting curves. Establish a standard curve when quantitative analysis is necessary.
8. If our protocol recommendations do not produce the desired results, the reaction conditions will need to be optimized for your particular system. Template and primer concentrations can be adjusted within the recommended parameters. In addition, the PCR cycling conditions may need to be modified. If a three-step protocol is necessary (e.g., when using primers with low Tm values) refer to Table IV: Recommended Cycling Conditions for Two- and Three-Step PCR for more information.

Table IV. Recommended Cycling Conditions for Two- and Three-Step PCR

Two-Step PCR				
Step	**Temp.**	**Time**	**Detection**	**Remarks**
Denaturation	95°C	3–5 sec.	Off	Real-time PCR targets are generally <300 bp, so denaturation at 95°C for 3–5 sec is sufficient.
Annealing/ Extension*	60–66°C	20–30 sec.	On*	First perform this step at 60°C for 20 sec. If optimization is required, increase the temperature incrementally from 60–66°C. If the reaction still does not proceed efficiently, extend the time or try three-step PCR.
Three-Step PCR				
Denaturation	95°C	3–5 sec.	Off	Real-time PCR targets are generally <300 bp, so denaturation at 95°C for 3–5 sec is sufficient.
Annealing	55–60°C	10–20 sec.	Off	Try annealing at 55°C for 10 sec. If non-specific products are generated or if the amplification efficiency is low, try increasing the annealing time.
Extension*	72°C	6–15 sec.	On*	When the target size is less than 300 bp, the extension time should be between 6–15 sec. Longer extension times can cause non-specific amplification.

*If you are using one of the following Applied Biosystems real-time instruments, the detection step must be longer than 30 sec: on the ABI PRISM 7700 and 7900HT, program a 30-sec detection step; on the ABI PRISM 7000 or the ABI 7300 Real-Time PCR System, program a 31-sec detection step; and on the ABI 7500 Fast Real-Time PCR System, program a 34-sec detection step.

VI. References

Mathews, D.H., Sabina, J., Zuker, M., & Turner, D.H. Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. *J. Mol. Biol.* **288**(5):911-940 (1999).

Sambrook, J. & D. W. Russell. *Molecular Cloning: A Laboratory Manual*, Third Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (2001).

Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**(13):3406-15 (2003).

Appendix A. Troubleshooting Guide

Table V. Troubleshooting Guide

Problem	Possible Explanation	Solution
No signal observed in fluorescence detector **(if a PCR product of the correct size is seen when the post-PCR reaction is electrophoresed on an agarose gel)**	The wrong fluorescence channel is being read.	Confirm that the instrument detection parameters were set properly according to the detection substrate used.
	The data is not being collected at the appropriate time.	Confirm that the data is being collected during the annealing and extension steps.
No signal observed in fluorescence detector **(if no PCR product is seen when the post-PCR reaction is electrophoresed on an agarose gel)**	A reaction component is missing or degraded.	Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional.
	The annealing temperature is too high.	Decrease the annealing temperature in increments of 2–4°C.
	The template quality is poor.	Check template integrity by electrophoresis or other preferred methods.
	The template contains secondary structure.	Incubate the template for 5 min at 70°C, then for at least 2 min at 4°C. Set reactions up on ice to prevent these structures from re-forming.
	There is insufficient template.	Increase the amount of template; use up to 100 ng per reaction. Alternatively, your template may be sticking to the plastic ware. We recommend diluting with EASY Dilution Buffer (Cat. No. 9160) instead of water.
	You are amplifying a rare target.	When amplifying rare targets, increase the number of amplification cycles up to 50 per assay.
	The primer concentration is too low.	Increase the amount of each primer up to 1 µM.
	The primer design is suboptimal.	Redesign your primer(s) after confirming the accuracy of the sequence information.

Problem	Possible Explanation	Solution
Nonspecific amplification; multiple products observed on agarose gel	Contamination, which most often results in extra bands or smearing	It is important to include a No Template Control (in which the RNA template is replaced with PCR-grade H_2O) and a no RT-control (in which the reverse transcriptase is replaced with PCR-grade H_2O) in every experiment to determine if the reagents, pipettors, or reaction tubes are contaminated.
	Annealing temperature is too low	Increase the annealing/extension temperature in increments of 2–3°C. Make sure you don't use an annealing temperature that is incompatible with your chemistry, especially if the data collection is done at the annealing step.
	Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information.
	Primer/probe design is not able to distinguish between gene families or splice variants	Redesign your primer(s) after confirming the accuracy of the sequence information and all of the expressed sequence tags (ESTs) described for that gene.

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