SideStep SYBR® Green QPCR Master Mix

INSTRUCTION MANUAL

Catalog #400904

Revision B.01

For In Vitro Use Only

400904-12

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SideStep SYBR® Green QPCR Master Mix

Contents

SideStep SYBR® Green QPCR Master Mix

MATERIALS PROVIDED

Catalog #400904

Materials provided	Quantity[a,b]
SideStep Lysis and Stabilization Buffer	10 ml
2× Brilliant SYBR® Green QPCR Master Mix	2 × 2.5 ml
Reference dye[c], 1 mM	100 µl

[a] Sufficient SideStep Lysis and Stabilization Buffer is provided for the preparation of 100 cell lysate samples.

[b] Sufficient PCR reagents are provided for 400 reactions (25 µl each).

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

STORAGE CONDITIONS

SideStep Lysis and Stabilization Buffer: Store at –20°C upon receipt. After thawing, store at 4°C.

Brilliant SYBR® Green QPCR Master Mix Kit: Upon receipt, store all components at –20°C. After thawing, store the 2× QPCR master mix at 4°C. Continue storing the reference dye at –20°C. 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
PBS, cold (see *Preparation of Media and Reagents*)
Microcentrifuge tubes

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Revision B.01

INTRODUCTION

The SideStep SYBR® Green QPCR Master Mix provides the reagents necessary to analyze mammalian cells by QPCR using a rapid protocol that skips the conventional nucleic acid purification steps.

Overview of SideStep Technology

Conventional QPCR experiments include nucleic acid isolation steps. The process of nucleic acid isolation is tedious and time-consuming, particularly when processing large numbers of samples. In addition, genomic DNA is subject to loss during isolation procedures, which is especially problematic when working with small samples and low-abundance targets.

Using SideStep technology,* you can skip the nucleic acid purification steps in your QPCR experiments, making analysis of a large number of samples much faster and simpler. The SideStep technology achieves cell lysis and nucleic acid stabilization in the same buffer, eliminating the need for nucleic acid purification. The simple lysate preparation protocol takes approximately 10 minutes to perform and includes a single PBS wash followed by cell lysis in the SideStep lysis and stabilization buffer. This buffer inactivates cellular nucleases and other enzymes, and the nucleic acids released into the buffer are stabilized and suitable for QPCR analysis for at least 20 months when stored at –80°C. The long-range stability of SideStep lysates offers the potential to perform multiple experiments using the same sample and to archive samples of interest for further analysis or nucleic acid isolation.

Overview of the Brilliant SYBR® Green QPCR Master Mix

Using the SideStep lysate as template, the Brilliant SYBR Green QPCR master mix is used for QPCR analysis of genomic DNA with SYBR Green dye-based detection.

The Brilliant SYBR Green QPCR master mix is a single-tube reagent, ideal for high-throughput QPCR applications. The Brilliant SYBR Green QPCR master mix includes the components necessary to carry out PCR amplification (primers and template are not included), 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 the AffinityScript QPCR cDNA Synthesis Kit.

The passive reference dye is provided in a separate tube, making the Brilliant master mix adaptable for many real-time QPCR platforms. The Brilliant SYBR Green QPCR master mix has been optimized for maximum performance on Stratagene Mx3000P, Mx3005P, and Mx4000 real-time PCR systems, as well as on the ABI PRISM® 7700 and GeneAmp® 5700 instruments. In addition, excellent results have been observed using most other QPCR platforms.

* Patent pending.

PROTOCOLS

Storage of SideStep Lysates and Dilutions

The SideStep system allows long-term storage of cell lysates. The **undiluted** lysates may be stored at 4°C for 1 month, at –20°C for 6 months, or at –80°C for 20 months. When dilution of SideStep lysates is necessary for use in downstream applications, dilute lysates in nuclease-free water and use immediately. Since nucleic acids are no longer stabilized after dilution of the SideStep buffer, do not store the lysate dilutions for future analysis.

Preparation of SideStep Cell Lysates

Note *The SideStep lysis and stabilization buffer may be used to prepare lysates from a variety of mammalian cell lines. Commonly used cell culture and harvesting methods are compatible with the SideStep buffer protocols, and those methods routinely used by your laboratory for the specific cell line should be employed.*

Cell Density Considerations

Lysates may be prepared with cell densities of up to 10^4 cell equivalents/µl of lysis buffer. When sufficient cultured cells are available, we recommend preparing the lysate at the maximum cell density (10^4 cells/µl) for maximum flexibility in downstream applications. The lysate may be diluted in SideStep buffer prior to addition to the QPCR reaction.

Cells are washed once in cold PBS in the protocol below. The density of the PBS suspension in step 4 will equal the final cell density of the lysate.

Prior to performing a large-scale experiment or screen using QPCR analysis of lysates, perform a pilot standard curve to determine the cell number range that gives linear amplification of the specific target under your specific reaction conditions.

Preparation of Lysates from Cultured Cells

1. Harvest the cultured cells, using a method appropriate to the properties of the cell line and the growth vessel.

   Note *If trypsin is used for cell harvesting, it must be inactivated before proceeding.*

2. Count the cells in an aliquot of the cell suspension.

3. Pellet the cells at 500–1,000 × *g* at 4°C for 5 minutes. Aspirate the supernatant.

4. Resuspend the pelleted cells in at least 100 µl of cold PBS to a final concentration of $\leq 10^4$ cells/µl.

   Note *The density of the PBS cell suspension will equal the final cell density in the lysate. We recommend using the maximum cell density of 10^4 cells/µl, for maximum flexibility in downstream applications.*

5. Place 100 µl of the cell suspension ($\leq 10^6$ cells) in a 1.5-ml microfuge tube.
6. Pellet the cells at 500–1,000 × *g* at 4°C for 5 minutes. Aspirate the supernatant. Remove as much of the PBS as possible without disturbing the pellet.
7. Add 100 µl of SideStep lysis and stabilization buffer to the cell pellet.
8. Vortex for 1 minute to lyse the cells.
9. Analyze the lysate by QPCR (see below for protocol) or store the lysate according to the following considerations. Nucleic acids in the lysate are stable at room temperature for 4 hours, at 4°C for 1 month, at –20°C for 6 months, and at –80°C for at least 20 months.

QPCR Analysis of SideStep Lysates

Determining the Amount of Lysate to Analyze

The amount of lysate added to the QPCR reaction depends on the experimental design (target abundance and the desired cell equivalents per reaction). Keep the following considerations in mind when planning QPCR analysis using SideStep lysates.

High concentrations of either cellular materials or lysis buffer may inhibit the QPCR reaction. The number of cell equivalents added to a 25-µl QPCR reaction should not exceed 100 and the total volume of **undiluted** lysate should not exceed 1 µl. However, for some cell lines, up to 200 cell equivalents may be used. Run a standard curve, analyzing serial dilutions of cell equivalents, to determine the maximum number of cells for your cell line Typically, lysates prepared at 10^4 cells/µl are serially diluted in water prior to addition to QPCR reactions The chart below illustrates options for addition of different cell number equivalents.

Desired Cell Equivalents	Cell Density of Undiluted Lysate		
	10^4 cells/µl	10^3 cells/µl	10^2 cells/µl
200	1 µl of 1:50 dilution	1 µl of 1:5 dilution	—
100	1 µl of 1:100 dilution	1 µl of 1:10 dilution	1 µl (undiluted lysate)
50	1 µl of 1:200 dilution	1 µl of 1:20 dilution	1 µl of 1:2 dilution
25	1 µl of 1:400 dilution	1 µl of 1:40 dilution	1 µl of 1:4 dilution
12.5	1 µl of 1:800 dilution	1 µl of 1:80 dilution	1 µl of 1:8 dilution
6.25	1 µl of 1:1,600 dilution	1 µl of 1:160 dilution	1 µl of 1:16 dilution
3.125	1 µl of 1:3,200 dilution	1 µl of 1:320 dilution	1 µl of 1:32 dilution

Note *Storage of diluted cell lysates is not recommended.*

The lower limit for SideStep lysate addition to QPCR reactions is determined by the abundance of the target and the sensitivity of the assay system used.

Preparing the QPCR Reactions

Notes *Once the tube containing the Brilliant SYBR Green QPCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.* ***SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.***

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

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI PRISM 7700 instrument or the GeneAmp 5700 instrument)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in the *Reference Dye* section found in *Appendix I*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM when using a 1:500 dilution or 300 nM when using a 1:50 dilution. **Keep all solutions containing the reference dye protected from light.**

   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 experimental reaction by adding the following components *in order*:

   ### Experimental Reaction

   Nuclease-free PCR-grade water to adjust the final volume to 25 µl (including cell lysate)
   12.5 µl of 2× master mix
   X µl of upstream primer (50–150 nM final concentration is recommended)
   X µl of downstream primer (50–150 nM final concentration is recommended)
   0.375 µl of diluted reference dye (optional, see step 1 for instructions)

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

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

4. Add 1 µl of SideStep cell lysate. See *Determining the Amount of Lysate to Analyze*, above, for guidelines.

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

   Note *Bubbles interfere with fluorescence detection.*

6. Centrifuge the reaction briefly.

PCR Cycling Programs

7. Place the reaction 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.

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

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]	55–60°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]	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 both the annealing step and the extension step of each cycle.

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

Dissociation Program

Mx3000P and Mx3005P Instruments

If using the 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.

APPENDIX I: QPCR ASSAY CONSIDERATIONS

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 you are using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, 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

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 assay sensitivity, use the lowest concentration of primers possible without compromising the PCR efficiency. 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.

* 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

The optimal $MgCl_2$ concentration promotes maximal amplification of the specific target amplicon with minimal non-specific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of non-specific dsDNA, including primer-dimers. Therefore, when a SYBR Green-based QPCR assay is being optimized, the $MgCl_2$ levels should be as low as possible, as long as the efficiency of amplification of the specific target is not compromised (typically between 1.5 and 2.5 mM $MgCl_2$). The Brilliant SYBR Green 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 at the time of set up.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[1] Because SYBR Green I dye fluoresces in the presence of any dsDNA, multiplexing in the presence of SYBR Green I dye is not recommended.

Preventing Template Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

dUTP is used instead of dTTP in the Brilliant SYBR Green QPCR master mix. When dUTP replaces dTTP in PCR amplification, UNG treatment (Uracil-N-glycosylase, not provided in this kit) can prevent the subsequent reamplification of dU-containing PCR products. UNG acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites. When this strategy is put to use, carry-over contamination will be eliminated while template DNA (DNA containing T) will be left intact.

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.

TROUBLESHOOTING

Amplification Plot

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling	The $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant SYBR Green QPCR master mix is 2.5 mM. It is possible to add small amounts of concentrated $MgCl_2$ to the experimental reactions to increase the $MgCl_2$ concentration, if desired.
	SureStart Taq DNA polymerase was not activated. Ensure that the 10 minute incubation at 95°C was performed as part of the cycling parameters.
	Ensure the correct dilution of reference dye was used.
	Optimize the primer concentration.
	The addition of too many cell-equivalents or too much lysis buffer to the QPCR reaction may be inhibitory. Prepare the lysates at $\leq 10^4$ cells/μl, and follow the guidelines for the upper limits of lysate addition to the QPCR reaction. If inhibition of QPCR by the addition of too many cell equivalents is suspected, the cell lysates may be diluted in additional SideStep buffer.
	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.

Dissociation Plot

Observation	Suggestion(s)
There is a large abundance of primer-dimer and non-specific PCR products	Increase the annealing temperature.
	Re-design the primers.
	For products <300 bp, increase extension temperature 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.

PREPARATION OF MEDIA AND REAGENTS

PBS (Phosphate Buffered Saline)

150 mM NaCl
20 mM Na_2HPO_4
adjust to pH 7.4 with HCl

REFERENCES

1. 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

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SIDESTEP SYBR® GREEN QPCR MASTER MIX

Catalog #400904

QUICK-REFERENCE PROTOCOL

Lysate Preparation from Cultured Cells

1. Harvest the cultured cells, using a method appropriate to the properties of the cell line and the growth vessel.

2. Count the cells in an aliquot of the cell suspension.

3. Pellet the cells at 500–1,000 × *g* at 4°C for 5 minutes. Aspirate the supernatant.

4. Resuspend the pelleted cells in at least 100 µl of cold PBS to a final concentration of $\leq 10^4$ cells/µl.

5. Place 100 µl of the cell suspension ($\leq 10^6$ cells) in a 1.5-ml microfuge tube.

6. Pellet the cells at 500–1,000 × *g* at 4°C for 5 minutes. Aspirate the supernatant. Remove as much of the PBS as possible without disturbing the pellet.

7. Add 100 µl of SideStep lysis and stabilization buffer to the cell pellet.

8. Vortex for 1 minute to lyse the cells.

9. Analyze the lysate by QPCR (see below) or store the lysate at room temperature for up to 4 hours, at 4°C for 1 month, at –20°C for 6 months, or at –80°C for at least 20 months.

QPCR Analysis

Note *This protocol has been optimized for Stratagene Mx3000P, Mx3005P, and Mx4000 instruments, the ABI PRISM 7700 instrument and the GeneAmp 5700 instrument. The protocol may be adapted for use with most other instruments by changing the reference dye dilution according to the guidelines in the manual and following the instrument manufacturer's recommendations for PCR cycling and dissociation programs.*

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx3000P, Mx3005P or Mx4000 instrument) or 1:50 (ABI PRISM 7700/GeneAmp 5700 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 SYBR Green QPCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

   Note *Multiple freeze-thaw cycles should be avoided.* ***SYBR Green I dye (present in the master mix) is light-sensitive, solutions containing the master mix should be protected from light whenever possible.***

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 cell lysate)
   12.5 µl of 2× master mix
   x µl of upstream primer (50–150 nM final concentration is recommended)
   x µl of downstream primer (50–150 nM final concentration is recommended)
   0.375 µl of **diluted** reference dye from step 1 (optional)

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

5. Add 1 µl of SideStep cell lysate. If needed, the lysate should be diluted in water to achieve the desired cell equivalents/µl prior to addition. Do not exceed 100 cell equivalents or 1 µl of undiluted SideStep lysate per 25 µl reaction.

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 PCR program below.

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

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

[a] For a PCR program for longer targets (400–900 bp), see the *Protocols* section of the manual.

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

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

Dissociation Programs

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.

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.

Other Instruments: Follow the manufacturer's guidelines for setting up dissociation curves.