Brilliant II SYBR® Green QPCR Master Mix with ROX

INSTRUCTION MANUAL

Catalog #600829 (Brilliant II SYBR® Green QPCR High ROX Master Mix)
#600830 (Brilliant II SYBR® Green QPCR Low ROX Master Mix)
#600832 (Brilliant II SYBR® Green QPCR High ROX Master Mix, 10-pack)
#600833 (Brilliant II SYBR® Green QPCR Low ROX Master Mix, 10-pack)

Revision A.01

For In Vitro Use Only

600829-12

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Brilliant II SYBR® Green QPCR Master Mix

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Brilliant II SYBR® Green QPCR Master Mix

MATERIALS PROVIDED

Materials provided	Catalog #600829[a]	Catalog #600830[a]	Catalog #600832	Catalog #600833
2× Brilliant II SYBR® Green QPCR High ROX Master Mix[b]	2 × 2.5 ml	—	20 × 2.5 ml	—
2× Brilliant II SYBR® Green QPCR Low ROX Master Mix[b]	—	2 × 2.5 ml	—	20 × 2.5 ml

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

[b] The master mix contains nucleotide mix GATC.

STORAGE CONDITIONS

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

Note *The Brilliant II SYBR® Green QPCR master mix is light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

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Notice to Purchaser: Limited License

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Revision A.01

INTRODUCTION

Quantitative PCR is becoming increasingly important for gene expression analysis. 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 QPCR Master Mix with ROX includes the components necessary to carry out QPCR amplifications with SYBR Green detection.* The improved Brilliant II formulation yields higher levels of final fluorescence and earlier Ct values for many genomic DNA and cDNA targets and the master mix format is ideal for high-throughput applications. The Brilliant II master mix has been optimized for a faster two-step cycling protocol that is 25% shorter than the protocol used with the original Brilliant SYBR Green QPCR master mix. The Brilliant II master mix is also ideal for quantification of cDNA in two-step QRT-PCR reactions when combined with the AffinityScript QPCR cDNA Synthesis Kit.

The Brilliant II SYBR Green QPCR master mix with ROX is available in two formulations: a high ROX master mix and a low ROX master mix. The final concentration of ROX reference dye in QPCR reactions prepared with the high ROX master mix is 500 nM. Reactions prepared with the low ROX master mix contain 30 nM of ROX. The two formulations are recommended for different real-time PCR platforms. Refer to the table below for catalog information and a list of compatible instruments for each product.

Catalog #	Product Name	Final ROX concentration	Compatible instruments
600829 (10-pack, 600832)	Brilliant II SYBR® Green QPCR High ROX Master Mix	500 nM	ABI PRISM® 7000 and 7700; ABI 7300, 7900HT and 7900HT Fast systems
600830 (10-pack, 600833)	Brilliant II SYBR® Green QPCR Low ROX Master Mix	30 nM	Stratagene Mx3000P, Mx3005P and Mx4000 systems; ABI 7500 system

ROX dye is included in the master mix to compensate for non-PCR related variations in fluorescence. Fluorescence from ROX dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, ROX detection compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of ROX dye are 584 nm and 612 nm, respectively.

In addition to ROX, the QPCR master mix includes SureStart *Taq* DNA polymerase, nucleotides (GATC), and an optimized QPCR buffer. SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability, improves PCR amplification reactions by decreasing background from nonspecific amplification and increasing amplification of desired products. Using SureStart *Taq*, hot start is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with little modification of cycling parameters or conditions.

* Primers and template are not included.

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). 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.

Because SYBR Green fluorescence depends on the presence of dsDNA, any double-stranded products generated during the PCR amplification will be detected by the instrument. Therefore, the specificity of the reaction is determined entirely by the specificity of the primers. 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.

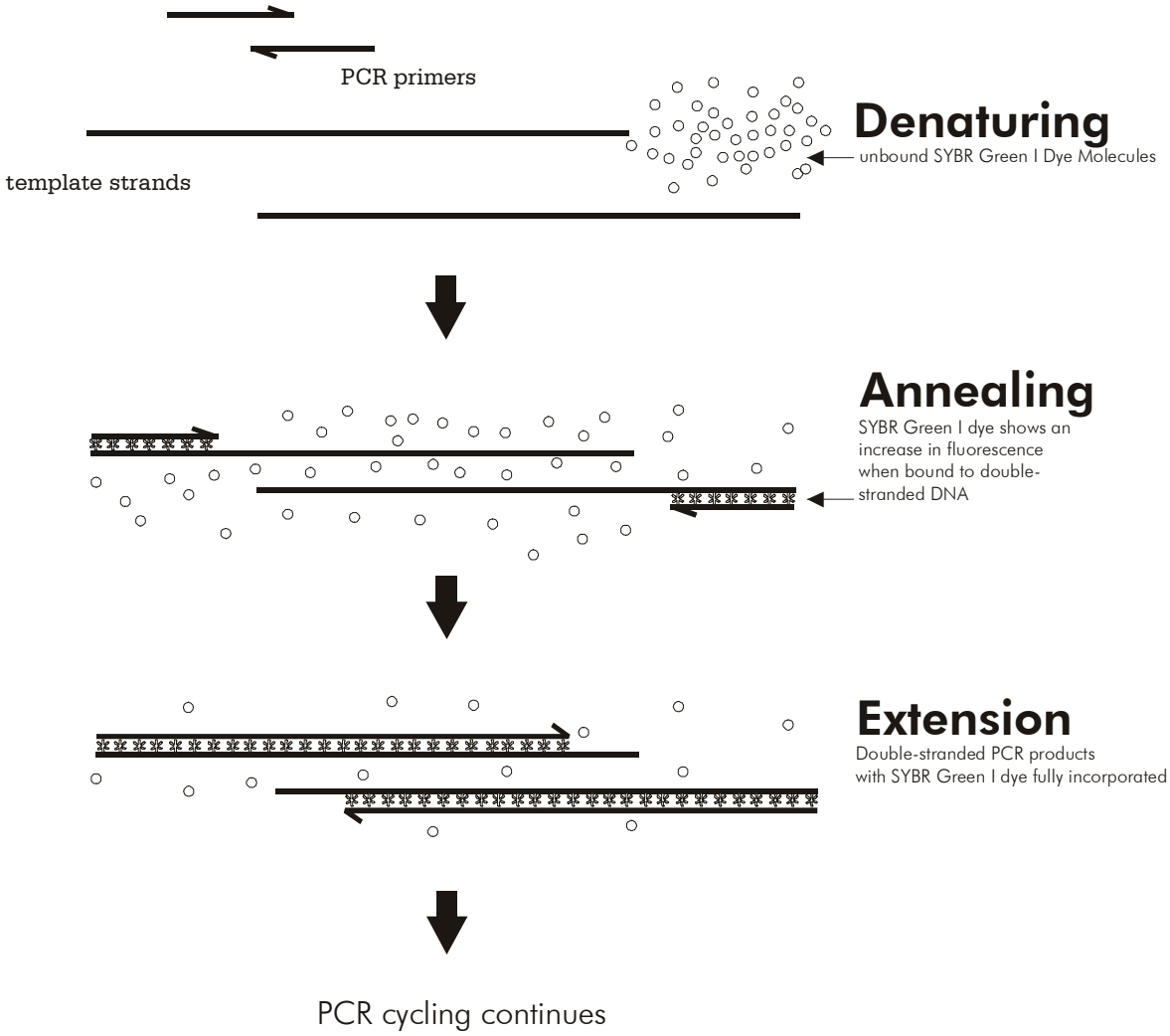

Figure 1 SYBR® Green I dye has a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA or RNA. Upon binding dsDNA, the fluorescence of SYBR® Green I increases by approximately 1000 fold, making it ideal for detecting the accumulation of dsDNA.

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, top panel), which reflects the change in fluorescence during cycling. Studies have shown that initial copy number can be quantitated during real-time PCR analysis 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, top panel, the Ct of the "+ template" reaction is 24 and the Ct of the "– template" reaction is 34). 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 get to a 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 it is based on a measurement taken during the exponential phase of PCR amplification when the PCR efficiency has yet to be influenced by limiting reagents, small differences in reaction components, or cycling conditions.

In Figure 2, SYBR Green detection was used in QPCR reactions containing genomic DNA template and a no-template control reaction. In the amplification plot (Figure 2, top panel) the reaction containing template shows a significant increase in fluorescence and has a Ct value of ≅ 24. The reaction without template shows a slight increase in fluorescence and has a Ct of 34. To determine if the increase in fluorescence in the no-template control reaction is true amplification due to template contamination or nonspecific amplification due to formation of primer-dimer (or some other nonspecific product), a dissociation profile is generated (Figure 2, bottom panel). 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. After completion of the dissociation segment, fluorescence is plotted versus temperature. For an easy interpretation of the dissociation profile the first derivative should be displayed, i.e. $-R'(T)$ or $-Rn'(T)$. As the temperature increases, the amplification products in each tube will 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 (defined as the T_m) than the desired products. The melting of products results in a drop of fluorescence, which is due to SYBR Green dissociation. The dissociation curve plot of these samples shows two fluorescence peaks: one in the "– template" reaction centered around 76°C (which corresponds to primer-dimer); and the other, in the "+ template" reaction, centered around 81°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.

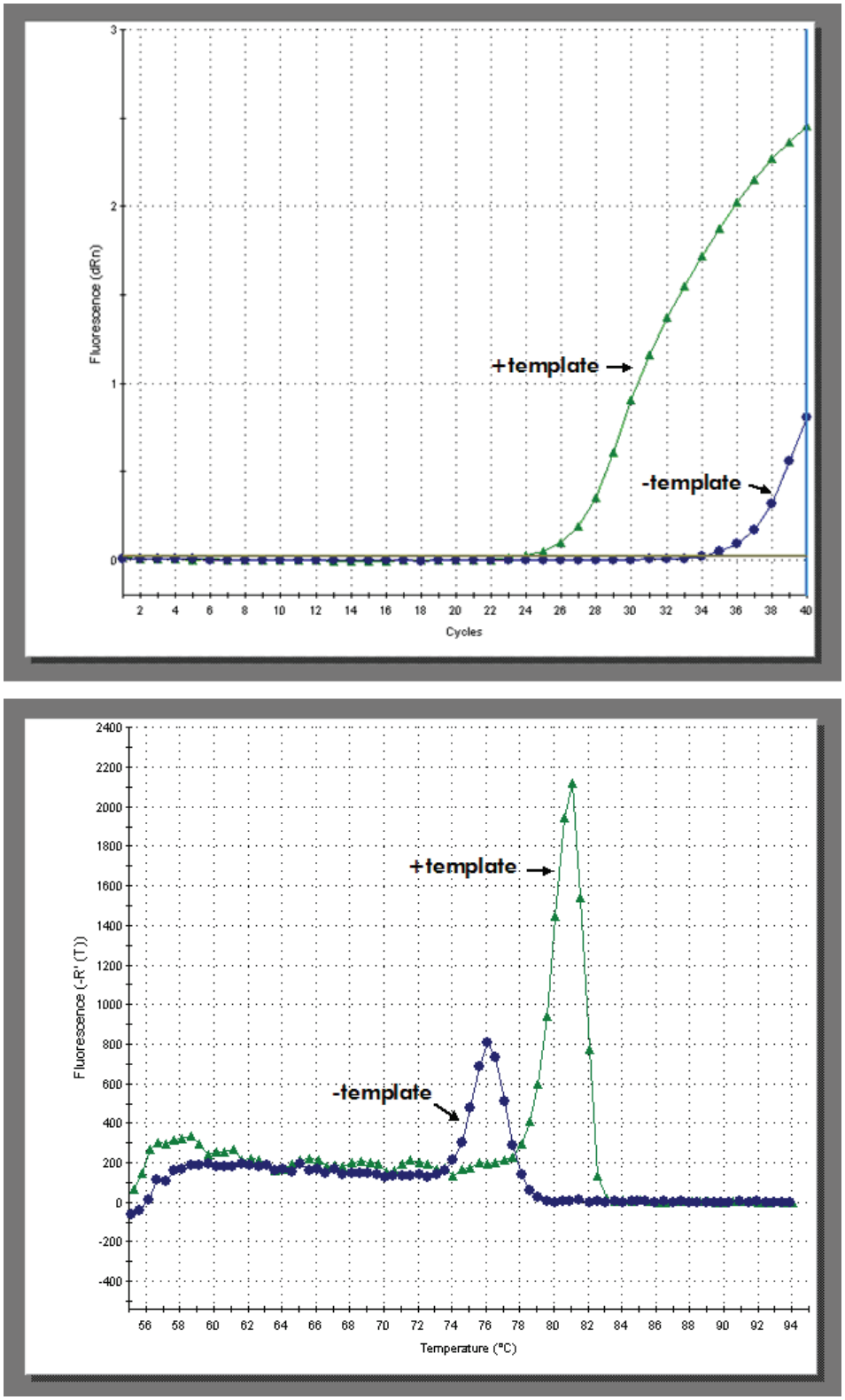

Figure 2 Mx3000P QPCR instrument amplification plot (top panel) and dissociation curve (bottom panel) of a reaction with and without template DNA. When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the amplicon (centered around 81°C) is distinguishable from the peak due to primer-dimer (centered around 76°C).

PREPROTOCOL CONSIDERATIONS

PCR Primers

It is critical in SYBR Green-based QPCR to minimize the formation of non-specific amplification products. This issue becomes more prominent at low target concentrations. Therefore, to maximize the sensitivity of the assay, it is necessary to use the lowest concentration of primers possible without compromising the efficiency of PCR. It is important to consider both the relative concentrations of forward and reverse primers and the total primer concentration. 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 200–600 nM are satisfactory.

Magnesium Chloride

The optimal $MgCl_2$ concentration promotes robust 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 QPCR 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 a concentrated $MgCl_2$ solution to the 1× experimental reaction at the time of setup.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[3] Because SYBR Green I dye fluoresces in the presence of any dsDNA, multiplexing with the Brilliant II SYBR Green QPCR master mix is not recommended.

Preventing Template Cross-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.

Data Acquisition with a Spectrofluorometric Thermal Cycler

The instrument should be set to collect SYBR Green I data in real-time at each cycle. How this is accomplished will depend on the software that commands the particular instrument you are using. Consult the manufacturer's instruction manual for the instrument and software version you are using.

When developing an assay, it is necessary to decide whether to use a 2-step or a 3-step PCR cycling protocol. We recommend a 2-step protocol for the Brilliant II SYBR Green QPCR master mix, 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 no-template controls should be chosen for analysis. For longer amplicons, fluorescence measurements taken during the extension step generally yield more useful data.

PROTOCOL

Preparing the Reactions

Notes *Once the tube containing the Brilliant II SYBR Green QPCR master mix is thawed, store it on ice while setting up the reactions. 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.

1. Prepare the experimental reactions by combining the following components *in order*. 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.

Experimental Reaction

Nuclease-free PCR-grade water to adjust the final volume to 25 µl (including experimental DNA)
12.5 µl of 2× Brilliant II SYBR Green QPCR master mix
x µl of upstream primer (200–600 nM final concentration)
x µl of downstream primer (200–600 nM final concentration)

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

2. Gently mix without creating bubbles (do not vortex), then distribute the mixture to the individual PCR reaction tubes.

3. Add *x* µl of experimental gDNA, cDNA, or plasmid DNA to each reaction.

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

Note *Bubbles interfere with fluorescence detection.*

5. Centrifuge the reactions briefly.

PCR Cycling Programs

6. Place the reactions in the instrument and run one of the PCR programs listed below. We recommend a two-step cycling protocol for most primer/template systems. For targets <150 bp in length, the fast protocol with two-step cycling may be used to decrease run times without compromising amplification efficiency. For primers with low melting temperatures, the three-step cycling protocol may be optimal.

Recommended Protocol with Two-Step Cycling (All Targets)

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

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

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

Fast Protocol with Two-Step Cycling (Targets <150 bp)

Cycles	Duration of cycle	Temperature
1	15 minutes[a]	95°C
40	10 seconds	95°C
	30 seconds[b]	60°C

[a] Initial 15 minute incubation is required to 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 (All Targets)

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

[a] Initial 10 minute incubation is required to 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 Programs

7. 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 (Mx3000P, Mx3005P)

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 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. Figure 3 shows how to set the *Thermal Profile* for the dissociation curve program on the Mx3000P and Mx3005P instruments.

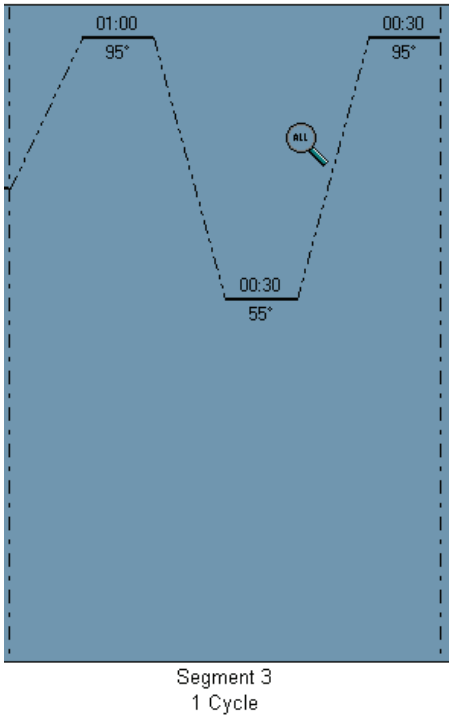

Figure 3 Dissociation program settings on the Mx3000P and Mx3005P real-time PCR instruments.

Dissociation Program for All Targets (Mx4000)

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. The duration of each cycle should be set to 30 seconds. Figure 4 shows how to correctly set the *Plateau Properties* 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.

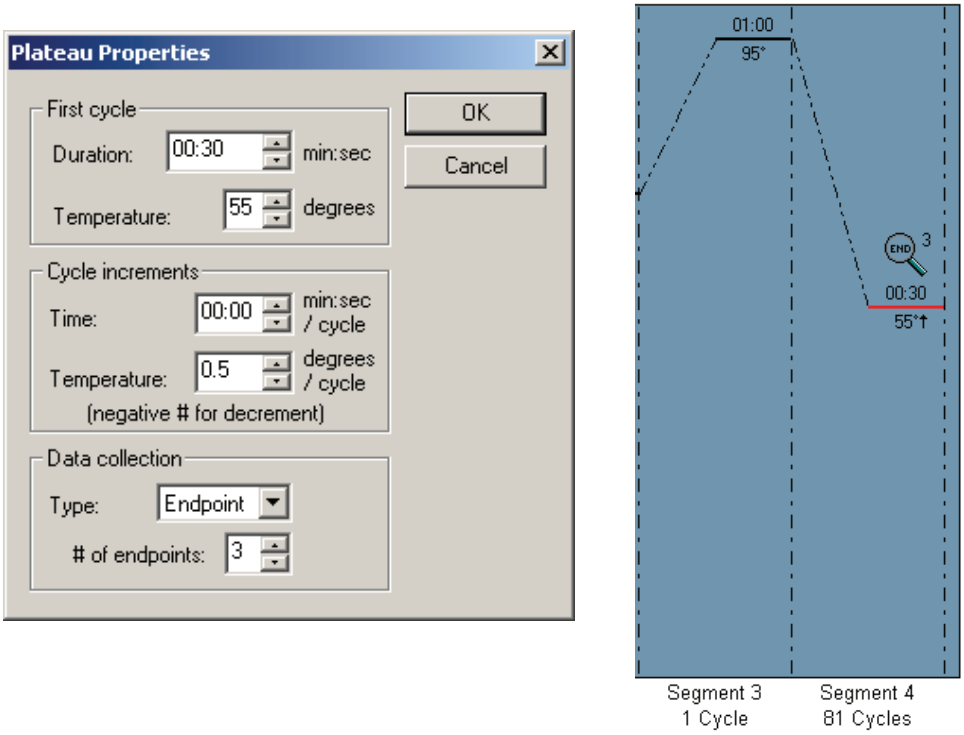

Figure 4 Settings for the dissociation program plateau properties on the Mx4000 multiplex quantitative PCR instrument.

TROUBLESHOOTING

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling	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 serial dilutions of template before repeating PCR. It may also be possible to check for PCR inhibitors by adding this target into an assay that is known to work.
	Ensure the annealing/extension time (2-step cycling protocol) or extension time (3-step cycling protocol) is sufficient. Check the length of the amplicon and increase the time if necessary.
	Use a sufficient number of cycles in the PCR reaction.
	If using a 3-step cycling protocol, ensure the annealing temperature is appropriate for the primers used.
	Gel analyze PCR product to determine if there was successful amplification.
	SureStart *Taq* DNA polymerase was not activated. Ensure that the 10 minute incubation (15 minute for fast cycling protocol) at 95°C was performed as part of the cycling parameters.
	The $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant II SYBR Green QPCR 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.
No (or little) increase in fluorescence with long amplicons (>400 bp)	Increase the length of the annealing/extension step (2-step cycling protocol) or the extension step (3-step cycling protocol).
There is a large abundance of primer-dimer and nonspecific PCR products	Increase the annealing temperature (3-step cycling protocol) above the Tm of the primer-dimer and/or nonspecific products.
	Re-design primers.

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. Edwards, M. and Gibbs, R. (1995). Multiplex PCR. In *PCR Primer: A Laboratory Manual*, C. W. Dieffenbach and G. S. Dveksler (Eds.), pp. 157-171. Cold Spring Harbor Laboratory Press, Plainview, NY.

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 QPCR MASTER MIX WITH ROX

Catalog #600829, #600830, #600832, #600833

QUICK-REFERENCE PROTOCOL

Prior to the experiment, it is prudent to carefully optimize experimental conditions and to include controls at every stage. See *Preprotocol Considerations* for details.

1. Thaw the Brilliant II SYBR Green QPCR 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.* ***SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.***

2. Prepare the experimental reaction by adding the following components *in order*:

   ### Experimental Reaction

   Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental DNA)
   12.5 µl of 2× Brilliant II SYBR Green QPCR master mix
   x µl of upstream primer (200–600 nM final concentration is recommended)
   x µl of downstream primer (200–600 nM final concentration is recommended)

   Note *Total reaction volumes of 50 µl may also be used.*

3. Gently mix the reaction without creating bubbles **(bubbles interfere with fluorescence detection; do not vortex)**.

4. Add x µl of experimental gDNA, cDNA, or plasmid DNA.

5. Gently mix the reaction without creating bubbles **(do not vortex)**.

6. Centrifuge the reaction briefly.

7. Place the reaction in the instrument and run the appropriate PCR program below.

Recommended Protocol with Two-Step Cycling (All Targets)

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

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

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

Fast Protocol with Two-Step Cycling (Targets <150 bp)

Cycles	Duration of cycle	Temperature
1	15 minutes[a]	95°C
40	10 seconds	95°C
	30 seconds[b]	60°C

[a] Initial 15 minute incubation is required to 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 (All Targets)

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

[a] Initial 10 minute incubation is required to 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.

8. Follow the dissociation guidelines below for the instrument used.

Dissociation Program for All Targets (Mx3000P and Mx3005P Instruments)

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 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, 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 for All Targets (Other Instruments)

Follow manufacturer's guidelines for setting up dissociation depending on the instrument's software version.