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Product Information

SYBR® Green Quantitative RT-PCR Kit

Catalog Number **QR0100**

TECHNICAL BULLETIN

Product Description

The SYBR® Green Quantitative RT-PCR Kit combines Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), JumpStart™ *Taq* DNA polymerase, and SYBR Green I fluorescent dye in a one-step RT-PCR kit designed for measurement of gene expression. This one step quantitative RT-PCR procedure provides high specificity, reduced risk of contamination, and increased reproducibility.

M-MLV RT efficiently transcribes RNA. This newly synthesized cDNA is amplified using SYBR Green *Taq* ReadyMix™, a PCR blend with increased specificity and sensitivity due to the antibody-mediated hot start enzyme. The ReadyMix includes SYBR Green I dye, JumpStart *Taq* DNA polymerase, 99% pure deoxynucleotides, buffer, glass passivator, and stabilizers, and is provided as a 2× concentrate for convenience. JumpStart *Taq* DNA polymerase uses JumpStart *Taq* antibody to inactivate the enzyme at temperatures below 70 °C, preventing primer-dimer and nonspecific product formation.

The double-strand DNA-specific SYBR Green I fluorescent reporter offers distinct advantages. SYBR Green I dye is inexpensive, easy to use, and sensitive. Well-designed primers must be used in SYBR Green quantitative RT-PCR reactions because SYBR Green I dye will detect nonspecific products, resulting in an overestimation of the target concentration. This kit is not suitable for multiplex quantitative RT-PCR reactions because SYBR Green I dye cannot distinguish between the different targets.

Reagents provided

Suitable for 100 reactions (50 μL reaction volume)

- SYBR Green *Taq* ReadyMix for Quantitative RT-PCR, Catalog No. D5191, 2 × 50 RXN 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers and glass passivator, 1 U/μL RNase Inhibitor, *Taq* DNA Polymerase, JumpStart *Taq* antibody, and SYBR Green I dye.
- M-MLV Reverse Transcriptase, Catalog Number M1427, 5000 units, 200 units/μL
- 10× PCR Buffer, Catalog Number P2192, 1 vial 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin, 1.5 ml/vial
- $MgCl_2$ solution, 25 mM, Catalog Number M8787, 1 vial, 1.5 ml/vial
- Reference Dye for Quantitative PCR, Catalog Number R4526, 100× dye. 1 vial, 0.3 ml/vial

Reagents and equipment required, not provided

- RNA for transcription and amplification
- Specific primers
- Dedicated pipettes
- Aerosol resistant pipette tips
- Plastic PCR tubes, plastic PCR plates, or glass capillary tubes recommended by the manufacturer of the spectrofluorometric thermal cycler
- Spectrofluorometric thermal cycler

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in this kit should be stored at –20 °C. All other components can be stored at –20 °C, or may be stored at 2-8 °C for up to 6 months. Protect the SYBR Green *Taq* ReadyMix and reference dye from light.

Preliminary Considerations

RNA Preparation

The single most important step in assuring success with RT-PCR is high quality RNA preparation. Integrity and purity of RNA template is essential. RNA must be entirely free of RNase contamination. SYBR Green RT-PCR involves multiple cycles of enzymatic reactions and is therefore more sensitive to impurities such as proteins, phenol/chloroform, salts, EDTA, and other chemical solvents.

Contaminants can also interfere with fluorescence detection. The ratio between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1. Lower ratios indicate the presence of contaminants such as proteins. Either total or poly(A)$^+$ RNA can be used as template for the reverse transcription reaction. All RNA preparations should be DNA-free to assure that product is derived from RNA. DNase I, Catalog Number AMPD1, is recommended for the digestion of contaminating DNA in the RNA preparation before SYBR Green quantitative RT-PCR. Purified RNA should be stored at –20 °C or –70 °C.

Primer Design

Since SYBR Green I dye will detect all nonspecific quantitative RT-PCR products, only well designed specific primers are recommended for this system to ensure the highest possible specificity. Specific primers for RT and PCR should be designed with the aid of primer design software to eliminate the complications introduced with primer-dimers and secondary structures. Lower primer concentrations decrease the accumulation of primer-dimer formation and nonspecific product formation, which is critical in SYBR Green quantitative RT-PCR. If genomic DNA contamination is possible, primers spanning an intron will reduce the possibility of amplifying DNA and overestimating RNA message.

$MgCl_2$ Concentration

Lower magnesium chloride concentrations usually result in the formation of fewer nonspecific products. The ReadyMix solution is provided at a 2× concentration of 6 mM magnesium chloride (final concentration 3 mM). A vial of 25 mM magnesium chloride is provided for further optimization of the final magnesium chloride concentration if necessary.

Internal Reference Dye

An internal reference dye is included in a separate vial for reaction normalization when using real-time thermal cyclers that recommend addition of a reference dye. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm. Standard instrument settings for ROX reference dye are satisfactory for the measurement of the internal reference dye. This internal reference dye is necessary for ABI Sequence Detection Systems, and it does not interfere with LightCycler® SYBR Green quantitative RT-PCR results.

Controls

A positive control is always helpful to make sure all of the kit components are working properly. Two negative controls, no template and no reverse transcriptase, are necessary to determine if contamination is present. A signal in the no template control demonstrates the presence of DNA contamination or primer dimer formation. A signal in the no reverse transcriptase reaction demonstrates the presence of significant DNA that can be amplified. See Lovatt *et al.* for a thorough discussion of qRT-PCR controls.[1]

Data Analysis

Follow the recommendations of the real time instrument used to perform quantitative SYBR Green RT-PCR. The following may help new instrument users. Generally the number of cycles is plotted against the fluorescence. Threshold cycles (C_T) or crossing points are used to determine the template amount in each sample. Threshold cycle or crossing point is the first cycle that shows a detectable increase in fluorescence due to the formation of RT-PCR products. The cycles before the crossing point are the baseline cycles. The baseline cycles show no detectable increase in fluorescence due to RT-PCR products. The threshold used to determine when the first detectable increase in fluorescence occurs may also be adjusted manually. The threshold should always be done on a logarithmic amplification plot. In a logarithmic amplification plot the threshold should be set in the log-linear range and not the plateau phase.

Melting Curves

Performing a melting curve analysis at the end of the run will help to analyze only the RT-PCR product of interest. Follow the real time instrument manufacturer's instructions for melting curve analysis. Successive runs with the same primers can be modified to remove the contribution of primer dimer formation to product signal by collecting data in an additional cycling step, the temperature of which must lie between the already determined dimer and product melting temperatures (T_m).

Methods of Quantification

Standard Curves

Standard curves are necessary for both absolute and relative quantification. When generating standard curves, different concentrations of RNA (typically five) should be used to generate a standard curve that will bracket the concentration of the unknown. Each concentration should be run in duplicate.

Absolute and Relative Quantification

This SYBR Green RT-PCR kit may be used to quantify target RNA using either absolute or relative quantification. Absolute quantification techniques are used to determine the amount of target RNA in the initial sample, while relative quantification determines the ratio between the amount of target RNA and a reference amplicon. The ideal reference amplicon would have invariant, constitutive expression. In practice, a housekeeping gene is chosen for this function, but there are other reference choices that better adhere to the above requirements.[2]

Absolute quantification uses external standards to determine the absolute amount of target nucleic acid of interest. To remove the differences in quantification due to annealing, the primer binding sites of the external standards must be the same as those in the target sequence. The ideal external standard contains sequences that are the same as the target sequence or which vary only slightly from the target sequence. Equivalent amplification efficiencies between the target and external standard are necessary for absolute quantification. Once a suitable construct or amplicon is identified, a standard curve of external standard dilutions is generated and used to determine the concentrations of unknown target samples.

Relative quantification allows calculation of the ratio between the amount of target template and a reference template in a sample. Since this method measures the amount of target relative to a presumably invariant control, relative qRT-PCR is most often used to measure genetic polymorphism differences, for instance, between tissues or between healthy and diseased samples. The advantage of this technique is that using an internal standard can minimize the variations in sample preparation and handling. When using SYBR systems, the target and internal reference quantification must be run in separate reactions.

The accuracy of relative quantification depends on the appropriate choice of a reference template for standards. Variability of the standard will influence the results and so it is most important that standards be appropriate.[2] Some researchers choose not to run a standard curve and report target quantities as a fraction of the reference, a technique termed comparative quantitation. Alternatively, one may assume that the difference in amplification efficiencies of target and reference is negligible, and quantify target based solely on the standard curve determined for the reference sequence.

Finally, in the most accurate of the relative quantification techniques, the amplification efficiencies of both the reference and target are measured, and a correction factor is determined. This process, termed normalization,[2] requires a sample containing known concentrations of both target and reference and the generation of two standard curves.

Determination of RT-PCR Reaction Efficiencies

The RT-PCR efficiency between a reference sample and a target sample is determined by preparing a dilution series for each target. The C_T values of the reference are subtracted from the target and this difference in C_T values is plotted against the logarithm of the template amount. If the resulting slope of the straight line is less than ±0.1 the amplification efficiencies are judged to be similar.

References

1. Lovatt, A., et al. Validation of Quantitative PCR Assays, *BioPharm*, March 2002, p. 22-32.
2. Bustin, S.A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, *J. Molecular Endocrinology*, **29**, 23-29 (2002).
3. Morrison, T.B., et al., Quantification of Low-Copy Transcripts by Continuous SYBR® Green I Monitoring during Amplification. *BioTechniques,* **24,** 954-962 (1998).

Related Products

- Dnase I, Amplification Grade, for removing DNA from RNA preps, Catalog Number AMPDI
- GenElute Direct mRNA Miniprep Kit, for isolating mRNA from cells or tissue, Catalog Numbers DMN10 and DMN70
- GenElute Mammalian Total RNA Miniprep Kit, for isolating total RNA from tissue or cells, Catalog Numbers RTN10, RTN70, and RTN350
- GenElute mRNA Miniprep Kit, for isolating mRNA from total RNA, Catalog Numbers MRN10 and MRN70
- TRI Reagent®, for isolating total RNA from tissue Catalog Number T9424
- TRI Reagent BD, for isolating total RNA from whole blood, Catalog Number T3809
- TRI Reagent LS, for isolating total RNA from fluid samples, Catalog Number T3934

Procedure

Note: Because SYBR Green I fluorescent dye binds to all double stranded DNA, it is important to test primers, cycling conditions and $MgCl_2$ concentration to insure the RT-PCR product is a single band or the results will not be interpretable. RT-PCR amplicons should be between 100-150 bp in length and should not exceed 500 bp. If primer-dimer or nonspecific product formation is a problem, a melting curve analysis is highly recommended, which increases the dynamic range and reliability of quantification.[3] Follow the spectrofluorometric thermal cycler manufacturer's instructions for melting curve analysis. RT-PCR specificity may be checked on a non-quantitative thermocycler by analyzing the results using agarose gel separation before performing quantitative RT-PCR with SYBR Green dye.

For best results, optimal concentrations of primers, $MgCl_2$, KCl, and PCR adjuncts need to be determined. Testing various combinations of primer concentrations (50-1000 nM) is most efficient for primer optimization. If maximum sensitivity is not required and your PCR target is abundant, satisfactory results for SYBR Green based qPCR are often obtained with final concentrations of both primers 200-400 nM.

The following procedure serves as a guideline to establish optimal primer concentrations. Further optimization may be necessary due to primer specificity. For more optimization information, please read the qPCR user guide available online at www.sigmaaldrich.com.

Note 1: The use of up to 5% (v/v) dimethyl sulfoxide (DMSO) will not disturb the enzyme-antibody complex. Other co-solvents, solutes (salts) and extremes in pH or other reaction conditions may reduce the affinity of the JumpStart *Taq* antibody for the *Taq* polymerase and thereby compromise its effectiveness.

Note 2: Optimization of primer concentrations is highly recommend if this has not been previously performed for your application. See section B, immediately following.

A. Procedure for Routine (previously optimized) Analysis

1. Preparation of a reaction master mix is highly recommended to give best reproducibility. Use a thin walled RNase free PCR tube or plate recommended by the manufacturer of the spectrofluorometric thermal cycler. Mix all reagents but template in a common mix, using ~10% more than needed. Once template is diluted into the reaction vessel, master mix is aliquoted into the proper tube or plate for thermocycling.

Volume	Reagent	Final Concentration
25 µL	2× SYBR Green *Taq* ReadyMix for Quantitative RT-PCR	1× *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP, stabilizers
(0.5 µL)	Reference Dye** (optional)	1x
--- µL	25 mM $MgCl_2$ (optional)	3.0 mM (without addition; optimize as required)
x µL	RNA template	10-100 ng/µL total RNA or 0.5 - 10 ng mRNA
--- µL	Forward Primer	Optimal Conc. from Sec. B
--- µL	Reverse Primer	Optimal Conc. from Sec. B
0.25 µL	200unit/µl M-MLV RT	1 unit/µL See Note 1
q.s. to 50 µL	Water	
50 µL	Total Volume	

* Volume for 50 µL reaction, however volumes may be scaled to give the desired reaction volumes.

** Use 0.1× for ABI 7500 and Stratagene instruments; replace with FITC for BioRad iCycler.

Note 1: Most applications will require the formulation of more than one reaction, and in these cases it is recommended that a master mix of ReadyMix, M-MLV RT and $MgCl_2$ solution be formulated and aliquotted. If performing fewer than four reactions, it is recommended that the researcher dilute the M-MLV RT four-fold in PCR buffer (2.5 µL water, 0.5 µL PCR reagent, 1 µL M-MLV RT). This course is recommended because accurate transfer of less than 1µL is difficult and a significant source of irreproducibility when pipetting critical reagents.

2. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.
3. Perform Thermal cycling

Typical cycling parameters for 100–600 bp fragments:

Reverse Transcription	42–44 °C	30 min
Initial denaturation	94 °C	2 min
40 cycles		
Denaturation	94 °C	15 sec
Annealing, extension, and read fluorescence	60 °C or 5 °C below lowest primer T_M	1 min
(Optional) Hold	4 °C - only if products will be run out on a gel	

For Real-Time Instruments using Capillary Tubes (20 µL reaction volume)

1. Add the following reagents to a thin walled plastic PCR tube for transfer to a glass capillary or directly to a capillary tube recommended by the manufacturer of the spectrofluorometric thermal cycler.

Volume *	Reagent	Final Concentration
10 µL	2× SYBR Green *Taq* ReadyMix for Quantitative RT-PCR	1× *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP, stabilizers
--- µL	25 mM $MgCl_2$ (optional)	3.0 mM (without addition; optimize as required)
--- µL	Forward Primer	Optimal Conc. from Sec. A
--- µL	Reverse Primer	Optimal Conc. from Sec. A
--- µL	RNA template	10-100 ng/µL total RNA or 0.5 - 10 ng mRNA
0.1 µL	200 unit/µL M-MLV RT	1 unit/µL See Note 1
q.s. to 20 µL	Water	
20 µL	Total Volume	

Note 1: Most applications will require the formulation of more than one reaction, and in these cases it is recommended that a master mix of ReadyMix, M-MLV RT and magnesium chloride solution be formulated and aliquotted. If performing fewer than ten reactions, it is recommended that the researcher dilute the M-MLV RT ten-fold in PCR buffer (8 µL Water, PCR reagent, 1 µL PCR reagent, 1 µL M-MLV RT). This course is recommended because accurate transfer of less than 1 µL is usually impossible and a significant source of irreproducibility when pipetting critical reagents.

2. If the reactions were not set up in the glass capillaries, gently mix the master mix before adding to the glass capillary tubes. Transfer the mix to the capillaries and centrifuge them with the metal adaptors according to the instrument manufacturer's recommendations.
3. Amplification parameters will vary depending on the primers. It may be necessary to optimize the system for individual primers and template. A typical 3 step PCR cycling protocol is recommended, when using SYBR Green as the detection method, after the reverse transcription reaction.
4. Follow the spectrofluorometric thermal cycler manufacturer's instructions for SYBR Green analysis.

Data acquisition is performed during the extension step or at a detection step after the extension step. If data acquisition is performed at a detection step after the extension step, the temperature of this detection step may be derived using melting curve analysis software.[3]

The following cycling parameters are recommended for use with the Roche LightCycler. Other instruments may require optimization of amplification parameters.

Step	Temperature	Time	Temperature Transition Rate
First Strand Synthesis	42 - 44 °C (See Note 2 below)	30 minutes	20 °C/second
Denaturation/ RT Inactivation	94 °C	30 seconds	20 °C/second
For Cycles 1 - 40+			
Denaturation	94 °C	0 second	20 °C/second
Annealing	5 °C below T_m of primers	5-10 seconds	20 °C/second
Extension	72 °C	1 second per 25 bp of amplified product	Between 2-20 °C/second (See Note 3 below)
Detection (Optional)	Approx. 3 °C below T_m of RT-PCR amplicon[3]	5 seconds	20 °C/second

Note 2: Initial reverse transcription at 42-22 °C is recommended, but some poorly transcribing templates may benefit from a 55 °C reaction temperature. Such cases will need to be empirically determined.

Note 3: For primers with a T_m below 55 °C, a lower ramp time is recommended.

B. Optimizing Primer Concentrations

This optimization protocol is highly recommended for reactions that have not been previously performed in your lab.

1. Prepare and dispense diluted primers (Fig 1).

a. Prepare 60 µL of 8 µM working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
b. Dispense 30 µL of water into tubes 2-5.
c. Transfer 30 µL of the 8 µM primer solution from tube 1 into tube 2. Mix thoroughly by pipetting up and down at least 5 times.
d. Repeat transfer and mixing from tube 2 to 3, 3 to 4, and 4 to 5.
e. Using a multichannel pipettor, transfer 5 µL from the strip-tubes containing diluted fwd primer into the first 5 wells down columns 1-5 of a 96-well PCR plate. After adding fwd primer, PCR mix and template, final concentrations of fwd primer will be 1000, 500, 250, 125, 62.5 nM.
f. Similarly transfer 5 µL from the strip-tubes containing diluted rev primer into the first 5 wells across rows A-E. After adding PCR mix and template, final concentrations of rev primer will be 1000, 500, 250, 125 and 62.5 nM

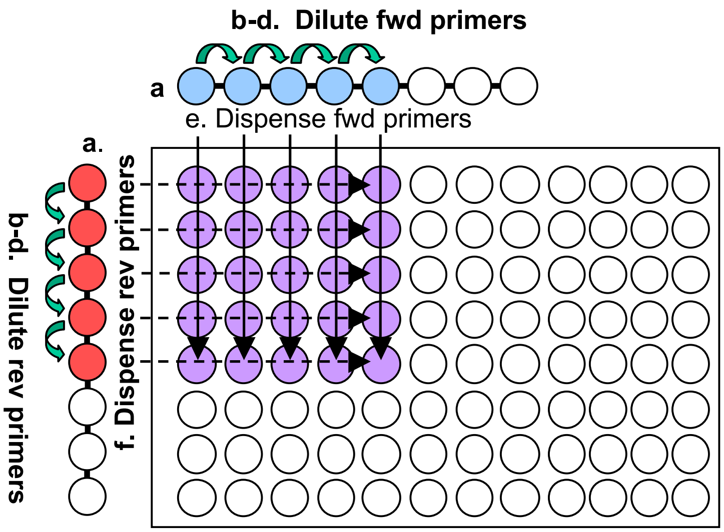

Fig 1: Follow steps 1a – 1f using diagram above

2. Prepare qPCR master mix:
   Add reagents below in an appropriate sized DNase-free tube. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

Volume	**Reagent**	**Final Concentration**
520 µL	2× SYBR Green *Taq* ReadyMix for Quantitative RT-PCR	1× *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP, stabilizers
(7 µL)	Reference Dye* (optional)	1x
q.s. to 676 µL	Water	
5.2 µL	200unit/µl M-MLV RT	
676 µL	Total Volume	

*Use 0.1× for ABI 7500 and Stratagene instruments; replace with FITC for BioRad iCycler.

3. Aliquot 26 µL master mix into all wells in the PCR plate that contain primers (A1-E5)
4. Mix Thoroughly and transfer 18 µL from each of wells A1 through E5 to wells A8 through E12.
5. Add 2 µL template RNA (10-100 ng total RNA or 0.5-10 ng mRNA) to one set of reactions (columns 1-5) and 2 µL of water to the other columns (8-12).
6. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.
7. Perform Thermal cycling:

Optimal cycling parameters vary with primer design and thermal cycler. Consult your thermal cycler manual. It may be necessary to optimize the cycling parameters to achieve maximum product yield and/or quality.

Typical cycling parameters for 100-600 bp fragments:

This protocol has been successfully tested on the following thermal cyclers: Stratagene MX 3000P, BioRad iCycler, MJ Opticon and ABI 7700.

Reverse Transcription	42-44 °C	30 min
Initial denaturation	94 °C	2 min
40 cycles:		
Denaturation	94 °C	15 sec
Annealing, extension, and read fluorescence	60 °C or 5 °C below lowest primer T_M	1 min
(Optional) Hold	4 °C - only if products will be run out on a gel	

8. Evaluate fluorescence plots (ΔRn) for reactions containing target nucleic acid (columns 1-5). Primer combinations with the lowest C_T and the highest fluorescence will give the most sensitive and reproducible assays.

9. Follow the spectrofluorometric thermal cycler manufacturer's instructions for SYBR Green analysis. Data acquisition is performed during the extension step or at a detection step after the extension step. If data acquisition is performed at a detection step after the extension step, the temperature of this detection step may be derived using melting curve analysis software.[3]

Troubleshooting Guide

Problem	Possible Cause	Solution
No RT-PCR product (signal) is observed, RT-PCR product is detected late in PCR, or only primer-dimers are detected.	The RNA is degraded.	Check the RNA by denaturing agarose gel electrophoresis. Poly(A)$^+$ RNA should appear as a smear between 0.5 kb and 2 kb. The total RNA should have two sharp ribosomal RNA bands without notable degradation. For purifying RNA, use TRI Reagent or GenElute™ RNA isolation kits (See Related Products section).
	There is not enough RNA template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	There is incomplete removal of guanidinium during RNA isolation.	For any procedure using guanidinium-based lysis solution, remove as much of the residual liquid as possible after the first precipitation and then wash once with 70% alcohol.
	There is incomplete removal of the protease (such as Proteinase K) during RNA isolation.	Proteases used during RNA isolation may be removed by phenol/chloroform extraction and alcohol precipitation.
	Reverse transcription reaction temperature is incorrect.	An optimal reverse transcription reaction temperature between 42-65 °C needs to be experimentally determined.
	The ratio of SYBR Green RT-PCR master mix to M-MLV RT is not correct.	If the reaction volume is reduced or increased then the ratio of SYBR Green master mix to M-MLV RT has to be adjusted accordingly.
	RT-PCR product is too long.	The best results are obtained when RT-PCR products are between 100-150 bp and do not exceed 500 bp.
	Primer concentration is not optimal.	Lower concentrations of primers give less non-specific products and primer-dimers.
	Primers are degraded.	Check for possible degradation of primers on a denaturing polyacrylamide gel.
	A PCR component is missing or degraded.	A positive control should always be run to insure components are functioning. Check concentrations and storage conditions of reagents, including primers and template RNA. A checklist is also recommended when assembling reactions.
	JumpStart *Taq* activated too early.	Complete the RT reaction before the denaturation step, which inactivates the JumpStart antibody.
	There are too few cycles performed.	Increase the number of cycles (3-5 additional cycles at a time). Some spectrofluorometric thermal cyclers including the Roche LightCycler allow extra cycles to be added during the run.
	The annealing temperature is too high.	Decrease the annealing temperature in 1 °C increments.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of the primer sequence to non-target sequences.
	The denaturation temperature is too high or too low.	Optimize the denaturation temperature by increasing or decreasing the temperature in 1 °C increments.
	The denaturation time is too long or too short.	Optimize the denaturation time by increasing or decreasing it in 10 second increments. The Roche LightCycler only recommends a 0 second denaturation time for normal templates and should only be increased in 5 second or less increments.
	Magnesium concentration is not optimal.	Start with the magnesium concentration provided in SYBR Green RT-PCR master mix (3 mM final concentration). A 25 mM vial of $MgCl_2$ is provided if increased Mg^{2+} concentration is needed for optimal results.

Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
No RT-PCR product (signal) is observed, RT-PCR product is detected late in PCR, or only primer-dimers are detected (continued).	Detection was not activated.	Activate SYBR Green fluorescence detection in the cycling program.
	The correct detection parameters were not activated.	Perform fluorescence detection during the extension or extra detection step of the PCR cycling program.
Multiple RT-PCR products	Reactions set up at room temperature.	Set up RT-PCR reactions on ice to avoid premature cDNA synthesis from nonspecific primer annealing.
	JumpStart *Taq* activated too early.	Complete the reverse transcription reaction before the denaturation step, which inactivates the JumpStart antibody.
	Magnesium concentration is not optimal.	Start with the magnesium concentration provided in SYBR Green RT-PCR master mix (3 mM final concentration). A 25 mM vial of $MgCl_2$ is provided if increased Mg^{2+} concentration is needed for optimal results.
	The annealing temperature is too low.	Increase the annealing temperature in increments of 1 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of primer sequence to non-target sequences.
	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase I, Catalog Number AMPD1. Try and use primers that span an intron so amplification from genomic DNA is minimized.
	Primer-dimers are co-amplified	Include an additional detection step in the cycling program to avoid detection of primer-dimers.
	Primer concentration is too high.	Reduce the primer concentration in a series of two-fold dilutions (i.e., 0.1 μM, 0.05 μM, 0.025 μM) and test in a trial set of PCR reactions.
	Reverse transcription reaction temperature too low.	Start out at a reaction temperature between 42-50 °C. The reverse transcription reaction temperature may be increased if mispriming is detected.
	Primers are degraded.	Check for primer degradation on a polyacrylamide gel.
No linearity in ratio of C_T value to log of the template amount.	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase I, Catalog Number AMPD1.
	Template amount too high.	Do not exceed the maximum recommended amounts of template RNA.
	Template amount too low.	Increase amount of template RNA.
	Primer-dimers were co-amplified.	Include an additional detection step in the cycling program to avoid detection of primer-dimers, or redesign primers that do not produce primer-dimers.

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