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

Instruction Manual

Catalog #400909

Revision E

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400909-12

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

Contents

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MATERIALS PROVIDED

Catalog #400909

Materials Provided	Concentration	Quantity
SideStep II QPCR cDNA Synthesis Kit[a]		
SideStep Lysis & Stabilization Buffer	1×	10 ml
SideStep II Neutralization Buffer	10×	100 µl
SideStep II DNase I	10 U/µl	50 µl
SideStep II DNase Digestion Buffer	10×	100 µl
cDNA Synthesis Master Mix	2×	500 µl
AffinityScript RT/ RNase Block Enzyme Mixture	—	50 µl
Oligo(dT) primer	100 ng/µl	15 µg
Random primers (9-mers)	100 ng/µl	15 µg
RNase-free H_2O	—	1.2 ml
QPCR Normalization Primers, Set 1	45 µM (100×)	12.5 µl
QPCR Normalization Primers, Set 2	30 µM (100×)	12.5 µl
QPCR Normalization Primers, Set 3	45 µM (100×)	12.5 µl
Brilliant SYBR® Green QPCR Master Mix[b]		
Brilliant SYBR® Green QPCR Master Mix	2×	2 × 2.5 ml
Reference dye	1 mM	100 µl

[a] Sufficient reagents are provided for the preparation of 100 cell lysate samples (or 25 DNase-treated lysate samples) and 50 cDNA synthesis reactions (20-µl reaction volume). Each set of QPCR normalization primers provides for fifty, 25-µl QPCR reactions.

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

STORAGE CONDITIONS

SideStep II QPCR cDNA Synthesis Kit: Store all components at –20°C upon receipt. After thawing, store the SideStep lysis and stabilization buffer, the neutralization buffer, and the DNase digestion buffer at 4°C. Continue storing the remaining components at –20°C.

Brilliant SYBR® Green QPCR Master Mix: Store all components at –20°C upon receipt. After thawing, store the 2× QPCR master mix at 4°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

PBS, cold (see *Preparation of Media and Reagents*)
Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

Revision E

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INTRODUCTION

Quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The SideStep II SYBR® Green QRT-PCR Master Mix, 2-Step combines SideStep technology, for the rapid preparation of mammalian cell lysates, with AffinityScript reverse transcription reagents and Brilliant SYBR Green QPCR Master Mix. Together, these reagents provide a streamlined, fully optimized system for two-step QRT-PCR analysis of gene expression directly from mammalian cells.

The kit can be divided into two modules. The SideStep II QPCR cDNA Synthesis Kit provides reagents to rapidly lyse mammalian cells and prepare QPCR-ready cDNA that is free of genomic DNA. The cell lysis and DNA digestion protocols take approximately 25 minutes while the conversion of RNA to cDNA can be completed in another 25 minutes. The second module of the kit is the Brilliant SYBR Green QPCR master mix to quantify your cDNA of interest.

Overview of the SideStep II QPCR cDNA Synthesis Kit

SideStep Lysis and Stabilization Buffer

Using SideStep Lysis and Stabilization Buffer, you can skip the nucleic acid purification steps in your QRT-PCR 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 RNA purification. This buffer inactivates cellular nucleases and other enzymes, and the nucleic acids released into the buffer are stabilized and suitable for QRT-PCR 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 RNA isolation. RNA may be isolated from SideStep lysates using most standard RNA purification methods.

Reagents for DNase Treatment of SideStep Lysates

When using the double-stranded DNA-binding dye SYBR Green for detection in QPCR assays, the presence of genomic DNA in the cDNA samples may promote nonspecific amplification. The kit includes all the reagents necessary for DNase treatment of your SideStep lysates prior to cDNA synthesis. The SideStep II neutralization buffer and DNase digestion buffer permit activity of the RNase-free DNase I enzyme, and at the end of the 10-minute incubation, the DNase is inactivated by simply diluting the reactions in additional SideStep lysis buffer.

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 also contains $MgCl_2$ and dNTPs.

AffinityScript RT/RNase Block Enzyme Mixture

AffinityScript Multiple Temperature Reverse Transcriptase is a genetically engineered version of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and is highly thermostable, allowing you to reverse transcribe at your preferred reaction temperature. It has been tested 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. 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.

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 9-mers 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 Normalization Primer Sets

The QPCR Normalization Primers are pre-mixed primer sets that amplify distinct, single-copy regions of noncoding human genomic DNA. Because both RNA and DNA are stabilized in SideStep lysis buffer, the primers can be used in QPCR reactions to quantitate genomic DNA content. Quantitation of genomic DNA can be useful for analysis of SideStep lysates in the following applications.

A) Normalizing QRT-PCR Gene Expression Data

When using QRT-PCR to compare gene expression levels across multiple SideStep lysates, quantitation of an endogenous control helps correct for any variations in cell density among lysate preparations. With the QPCR normalization primer sets, you can use a genomic DNA target as this endogenous control. Genomic DNA content is constant from cell to cell regardless of experimental conditions, providing a stable baseline for normalization of your QRT-PCR gene expression data. A QPCR protocol using the primers and formulas for normalizing your QRT-PCR results to genomic DNA content are provided in *Appendix II*.

B) Determining Cell Concentration of Lysate Preparations

As described in the lysate preparation protocol, before cells are lysed, a cell count should be performed to determine the number of cell equivalents/µl of lysis buffer. However, as long as the number of cells does not exceed the maximum of 10^4/µl, the density can be determined after lysate preparation is complete, allowing you to skip the cell count step. Simply use the QPCR normalization primers to generate a standard curve with one lysate preparation of the same cell type and of known cell density. The density of the unknown lysates can then be determined through QPCR. This application is particularly useful when preparing lysates from a large number of cultures of the same cell type. See *Appendix III* for a protocol.

Locations of QPCR Normalization Targets

Although DNA copy number should be invariable in diploid cells, immortalized or tumor-derived cell lines may carry genomic deletions or duplications that alter the DNA copy number in localized regions of the genome. For this reason, the SideStep II QPCR cDNA synthesis kit provides three sets of QPCR normalization primers, each covering a different human chromosome, so you can determine which primer set works best with your cells. All three primers sets have been successfully tested with DNA from multiple human cell lines. The table below lists the product size and chromosome for each primer set.

Primer Set	Chromosome	Product size
# 1	9	233 bp
# 2	20	244 bp
# 3	15	273 bp

Overview of the Brilliant SYBR® Green QPCR Master Mix

The Brilliant SYBR Green QPCR master mix is a single-tube reagent, ideal for most high-throughput QPCR applications. In addition to SYBR Green, the 2× master mix also includes SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR performance by decreasing background and increasing amplification of desired products. The master mix also contains an optimized QPCR buffer, dNTPs, and $MgCl_2$.

The passive reference dye (with excitation and emission wavelengths of 584 nm and 612 nm, respectively) is provided as an optional reagent that may be added to compensate for non-PCR related variations in fluorescence. See *Use of the Passive Reference Dye* in *Appendix I* for more information.

The Brilliant SYBR Green QPCR master mix has been optimized for maximum performance on the Agilent Mx3000P, Mx3005P, and Mx4000 instruments, as well as on the ABI PRISM® 7700 instrument. In addition, excellent results have been observed using most other QPCR platforms.

PREPROTOCOL CONSIDERATIONS

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 lysis buffer, do not store the lysate dilutions for future analysis.

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.

Primer Selection

The optimum primer type [oligo(dT) or random primer] for cDNA priming varies for different targets and should be determined empirically with each target. Agilent's QPCR Reference Total RNA (available separately) can be used for this step. 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.

Another option for cDNA synthesis priming is to design a gene-specific primer that anneals to a particular mRNA transcript of interest rather than the entire mRNA population. For gene-specific primers, a concentration of 5 ng/µl in the first-strand reaction is generally recommended, but primer specificity may be improved by optimizing the concentration and reaction temperature.

Performing No-RT Control Reactions

We recommend performing *no-RT control* reactions for each lysate sample by omitting AffinityScript reverse transcriptase from the reaction. The *no-RT control* is expected to generate no signal in subsequent QPCR if there is no amplification of genomic DNA.

PROTOCOLS

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.

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 QRT-PCR 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.*

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. Proceed to DNase treatment or store the lysate at room temperature for up to 4 hours, at 4°C for 1 month, or at –20°C for 6 months.

DNase Treatment Protocol

Note *The protocol below is for DNase treatment of 4 µl of SideStep lysate, containing up to 4 × 10^4 cell equivalents. This protocol may be scaled up using the following guidelines:*

- Use equal volumes of SideStep lysate and neutralization buffer
- Adjust the volume of the final DNase treatment reaction with water to 10× the original SideStep lysate volume
- Use 1 µl of DNase I per 10^5 cell equivalents

1. Mix the SideStep lysate and transfer 4 µl to a 1.5 ml tube.

   Note *Reserve a portion of the untreated lysate for use in QPCR reactions with the QPCR normalization primers.*

2. Add 4 µl of SideStep II neutralization buffer to the aliquot of lysate.

3. Add 4 µl of 10× DNase digestion buffer.

4. Add 27.6 µl of RNase-free water.

5. Add 0.4 µl of DNase I.

   Notes *Do not vortex the DNase I enzyme solution or cell lysate mixtures containing DNase I.*

   The amount of DNase I may be increased to up to 0.8 µl if additional DNase digestion is required. Do not increase the digestion reaction time in step 8.

6. Mix gently (no vortexing).

7. Incubate the mixture at 37°C for 10 minutes.

8. Add 60 µl of RNase-free water and 300 µl of SideStep lysis buffer to bring the final volume to 400 µl. The additional SideStep lysis buffer serves to inactivate the DNase and stabilize the nucleic acids in the lysate.

9. The density of the cell lysate has now been diluted 1:100. DNase-treated lysates may be stored at 4°C for 1 month or at –20°C for 6 months. If further dilutions are needed for QRT-PCR amplification, dilute the lysate in water just before use.

cDNA Synthesis from SideStep Lysates

Amount of Lysate to Use as Template

For first-strand cDNA synthesis, the total number of cell equivalents added to a 20-µl reaction should not exceed 100 and the volume of **undiluted** lysate added to the reaction should not exceed 1 µl. The addition of more than 100 cell equivalents or more than 1 µl of 1× SideStep lysis buffer may inhibit reverse transcription. Additionally, a 25-µl QPCR reaction should contain no more than 2 µl of the cDNA synthesis reaction, or 10 cell equivalents. (This is a general guideline; for some cell lines, up to 200 cell equivalents may be added to the cDNA synthesis reaction without inhibiting reverse transcription and, subsequently, the QPCR reaction can then accommodate up to 20 cell equivalents. Analyze serial dilutions of your cDNA samples to determine the maximum number of cells that may be added to the first-strand synthesis reaction.)

Example *If lysates are prepared at 10^4 cell equivalents/µl, the following protocol may be used. After the DNase treatment protocol, the lysate will contain 100 cell equivalents/µl in 0.75× SideStep lysis buffer. Add 1 µl of the DNase-treated lysate to the 20-µl cDNA synthesis reaction. In this example, the cDNA synthesis reaction will contain 100 cell equivalents. The 25-µl QPCR reaction, containing 2 µl of the cDNA synthesis reaction, will then contain 10 cell equivalents. When running a standard curve, make 2-fold serial dilutions of the cDNA synthesis reaction such that cell equivalents of 10, 5, 2.5 and 1.25 are analyzed.*

cDNA Synthesis Protocol

Notes *Before use, mix each reaction component and then spin the components in a microcentrifuge.*

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

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

   RNase-free H_2O to achieve a final reaction 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 DNase-treated lysate or lysate dilution
   (≤100 cell equivalents)

   Notes *If required, dilute the lysate in RNase-free H_2O just prior to addition to the cDNA synthesis reaction. Do not add more than 1 µl of* ***undiluted*** *lysate to the reaction.*

   The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically. See the Primer Selection *section 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.*

4. Incubate the reaction at 95ºC for 5 minutes to terminate cDNA synthesis.

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 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)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in *Use of the Passive Reference Dye* found in *Appendix I*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the 1:500 dilution and 300 nM for the 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 cDNA, added in step 4)
   12.5 µl of 2× SYBR Green QPCR master mix
   X µl of upstream primer (optimized concentration)
   X µl of downstream primer (optimized concentration)
   0.375 µl of diluted reference dye (optional)

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

   The optimal primer concentration (generally 50–150 nM final concentration) must be experimentally determined for each target.

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

4. Add 2 µl of the cDNA synthesis reaction.

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 an Agilent 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.

Normalizing Variations in Nucleic Acid Concentration

Individual preparations of SideStep cell lysates can vary slightly in cell concentration. In order to accurately compare gene transcript levels in cDNA samples prepared from different lysate preparations, these variations need to be normalized. The QPCR normalization primers enable you to use the genomic DNA present in your SideStep lysates as a reference target in QPCR normalization reactions. See *Appendix III* for a protocol.

Although QPCR with the normalization primers is useful for normalizing variations in nucleic acid concentration caused by differences in cell density among lysates, other sources of variation may exist in the final cDNA samples. Therefore, in addition to running QPCR reactions on your SideStep lysates to amplify genomic DNA, it may also be useful to perform QRT-PCR with an endogenous RNA control, such as a housekeeping gene transcript whose expression is not affected by your experimental conditions.

APPENDIX I: QPCR ASSAY CONSIDERATIONS

Optimizing QPCR Assays

Prior to performing a large-scale experiment using QRT-PCR analysis of lysates, the assay should be optimized for the specific target and primer system using purified RNA. Important optimization parameters include primer design and concentrations and PCR cycling conditions. Agilent's QPCR Reference Total RNA, purchased separately, provides an ideal source of RNA for assay optimization (human reference RNA, Catalog #750500 and mouse reference RNA, Catalog #750600).

Primer Design and Optimal Concentrations

Design QPCR primers to generate amplicons of ≤150 bp and, when possible, avoid regions of secondary structure in the mRNA.

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 50–150 nM.

Use of 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 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 an Agilent Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If you are 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.

Magnesium Chloride Concentration

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 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 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.

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.

* 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.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction. 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 fluorescence data at both the annealing and extension steps of each cycle. Consult the manufacturer's instruction manual for the instrument/software version you are using for data acquisition guidelines.

APPENDIX II: QPCR WITH THE QPCR NORMALIZATION PRIMERS

The QPCR normalization primers allow you to amplify a single-copy genomic DNA target from your SideStep lysates, providing a means for detecting and normalizing differences in cell concentration in human cell lysate preparations. SYBR Green should be used for detection in the QPCR reactions, but quantitation of your cDNA target of interest can be performed with the protocol of your choice.

Selecting a QPCR Normalization Primer Set

Three sets of QPCR normalization primers are provided. If the genomic integrity of the cells is unknown, determine empirically by QPCR which primer sets amplify an intact region of the genome using the approach outlined below. If your cell lines do not carry any chromosomal lesions, use primer set #1 for your QPCR normalization reactions, but continue with the procedure below in order to determine the amplification efficiency of primer set #1 with your lysates.

1. Select a representative SideStep lysate sample for each of your cell lines under investigation. All cell lines that will be included in your studies need to be analyzed.

2. Prepare serial dilutions of the representative lysate samples. At least four 2-fold serial dilutions are needed to generate a standard curve. A good range of lysate concentrations to test is: 100, 50, 25, and 12.5 cell equivalents per µl.

3. Perform QPCR on your diluted lysates with all three primer sets. (Refer to the next section, *QPCR Protocol for Normalization Primers*.)

4. Analyze the standard curves. Select the primer set that yields the most consistent results from sample to sample. Eliminate any primer sets that fail to produce a product in one or more cell lines. Record the amplification efficiency of the optimal primer set for use in the normalization calculations.

QPCR Protocol for Normalization Primers

Notes *The following protocol uses the Brilliant SYBR Green QPCR master mix (provided in this kit and sold separately as catalog #600548), but this protocol could be adapted for use with other QPCR reagents.*

Because the QPCR reactions with your cDNA of interest may use a different PCR cycling program than that required for the QPCR normalization primers, the normalization reactions may need to be run on a separate plate. Multiplex PCR with the normalization primers is not recommended.

1. Complete the SideStep lysate preparation protocol as described in the *Protocols* section. If DNase treatment of your lysate is necessary, reserve an untreated portion for use in this protocol.

2. 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 Mx3000P, Mx3005P or Mx4000 instruments, the use of the reference dye may be required for optimal results.*

3. If necessary, dilute SideStep lysate with nuclease-free water to a density ≤100 cell equivalents/µl.

   Note *Before pipetting the cell lysate, always ensure the sample is well mixed. Vortex lysate briefly and do not centrifuge prior to removing an aliquot.*

4. Prepare the reaction by adding the following components *in order*. Each reaction should be set up in triplicate.

   10.875 µl of Nuclease-free PCR-grade water
   12.5 µl of 2× Brilliant SYBR Green QPCR Master Mix
   0.25 µl of selected normalization primer set
   0.375 µl of diluted reference dye (optional)

   Note *Although the three QPCR normalization primer sets are provided in different molar concentrations, they are all 100× stocks.*

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

6. Vortex the lysate or lysate dilution briefly and then add 1 µl of the lysate sample to the reaction mixture.

   Note *Use your original cell lysates as template. Do not use lysates converted to cDNA or DNase-treated lysates.*

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

PCR Cycling Program

8. Centrifuge the reaction briefly. Place the reaction in the instrument and run the PCR program below. Set the temperature cycler to detect and report fluorescence during both the annealing step and the extension step of each cycle. At the end of the program, generate a dissociation curve to verify that the QPCR normalization primers have amplified a single product.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	30 seconds	95°C
	1.0 minute	60°C
	30 seconds	72°C

Example of Normalization Calculations and Formulas

If the reactions were ran on a separate plate and your QPCR instrument software does not allow multi-plate analysis, you can normalize your QRT-PCR gene expression data to the normalization results using the formulas described in the following example. The amplification efficiencies of both the normalization target and your target of interest need to be determined with a standard curve before completing the normalization calculations.

In this example experiment, SideStep lysates were prepared from control cells and from an otherwise identical culture of cells exposed to an experimental treatment to determine if expression of a gene of interest (GOI) is affected by this treatment. A portion of each lysate sample was used for QPCR with a normalization primer set and another portion was processed separately for QRT-PCR reactions to quantitate the GOI mRNA. The Ct data for the two targets are displayed in the table.

	Ct_{GOI}	Ct_{NORM}
Control Cells	31.00	25.95
Treated Cells	28.87	23.79

Note *Consider exporting your Ct data to a spreadsheet application with mathematical function capabilities, such as Microsoft® Excel.*

Normalization is based on the Ct data for the two sets of reactions: the QPCR normalization genomic DNA reactions (Ct_{NORM}) and the GOI reactions (Ct_{GOI}). First, the ΔCt value is calculated for each target:

ΔCt_{GOI} = (Ct_{GOI} from control cells) – (Ct_{GOI} from treated cells)

In this example: $\Delta Ct_{GOI} = 31.00 - 28.87 = 2.13$

ΔCt_{NORM} = (Ct_{NORM} from control cells) – (Ct_{NORM} from treated cells)

In this example: $\Delta Ct_{NORM} = 25.95 - 23.79 = 2.16$

From the ΔCt values, the fold change in GOI expression is found using the Pfaffl formula shown below.[3] The term *Eff* in the equation represents PCR amplification efficiency. In this example, the amplification efficiency is 100% for the GOI and 84.3% for the normalization product. In your own experiments, the amplification efficiency needs to be empirically determined for each target.

$$\text{Fold change of GOI} = \frac{(1 + \text{Eff}_{\text{GOI}})^{\Delta\text{Ct}_{\text{GOI}}}}{(1 + \text{Eff}_{\text{NORM}})^{\Delta\text{Ct}_{\text{NORM}}}}$$

In this example:

$$\text{Fold change of GOI} = \frac{(1+1)^{2.13}}{(1+0.843)^{2.16}} = \frac{4.38}{3.75} = 1.17$$

Fold change is the ratio of the quantity of template in an experimental sample, or unknown, relative to a control sample, or calibrator. Use the guidelines illustrated here to apply this formula to your own QRT-PCR gene expression experiments.

APPENDIX III: DETERMINING CELL DENSITY OF SIDESTEP LYSATES

When preparing multiple cell lysate samples of the same human cell type using SideStep lysis buffer, it may be more convenient to determine the precise cell density of the samples after preparation is complete rather than counting the cells from an aliquot of each cell suspension during the lysate preparation protocol (provided the density of cells does not exceed 10^4/µl). Evaluation of cell density can be performed using the QPCR normalization primers and QPCR. Simply use the primers to generate a standard curve with one lysate preparation of known cell density and the density of lysates of the same cell type can be determined with a QPCR reaction.

Preparing a Reference Lysate of Known Cell Density

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. Repeat with 2 more aliquots and calculate the average of the three counts. It is critical that the cell count be as accurate as possible.

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 10^4 cells/µl.

   Note *The density of the PBS cell suspension will equal the final cell density in the lysate.*

5. Place 100 µl of the cell suspension (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. This sample will serve as the standard for determining cell density of any lysate preparations of the same cell type. Store the lysate at 4°C for 1 month or at –20°C for 6 months. Avoid repeated freeze-thaw cycles to maintain maximum stability of the genomic DNA.

QPCR Reactions with Lysates of Known and Unknown Density

Notes *The following protocol uses the Brilliant SYBR Green QPCR Master Mix (provided in this kit and sold separately as catalog #600548), but this protocol could be adapted for use with other QPCR reagents.*

Select one of the three sets of QPCR normalization primer sets for use in this protocol. See Selecting a QPCR Normalization Primer Set *in* Appendix II *for instructions.*

When amplifying genomic DNA from SideStep lysates with the QPCR normalization primers, we recommend using lysates that have undergone no more than two freeze-thaw cycles. Ensure SideStep lysates are well-mixed before pipetting. Vortex lysate briefly and do not spin down prior to removing an aliquot.

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)** using nuclease-free PCR-grade H_2O. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx4000 instrument and 300 nM for the ABI PRISM 7700 instrument. **Keep all solutions containing the reference dye protected from light.**

   Thaw the 2× QPCR master mix and store the solution on ice. Gently mix by inversion prior to pipetting.

   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 dilutions of the reference lysate for QPCR. Dilute the reference lysate in nuclease-free water to prepare a set of 2-fold serial dilutions of the following concentrations: 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 cell equivalents/µl. These samples will be the lysates added to the QPCR reactions to generate a standard curve. Making a 1:25 dilution of the original reference lysate will generate a lysate with a density of 400 cell equivalents/µl.

   Note *Consider preparing an additional set of dilutions using purified human genomic DNA to be ran on the same QPCR plate. The result is two standard curves: one curve relates Ct value to number of cell equivalents and the other relates Ct value to ng of human genomic DNA. These curves can then be used to correlate number of cell equivalents to DNA mass. Then, if a new standard curve is needed in the future for the same cell line, the purified human genomic DNA can be used as template instead of preparing a fresh lysate sample of known cell density. Convert ng of DNA to cell equivalents to generate a standard curve that relates Ct to number of cell equivalents.*

3. Prepare dilutions of lysates of unknown density for QPCR. Dilute SideStep lysates of unknown density in nuclease-free water in order to generate samples that contain somewhere between 10 to 100 cell equivalents/µl. For each lysate sample under investigation, at least one dilution needs to be tested to verify cell density, but using 2 or more dilutions could improve the accuracy of the density determination.

4. Prepare the QPCR reactions by combining the following components *in order*. We recommend preparing a single reagent mixture for all of the reactions (in triplicate), plus one reaction volume excess, using multiples of each component listed below.

   10.875 µl of Nuclease-free PCR-grade water
   12.5 µl of 2× Brilliant SYBR Green QPCR Master Mix
   0.25 µl of selected normalization primer set
   0.375 µl of diluted reference dye (optional)

5. Gently mix the reagent mixture without creating bubbles (do not vortex), then distribute the mixture to the reaction tubes.

6. Add 1 µl of diluted lysate to each reaction. Triplicate reactions should be set up for each of the reference lysate dilutions and the dilutions of the lysates of unknown density.

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

   Note *Bubbles interfere with fluorescence detection.*

8. Centrifuge the reactions briefly.

9. Place the reactions in the instrument and run the PCR program below. Set the temperature cycler to detect and report fluorescence during both the annealing step and the extension step of each cycle. After cycling, generate a dissociation curve to verify specificity of amplification.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	30 seconds	95°C
	1.0 minute	60°C
	30 seconds	72°C

10. With the QPCR instrumentation software, use the fluorescence values collected from the reference lysate dilutions to generate a standard curve that relates Ct to the number of cell equivalents added to the reaction. A good standard curve should have an R^2-value between 0.980 and 1.000 and a slope between –3.5 and –3.2.

11. Compare the Ct values for the unknown lysate samples to the standard curve to determine the number of cell equivalents that were in the reaction.

TROUBLESHOOTING

Observation	Suggestion
No or low yield of first-strand cDNA	RNA may have been degraded prior to the addition of SideStep lysis buffer. To prevent degradation, ensure that cold PBS is used to wash the cells, and that the cells are kept on ice prior to lysis buffer addition.
	Cells should be harvested during active growth on fresh media for optimal RNA expression.
	Optimize the reaction using Agilent's QPCR Reference Total RNAs, which can then be used as a calibrator for subsequent experiments.
	Increase the incubation time of the cDNA synthesis reaction to 45 minutes for long targets.
	Increase the incubation temperature of the cDNA synthesis reaction from 42°C to 55°C for secondary structure-rich targets.
	The addition of too many cell-equivalents to the cDNA synthesis reaction may be inhibitory. Prepare the lysates at $\leq 10^4$ cells/µl and follow the guidelines in *Amount of Lysate to Use as Template* in the *Protocols* section.
	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.
No or low yield of amplification product in QPCR	See the discussion under *No or low yield of first-strand cDNA* for suggestions related to insufficient first strand synthesis.
	Optimize the QPCR primer concentration, annealing temperature, and/or extension time, varying each individually and in increments. Agilent's 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 for the length of the amplicon.
	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 at 95°C in order to activate SureStart *Taq* DNA polymerase.
	Verify the correct dilution of reference dye was used, based on the type of QPCR instrument.
	The $MgCl_2$ concentration may not be optimal. The $MgCl_2$ concentration in the 1× Brilliant 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.
An abundance of primer-dimer and non-specific PCR products are observed in the dissociation curve	Increase the annealing temperature.
	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.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 4 to minimize contamination.
	Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See *Preventing Template Cross-Contamination* in *Appendix I*.
All three sets of QPCR normalization primers fail to amplify a product.	Ensure the lysate sample is well mixed before adding it to the QPCR reaction. Vortexing the lysate just prior to addition to the reaction is recommended. Do not centrifuge the lysate before removing an aliquot.
	The primers recognize human genomic DNA. Verify the lysate is made from a human cell line.

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

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3. Pfaffl, M. W. (2001) *Nucleic Acids Res.* 29(9):e45.
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ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

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

Catalog #400909

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 and vortex for 1 minute to lyse the cells.

8. Treat lysate with DNase or store at room temperature for 4 hours, at 4°C for 1 month, at –20°C for 6 months, or at –80°C for 20 months.

DNase Treatment of Lysates

1. Add the following components to a 1.5 ml microcentrifuge tube *in order:*
   4.0 µl of SideStep lysate (up to 4 × 10^4 cell equivalents)
   4.0 µl of 10× neutralization buffer
   4.0 µl of 10× DNase digestion buffer
   27.6 µl of RNase-free H_2O
   0.4 µl of DNase I

2. Mix gently (no vortexing).

3. Incubate the mixture at 37°C for 10 minutes.

4. Add 60 µl RNase-free water and 300 µl SideStep lysis buffer to bring final volume to 400 µl.

5. Proceed to cDNA synthesis or store the lysate as recommended in Step 8 above.

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 DNase-treated lysate or lysate dilution (≤100 cell equivalents)
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.
4. Incubate the reaction at 95°C for 5 minutes to terminate cDNA synthesis.
5. Place the reaction on ice for immediate use in QPCR or at –20°C for long-term storage.

QPCR Reaction

1. If the reference dye will be included (optional), dilute 1:500 (Agilent Mx instruments) or 1:50 (ABI PRISM 7700 instrument). Keep all solutions containing the reference dye protected from light. *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. Keep the master mix (containing SYBR Green I dye) protected from light.
3. Prepare the 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× SYBR Green QPCR 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 (**do not vortex**).
5. Add 2 µl of cDNA and gently mix without creating bubbles (**do not vortex**).
6. Centrifuge the reaction briefly, then place the reaction in the instrument and run the PCR program below. This program is suitable for targets 50–400 bp in length.

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] Collect fluorescence data during both the annealing step and the extension step of each cycle.
[b] Choose an appropriate annealing temperature for the primer set used.

7. Following amplification, perform a dissociation curve, according to the type of QPCR instrument used. See *Dissociation Program* in the *Protocols* section of the main manual for more information.