Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step

INSTRUCTION MANUAL

Catalog #600825 (single kit)
#600826 (10-pack kit)
Revision B.01

For In Vitro Use Only
600825-12

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Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step

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Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step

MATERIALS PROVIDED

Catalog #600825 (single kit), #600826 (10-pack kit)

Materials provided	Quantity[a,b]
2× Brilliant II SYBR® Green QRT-PCR Master Mix	2 × 2.5 ml
RT/RNase Block Enzyme Mixture	400 µl
Reference Dye[c], 1 mM	100 µl

[a] Sufficient PCR reagents are provided for four hundred, 25-µl reactions.

[b] Quantities listed are for a single kit. For 10-pack kits, each item is provided at 10 times the listed quantity.

[c] The reference dye and master mix are light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Upon receipt, store all components at –20°C. Store the 2× master mix at 4°C after thawing. Once thawed, full activity is guaranteed for 6 months.

Note *The reference dye and master mix are light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICES TO PURCHASER

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Revision B.01

INTRODUCTION

Quantitative PCR is a powerful tool for gene expression analysis. Many fluorescent chemistries are used to detect and quantitate gene transcripts. One method for real-time quantitation uses SYBR® Green I, a dye that fluoresces when bound nonspecifically to double-stranded DNA. The fluorescence response may be monitored in a linear fashion as PCR product is generated over a range of PCR cycles. The Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step can be used to perform absolute or relative quantitation of gene expression. The single-step master mix format is ideal for most high-throughput QPCR applications where it is not necessary to archive cDNA.

The Brilliant II SYBR Green QRT-PCR master mix kit includes the components necessary to carry out cDNA synthesis and PCR amplification in one tube and one buffer.* The kit has been successfully used to amplify and detect a variety of high- and low-abundance RNA targets, from experimental samples including total RNA and synthetic RNA. The kit is ideal for amplicons of up to 300 bp.

The Brilliant II SYBR Green QRT-PCR master mix kit has been optimized for maximum performance on Stratagene Mx3000P, Mx3005P, and Mx4000 instruments, as well as on the ABI 7900HT instrument.

Features of Kit Components

Brilliant II SYBR Green QRT-PCR master mix kit reagents are supplied in three separate tubes for increased flexibility in experimental design and platform options. The 2× master mix contains an optimized RT-PCR buffer, $MgCl_2$, nucleotides (GAUC), SureStart *Taq* DNA polymerase, SYBR Green and stabilizers. Reverse transcriptase (RT) is provided, in combination with RNase block, in a separate tube so that *no-RT control* reactions may be included in the QRT-PCR experiments. A passive reference dye is provided in a third tube; providing this reagent separately allows the user to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms.

SureStart *Taq* DNA polymerase, included in the 2× master mix, is a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR performance by decreasing background and increasing amplification of desired products. Using SureStart *Taq*, hot start is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with minimal modification of cycling parameters or reaction conditions.

* Primers and template are not included.

The reverse transcriptase (RT) provided in the kit is a Moloney-based RT specifically formulated for Stratagene Brilliant II kits. This RT performs optimally at a reaction temperature of 50°C when used in 1-step QRT-PCR with the Brilliant II master mix. It is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis and to ensure sensitive and reproducible performance in QRT-PCR experiments with a broad range of RNA template amounts and a variety of RNA targets that vary in size, abundance, and GC-content. The RNase block, provided in the same tube, serves as a safeguard against contaminating RNases.

The passive reference dye (with excitation and emission wavelengths of 584 nm and 612 nm, respectively) is provided as an optional reagent that may be added to compensate for non-PCR related variations in fluorescence. Providing the reference dye in a separate tube makes the Brilliant II SYBR Green QRT-PCR master mix kit adaptable for many real-time QPCR platforms (see *Reference Dye* in *Preprotocol Considerations* for more information).

SYBR® Green I Dye

The SYBR Green I dye[1] has a high binding affinity to the minor groove of double-stranded DNA (dsDNA). It has an excitation maximum at 497 nm and an emission maximum at 520 nm. In the unbound state the dye exhibits little fluorescence; however, when bound to dsDNA, the fluorescence greatly increases, making it useful for the detection of product accumulation during real-time PCR.

The presence of SYBR Green I allows the user to monitor the accumulation of PCR products in real-time. During denaturation, all DNA becomes single-stranded. At this stage, SYBR Green is free in solution and produces little fluorescence. During the annealing step, the primers will hybridize to the target sequence, resulting in dsDNA to which SYBR Green I can bind. As the PCR primers are extended in the elongation phase, more DNA becomes double-stranded, and a maximum amount of SYBR Green I is bound (see Figure 1). The increase in fluorescence signal intensity depends on the initial concentration of target present in the PCR reaction.

Because SYBR Green fluorescence depends on the presence of dsDNA, the specificity of the reaction is determined entirely by the specificity of the primers. Therefore, any double-stranded products generated during the PCR amplification will be detected by the instrument. The detection of the target amplicon will not be initially distinguishable from the spurious PCR products, such as the extension of primers bound to non-target DNA sequences, primer-dimer formation, etc. Thus, although an increase in fluorescence may be detected, it is not necessarily due to an increase in the concentration of the intended PCR product. Careful primer design and purification can minimize the effects of any side-reaction products, leading to more reliable DNA quantification. For some applications, HPLC-purified primers may generate better results. During the initial stages of assay optimization, it is recommended that the PCR products are analyzed on a gel to verify that the product of interest is being generated and that there is a correlation between the gel and fluorescence data.

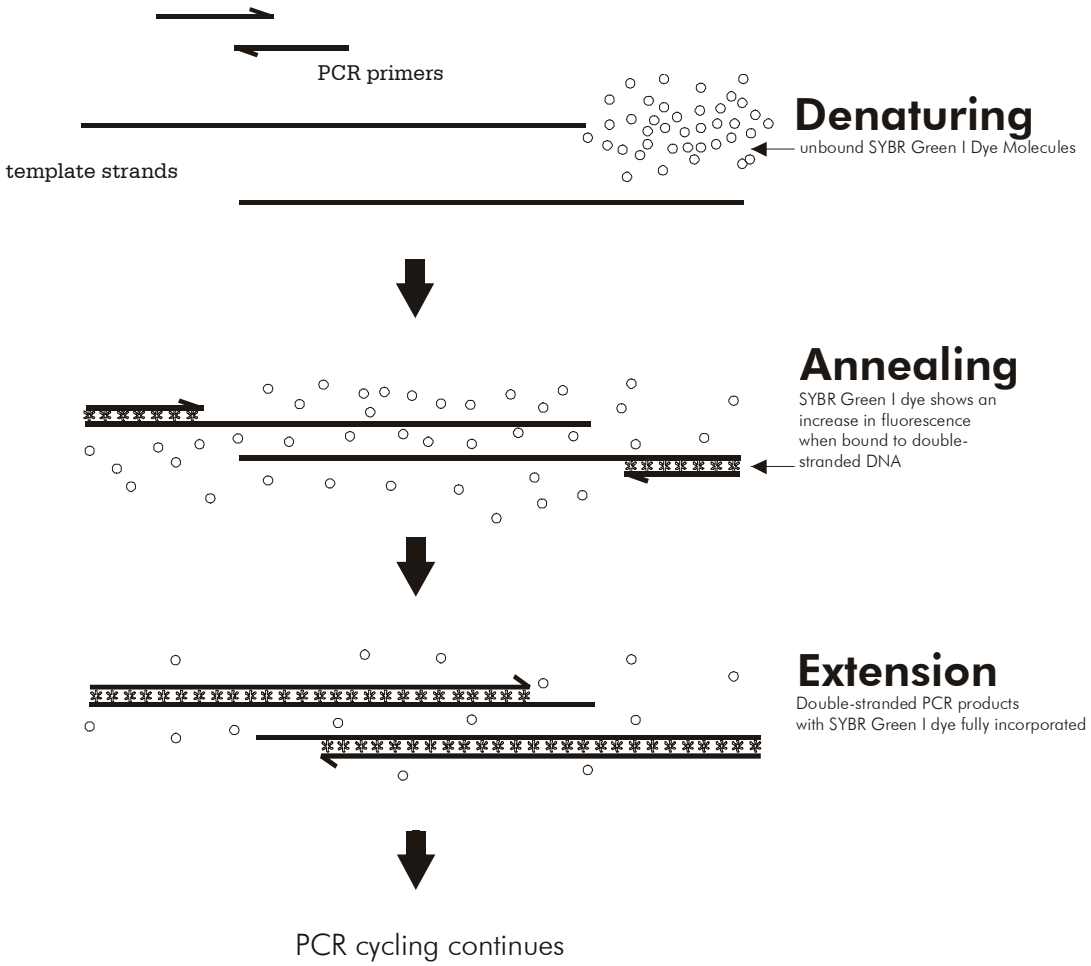


Figure 1 SYBR Green I dye has a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA or RNA. Upon binding dsDNA, the fluorescence yield of SYBR Green I increases by approximately 1000 fold, making it ideal for detecting the accumulation of dsDNA.

Fluorescence Monitoring in Real-Time

When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot (see Figure 2), which reflects the change in fluorescence during cycling. This information can be used to quantitate initial copy number based on threshold cycle (Ct).[2] Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background (e.g., in Figure 2, the Ct of the "+ template" reaction is 23 and the Ct of the "– template" reaction is 39). The threshold cycle has been shown to be inversely proportional to the log of the initial copy number.[2] The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations as Ct is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents and small differences in reaction components or cycling conditions.

Figure 2 shows Mx3005P system amplification and dissociation curve plots for reactions to detect a 89-bp amplicon using the Brilliant II SYBR Green QRT-PCR master mix kit, 1-step. A no-template control reaction is shown for comparison. In the amplification plots, the reaction containing template shows a significant increase in fluorescence and has a Ct value of ≅ 23. The reaction without template has a Ct of 39. To determine if this is true amplification, and whether the fluorescence in the no-template control is due to contamination of the reaction with template or due to the formation of primer-dimers (or other nonspecific products), a dissociation profile is generated. In the dissociation curve, PCR samples are subjected to a stepwise increase in temperature from 55°C to 95°C and fluorescence measurements are taken at every temperature increment. The melting of products causes SYBR Green dissociation, resulting in decreased fluorescence. After completion of the dissociation segment, fluorescence is plotted versus temperature. To simplify interpretation, the first derivative [–R´(T) or –Rn´(T)] should be plotted. As the temperature increases, the amplification products melt according to their composition. If primer-dimer or nonspecific products were made during the amplification step, they will generally melt at a lower temperature (T_m) than the desired products. The dissociation curve plot of these samples shows two fluorescence peaks: one in the "– template" reaction centered around 74°C (which corresponds to primer-dimer); and the other, in the "+ template" reaction, centered around 79°C (which corresponds to amplicon). In this way, the dissociation curve analysis of PCR products amplified in the presence of SYBR Green I dye can be a very powerful tool in the interpretation of fluorescence data. The results obtained from the dissociation plot can also be used for the modification of cycling conditions for future experiments. For example, if a primer-dimer was observed with a T_m of 72°C, the extension step of the PCR can be raised to 74°C, thereby reducing the signal from primer-dimers. This adjustment may not, however, work with all targets, especially long amplicons.

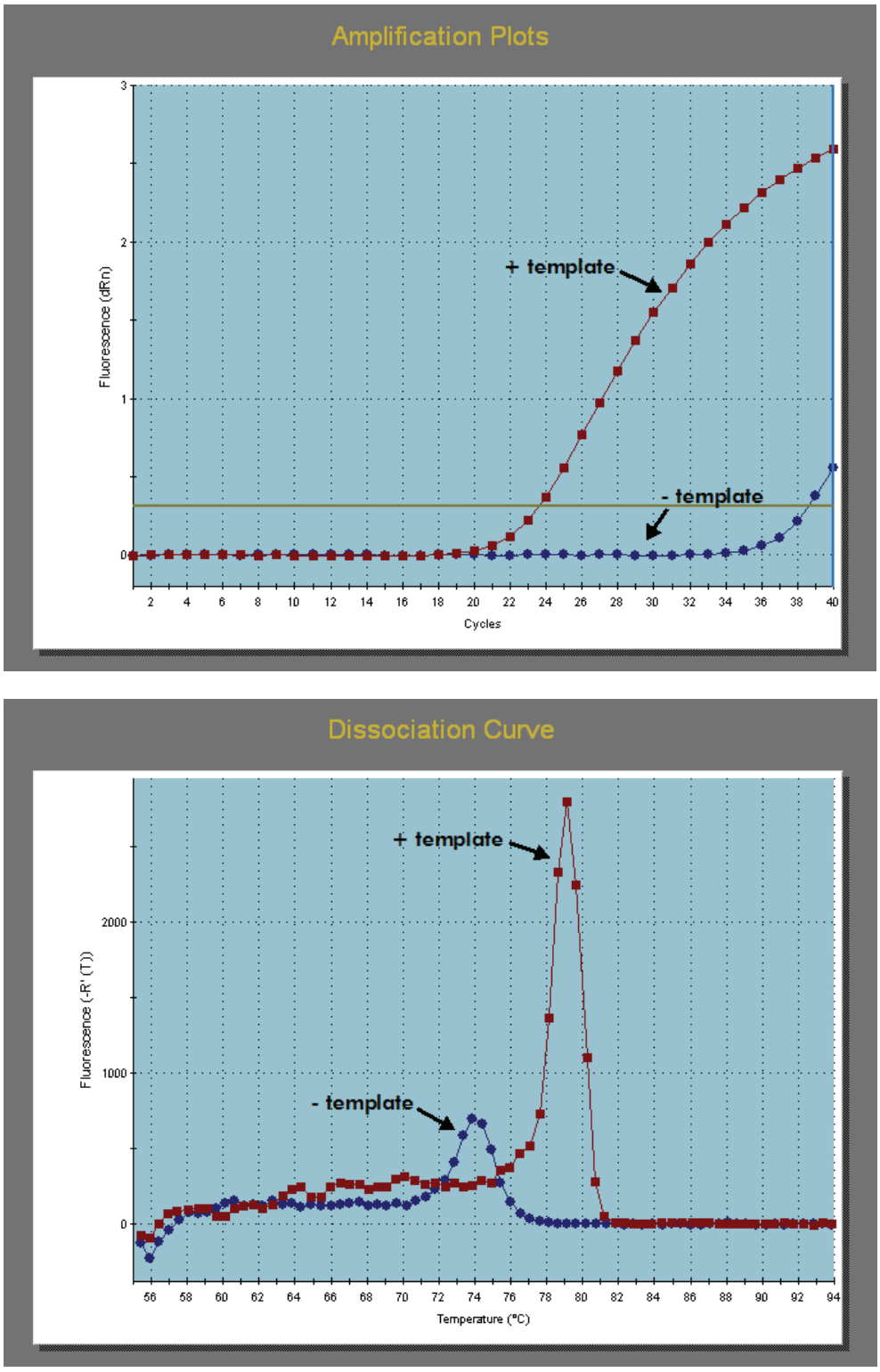


Figure 2 Mx3005P quantitative PCR instrument amplification plots (top panel) and dissociation curve plots (bottom panel) of reactions with and without template RNA. When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the amplicon (centered around 79°C) is distinguishable from the peak due to primer-dimer (centered around 74°C).

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total and poly(A)$^+$ RNA can be rapidly isolated and purified using Stratagene Absolutely RNA isolation kits. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. RNA samples with $OD_{260/280}$ ratios of 1.8–2.0 are desired for QRT-PCR.

Preventing RNase Contamination

Take precautions to minimize the potential for contamination by ribonucleases (RNases). RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water. Do not use DEPC-treated water, which can inhibit PCR. The RNase inhibitor that is included in the RT/RNase block enzyme mixture provides additional protection against RNase contamination.

Preventing Genomic DNA Contamination

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

RT-PCR Primer Concentration

It is critical to minimize the formation of nonspecific amplification products when performing single-step QRT-PCR reactions using SYBR Green detection. This issue becomes more prominent at low target concentrations.

Use the lowest concentration of primers possible without compromising the efficiency of PCR. The optimal concentration of the upstream and downstream PCR primers is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration, with minimal or no formation of primer-dimer. This concentration should be determined empirically, but primer concentrations in the range of 50–200 nM are generally satisfactory (with especially difficult targets, higher primer concentrations may improve detection). It is important to consider both the relative concentrations of forward and reverse primers and the total primer concentration.

Quantitative PCR Human Reference Total RNA

Stratagene Quantitative PCR (QPCR) Human Reference Total RNA (Catalog #750500) is a high-quality control for quantitative PCR gene-expression analysis. QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines (see the table below), with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The reference RNA is carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

QPCR Human Reference Total RNA Cell Line Derivations
Adenocarcinoma, mammary gland
Hepatoblastoma, liver
Adenocarcinoma, cervix
Embryonal carcinoma, testis
Glioblastoma, brain
Melanoma, skin
Liposarcoma
Histiocytic lymphoma; macrophage; histocyte
Lymphoblastic leukemia, T lymphoblast
Plasmacytoma; myeloma; B lymphocyte

The QPCR Human Reference Total RNA is ideally suited for optimizing SYBR Green QRT-PCR assays. Often only small amounts of experimental RNA template are available for setting up an expression profiling study. Using the extensive representation of specific mRNA species in the generic template, assays may be optimized for a variety of targets. This eliminates the use of precious experimental RNA samples for assay optimization.

Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although addition of the reference dye is optional when using the Mx4000, Mx3000P or Mx3005P system, with other instruments (including the ABI 7900HT and ABI PRISM® 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If using the ABI 7900HT instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

Magnesium Chloride

The optimal $MgCl_2$ concentration promotes maximal amplification of the specific target amplicon with minimal nonspecific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of nonspecific dsDNA, including primer-dimers. Therefore, when a SYBR Green-based QPCR assay is being optimized, the $MgCl_2$ levels should be as low as possible, as long as the efficiency of amplification of the specific target is not compromised (typically between 1.5 and 2.5 mM $MgCl_2$). The Brilliant II SYBR Green QRT-PCR master mix contains $MgCl_2$ at a concentration of 2.5 mM (in the 1× solution), which is suitable for most targets. The concentration may be increased, if desired, by adding a small amount of concentrated $MgCl_2$ to the 1× experimental reaction at the time of set up.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

Preparing a Single Mixture for Multiple Samples

If running multiple samples containing the same primers, we recommend preparing a single mixture of reaction components and then aliquoting the mixture into individual reaction tubes using a fresh pipet tip for each addition. Preparing a common mixture facilitates the accurate dispensing of reagents, minimizes the loss of reagents during pipetting, and helps to minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Solutions that contain enzymes (including reverse transcriptase and SureStart *Taq* DNA polymerase) should be mixed gently by inversion or gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the buffer, which contains 50% glycerol, can lead to pipetting errors.

Temperature and Duration of cDNA Synthesis Reaction

For cDNA synthesis, a 50°C incubation is recommended for most targets using the Brilliant II SYBR Green QRT-PCR master mix kit. However, incubation up to 55°C can be employed to reduce secondary structures or to improve specificity. A 30-minute incubation for the first-strand synthesis reaction is sufficient for most targets. Rare RNA sequences or long amplicons may benefit from an extended incubation time (up to 60 minutes) at a lower temperature (42°C).

Preventing Sample Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Treatment with Uracil-N-glycosylase (UNG) is NOT recommended for decontamination of single tube RT-PCR reactions since UNG would be active during the 50°C incubation necessary for reverse transcription.

Recommended Control Reactions

No-Template Control (NTC)

We recommend performing no-template control reactions for each experimental sample to screen for contamination of reagents or false amplification.

No-RT Control

We recommend performing no-RT control reactions for each experimental sample by omitting the RT/RNase block enzyme mixture from the reaction. The no-RT control is expected to generate no signal if there is no amplification of genomic DNA. No signal indicates that the RNA preparation is free of contaminating genomic DNA or that the primers are specific for the cDNA. See *Preventing Genomic DNA Contamination* in *RNA Isolation*.

Endogenous Control

Consider performing an endogenous control reaction to normalize variation in the amount of RNA template across samples. See Reference 3 for guidelines on the use of endogenous controls for QPCR.

Fluorescence Detection

Fluorescence may be detected either at the endpoint of cycling or in real-time using a real-time spectrofluorometric thermal cycler. For endpoint analysis, PCR reactions can be run on any thermal cycler and can then be analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and that is optimized for the detection of PCR reactions that include SYBR Green dye.

If using a fluorescence plate reader, it is recommended that readings be taken both before and after PCR for comparison.

Data Acquisition with a Spectrofluorometric Thermal Cycler

Acquisition of real-time data generated by fluorogenic dye should be performed as recommended by the instrument's manufacturer.

When developing an assay, it is necessary to decide whether to use a 2-step or a 3-step PCR protocol. We recommend a 2-step protocol for the Brilliant II SYBR Green QRT-PCR master mix kit, but a 3-step protocol may be helpful when using primers with low melting temperatures. In a 2-step cycling protocol, fluorescence data are collected during the combined annealing/extension step. When using a 3-step protocol, it is prudent to collect fluorescence data at both the annealing step and the extension step of the PCR reaction. For subsequent experiments, the plateau resulting in low Ct values for the samples containing target and high Ct values (or “no Ct” values) for the controls containing no target should be chosen for analysis. For longer amplicons, fluorescence measurements taken during the extension step generally yield more useful data.

Notes *Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.* ***SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.***

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification. Similarly, a no-RT control should be included to verify that the fluorescence signal is due to the amplification of cDNA and not of contaminating genomic DNA.

Consider performing an endogenous control reaction to normalize variations in the amount of RNA template across samples. For information on the use and production of endogenous controls for QPCR, see Reference 3.

Preparing the Reactions

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI 7900HT instrument)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in the *Reference Dye* section under *Preprotocol Considerations*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx3000P, Mx3005P, and Mx4000 instruments and 300 nM for the ABI 7900HT instrument. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× SYBR Green QRT-PCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.

3. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for duplicate experimental reactions and duplicate no-template-controls (plus at least one reaction volume excess), using multiples of each component listed below.

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental RNA)
12.5 µl of 2× SYBR Green QRT-PCR master mix
x µl of upstream primer (50–200nM final concentration is recommended)
x µl of downstream primer (50–200nM final concentration is recommended)
0.375 µl of the **diluted** reference dye (optional)
1.0 µl of RT/RNase block enzyme mixture

Note *A total reaction volume of 50 µl may also be used.*

4. Gently mix the reactions without creating bubbles (do not vortex), then distribute the mixture to the experimental reaction tubes.

5. Add *x* µl of experimental RNA to each reaction. The quantity of RNA depends on the RNA purity and the specific mRNA abundance. As a guideline, use 1 pg–400 ng of total RNA or 0.1 pg–1 ng of mRNA.

6. Gently mix the reactions without creating bubbles (do not vortex).

Note *Bubbles interfere with fluorescence detection.*

7. Centrifuge the reactions briefly.

RT-PCR Cycling Program

8. Place the reactions in the QPCR instrument and run the appropriate RT-PCR program using the guidelines in the tables below. The 2-step cycling protocol is preferred for most primer/template systems. For primers with low melting temperatures, consider using the alternative 3-step cycling protocol.

Two-Step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	60°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[b] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

Alternative Protocol with Three-Step Cycling

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	50–60°C[c]
	30 seconds	72°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[b] Set the temperature cycler to detect and report fluorescence during the annealing and extension step of each cycle.

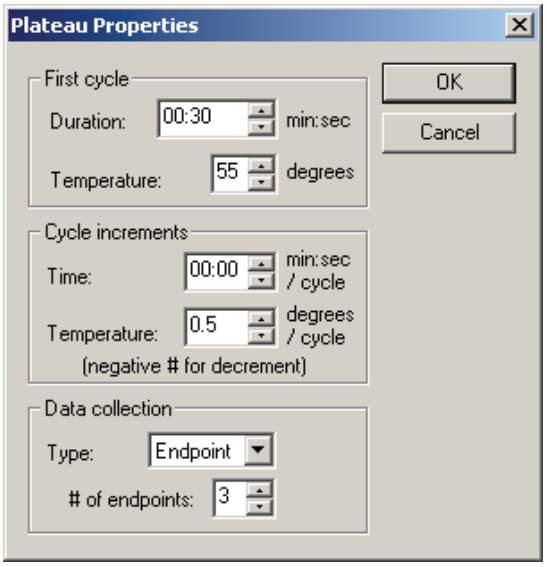
[c] Choose an appropriate annealing temperature for the primer set used.

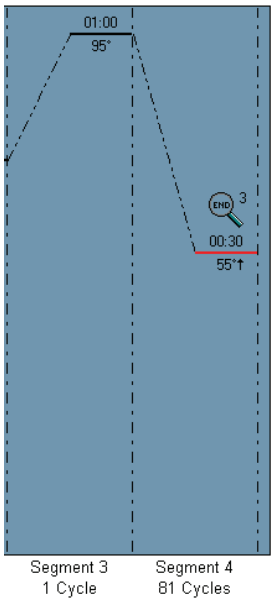
Dissociation Program

9. If using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, follow the dissociation guidelines below. If using another instrument follow the manufacturer's guidelines for generating dissociation curves.

Dissociation Program for all targets (Mx4000 Instrument)

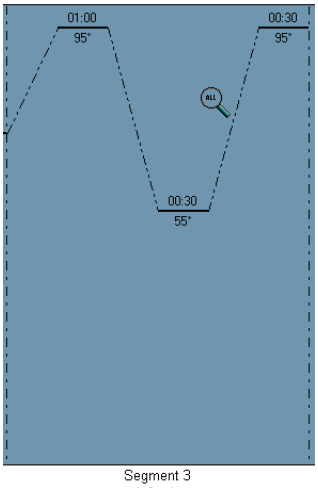
Incubate the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec. For the dissociation curve, complete 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set the duration of each cycle to 30 seconds. See below for the *Plateau Properties* and *Thermal Profile Setup* settings for the dissociation curve program on the Mx4000 instrument. To access the *Plateau Properties* dialog box for the dissociation curve segment, double-click on the solid line corresponding to the 55° plateau in Segment 4 of the *Thermal Profile Setup* window.





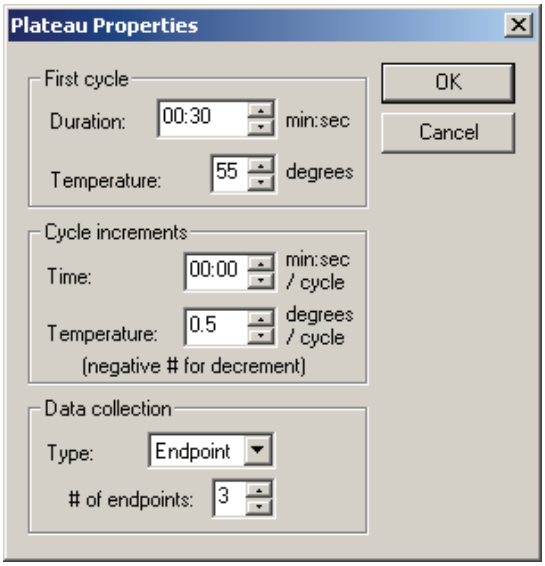
Dissociation Program for All Targets (Mx3000P and Mx3005P Instruments)

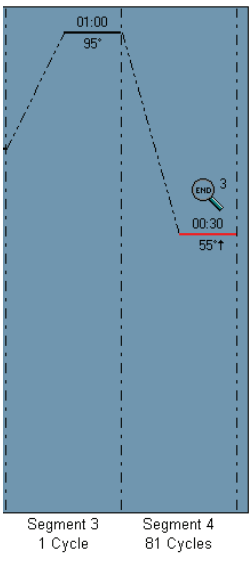
Prior to the dissociation curve, incubate the reactions for 1 minute at 95°C to denature the PCR products. Ramp down to 55°C. For the dissociation curve, ramp the temperature from 55°C to 95°C (at the instrument default rate of 0.2°C/sec) and collect fluorescence data continuously on the 55-95°C ramp. The thermal profile for the default dissociation curve is shown below.



Custom Dissociation Curve with Plateau Data Collection

The dissociation curve may also be set up using plateau stepping, with the instrument software settings shown below. Incubate the amplified product for 1 minute at 95°C, then ramp down to 55°C. For the dissociation curve, complete 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set the duration of each cycle to 30 seconds. To access the *Plateau Properties* dialog box for the dissociation curve segment, double-click on the solid line corresponding to the 55° plateau in the *Thermal Profile Setup* window.





TROUBLESHOOTING

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling in the amplification plots	Ensure that the correct amount of template was used and that the template sample is of good quality. If unsure, make new dilutions of template before repeating PCR. It may also be helpful to check for PCR inhibitors by adding this target into an assay that is known to work.
	SureStart *Taq* DNA polymerase was not activated. Ensure that the 10 minute incubation at 95°C was performed as part of the cycling parameters.
	Ensure the correct dilution of reference dye was used.
	Optimize the primer concentration.
	The $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant II SYBR Green QRT-PCR master mix is 2.5 mM. It is possible to add small amounts of concentrated $MgCl_2$ to the experimental reactions to increase the $MgCl_2$ concentration, if desired.
	The RNA template may be degraded. Ensure that the template RNA is stored properly (at –20°C or –80°C) and is not subjected to multiple freeze-thaw cycles. Check the quality of the RNA in the sample by gel electrophoresis or using an automated RNA population analysis system such as the Agilent 2100 Bioanalyzer.
	If the target RNA contains extensive secondary structure, increase the incubation temperature used during the first step of the RT-PCR program up to 55°C.
	For low-abundance targets or long amplicons, increase the duration of the cDNA synthesis step to 60-minutes while lowering the incubation temperature down to 42°C.
	Verify that all reagents and supplies are RNase-free.
	Where possible, increase the amount of template RNA. (Do not exceed the recommended amount of template.)
	Ensure the annealing/extension time is sufficient. Check the length of the amplicon and increase the annealing/extension time if necessary.
	Use a sufficient number of cycles in the PCR reaction.
	Ensure the annealing/extension temperature is appropriate for the primers used.
	Gel analyze PCR product to determine if there was successful amplification.
In the dissociation plot, there is a large abundance of primer-dimer and nonspecific PCR products	Increase the annealing/extension temp above the Tm of the primer-dimer and/or nonspecific products.
	Re-design primers.
	Optimize primer concentration by performing primer titration.
Increasing fluorescence in no-template control (NTC) reactions with cycling	Evaluate the dissociation profile. If Tm of the NTC peak is similar to the target peak, the reaction has been contaminated. Follow the procedures outlined in reference 4 to minimize contamination. If the Tm of the NTC peak is lower than the Tm of the target peak, primer-dimers are formed. Optimize primer concentration. Re-design primer set.
Ct reported for NTC sample is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

REFERENCES

1. Molecular Probes, Inc., at http://www.probes.com/media/pis/mp07567.pdf.
2. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology (N Y)* 11(9):1026-30.
3. Bustin, S. A. (2000) *Journal of Molecular Endocrinology* 25:169-193.
4. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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BRILLIANT II SYBR® GREEN QRT-PCR MASTER MIX KIT, 1-STEP

Catalog #600825, 600826

QUICK-REFERENCE PROTOCOL

Note *This protocol has been optimized for the Mx3000P, Mx3005P, and Mx4000 instruments and the ABI 7900HT instrument. The protocol may be adapted for use with most other instruments by changing the reference dye dilution according to the guidelines in the manual and following the instruction manufacturer's recommendations for RT-PCR cycling programs.*

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx3000P, Mx3005P, or Mx4000 instrument) or 1:50 (ABI 7900HT instrument). Keep all solutions containing the reference dye protected from light.

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× SYBR Green QRT-PCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

   Note *Multiple freeze-thaw cycles should be avoided.*

3. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture for multiple reactions using multiples of each component listed below.

   Reagent Mixture

   Nuclease-free PCR-grade H_2O to bring the final volume to 25 µl (including experimental RNA)
   - 12.5 µl of 2× SYBR Green QRT-PCR master mix
   - x µl of upstream primer (optimized concentration)
   - x µl of downstream primer (optimized concentration)
   - 0.375 µl of diluted reference dye from step 1 (optional)
   - 1.0 µl of RT/RNase block enzyme mixture

   Note *A total reaction volume of 50µl may also be used.*

4. Gently mix the reactions without creating bubbles (**do not vortex**), then distribute the mixture to the experimental reaction tubes.

5. Add x µl of experimental RNA to each reaction.

6. Gently mix the reactions without creating bubbles (**do not vortex**).

7. Centrifuge the reactions briefly.

8. Place the reactions in the instrument and run the PCR program below.

Two-Step Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[b]	95°C
40	30 seconds	95°C
	1 minute[c]	60°C

[a] A protocol for three-step cycling is provided in the *Protocol* section.

[b] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[c] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

9. Run a dissociation curve according to the QPCR instrument:

Dissociation Program (Mx4000 Instrument)

Incubate the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec, followed by 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set the cycle duration to 30 seconds/cycle.

Dissociation Program (Mx3000P or Mx3005P Instrument)

Incubate the reactions for 1 minute at 95°C, ramping down to 55°C. For the dissociation curve, ramp up the temperature from 55°C to 95°C (at the instrument default rate of 0.2°C/sec) and collect fluorescence data continuously on the 55–95°C ramp.

Dissociation Program (Other Instruments)

Follow manufacturer's guidelines for setting up a dissociation curve.