Brilliant QPCR Core Reagent Kit

Instruction Manual

Catalog #600530 (single kit)
#929530 (10-pack kit)
Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600530-12

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Brilliant QPCR Core Reagent Kit

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Brilliant QPCR Core Reagent Kit

MATERIALS PROVIDED

Catalog #600530 (single kit), #929530 (10-pack kit)

Materials provided (per kit)	Concentration	Quantity[a,b]
SureStart *Taq* DNA polymerase	5 U/µl	500 U
Core PCR buffer	10×	1.7 ml
Magnesium chloride	50 mM	1500 µl
dNTP mix	20 mM (5 mM each of dATP, dTTP, dGTP, and dCTP)	400 µl
Reference dye[c]	1 mM	100 µl

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

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

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

STORAGE CONDITIONS

All Components: –20°C

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

ADDITIONAL MATERIALS REQUIRED

Thermal cycler and fluorescence reader
or
Spectrofluorometric thermal cycler

Note *A heated lid for the thermal cycler is strongly recommended because it allows optimal detection of the fluorescence. An overlay of mineral oil slightly attenuates the fluorescence.*

Nuclease-free PCR-grade water

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INTRODUCTION

Quantitative PCR is a powerful tool for gene expression analysis. Many fluorescent chemistries are used to detect and quantitate gene transcripts. The use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed, and high throughput screening capabilities. The Brilliant QPCR Core Reagent Kit includes the components necessary to carry out PCR amplifications (primers, probe and template are not included). The Brilliant QPCR core reagent kit has been successfully used to amplify and detect a variety of DNA targets, including genomic DNA and plasmid DNA.[1] It is also suitable for use with cDNA targets and can be combined with the Stratagene AffinityScript Multiple Temperature cDNA Synthesis Kit, which provides sensitive production of full-length cDNA. Brilliant kits support quantitative amplification and detection with multiplex capability and show consistent high performance with various fluorescent detection systems, including molecular beacons, TaqMan probes, single-dye primers, and Scorpions probes. A separate reference dye is included for normalization of fluorescent signals.

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

Features of Kit Components

The Brilliant 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 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 or no modification of cycling parameters or reaction conditions.

Molecular Beacons

Molecular beacons are hairpin-shaped fluorescent hybridization probes that can be used to monitor the accumulation of specific product during or after PCR.[1–5] Molecular beacons have a fluorophore and a quencher molecule at opposite ends of an oligonucleotide. The ends of the oligonucleotide are designed to be complementary to each other. When the unhybridized probe is in solution, it adopts a hairpin structure that brings the fluorophore and quencher sufficiently close to each other to allow efficient quenching of the fluorophore. If, however, the molecular beacon is bound to its complementary target, the fluorophore and quencher are far enough apart that the fluorophore cannot be quenched and the molecular beacon fluoresces (see Figure 1). As PCR proceeds, product accumulates and the molecular beacon fluoresces at a wavelength characteristic of the particular fluorophore used. The amount of fluorescence at any given cycle depends on the amount of specific product present at that time.

TaqMan® Probes (Hydrolysis Probes)

TaqMan probes are linear.[6, 7] The fluorophore is usually at the 5´ end of the probe, and the quencher is either internal or is at the 3´ end. As long as the probe is intact, regardless of whether it is hybridized with the target or free in solution, no fluorescence is observed from the fluorophore. During the combined annealing–extension step of PCR, the primers and the TaqMan probe hybridize with the target (see Figure 2). The DNA polymerase displaces the TaqMan probe by 3 or 4 nucleotides, and the 5´-nuclease activity of the DNA polymerase separates the fluorophore from the quencher. Fluorescence can be detected during each PCR cycle, and fluorescence accumulates during the course of PCR.

Endpoint vs. Real-Time Measurements

The probes can be used in a variety of PCR applications, including infectious agent detection, genotyping, allelic discrimination, and quantitative gene expression analysis. The fluorescence of the probe can be monitored either when cycling is complete (endpoint analysis) or as the reaction is occurring (real-time analysis). For endpoint analysis, PCR reactions can be run on any thermal cycler and can then be analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and that is optimized for the detection of PCR reactions that include fluorescent probes. Real-time experiments are typically performed on an instrument capable of detecting fluorescence from samples during each cycle of a PCR protocol.

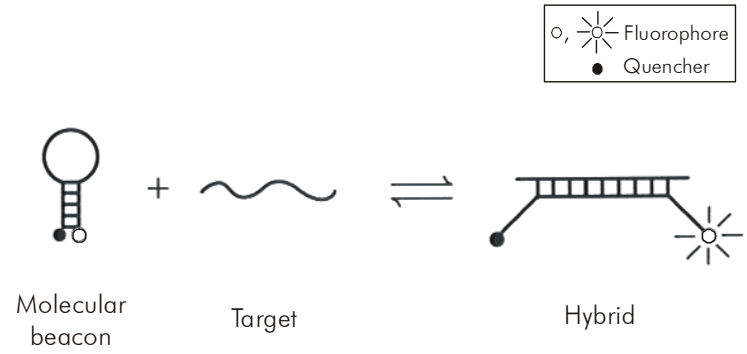

FIGURE 1 The molecular beacon binds to a complementary target and fluoresces.

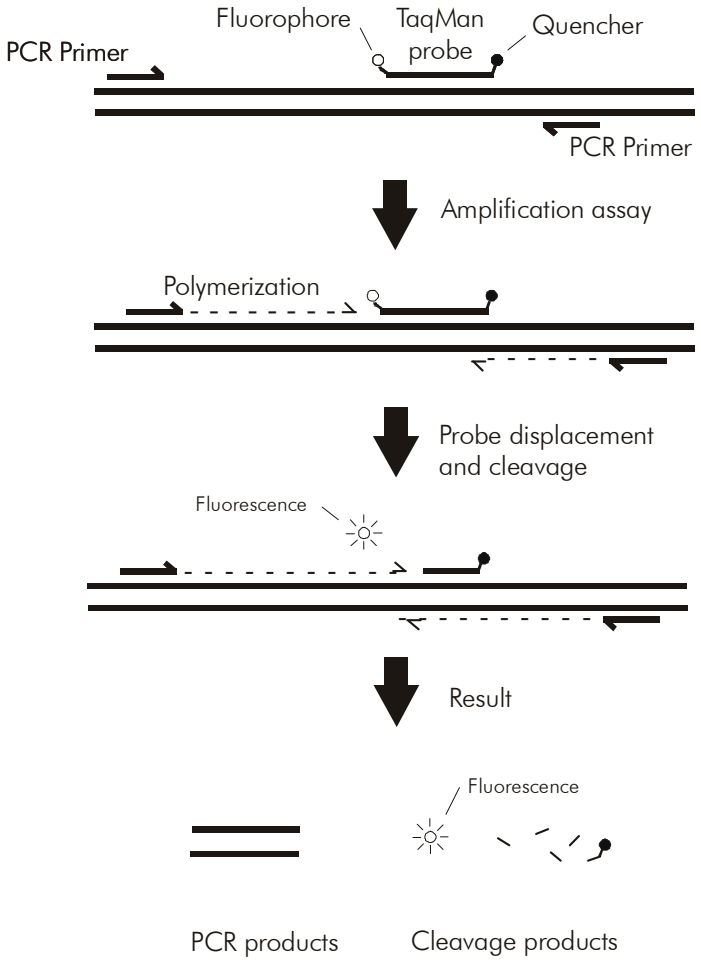

FIGURE 2 TaqMan probe fluoresces when the 5′-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

PREPROTOCOL CONSIDERATIONS

Probe Design

For considerations in designing TaqMan probes, refer to Primer Express® oligo design software from Applied Biosystems.

Resuspend lyophilized custom molecular beacons in TE buffer.

Fluorescence Detection

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

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

Optimal Concentrations for Experimental Probes and PCR Primers

Probes

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the primers. The optimal concentration of the probe should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration.

A) Molecular Beacons

The molecular beacon concentration can be optimized by varying the final concentration from 200 to 500 nM in increments of 100 nM.

B) TaqMan Probes

The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 nM.

PCR Primers

The optimal concentration of the upstream and downstream PCR primers should also be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The primer concentration for use with molecular beacons can be optimized by varying the concentration from 200 to 600 nM. The primer concentration for use with TaqMan probes can be optimized by varying the concentration from 50 to 300 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity. The conditions that produce the lowest amounts of primer dimer and nonspecific product are recommended.

Magnesium Chloride

We recommend optimizing the magnesium chloride concentration for each molecular beacon or TaqMan probe. The optimal concentration is the concentration that results in the lowest Ct and highest fluorescence for a given target concentration. The $MgCl_2$ concentration can be optimized by varying the concentration from 2 to 6 mM in increments of 0.5 mM.

Endogenous Control

Consider performing an endogenous control reaction to distinguish true negative results from PCR inhibition or failure. Follow the manufacturer's protocol.

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 Mx3005P, Mx3000P or Mx4000 system, with other instruments (including the ABI 7900HT and ABI PRISM 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If using the ABI PRISM 7700 instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

* 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.

Data Acquisition with a Spectrofluorometric Thermal Cycler

Acquisition of real-time data generated by fluorogenic probes should be performed as recommended by the instrument's manufacturer. Data should be collected at the annealing step of each cycle (3-step cycling protocol) or the annealing/extension step (2-step cycling protocol).

Changes may be required in the analysis portion of the software.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[8] In a typical multiplex PCR, one primer pair primes the amplification of the target of interest and another primer pair primes the amplification of an endogenous control. For accurate analysis, it is important to minimize competition between concurrent amplifications for common reagents. To minimize competition, the limiting primer concentrations need to be determined.[9] Consideration should also be given to optimization of the other reaction components. The number of fluorophores in each tube can influence the analysis. The use of a dark quencher might enhance the quality of multiplex PCR results. The following guidelines are useful for multiplex PCR.

PCR Primer Considerations for Multiplex PCR

- Design primer pairs with similar annealing temperatures for all targets to be amplified.
- To avoid duplex formation, analyze the sequences of primers and probes with primer analysis software.
- The limiting primer concentrations are the primer concentrations that result in the lowest fluorescence intensity without affecting the Ct. If the relative abundance of the two targets to be amplified is known, determine the limiting primer concentrations for the most abundant target. If the relative abundance of the two targets is unknown, determine the limiting primer concentrations for both targets. The limiting primer concentrations are determined by running 2-fold serial dilutions of those forward and reverse primer concentrations optimized for one-probe detection systems, but maintaining a constant target concentration. A range of primer concentrations of 20–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[9]

Magnesium Chloride Concentration in Multiplex PCR

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. Consider performing a titration to optimize the magnesium chloride concentration for multiplex PCR.

DNA Polymerase and dNTP Concentrations in Multiplex PCR

Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[10] The Brilliant QPCR core reagent kit has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase or dNTPs. If more than two targets are amplified and detected in a single reaction tube, optimal polymerase and dNTP concentrations may need to be determined empirically.

Probe Considerations for Multiplex PCR

A) Molecular Beacons

- Label each molecular beacon with a spectrally distinct fluorophore.[11]
- Design molecular beacons for different targets to have different stem sequences.

B) TaqMan Probes

Label each TaqMan probe with a spectrally distinct fluorophore.

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. Individual samples can then be prepared by aliquoting the master mix into individual tubes using a fresh pipet tip for each addition. 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.

PROTOCOL

Preparing the Reactions

Notes *Gently mix and spin each component in a microcentrifuge before use.*

Prepare all reactions on ice.

The preferred method for preparing reactions is making master mixes for the reactions by combining the desired multiple of each component listed in step 2. Individual samples can then be prepared by pipetting the master mixes into sterile thin-walled PCR tubes, PCR plates or 0.5-ml microcentrifuge tubes and then adding the DNA template. If using a spectrofluorometric thermal cycler, use PCR tubes and plates recommended by the manufacturer of the instrument.

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 or 1:50 depending on the detection instrument used. See guidelines in *Reference Dye* in the *Preprotocol Considerations* section.

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

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

   ### Reagent Mixture

   Nuclease-free PCR-grade water to adjust the final volume to 25 µl (including DNA)
   2.5 µl of 10× core PCR buffer
   x µl of 50 mM magnesium chloride (optimize for each primer-probe system)
   x µl of upstream primer
   x µl of downstream primer
   x µl of experimental probe
   1.0 µl of 20 mM dNTP mix
   0.375 µl of diluted reference dye (optional)
   0.25 µl of 5 U/µl SureStart *Taq* DNA polymerase

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

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

4. Centrifuge briefly and aliquot the master mixes into PCR reaction tubes or plates.

5. Add *x* µl of gDNA or cDNA to each reaction.

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

   Note *Bubbles interfere with fluorescence detection.*

7. Centrifuge the reactions briefly.

8. If the thermal cycler does not have a heated lid, carefully overlay each reaction with one drop of mineral oil to prevent evaporation and condensation during thermal cycling.

   Note *A mineral oil overlay slightly attenuates the fluorescence.*

PCR Cycling Programs

9. Place the reactions in a thermal cycler. For molecular beacons, either the three-step or two-step thermal-cycling program can be run. For TaqMan probes, run the two-step thermal-cycling program.

   Note *Increase cycle durations by 30 seconds when using the Stratagene RoboCycler temperature cycler*

Three-Step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	55°C[c]
	30 seconds	72°C

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

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

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

Two-Step Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	10 minutes[b]	95°C
40	15 seconds	95°C
	1 minute[c]	60°C[d]

[a] This two-step protocol is compatible with a number of molecular beacon detection systems, but it may not be optimal for all molecular beacons and primers.

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

[c] If applicable, set the temperature cycler to detect and report fluorescence during the annealing–extension step of each cycle.

[d] Choose an appropriate annealing temperature for the probe used.

TROUBLESHOOTING: MOLECULAR BEACONS

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The molecular beacon is not binding to the target efficiently because the loop portion is not completely complementary. Perform a melting curve analysis to determine if the probe binds to a perfectly complementary target.
	The molecular beacon is not binding to the target efficiently because the annealing temperature is too high. Perform a melting curve analysis to determine the optimal annealing temperature.
	The molecular beacon is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	The molecular beacon is not binding to the target efficiently because the magnesium concentration is too low. Perform a magnesium titration to optimize the concentration.
	Not enough or no specific product is formed during PCR. Verify product formation by gel electrophoresis.
	The molecular beacon has a nonfunctioning fluorophore. Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, resynthesize the molecular beacon.
	Resynthesize the molecular beacon using a different fluorophore.
	Redesign the molecular beacon.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Incorporate a contamination control system such as dUTP/UNG.
Ct reported for the no-target control sample (NTC) in experimental report is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

TROUBLESHOOTING: TAQMAN® PROBES

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The probe is not binding to the target efficiently because the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The probe is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	The probe is not binding to the target efficiently because the magnesium concentration is too low. Perform a magnesium titration to optimize the concentration.
	Not enough or no specific product is formed during PCR. Verify product formation by gel electrophoresis.
	The probe has a nonfunctioning fluorophore. Verify that the fluorophore functions by digesting the probe (100 nM probe in 25 µl 1× buffer with 10 U DNase or S1 nuclease) at room temperature for 30 minutes to confirm an increase in fluorescence following digestion.
	Redesign the probe.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Incorporate a contamination control system such as dUTP/UNG. We offer the Brilliant QPCR *Plus* Core Reagent Kit (Stratagene Catalog #600540) that includes the dUTP/UNG contamination control system.
Ct reported for the no-target control sample (NTC) in experimental report is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

APPENDIX: REAL-TIME PCR METHODS FOR QUANTITATING GENOMIC DNA

Relative Quantitation Using the Standard Curve Method

Note *Generate a separate standard curve for each target.*

1. Generate standards by preparing 10-fold serial dilutions of a gDNA sample of known quantity, starting with 100 ng.

2. Prepare PCR reactions using primers and molecular beacon or TaqMan probes for the experimental DNA target and for an endogenous control target.

3. Place the reactions in a spectrofluorometric thermal cycler and run the two-step or three-step thermal-cycling program given in the *Protocol*.

4. Calculate the Ct for each dilution of the standard and for the unknown experimental and endogenous control samples.

5. Generate standard curves for each target by plotting the Ct of each standard dilution against the logarithm of the initial amount of DNA. Calculate the regression line for each target.

6. Calculate the logarithms of the initial concentrations of both the experimental DNA target and the endogenous control target using the following formula:

   $$\log(\textit{initial target concentration}) = (Ct - b)/a$$

   where Ct = threshold cycle, b = y-intercept of the standard curve, and a = slope of the standard curve.

7. Divide the amount of experimental DNA by the amount of endogenous control to normalize the amount of experimental DNA.

Absolute Quantitation

The Brilliant QPCR core reagent kit can be used for absolute gene quantitation with both molecular beacons and TaqMan probes. See reference 13 for methods.

REFERENCES

1. Padmabandu, G., Grismer, L. and Mueller, R. (2000) *Strategies* 13(3):88-92.
2. Kostrikis, L. G., Tyagi, S., Mhlanga, M. M., Ho, D. D. and Kramer, F. R. (1998) *Science* 279(5354):1228-9.
3. Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P. et al. (1998) *Nat Biotechnol* 16(4):359-63.
4. Tyagi, S., Bratu, D. P. and Kramer, F. R. (1998) *Nat Biotechnol* 16(1):49-53.
5. Tyagi, S. and Kramer, F. R. (1996) *Nat Biotechnol* 14(3):303-8.
6. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology* (N Y) 11(9):1026-30.
7. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. (1991) *Proc Natl Acad Sci U S A* 88(16):7276-80.
8. 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.
9. McBride, L., Livak, K., Lucero, M., Goodsaid, F., Carlson, D. et al. (1998). Quantitative PCR Technology. In *Gene Quantification*, F. Ferré (Ed.), pp. 97-110. Birkhauser Boston Press, Boston.
10. Chamberlain, J. S. and Chamberlain, J. R. (1994). Optimization of Multiplex PCR. In *The Polymerase Chain Reaction*, K. B. Mullis, F. Ferré and R. A. Gibbs (Eds.), pp. 38-46. Birkhauser Boston Press, Boston.
11. Marras, S. A., Kramer, F. R. and Tyagi, S. (1999) *Genet Anal* 14(5-6):151-6.
12. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.
13. Freeman, W. M., Walker, S. J. and Vrana, K. E. (1999) *Biotechniques* 26(1):112-22, 124-5.

ENDNOTES

ABI PRISM® and Primer Express® are registered trademarks of The Perkin-Elmer Corporation.

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

STRATAGENE

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BRILLIANT QPCR CORE REAGENT KIT

Catalog #600530 and #929530

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 or 1:50 depending on the detection instrument used. See guidelines in *Reference Dye* in the *Preprotocol Considerations* section.

   Notes *The preferred method for preparing reactions is making master mixes by combining the desired multiple of each component listed in step 2. Prepare all reactions on ice and ensure components are mixed well prior to use.*

   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 reactions by adding the following components *in order*:

   Reagent Mixture

   Nuclease-free PCR-grade water to adjust the final volume to 25 µl (including DNA)
   2.5 µl of 10× core PCR buffer
   x µl of 50 mM magnesium chloride (optimize for each primer-probe system)
   x µl of upstream primer
   x µl of downstream primer
   x µl of experimental probe
   1.0 µl of 20 mM dNTP mix
   0.375 µl of diluted reference dye (optional)
   0.25 µl of 5 U/µl SureStart *Taq* DNA polymerase

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

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

4. Centrifuge briefly and aliquot the master mixes into PCR reaction tubes or plates.

5. Add x µl of gDNA or cDNA to each reaction.

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

7. Centrifuge the reactions briefly.

8. If the thermal cycler does not have a heated lid, carefully overlay each reaction with one drop of mineral oil to prevent evaporation and condensation during thermal cycling. **Mineral oil slightly attenuates the fluorescence.**

9. Place the reactions in a thermal cycler. For molecular beacons, either the three-step or two-step thermal-cycling program can be run. For TaqMan probes, run the two-step thermal-cycling program.

   Note *Increase cycle durations by 30 seconds when using the Stratagene RoboCycler temperature cycler.*

Three-Step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	55°C[c]
	30 seconds	72°C

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

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

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

Two-Step Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	10 minutes[b]	95°C
40	15 seconds	95°C
	1 minute[c]	60°C[d]

[a] This two-step protocol is compatible with a number of molecular beacon detection systems, but it may not be optimal for all molecular beacons and primers.

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

[c] If applicable, set the temperature cycler to detect and report fluorescence during the annealing–extension step of each cycle.

[d] Choose an appropriate annealing temperature for the probe used.