Brilliant SYBR® Green QPCR Core Reagent Kit

INSTRUCTION MANUAL

Catalog #600546 (single kit)
#929546 (10-pack kit)
Revision B.01

For In Vitro Use Only
600546-12

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Brilliant SYBR® Green QPCR Core Reagent Kit

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Brilliant SYBR® Green QPCR Core Reagent Kit

MATERIALS PROVIDED

Catalog #600546 (single kit), #929546 (10-pack kit)

Materials provided (per kit)	Quantity [a,b]
SureStart *Taq* DNA polymerase, 5 U/ µl	500 U
Core PCR buffer, 10x	1.7 ml
Magnesium chloride, 50 mM	1.5 ml
dNTP mix, 20 mM (5 mM each of dATP, dTTP, dGTP, and dCTP)	400 µl
DMSO, 100%	500 µl
Glycerol, 50%	2.0 ml
SYBR® Green I stock solution [c], 10,000X	25 µl
Reference dye [c], 1 mM	100 µl

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

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

[c] The SYBR Green I stock solution and the reference dye are light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Upon receipt, store all components at –20°C.

Note *The SYBR Green I dye and the reference dye are light sensitive and should be kept away from light whenever possible*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

Revision B.01

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INTRODUCTION

Quantitative PCR is becoming increasingly important for gene expression analysis. A variety of 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 QPCR Core Reagent Kit conveniently combines SYBR Green reagents with the components of the Stratagene Brilliant core reagent kit, making it the most versatile and flexible kit on the market. This kit is ideal for users optimizing QPCR reactions, developing assays for new primer/probe systems, or screening samples in a high-throughput platform.

The Brilliant SYBR Green QPCR core reagent kit includes the components necessary to carry out PCR amplifications*, and has been successfully used to amplify and detect a variety of genomic and plasmid DNA targets. It is also ideal for quantification of cDNA in a 2-step QRT-PCR reaction when combined with our AffinityScript Multiple Temperature cDNA Synthesis Kit. The Brilliant SYBR Green QPCR core reagent kit has been formulated to perform optimally using the SYBR Green I dye included. It also provides the flexibility of using the kit with fluorescence-labeled probes, single-dye primers, and Scorpions probes. The Brilliant SYBR Green QPCR core reagent kit has been optimized for maximum performance on Stratagene Mx3000P and Mx3005P real-time instruments and the Mx4000 multiplex quantitative PCR instrument, as well as on the ABI PRISM® 7700 instrument. In addition, excellent results have been observed using most other real-time QPCR platforms.

The Brilliant SYBR Green QPCR core reagent kit includes SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR amplification reactions by decreasing background from non-specific 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 reaction conditions. For normalization of fluorescent signals, a separate reference dye is included.

* Primers and template are not included.

SYBR® Green I Dye

SYBR Green I dye[1] is a double-stranded (dsDNA) minor groove binding dye with a high affinity. 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. 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). 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 nonexistent 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.

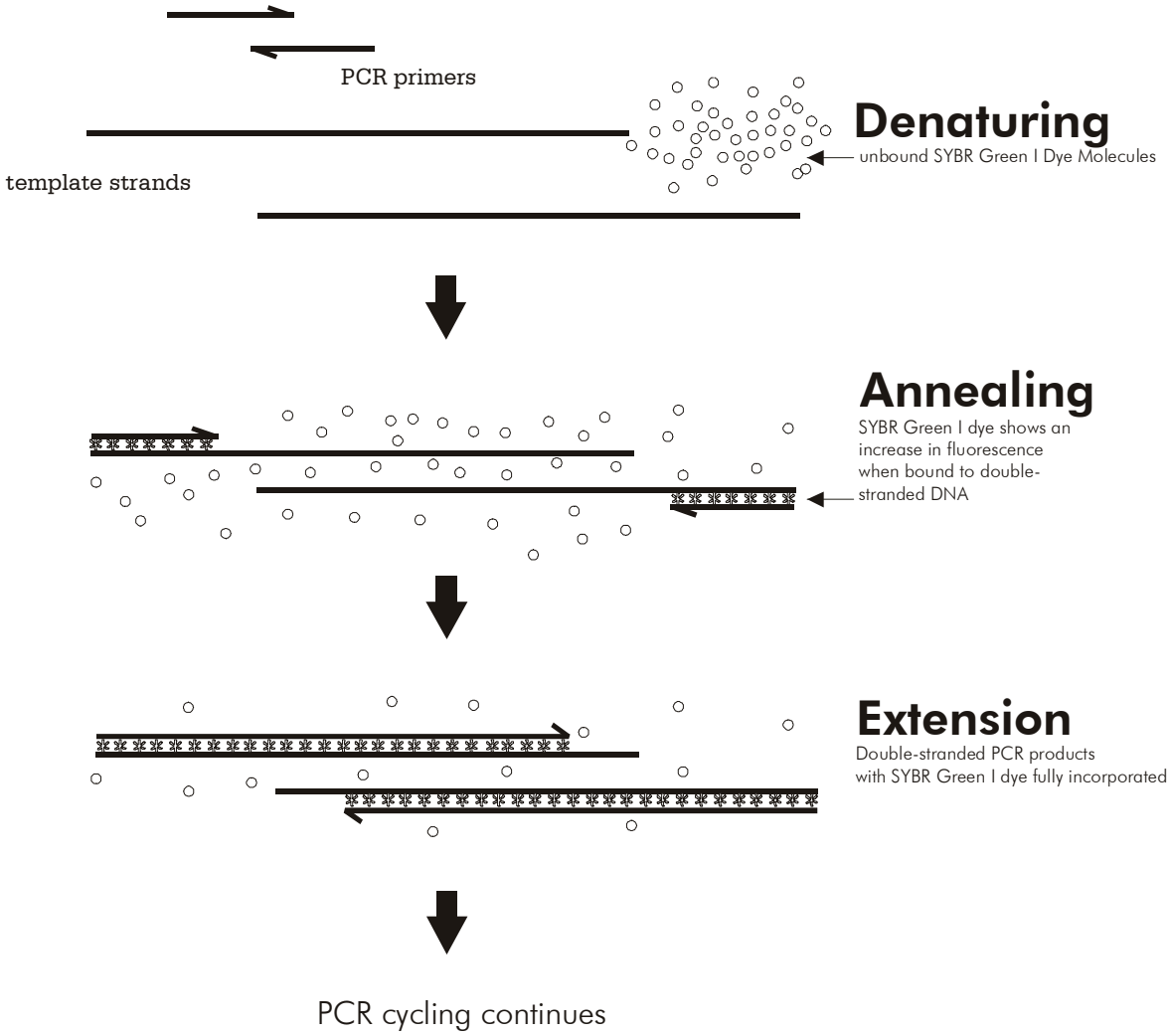


Figure 1 SYBR Green I dye has a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA or RNA. Upon binding dsDNA, the fluorescence yield of SYBR Green I increases by approximately 1000 fold, making it ideal for detecting the accumulation of dsDNA.

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 during PCR experiments to quantitate initial copy number. 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 limiting reagents and small differences in reaction components or cycling parameters. 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, the Ct of the "+ template" reaction is 21 and the Ct of the "– template" reaction is 38). 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.

Figure 2 shows Mx4000 instrument amplification and dissociation curve plots of a control reaction (no template) alongside a reaction containing a 550 bp amplicon in the presence of SYBR Green I dye. The reaction containing template shows a significant increase in fluorescence and has a Ct value ≅ 21. The reaction without template has a Ct of 38. To determine if this is true amplification due to template contamination of the reaction or an increase in fluorescence due to primer-dimer (or some other non-specific product) formation, the dissociation curve is performed. A dissociation profile is generated after amplification with SYBR Green I. In the dissociation curve, PCR samples are subjected to a stepwise increase in temperature from 55°C to 95°C; 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 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 75°C (which corresponds to primer-dimer); and the other, in the "+ template" reaction, centered around 83°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.

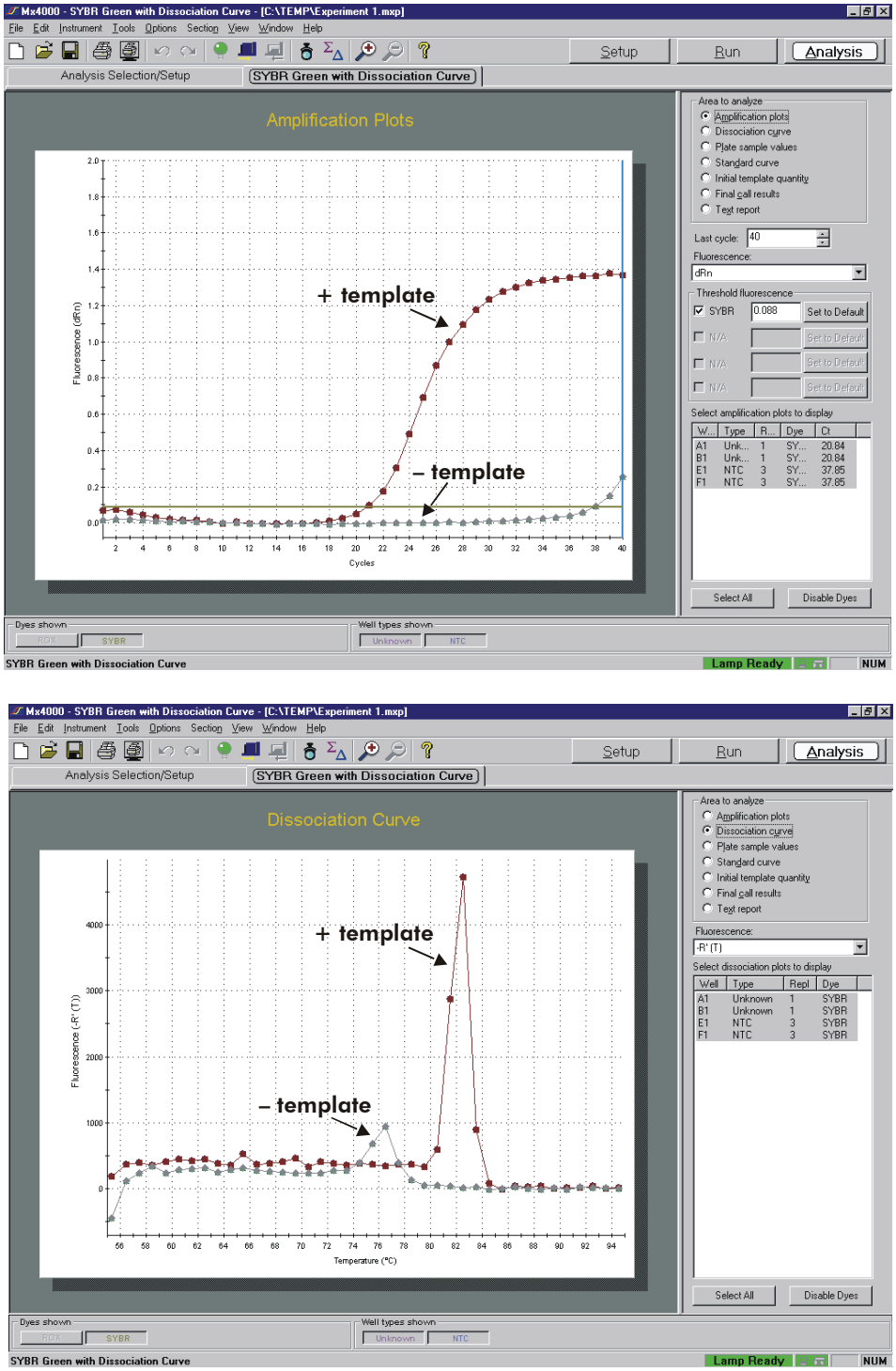


Figure 2 Mx4000 instrument amplification and dissociation curve plot of a reaction with and without template DNA. When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the amplicon (centered around 83°C) is distinguishable from the peak due to primer-dimer (centered around 75°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 50–150 nM are satisfactory.

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 this 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 $MgCl_2$).

SYBR® Green I Dye Dilution Recommendations

Prepare **fresh*** dilutions of the SYBR Green I dye prior to setting up the reactions, and **keep all tubes containing the SYBR Green protected from light as much as possible**. Make initial dilutions of SYBR Green I using nuclease-free PCR-grade H_2O. Use SYBR Green I at a final dilution of 0.167× in the reaction.

Glycerol and DMSO

Glycerol and DMSO are included in this kit, and should be added to the reactions to obtain optimal PCR performance. Start with 8% glycerol (final concentration) and 3% DMSO (final concentration). Further optimization of these concentrations may be necessary.

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

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 or between experiments caused by pipetting errors or instrument variability. 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 you are using a Stratagene Mx3000P or Mx3005P real-time PCR system or Mx4000 multiplex quantitative PCR system, use the reference dye at a final concentration of 30 nM. If you are using the ABI PRISM 7700 or the GeneAmp® 5700 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.

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 in the presence of SYBR Green I dye is not recommended unless the amplicons have very different melting temperatures (T_m). This difference in T_m may depend on the resolution capability of the instrument used, but in general should be approximately 5°C.

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

Data Acquisition with a Spectrofluorometric Thermal Cycler

The instrument should be set to collect SYBR Green I data in real-time at both the annealing step and the extension step of 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/software version you are using.

General Notes

Preventing 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.

Preparing a Master Mix for Multiple Samples

If running multiple samples, a master mix of reaction components can be prepared by combining the desired multiple of each component. Using a master mix facilitates accurate dispensing of reagents, minimizes loss of reagents during pipetting, and makes repeated dispensing of each reagent unnecessary, all of which help minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Enzymes (e.g. SureStart *Taq* DNA polymerase) should be mixed by gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

Preparing the Reactions

Notes *Once component tubes are thawed and mixed, store them on ice while setting up the reactions (except SYBR Green I stock and DMSO, which will immediately re-freeze). After SYBR Green I dilution is made, put the dilution on ice and return the stock tube to the freezer.*

The preferred method for preparing reactions is making master mixes for the experimental reaction by combining the desired multiple of each component listed in step 3. Individual samples can then be prepared by pipetting the master mixes into the PCR tubes/plates recommended by the manufacturer of the instrument. Template DNA can then be added to individual samples.

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.

1. If the reference dye will be included in the reaction, (optional), dilute 1:500 using nuclease-free PCR-grade H_2O. **Keep all solutions containing the reference dye protected from light.**

2. Prepare a fresh 1:3000 dilution of the SYBR Green I dye in nuclease-free PCR-grade H_2O. **Keep all solutions containing SYBR Green I dye protected from light.**

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

Component	Amount per reaction	Recommended final concentration
Nuclease-free PCR-grade H_2O	to a final volume of 25 µl (including experimental DNA)	—
10× core PCR buffer	2.5 µl	1×
50 mM magnesium chloride solution	*x* µl	1.5–2.5 mM (optimized amount)
20 mM dNTP mix	1.0 µl	0.8 mM
Upstream primer	*x* µl	50–150 nM (optimized amount)
Downstream primer	*x* µl	50–150 nM (optimized amount)
50% glycerol solution	4.0 µl	8% (may require optimization)
DMSO (100%)	0.75 µl	3% (may require optimization)
diluted reference dye (optional)	0.375 µl of **1:500 dilution**	30 nM
diluted SYBR® Green I dye	1.25 µl of **1:3000 dilution**	0.167×
SureStart *Taq* DNA polymerase (5 U/µl)	0.25 µl	1.25 U/25 µl reaction

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

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

5. Centrifuge briefly and aliquot the master mixes into the appropriate tubes or plates.

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

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

   Note *Bubbles interfere with fluorescence detection.*

8. Centrifuge the reactions briefly.

PCR Cycling Programs

9. Place the reactions in the instrument and run the appropriate PCR program below. This amplification protocol is recommended initially, but optimization may be necessary for some primer/template systems.

PCR Program for Amplification of Short Targets (50–400 bp)

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

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

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

PCR Program for Amplification of Long Targets (400–900 bp)

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	30 seconds	95°C
	1.0 minute[a]	50–60°C[b]
	1.5 minutes[a]	72°C
1	3.0 minutes	72°C

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

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

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 3 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.

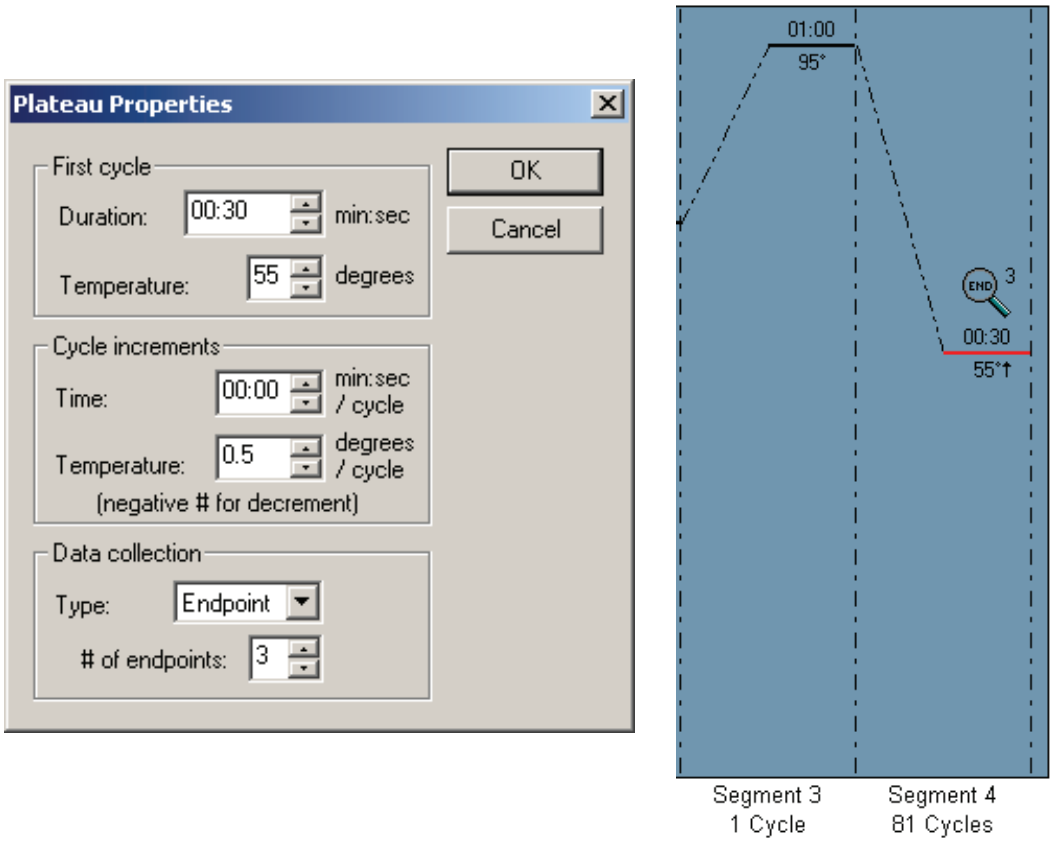
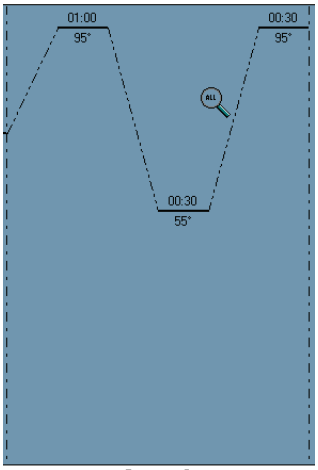


Figure 3 Settings for the dissociation program plateau properties on the Mx4000 multiplex quantitative PCR instrument.

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 4 shows how to set the *Thermal Profile* for the dissociation curve program on the Mx3000P instrument.

Figure 4 Dissociation program settings on the Mx3000P real-time PCR instrument.

PROTOCOL: ABI PRISM® 7700 INSTRUMENT

Preparing the Reactions

Notes *Once component tubes are thawed and mixed, store them on ice while setting up the reactions (except SYBR Green I stock and DMSO, which will immediately re-freeze). After SYBR Green I dilution is made, put the dilution on ice and return the stock tube to the freezer.*

The preferred method for preparing reactions is making master mixes for the experimental reaction by combining the desired multiple of each component listed in step 3. Individual samples can then be prepared by pipetting the master mixes into the PCR tubes/plates recommended by the manufacturer of the instrument. Template DNA can then be added to individual samples.

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.

1. If the reference dye will be included in the reaction, (optional), dilute 1:50 using nuclease-free PCR-grade H_2O. **Keep all solutions containing the reference dye protected from light.**

   Note *The use of the reference dye may be required for optimal results.*

2. Prepare a fresh 1:3000 dilution of the SYBR Green I dye in nuclease-free PCR-grade H_2O. **Keep all solutions containing SYBR Green I dye protected from light.**

3. Prepare the reactions by adding the following components *in order*:

Component	Amount per reaction	Recommended final concentration
Nuclease-free PCR-grade H_2O	to a final volume of 25 µl (including experimental DNA)	—
10× core PCR buffer	2.5 µl	1×
50 mM magnesium chloride solution	*x* µl	1.5–2.5 mM (optimized amount)
20 mM dNTP mix	1.0 µl	0.8 mM
Upstream primer	*x* µl	50–150 nM (optimized amount)
Downstream primer	*x* µl	50–150 nM (optimized amount)
50% glycerol solution	4.0 µl	8% (may require optimization)
DMSO (100%)	0.75 µl	3% (may require optimization)
diluted reference dye (optional)	0.375 µl of **1:50 dilution**	300 nM
diluted SYBR® Green I dye	1.25 µl of **1:3000 dilution**	0.167×
SureStart *Taq* DNA polymerase (5 U/µl)	0.25 µl	1.25 U/25 µl reaction

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

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

5. Centrifuge briefly and aliquot the master mixes into the appropriate tubes or plates.

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

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

   Note *Bubbles interfere with fluorescence detection.*

8. Centrifuge the reactions briefly.

PCR Cycling Programs

9. Place the reactions in the ABI PRISM instrument and run the appropriate PCR program below. This amplification protocol is recommended initially, but optimization may be necessary for some primer/template systems.

PCR Program for Amplification of Short Targets (50–400 bp)

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	15 seconds	95°C
	1.0 minute[b]	50–60°C[c]
	1.0 minute[b]	72°C

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

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

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

PCR Program for Amplification of Long Targets (400–900 bp)

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1.0 minute[b]	50–60°C[c]
	1.5 minutes[b]	72°C
1	3.0 minutes	72°C

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

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

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

Dissociation Program for All Targets

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

TROUBLESHOOTING: SYBR® GREEN I DYE

Amplification Plot

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling	A reagent is missing from the PCR reaction, repeat the PCR.
	The $MgCl_2$ concentration is not optimal. 1.5–2.5 mM is a recommended starting range. This may be increased up to 5.0 mM in 0.5 mM increments.
	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 SYBR Green was used.
	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 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 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.
No (or little) increase in fluorescence with long amplicons (> 400 bp)	Increase the length of the extension step.
	Ensure the SYBR green concentration is not too high.

Dissociation Plot

Observation	Suggestion(s)
There is a large abundance of primer-dimer and non-specific PCR products	Reduce the $MgCl_2$ concentration.
	Increase the annealing temperature.
	Design more optimal primers.
	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.

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

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

An **Agilent Technologies** Division

BRILLIANT SYBR® GREEN QPCR CORE REAGENT KIT

Catalog #600546, #929546

QUICK-REFERENCE PROTOCOL

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

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx3000P, Mx3005P and Mx4000 instruments) or 1:50 (ABI PRISM 7700 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. Dilute the SYBR Green I dye 1:3000. **Keep all solutions containing SYBR Green I dye protected from light.**

   Note *The preferred method for preparing reactions is making master mixes by combining the desired multiple of each component listed in step 3. Prepare all reactions on ice and ensure components are mixed well prior to use.*

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

Component	Amount per reaction	Recommended final concentration
Nuclease-free PCR-grade H_2O	to a final volume of 25 µl (including experimental DNA)	—
10× core PCR buffer	2.5 µl	1×
50 mM magnesium chloride solution	*x* µl	1.5–2.5 mM (optimized amount)
20 mM dNTP mix	1.0 µl	0.8 mM
Upstream primer	*x* µl	50–150 nM (optimized amount)
Downstream primer	*x* µl	50–150 nM (optimized amount)
50% glycerol solution	4.0 µl	8% (may require optimization)
DMSO (100%)	0.75 µl	3% (may require optimization)
diluted reference dye (optional)	0.375 µl of **1:500 dilution** for the Mx3000P, Mx3005P and Mx4000 instruments **OR** 0.375 µl of **1:50 dilution** for the ABI PRISM 7700 instrument	30 nM (Mx4000 instrument) **OR** 300 nM (ABI PRISM 7700 instrument)
diluted SYBR® Green I dye	1.25 µl of **1:3000 dilution**	0.167×
SureStart *Taq* DNA polymerase (5 U/µl)	0.25 µl	1.25 U/reaction

Note *Reaction volumes of 50 µl may also be used.*

4. Gently mix the master mixes without creating bubbles (**bubbles interfere with fluorescence detection; do not vortex**).

5. Centrifuge briefly and aliquot the master mixes into microcentrifuge tubes or plates.

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

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

8. Centrifuge the reactions briefly.

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

PCR Program for Amplification of Short Targets (50–400 bp)

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	30 seconds (Mx3000P, Mx3005P, Mx4000 instruments) **or** 15 seconds (ABI PRISM 7700 instrument)	95°C
	1.0 minute[a]	55–60°C[b]
	1.0 minute[a]	72°C

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

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

PCR Program for Amplification of Long Targets (400–900 bp)

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	30 seconds	95°C
	1.0 minute[a]	55–60°C[b]
	1.5 minutes[a]	72°C
1	3.0 minutes	72°C

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

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

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 (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 (Other Instruments)

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