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

INSTRUCTION MANUAL

Catalog #600552 (single kit)
#929552 (10-pack kit)
Revision #056003b

For In Vitro Use Only

LIMITED PRODUCT WARRANTY

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

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

MATERIALS PROVIDED

Catalog #600552 (single kit), #929552 (10-pack kit)

Materials provided	Quantity[a,b]
2× SYBR® QRT-PCR Master Mix	2 × 2.5 ml
StrataScript® RT/RNase Block Enzyme Mixture	25 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

Revision #056003b

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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 non-specifically 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® 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. Alternatively, the combination of Stratagene's StrataScript® First Strand cDNA Synthesis Kit (catalog #200420) and the Brilliant SYBR Green QPCR Master Mix (catalog #600548) provides RNA quantification in two steps to allow archival of cDNA for further analysis.

The Brilliant SYBR Green QRT-PCR master mix kit, 1-step includes the components necessary to carry out cDNA synthesis and PCR amplification in one tube and one buffer.* The Brilliant SYBR Green QRT-PCR master mix 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 400 bp.

The Brilliant SYBR Green QRT-PCR master mix kit has been optimized for maximum performance on Stratagene's Mx3000P® and Mx3005P™ real-time PCR systems and Stratagene's Mx4000® multiplex quantitative PCR system, as well as on the ABI PRISM® 7700 instrument. In addition, excellent results have been observed using most other QPCR platforms.

Features of Kit Components

Brilliant 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. StrataScript® reverse transcriptase 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 (an optional reaction component) 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.

StrataScript reverse transcriptase is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) without any detectable RNase H activity. Cloned StrataScript reverse transcriptase is purified from recombinant *E. coli* containing a genetically engineered mutant MMLV-RT gene. A point mutation in the highly conserved residue of the RNase H region results in the loss of undesired RNase H degradative activity without affecting the desired reverse transcriptase function. The result is a nuclease-free mutant of MMLV-RT that can produce larger yields of cDNA than yields obtained from wild-type MMLV-RT, which possesses substantial RNase H activity. RNase block is also included in the reverse transcriptase solution 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 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. An important consideration when using SYBR Green I, however, is that signal can also be generated from nonspecific dsDNA (e.g. primer-dimers and spurious PCR products). When setting up preliminary assays, it is prudent to collect fluorescence intensity values at 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.

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 (HPLC-purified primers are recommended) can minimize the effects of any side-reaction products, leading to more reliable DNA quantification. 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.

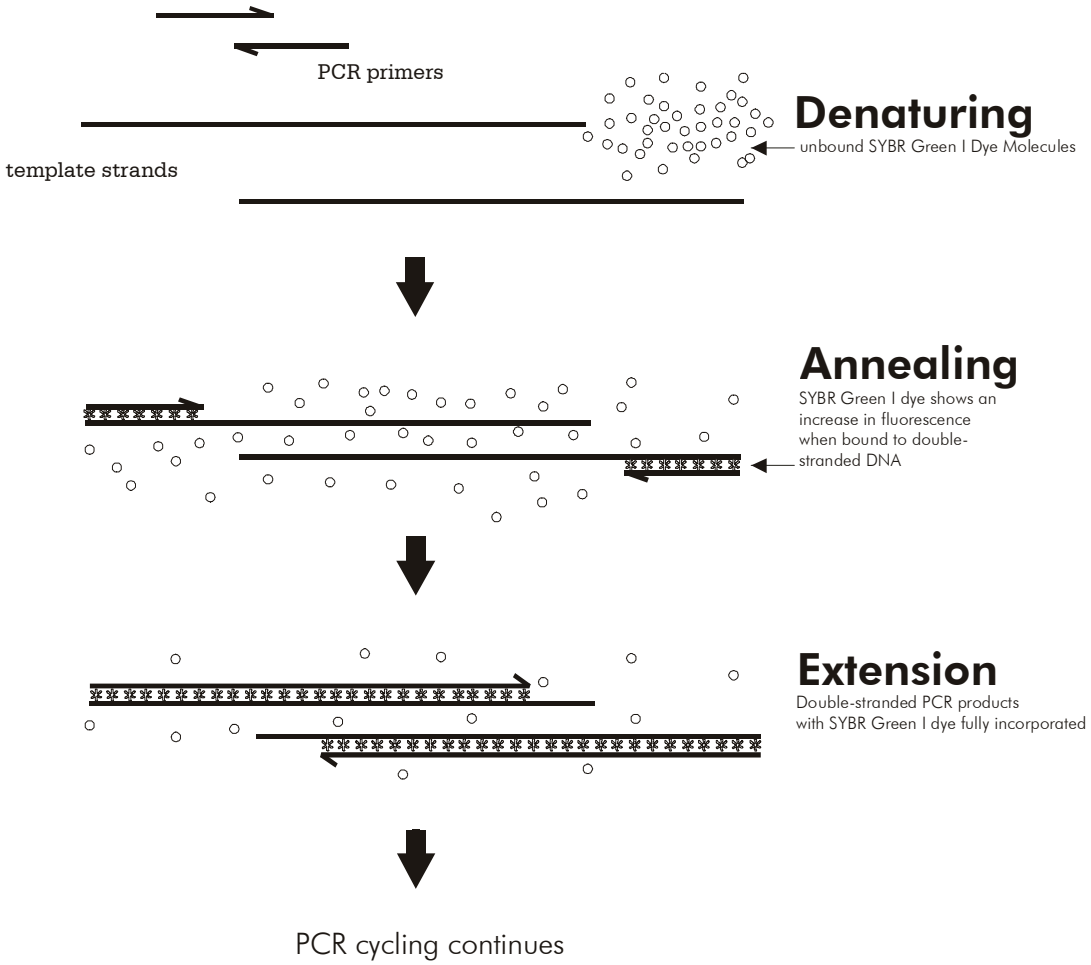


Figure 1 SYBR Green I dye has a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA or RNA. Upon binding dsDNA, the fluorescence yield of SYBR Green I increases by approximately 1000 fold, making it ideal for detecting the accumulation of dsDNA.

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 33). 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. Quantitative assessments based on endpoint fluorescence values (a single reading taken at the end of the PCR reaction) are inherently inaccurate because endpoint values can be greatly influenced by these factors.

Figure 2 shows Mx4000 system amplification and dissociation curve plots for reactions prepared using the Brilliant SYBR Green QRT-PCR master mix kit to detect a 135-bp amplicon. 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 33. 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 non-specific 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 75°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.

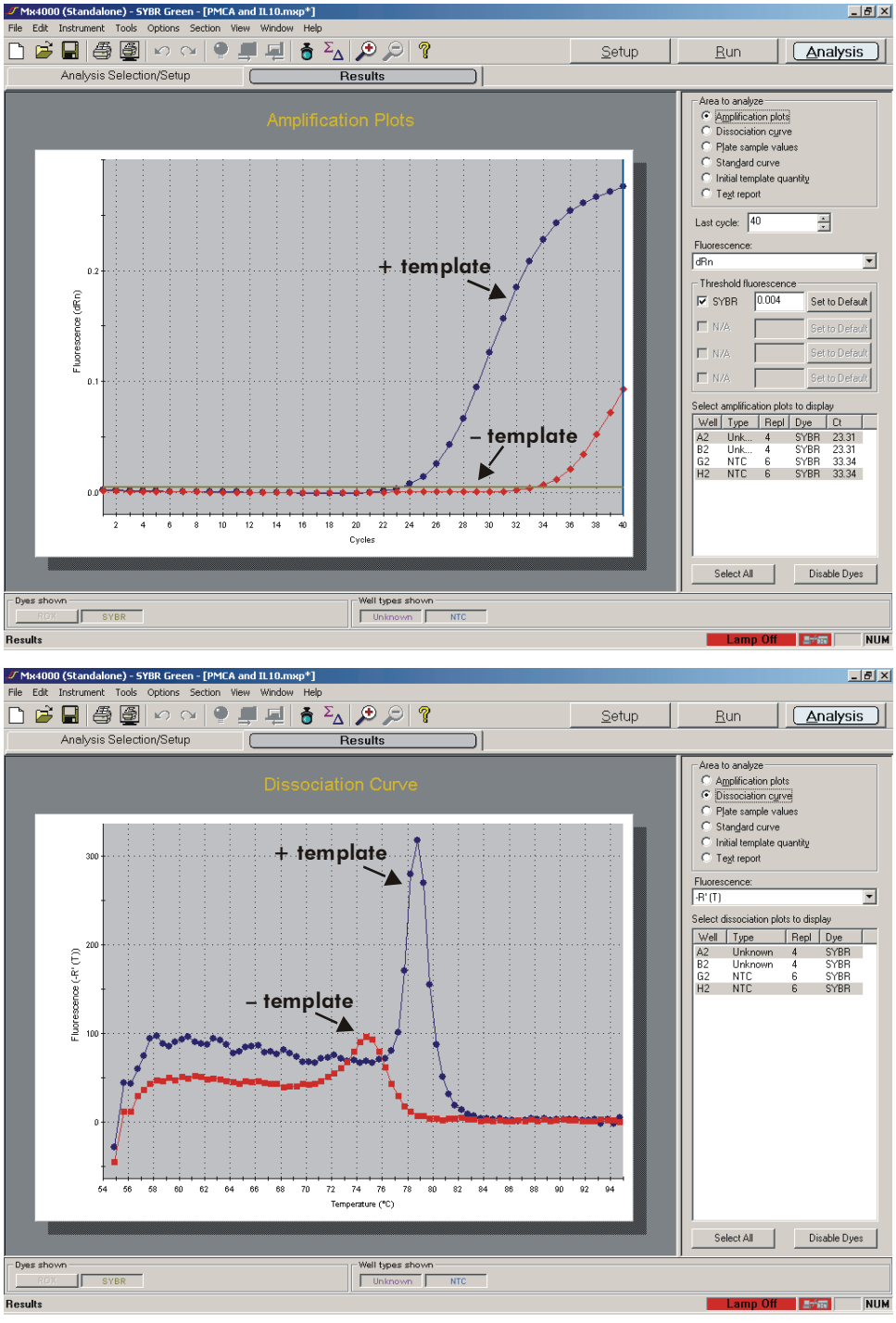


Figure 2 Mx4000 multiplex quantitative PCR instrument amplification and dissociation curve plots of reactions with and without template RNA. When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the amplicon (centered around 79°C) is distinguishable from the peak due to primer-dimer (centered around 75°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's 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 block that is included in the reaction mixture prior to RNA template addition 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 non-specific 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; generally, primer concentrations in the range of 50–100 nM are satisfactory. It is important to consider both the relative concentrations of forward and reverse primers and the total primer concentration.

Note *If using a given primer set for both QPCR and single-tube QRT-PCR assays that employ SYBR Green detection, it is important to optimize the primer concentration specifically for the QRT-PCR assay. Typically, for a given primer pair,* ***lower primer concentrations are used in a single-tube SYBR Green QRT-PCR assay compared with a SYBR Green QPCR assay****.*

Quantitative PCR Human Reference Total RNA

Stratagene's 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 Brilliant 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 primer/probe systems. 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. Addition of the reference dye is optional. 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.

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 Stratagene's Mx3000P, Mx3005P, or Mx4000 instruments, use the reference dye at a final concentration of 30 nM. If using the ABI PRISM 7700 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 non-specific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of non-specific 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 SYBR Green QRT-PCR master mix kit 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, Stratagene recommends 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 StrataScript RT 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.

cDNA Synthesis Reaction

Incubation Temperature and Duration

StrataScript RT is effective between 37°C and 60°C for short targets such as those used in quantitative real-time RT-PCR. Stratagene recommends a 50°C incubation for most targets using the Brilliant QRT-PCR master mix kit; however, incubation up to 60°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, long transcripts, or targets at the 5´ end of long transcripts may benefit from an extended incubation time (up to 90 minutes) at a lower temperature (45°C).

StrataScript® RT Inhibition of PCR

StrataScript RT can inhibit subsequent PCR and must be heat-inactivated in the first thermal cycle of PCR. Do not exceed the recommended amount of StrataScript RT/RNase block, as heat inactivation becomes difficult.

For low abundance RNA targets, increasing the incubation time for first-strand synthesis may be beneficial. Do not increase the amount of StrataScript RT/RNase block.

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)

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

No-RT Control

Stratagene recommends performing no-RT control reactions for each experimental sample by omitting StrataScript reverse transcriptase 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 in real-time or at the endpoint of cycling 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. The 3-step protocol offers more options for data acquisition, and 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. When using a 2-step cycling protocol, data are collected during the combined annealing/extension step.

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 distinguish true negative results from PCR inhibition or failure. 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 PRISM 7700 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 PRISM 7700 instrument. **Keep all solutions containing the reference dye protected from light.**

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*. Stratagene recommends preparing 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 QRT-PCR master mix
x µl of upstream primer (50 – 100nM final concentration is recommended)
x µl of downstream primer (50 – 100nM final concentration is recommended)
0.375 µl of the **diluted** reference dye (optional)
0.0625 µl of StrataScript 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–1 µg 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 below. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

   Note *For short targets (<300 bp), a 2-step PCR protocol may be considered.*

Three-Step Cycling Protocol for Amplification of Targets (50–400 bp)

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/extension step of each cycle.

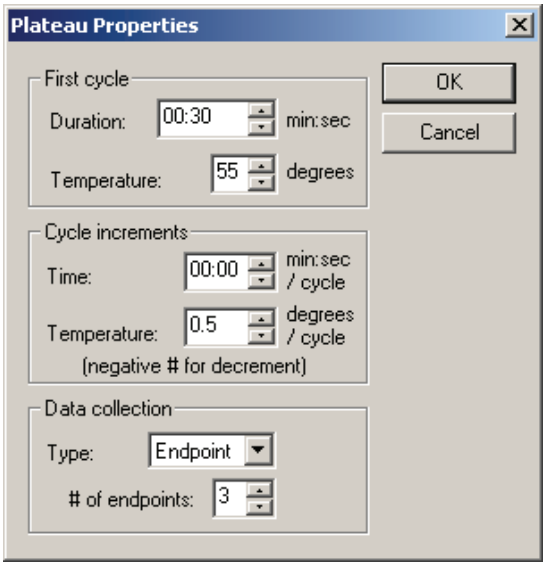
[c] Choose an appropriate annealing temperature for the primer set used.

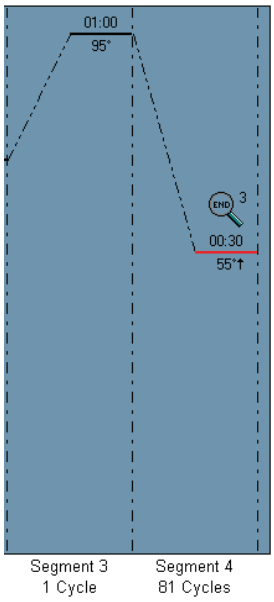
Dissociation Program

9. If using Stratagene's Mx3000P, Mx3005P, or Mx4000 instruments, 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)

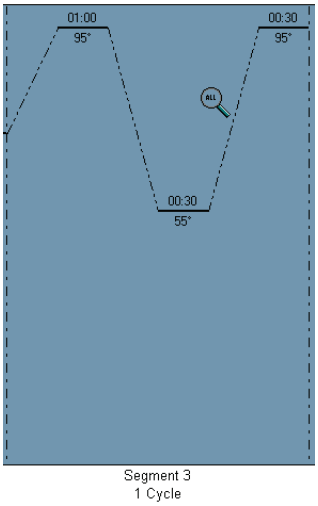
Incubate the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec. For the dissociation curve, complete 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set he duration of each cycle to 30 seconds. See below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





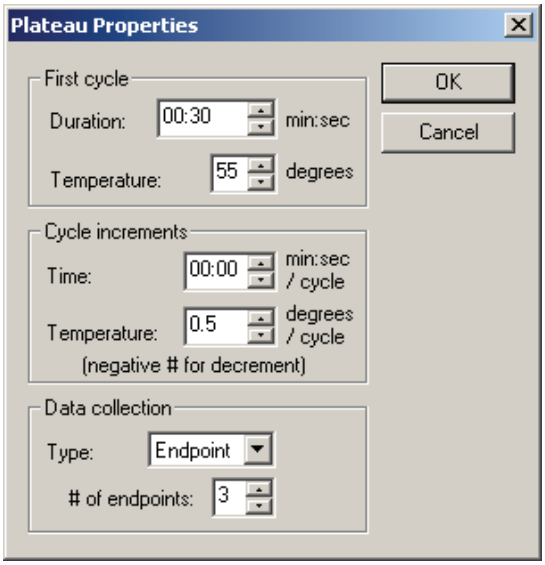
Dissociation Program for All Targets (Mx3000P® and Mx3005P™ Instruments)

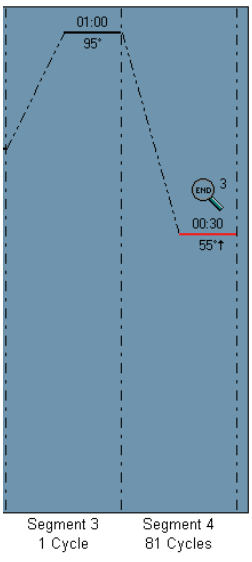
Prior to the dissociation curve, incubate the reactions for 1 minute at 95°C to denature the PCR products. Ramp down to 55°C. For the dissociation curve, ramp the temperature from 55°C to 95°C (at the instrument default rate of 0.2°C/sec) and collect fluorescence data continuously on the 55-95°C ramp. 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	A reagent is missing from this PCR, set up a new reaction.
	The $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant 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.
	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.
	Ensure that the correct concentration and 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.
	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 to up to 60°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 and extension times are sufficient. Check the length of the amplicon and increase the extension time if necessary.
	Use a sufficient number of cycles in the PCR reaction.
	Ensure the annealing 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 non-specific PCR products	Increase the annealing temperature.
	Re-design primers.
	Optimize primer concentration by performing primer titration.
	For products <300 bp, increase extension temp above the Tm of the primer-dimer and/or nonspecific products. Ensure the instrument is set to collect data during extension. Data analysis of extension step can be more useful in this case.
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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BRILLIANT® SYBR® GREEN QRT-PCR MASTER MIX KIT, 1-STEP

Catalog #600552, 929552

QUICK-REFERENCE PROTOCOL

Note *This protocol has been optimized for the Mx3000P, Mx3005P, and Mx4000 instruments and the ABI PRISM 7700 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 instrument 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 PRISM 7700 instrument). Keep all solutions containing the reference dye protected from light.

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 reaction 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 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)
   - 0.0625 µl of StrataScript 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. (For amplicons <300 bp, a two-step PCR program may be considered.)

Three-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]	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/extension step of each cycle.

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

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.