Brilliant II SYBR® Green QRT-PCR, AffinityScript Master Mix, 2-Step

INSTRUCTION MANUAL

Catalog #600834

Revision B.01

For In Vitro Use Only

600834-12

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Brilliant II SYBR® Green QRT-PCR, AffinityScript Master Mix, 2-Step

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Brilliant II SYBR® Green QRT-PCR, AffinityScript Master Mix, 2-Step

MATERIALS PROVIDED

Catalog #600834

Materials Provided	Concentration	Quantity
AffinityScript QPCR cDNA Synthesis Kit[a]		
cDNA Synthesis Master Mix	2×	500 µl
AffinityScript RT/ RNase Block Enzyme Mixture	—	50 µl
Oligo(dT) primer	100 ng/µl	20 µg
Random primers	100 ng/µl	20 µg
RNase-free H_2O	—	1.2 ml
Brilliant II SYBR® Green QPCR Master Mix[b]		
Brilliant II SYBR® Green QPCR Master Mix[c]	2×	2 × 2.5 ml
Reference dye[d]	1 mM	100 µl

[a] Sufficient reagents are provided for fifty, 20-µl cDNA synthesis reactions.

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

[c] The QPCR master mix contains nucleotide mix GATC.

[d] The reference dye is 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 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

Real-time quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The Brilliant II SYBR® Green QRT-PCR, AffinityScript Master Mix provides a streamlined, fully optimized system for two-step QRT-PCR applications using SYBR Green I dye detection. A two-step RT-PCR format is useful for amplifying multiple targets from a single cDNA source and for maintaining archival cDNA. The kit is composed of two modules, both of which are optimized for streamlined QRT-PCR experiments. First, RNA is reverse-transcribed using the AffinityScript QPCR cDNA Synthesis Kit with a master mix format and fast protocol time of just 25 minutes. Second, the cDNA of interest is quantified using the Brilliant II SYBR Green QPCR Master Mix.

The improved Brilliant II formulation of the SYBR Green QPCR master mix yields higher levels of final fluorescence and earlier Ct values for many cDNA targets. The Brilliant II QPCR master mix has also 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.

Reverse Transcription with the AffinityScript QPCR cDNA Synthesis Kit

The AffinityScript QPCR cDNA synthesis kit provides a comprehensive set of reagents necessary to generate high-quality, QPCR-ready cDNA templates up to 12 kb in length from either mRNA or total RNA. The first-strand synthesis step is completed using a convenient master mix with few pipetting steps, saving you time and ensuring experiment-to-experiment reproducibility. Each reaction accommodates a range of RNA amounts from fg to µg.

cDNA Synthesis Master Mix

The cDNA synthesis master mix contains a buffer that is specifically optimized for QRT-PCR performance, allowing a fast protocol and reducing variability in Ct measurements between reactions. In addition to the optimized buffer, the master mix contains $MgCl_2$ and dNTPs.

AffinityScript RT/RNase Block Enzyme Mixture

The kit includes our QPCR-grade AffinityScript Multiple Temperature Reverse Transcriptase* (RT), a genetically engineered version of Moloney murine leukemia virus RT that is highly thermostable, allowing you to reverse transcribe at your preferred reaction temperature. AffinityScript RT is provided, in combination with RNase block, in a separate tube so that *no-RT* control reactions may be included in the QRT-PCR experiments. The RNase block serves as a safeguard against contaminating RNases.

AffinityScript RT is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis, particularly from small input RNAs. It has been tested in QRT-PCR experiments to ensure sensitive and reproducible performance over a broad range of RNA template amounts and over a variety of RNA targets that vary in size, abundance, and GC-content.

* Patents pending.

cDNA Primers

The cDNA priming strategy can affect cDNA yield, sensitivity, and detection of certain targets, such as GC-rich targets or sequences located at the 5´ or 3´ end of a transcript.[1] For this reason, individually packaged random nonamers and oligo(dT) primers are provided separately from the master mix, allowing you to use the best priming strategy for your specific target.[2]

QPCR Analysis using the Brilliant II SYBR® Green QPCR Master Mix

The Brilliant II SYBR Green QPCR master mix includes all of the components necessary to carry out QPCR amplification of cDNA, including SureStart *Taq* DNA polymerase, an optimized buffer, dNTPs, $MgCl_2$, and stabilizers. The master mix also contains SYBR Green I dye for detection of the amplification products. (Gene-specific primers are supplied by the user.)

SureStart *Taq* DNA Polymerase

SureStart *Taq* DNA polymerase is 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. SureStart *Taq* is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with little modification of cycling parameters or reaction conditions.

Reference Dye

A passive reference dye (an optional reaction component) is provided in a separate tube; providing this reagent separately allows you to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms.

QPCR Platforms

The Brilliant II SYBR Green QPCR master mix has been optimized for maximum performance on the Stratagene Mx3000P and Mx3005P real-time PCR systems, as well as the ABI 7900HT real-time PCR instrument. In addition, excellent results have been observed using most other QPCR platforms.

Detection using SYBR Green I Dye

SYBR Green I dye[3] has an excitation maximum at 497 nm and an emission maximum at 520 nm. When free in solution the dye displays relatively low fluorescence, but when bound to double-stranded DNA (dsDNA) its fluorescence increases by over 1000-fold (Figure 1).

In reactions containing SYBR Green I dye, fluorescence increases proportionately to dsDNA concentration. As the target is amplified, the increasing concentration of dsDNA in the solution can be directly measured by the increase in fluorescence signal. Compared to probe-based methods, SYBR Green I assays are relatively easy to design and optimize. All that is necessary is to design a set of primers, optimize the amplification efficiency and specificity, and then run the PCR reaction in the presence of the dye. One limitation of assays based on DNA-binding dye chemistry is the inherent non-specificity of this method. SYBR Green I will increase in fluorescence when bound to any double-stranded DNA (dsDNA). Therefore, the reaction specificity is determined solely by the primers. Consequently, the primers should be designed to avoid nonspecific binding (e.g. primer-dimer formation). A nonspecific signal cannot always be prevented, but its presence can be easily and reliably detected by performing dissociation curve analysis on the reaction mixture following PCR in every run.

In dissociation curve analysis, as the temperature increases, the dsDNA melts and the fluorescence intensity decreases. If the PCR products consist of molecules of homogeneous length, a single thermal transition will be detected. If, however, the reaction contains more than one population of PCR products, multiple thermal transitions in the fluorescence intensity will be detected. In this way, the dissociation curve is used to differentiate between specific and nonspecific amplicons based on the T_m (melting temperature) of the reaction end-products.

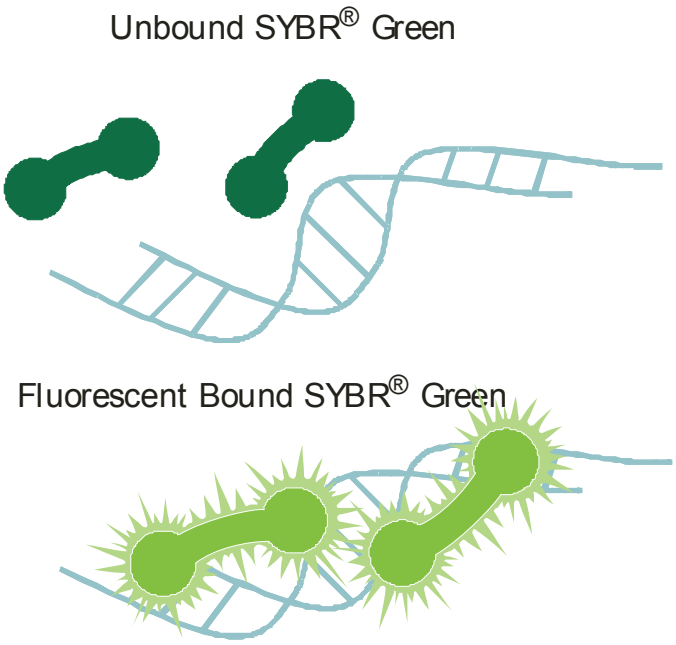

FIGURE 1 SYBR® Green I detection mechanism. Double-stranded DNA in the reaction is bound by the dye. In the bound state, SYBR Green I is 1000-fold more fluorescent than in the unbound state. As PCR amplification increases the amount of dsDNA present, the fluorescence signal increases proportionately.

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total RNA can be rapidly isolated and purified from cells using Stratagene Absolutely RNA isolation kits, which are available for nano-, micro- and miniprep scale RNA purifications (Catalog #400753, #400805, and #400800, respectively). Total RNA may also be isolated from formalin-fixed, paraffin-embedded (FFPE) tissues using the Absolutely RNA FFPE kit (Catalog #400809).

Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. The Absolutely mRNA purification kit (Catalog #400806) is recommended for this application.

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, RNase-free tubes, pipet tips, and water. The RNase block that is included in the cDNA synthesis reaction mixture provides additional protection against RNase contamination.

Preventing Genomic DNA Contamination

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. All of the Stratagene Absolutely RNA kits include RNase-free DNase which is used in a rapid on-column DNase treatment protocol. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing DNA.

cDNA Synthesis Reaction Considerations

Duration and Temperature of Incubation

For first-strand synthesis, a 15-minute incubation at 42°C is sufficient for most targets. Increasing the incubation time to 45 minutes at 42°C is optional and may increase cDNA yield for more challenging RNA targets, such as low-abundance targets or targets longer than 12 kb. For targets prone to secondary structure formation, raising the incubation temperature from 42°C to 55°C may improve cDNA yield.

Amount of AffinityScript RT

The cDNA synthesis reaction is fully optimized for high efficiency and dynamic range across a variety of targets and RNA input amounts. Do not try to address problems posed by low abundance or challenging targets by increasing the amount of AffinityScript RT/RNase block. (Use only 1 µl of AffinityScript RT/RNase block per 20-µl reaction.) See *Duration and Temperature of Incubation*, above, for recommendations on increasing cDNA synthesis incubation time or temperature to address yield problems for challenging targets. It is important to heat-inactivate the reverse transcriptase by incubating the reaction at 95°C for 5 minutes after cDNA synthesis.

cDNA Primer Selection

The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically with each target. Stratagene QPCR Reference Total RNA (available separately) can be used for this step. (See *Using a Reference RNA for QRT-PCR Experiments* for more details.) For most targets, the best results are achieved using either oligo(dT) or random primers. For some challenging targets (long or secondary structure-rich targets), however, using a mixture of oligo(dT) and random primers may increase cDNA yield. When testing the use of mixed primers, adding a mixture of 170 ng oligo(dT) primer and 30 ng random primers to each 20-µl reaction is a good starting point.

Using a Reference RNA for QRT-PCR Experiments

In order to reliably compare data across multiple experiments and instruments, it is essential to have a constant reference material to assess the performance of each QPCR run and to quantify gene expression levels. Stratagene QPCR Reference Total RNA is a high-quality control for quantitative PCR gene-expression analysis. Including a standard curve with the Stratagene QPCR Reference Total RNA in every experiment allows you to assess QPCR assay efficiency and precision, and to quantitate relative to an unchanging reference standard. In addition, the broad gene coverage allows you to use the reference material for nearly any human or mouse gene being investigated, thus eliminating the extra work required in generating new standards for each new gene target.

Stratagene QPCR Reference Total RNAs are available for both the human system (Catalog #750500) and the mouse system (Catalog #750600). The QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The QPCR Mouse Reference Total RNA is derived from RNA pooled from 11 mouse cell lines. These reference RNAs are carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

As an external RNA control, Stratagene Alien QRT-PCR Inhibitor Alert (catalog #300600) is also well suited for detecting inhibitors in RNA samples as well as QPCR assay standardization applications. The Alien RNA transcript is an *in vitro*-transcribed RNA molecule that has no significant homology to any known nucleic acids. Using the Alien RNA, with the primers included in the Alien inhibitor alert kit, as a reference control to generate standard curves allows data comparisons from multiple experiments, across platforms, and between laboratories. The Alien RNA is produced in large lots and subjected to stringent quality-control measures to ensure the availability of consistent reference RNA material over long-term experimental studies.

QPCR Reaction 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, 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. The optimal concentration should be determined empirically for each primer and is generally in the range of 200–600 nM.

Using the Passive Reference Dye

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

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If 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 7900HT or the ABI PRISM 7700 instruments, 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.

* 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.

Magnesium Chloride Concentration

The optimal $MgCl_2$ concentration promotes maximal amplification of the specific target amplicon with minimal nonspecific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of nonspecific dsDNA, including primer-dimers. Therefore, for SYBR Green-based QPCR assays, the $MgCl_2$ concentration 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 concentrated $MgCl_2$ to the 1× experimental reaction.

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.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[4] Because SYBR Green I dye fluoresces in the presence of any dsDNA, multiplexing in the presence of SYBR Green I dye is not recommended.

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 cycling protocol. We recommend a 2-step protocol for the Brilliant II SYBR Green 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 controls containing no target should be chosen for analysis. For longer amplicons, fluorescence measurements taken during the extension step generally yield more useful data.

Recommended Control Reactions

No-RT Control

We recommend performing no-RT control reactions for each experimental sample by omitting the RT/RNase block enzyme mixture from the cDNA synthesis reaction. The no-RT control is expected to generate no signal if there is no amplification of genomic DNA in the subsequent QPCR. 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*.

No-Template Control (NTC)

Perform QPCR with no-template control reactions for each experimental sample to screen for contamination of reagents or false amplification.

Endogenous Control

Consider performing QPCR on an endogenous control target to normalize variation in the amount of cDNA template across samples. See Reference 5 for guidelines on the use of endogenous controls for QPCR.

PROTOCOLS

First-Strand cDNA Synthesis

Note *Before use, mix each component and briefly spin in a microcentrifuge.*

It is prudent to include a No-RT *control reaction for each RNA sample by omitting the AffinityScript RT-RNase Block enzyme mixture. This control verifies that signal detected in the subsequent QPCR is not due to genomic DNA contamination.*

1. Prepare the first-strand cDNA synthesis reaction in a microcentrifuge tube by adding the following components *in order*:

   RNase-free H_2O to a total volume of 20 µl
   10.0 µl of cDNA synthesis master mix (2×)
   3.0 µl of oligo(dT) primer OR random primers (0.1 µg/µl)
   1.0 µl of AffinityScript RT/RNase Block enzyme mixture
   x µl of RNA (0.3 pg–3 µg total RNA)

   Note *The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically. See* cDNA Primer Selection *in* Preprotocol Considerations *for more information.*

2. Incubate the reaction at 25°C for 5 minutes to allow primer annealing.

3. Incubate the reaction at 42°C for 15 minutes to allow cDNA synthesis.

   Note *This protocol is ideal for most targets up to 12 kb. Increasing the incubation time to 45 minutes at 42°C or raising the incubation temperature from 42°C to 55°C may increase cDNA yield for longer or secondary structure-rich targets, respectively.*

   When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.

4. Incubate the reaction at 95ºC for 5 minutes to terminate reverse transcription.

5. Place the completed first-strand cDNA synthesis reaction on ice for immediate use in QPCR. For long-term storage, place the reaction at –20°C.

QPCR Amplification of cDNA

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.

Setting Up the QPCR 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 and ABI 7900HT instruments)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in the *Reference Dye* section under *Preprotocol Considerations*. **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. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for duplicate experimental reactions and duplicate no-template controls (plus at least one reaction volume excess), using multiples of each component listed below.

   #### Experimental Reaction

   Nuclease-free PCR-grade water to adjust the final volume to 25 µl (including experimental cDNA)
   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)
   0.375 µl of diluted reference dye (optional)

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

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

4. Add 2 µl of the cDNA synthesis reaction to each PCR reaction tube.

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

   Note *Bubbles interfere with fluorescence detection.*

6. Centrifuge the reactions briefly.

PCR Cycling Programs

7. 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 Program

Mx3000P and Mx3005P Instruments

If using a Stratagene Mx3000P or Mx3005P instrument, use the default dissociation curve for SYBR Green experiments. This default profile dissociation curve begins with a 1-minute incubation at 95°C to melt the DNA and then a 30-second incubation at 55°C. This is followed by a ramp up to 95°C with *Allpoints data collection* performed during the ramp.

Mx4000 Instrument

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

Other Instruments

If using another instrument, follow the manufacturer's guidelines for generating dissociation curves.

TROUBLESHOOTING

Observation	Suggestion
No or low yield of first-strand cDNA	Verify the integrity of the input RNA by denaturing agarose gel electrophoresis to ensure it is not degraded.
	Optimize the reaction using Stratagene QPCR Reference Total RNAs, which can then be used as a calibrator for subsequent experiments.
	Replace the RNA. Use Stratagene RNA isolation kits to isolate intact total RNA or mRNA.
	Isolate the RNA in the presence of a ribonuclease inhibitor, and ensure that all RT-PCR reagents and labware are free of RNases.
	Inhibitors of reverse transcription (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4, or spermidine) may be present in the RNA sample. Reduce the volume of the input RNA or remove RT inhibitors with an additional 70% (v/v) ethanol wash following ethanol precipitation. Use Stratagene Alien QRT-PCR Inhibitor Alert for detecting inhibitors in RNA samples.
	Increase the length of the 42°C cDNA synthesis reaction to 45 minutes for longer RNA targets.
	Increase the incubation temperature from 42°C to 55°C for secondary structure-rich targets.
	Increase the concentration of the template RNA.
	Try switching the cDNA primer composition [oligo(dT) vs. random primers]. For challenging targets, a mixture of the two primer types may also be tested.
	When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.
No or low yield of amplification product in QPCR	See the discussion under *No or low yield of the first-strand cDNA* for suggestions related to insufficient first strand synthesis.
	Add more cDNA synthesis product to the PCR. Up to 10% of the cDNA synthesis reaction may be added to each 25-µl QPCR reaction.
	Optimize the QPCR primer concentration, annealing temperature, and/or extension time, varying each individually and in increments. Stratagene QPCR Reference Total RNAs may be used for this step to conserve experimental samples.
	Increase the number of thermal cycles.
	Ensure the extension time is sufficient. Check the length of the amplicon and increase the extension time if necessary.
	Re-examine the QPCR primer design. Make sure primers are not self-complementary or complementary to each other. Verify that the primers are designed to be complementary to the appropriate strands. Try using longer primers.
	Ensure that the cycling program includes the 10 minute incubation (15 minute for fast cycling) at 95°C in order to activate SureStart *Taq* DNA polymerase.
	Verify that the correct dilution of reference dye was used, based on the type of QPCR instrument used.
	The $MgCl_2$ concentration may not be optimal. The $MgCl_2$ concentration in the 1× Brilliant II SYBR Green QPCR master mix is 2.5 mM. A small amount of concentrated $MgCl_2$ may be added to the experimental reactions, if desired.

Table continues on following page

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An abundance of primer-dimer and nonspecific PCR products are observed in the dissociation curve	Increase the annealing temperature if using a 3-step cycling protocol.
	Re-design 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 collected during the extension step can be more useful in this case.
Size of the amplification product is greater than expected	The RNA preparation may be contaminated with genomic DNA. Test for the presence of contaminating DNA by performing RT-PCR in the absence of AffinityScript RT. If DNA contamination is confirmed, treat the RNA preparation with RNase-free DNase I. Alternatively, redesign the PCR primers to anneal to sequences in the exon–exon boundary of the target gene.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 6 to minimize contamination.

REFERENCES

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3. Molecular Probes, Inc., at *http://www.probes.com/media/pis/mp07567.pdf*.
4. 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.
5. Bustin, S. A. (2000) *Journal of Molecular Endocrinology* 25:169-193.
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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

An **Agilent Technologies** Division

BRILLIANT II SYBR® GREEN QRT-PCR, AFFINITYSCRIPT MASTER MIX, 2-STEP

Catalog #600834

QUICK-REFERENCE PROTOCOL

cDNA Synthesis Reaction

1. Add the following components to a microcentrifuge tube *in order:*

   RNase-free H_2O to a total volume of 20 µl
   10.0 µl of cDNA synthesis master mix (2×)
   3.0 µl of oligo(dT) primer OR random primers (0.1 µg/µl)
   1.0 µl of AffinityScript RT/RNase Block enzyme mixture
   x µl of RNA (0.3 pg–3 µg total RNA)

2. Incubate the reaction at 25°C for 5 minutes to allow primer annealing.

3. Incubate the reaction at 42°C for 15 minutes to allow cDNA synthesis.

   Notes *For challenging targets, increasing the incubation time to 45 minutes at 42°C or raising the incubation temperature from 42°C to 55°C may increase cDNA yield.*

   When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C.

4. Incubate the reactions at 95°C for 5 minutes to terminate reverse transcription.

5. Place the reactions on ice for immediate use in QPCR or at –20°C for long-term storage.

QPCR Reaction

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

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

   Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including cDNA)
   12.5 µl of 2× 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)
   0.375 µl of **diluted** reference dye from step 1 (optional)

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

5. Add 2 µl of experimental cDNA.

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

7. Centrifuge the reaction briefly.

8. Place the reaction in the instrument and run the appropriate PCR program below. See the *Protocols* section of the manual for an alternative protocol with three-step cycling.

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.

9. Follow the dissoication 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.