SideStep II QRT-PCR Master Mix Kit, 1-Step

Instruction Manual

Catalog #400917

Revision E

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SideStep II QRT-PCR Master Mix Kit, 1-Step

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MATERIALS PROVIDED

Catalog # 400917

Materials provided	Concentration	Quantity
SideStep II Cell Lysis Analysis Kit[a]		
SideStep Lysis and 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
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 II QRT-PCR Master Mix Kit, 1-Step[b]		
2× Brilliant II QRT-PCR Master Mix	2×	2 × 2.5 ml
RT/RNase Block Enzyme Mixture	—	400 µl
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). Each set of QPCR normalization primers provides for fifty, 25-µl QPCR reactions.

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

STORAGE CONDITIONS

SideStep II Cell Lysis Analysis Kit: Upon receipt, store all components at –20°C. After thawing, store the SideStep lysis and stabilization buffer, the neutralization buffer, and the DNase digestion buffer at 4°C. Continue storing the DNase I and QPCR normalization primer sets at –20°C.

Brilliant II QRT-PCR Master Mix Kit: Upon receipt, store all components at –20°C. After thawing, store the 2× QRT-PCR master mix at 4°C. Continue storing the RT/RNase block mixture and the reference dye at –20°C. Once thawed, full activity is guaranteed for 6 months.

Note *The reference dye is 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

Revision E

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INTRODUCTION

Quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The SideStep II QRT-PCR Master Mix Kit, 1-Step, is composed of two modules that, when used in combination, provide all the reagents necessary to analyze gene expression in mammalian cells by QRT-PCR. With the reagents in the SideStep II Cell Lysis Analysis Kit, mammalian cell lysates are prepared using a rapid protocol that skips the conventional nucleic acid purification steps. The lysates are then ready for gene expression analysis with the Brilliant II QRT-PCR Master Mix Kit, 1-Step for probe-based detection protocols.

Overview of the SideStep II Cell Lysis Analysis Kit

SideStep Lysis and Stabilization Buffer

Conventional QRT-PCR experiments include RNA isolation steps in order to protect the mRNA of interest from degradation by RNases and to remove inhibitors of reverse transcriptase from the sample. The process of RNA isolation is tedious and time-consuming, particularly when processing large numbers of samples. In addition, RNA is subject to loss during isolation procedures, which is especially problematic when working with small samples and low-abundance RNAs.

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. 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 buffer. 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

Removal of genomic DNA from your SideStep lysates is helpful for certain downstream applications, so the SideStep II cell lysis analysis kit includes all the reagents necessary for DNase treatment. The SideStep II neutralization buffer and DNase digestion buffer permit activity of the RNase-free DNase I enzyme. At the end of the 10-minute incubation, the DNase is inactivated by simply diluting the reactions in additional SideStep buffer. Refer to *Appendix I* for a complete protocol.

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 buffer, the QPCR normalization 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 III*.

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 IV* for a protocol.

Locations of 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 II QRT-PCR Master Mix Kit, 1-Step

Using the SideStep lysate as template, the Brilliant II QRT-PCR master mix is used for one-step QRT-PCR analysis of cellular RNAs with probe-based detection. Using this kit, cDNA synthesis and PCR amplification are carried out in one tube and one buffer. The kit supports quantitative amplification and detection and shows consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The Brilliant II QRT-PCR master mix kit has been successfully used to amplify and detect a variety of high- and low-abundance RNA targets from mammalian cell lysates prepared using the SideStep lysis and stabilization buffer.

The Brilliant II QRT-PCR master mix kit has been optimized for maximum performance on the Agilent Mx3000P and Mx3005P real-time PCR systems and the Agilent 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.

Components of the Brilliant II QRT-PCR Master Mix Kit

Brilliant II QRT-PCR 2× Master Mix

The 2× master mix contains an optimized RT-PCR buffer, $MgCl_2$, nucleotides (GAUC), stabilizers, and SureStart *Taq* DNA polymerase. SureStart *Taq* DNA polymerase is a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR performance 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.

RT/RNase Block Enzyme Mixture

Reverse transcriptase (RT) is provided, in combination with RNase block, in a separate tube so that *no-RT control* reactions may be included in the QRT-PCR experiments. The reverse transcriptase provided in the kit is a Moloney-based RT specifically formulated for the Agilent Brilliant II kits. This RT performs optimally at a reaction temperature of 50°C when used in 1-step QRT-PCR with the Brilliant II master mix. It is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis and to ensure sensitive and reproducible performance in QRT-PCR experiments with a broad range of RNA template amounts and a variety of RNA targets that vary in size, abundance, and GC-content. The RNase block, provided in the same tube, serves as a safeguard against contaminating RNases.

Reference Dye

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.

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

cDNA Synthesis Reaction Considerations

Performing No-RT Control Reactions

We recommend performing *no-RT control* reactions for each experimental sample by omitting reverse transcriptase from the reaction. The *no-RT control* is expected to generate no signal if there is no amplification of genomic DNA. No signal indicates that the primers are specific for the cDNA.

Incubation Temperature and Duration

For cDNA synthesis, we recommend a 50°C incubation for most targets using the Brilliant II QRT-PCR master mix kit. However, incubation at up to 55°C can be employed to reduce secondary structures or to improve specificity. A 30-minute incubation for the first-strand synthesis reaction is sufficient for most targets. Rare RNA sequences or long amplicons may benefit from an extended incubation time (up to 60 minutes) at a lower temperature (42°C).

Reverse Transcriptase Inhibition of PCR

Reverse transcriptase can inhibit subsequent PCR and must be heat-inactivated in the first thermal cycle of PCR. Do not exceed the recommended amount of RT/RNase block, as heat inactivation becomes difficult. For low abundance RNA targets, increasing the incubation time for first-strand synthesis may be beneficial, but do not increase the amount of RT/RNase block.

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, prepare the lysate at the maximum cell density (10^4 cells/µl) for maximum flexibility in downstream applications. The lysate may be diluted in nuclease-free water just prior to addition to the QRT-PCR 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 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 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 RT-PCR (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 20 months.

One-Step QRT-PCR Analysis of SideStep Lysates

Optional DNase Treatment

It is not necessary to treat lysates with DNase if the QRT-PCR primers are designed to avoid amplification of genomic DNA (see *Primer Design and Optimal Concentration* in *Appendix I*). If your experimental design requires the use of primers that could amplify genomic DNA, lysates may be treated with DNase prior to the QRT-PCR reaction using the provided reagents and the protocol outlined in *Appendix II*.

Determining the Amount of Lysate to Analyze

The amount of lysate added to the QRT-PCR reaction depends on the experimental design (target abundance and the desired cell equivalents per reaction). Keep the following considerations in mind when planning one-step QRT-PCR 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 QRT-PCR 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 one-step QRT-PCR 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 QRT-PCR reactions is determined by the abundance of the target and the sensitivity of the assay system used.

Preparing the One-Step QRT-PCR Reactions

Notes *Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.*

Always include a no-template control reaction to screen for contamination of reagents or false amplification.

Include a no-RT control to verify that the fluorescence signal is due to the amplification of cDNA and not of genomic DNA. This control is especially important if DNase treatment is omitted.

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. Thaw the 2× Brilliant II QRT-PCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.

3. Prepare the experimental reactions by combining the following components *in order*. We recommend preparing a single reagent mixture for all your duplicate experimental reactions and duplicate no-template-controls (plus at least one reaction volume excess), by scaling up each of component listed below.

Reagent Mixture

Nuclease-free PCR-grade H_2O to achieve a final volume of 25 µl (including cell lysate)
12.5 µl of 2× Brilliant II QRT-PCR master mix
X µl of experimental probe (optimized concentration)
X µl of upstream primer (optimized concentration)
X µl of downstream primer (optimized concentration)
0.375 µl of the **diluted** reference dye (optional)
1.0 µl of RT/RNase block enzyme mixture

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

4. Gently mix the components without creating bubbles (do not vortex), then distribute the mixture to the experimental reaction tubes.

5. Add X µl of SideStep cell lysate or lysate dilution (up to 100 cell equivalents) to each reaction.

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

   Note *Bubbles interfere with fluorescence detection.*

Thermal Cycling Programs

7. Centrifuge the reactions briefly.

8. Place the reactions in the QPCR instrument and run the appropriate RT-PCR program using the guidelines in the tables below. The 2-step cycling protocol is preferred for most primer/template systems. For primers with low melting temperatures, consider using the alternative 3-step cycling protocol.

Two-Step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	15 seconds	95°C
	1 minute[b]	60°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/extension step of each cycle.

Alternative Protocol with Three-Step Cycling

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	50–60°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 and extension step of each cycle.

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

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 different lysate samples, 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: QRT-PCR ASSAY CONSIDERATIONS

Optimizing QRT-PCR 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/probe system using purified RNA. Important optimization parameters include primer and probe 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).

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 the Agilent Mx3000P, Mx3005P, or Mx4000 instruments, dilute the provided stock of reference dye 1:500 before setting up the QRT-PCR reaction. This dilution step will result in a final reference dye concentration of 30 nM. If you are using the ABI PRISM 7700 instrument, dilute the reference dye 1:50 in order to obtain a final concentration of 300 nM in the reaction. 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.

Probe Design and Optimal Concentrations

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the primers. For additional considerations in designing TaqMan probes, refer to Primer Express® oligo design software from Applied Biosystems.

The optimal concentration of the experimental 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.

Resuspend lyophilized custom molecular beacon or TaqMan probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

Primer Design and Optimal Concentrations

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

Primers should be designed to prevent amplification of genomic DNA. One approach is to include a primer that spans an exon-exon boundary in the target mRNA. This primer will not bind to genomic DNA sequences, where an intron interrupts the primer binding site. A second approach is to use primers that flank a large intron. Using this approach, a small amplicon (≤150 bp) is amplified from the intronless cDNA, but amplification of the large intron-containing genomic DNA amplicon does not occur under the cycling conditions used for QPCR. If the experimental design requires the use of QRT-PCR primers that could amplify genomic DNA, SideStep lysates may be treated with DNase using the provided reagents and the protocol outlined in *Appendix II*.

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 100 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

DNase Treatment

DNase treatment of SideStep lysates is not necessary if QRT-PCR primers are designed to avoid amplification of genomic DNA, as described in the *Primer Design and Optimal Concentrations* section above. If your experimental design requires the use of primers capable of amplifying genomic DNA, the lysates may be treated with DNase according the protocol in *Appendix II*.

For QPCR applications that require DNase treatment, keep a portion of the untreated lysates for use with the QPCR normalization primer sets.

Preparing a Single Mixture for Multiple Samples

If running multiple samples containing the same primers and probes, prepare a single mixture of reaction components and then aliquot the mixture into individual reaction tubes using a fresh pipet tip for each addition. Preparing a common mixture facilitates the accurate dispensing of reagents, minimizes the loss of reagents during pipetting, and helps to minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Solutions that contain enzymes (including RT/RNase Block and SureStart *Taq* DNA polymerase) should be mixed gently by inversion or gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the buffer, which contains 50% glycerol, can lead to pipetting errors.

Preventing Sample 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.

Treatment with Uracil-N-glycosylase (UNG) is NOT recommended for decontamination of single tube RT-PCR reactions since UNG would be active during the 50°C incubation necessary for reverse transcription.

Magnesium Chloride Concentration

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. The Brilliant II QRT-PCR master mix kit contains $MgCl_2$ at a concentration of 5.5 mM (in the 1× solution), which is suitable for most targets.

Fluorescence 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.

When developing an assay, it is necessary to decide whether to use a 2-step or a 3-step PCR protocol. We recommend a 2-step protocol for the Brilliant II QRT-PCR master mix kit, but a 3-step protocol may be helpful when using primers with low melting temperatures. In a 2-step cycling protocol, fluorescence data are collected during the combined annealing/extension step. When using a 3-step protocol, it is prudent to collect fluorescence data at both the annealing step and the extension step of the PCR reaction. For subsequent experiments, the plateau resulting in low Ct values for the samples containing target and high Ct values (or "no Ct" values) for the controls containing no target should be chosen for analysis. For longer amplicons, fluorescence measurements taken during the extension step generally yield more useful data.

APPENDIX II: DNASE TREATMENT OF SIDESTEP CELL LYSATES

DNase treatment of your SideStep lysates may be necessary if lysates are to be used in QRT-PCR experiments with primers that could amplify genomic DNA.

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. Complete the SideStep lysate preparation protocol as described in the *Protocols* section.

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

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

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

5. Add 27.6 µl of RNase-free water.

6. 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.

7. Mix gently (no vortexing).

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

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

10. 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.

APPENDIX III: QPCR WITH THE NORMALIZATION PRIMERS

The QPCR normalization primers allow you to amplify a human 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 normalization reactions, but quantitation of your gene transcript 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 normalization 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 Agilent's Brilliant SYBR Green QPCR Master Mix (catalog #600548), but this protocol could be adapted for use with other QPCR reagents.*

Because the QPCR reactions with your gene transcript of interest may use a different PCR cycling program than that required for the 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 reaction 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 run 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 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 normalization genomic DNA reactions (Ct_{NORM}) and the GOI reactions (Ct_{GOI}). First, the ΔCt value is calculated for each target:

$$\Delta Ct_{GOI} = (Ct_{GOI} \text{ from control cells}) - (Ct_{GOI} \text{ from treated cells})$$

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

$$\Delta Ct_{NORM} = (Ct_{NORM} \text{ from control cells}) - (Ct_{NORM} \text{ from treated cells})$$

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.[1] 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 IV: 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 normalization primers and QPCR. Simply use the normalization 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 Agilent's Brilliant SYBR Green QPCR Master Mix (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 III *for instructions.*

When amplifying genomic DNA from SideStep lysates with the normalization primers, use lysate preparations 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
Little or no increase in fluorescence with cycling	The probe is not binding to its target efficiently because the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The probe is not binding to its target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Design a probe that is compatible with 5.5 mM $MgCl_2$.
	Verify the function of the probe's fluorophore. For molecular beacons, fluorophore function is confirmed by an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. For TaqMan probes, 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. If increased fluorescence is not observed, resynthesize the probe or molecular beacon.
	Redesign the probe.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of the specific product by gel electrophoresis.
	The RNA template may be degraded. Ensure that the template RNA is stored properly (at –20°C or –80°C) and is not subjected to multiple freeze-thaw cycles. Check the quality of the RNA in the sample by gel electrophoresis or using an automated RNA population analysis system such as the Agilent 2100 Bioanalyzer.
	If the target RNA contains extensive secondary structure, increase the incubation temperature used during the first step of the RT-PCR program to up to 55°C.
	For low-abundance targets or long amplicons, increase the duration of the cDNA synthesis step to 60-minutes while lowering the incubation temperature down to 42°C.
	Verify that all reagents and supplies are RNase-free.
	The addition of too many cell-equivalents to the QRT-PCR reaction may be inhibitory. Prepare the lysates at $\leq 10^4$ cells/µl, and follow the guidelines in *Determining the Amount of Lysate to Analyze* in the *Protocols* section for the upper limits of lysate addition to the QRT-PCR reaction.
Increasing fluorescence in no-template control reactions	The reaction has been contaminated. Follow the procedures outlined in reference 2 to minimize contamination.
Ct for the no-template control is less than the total number of cycles but the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.
PCR products detected in no-RT negative controls	Genomic DNA in the lysate may be amplified in the absence of RT. Redesign primers to span an exon-exon boundary or to flank a large intron to avoid amplification of genomic DNA or treat the lysate with DNase I prior to QRT-PCR (see *Appendix II* for a protocol).
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

1. Pfaffl, M. W. (2001) *Nucleic Acids Res.* 29(9):e45.
2. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

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 QRT-PCR MASTER MIX KIT, 1-STEP

Catalog #400917

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 proceed directly to QRT-PCR. Store lysates 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 (optional)

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 buffer to bring final volume to 400 µl.

5. Proceed to QRT-PCR analysis or store the lysate as recommended in Step 8 above.

QRT-PCR Analysis

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 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 2× Brilliant II QRT-PCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C. (Avoid multiple freeze-thaw cycles.)

3. Prepare the experimental reaction by adding the following components *in order*. Prepare a single reagent mixture for multiple reactions using multiples of each component listed below.

   Nuclease-free PCR-grade H_2O to bring the final volume to 25 µl (including cell lysate)
   12.5 µl of 2× Brilliant II QRT-PCR master mix
   X µl of experimental probe (optimized concentration)
   X µl of upstream primer (optimized concentration)
   X µl of downstream primer (optimized concentration)
   0.375 µl of diluted reference dye from step 1 (optional)
   1.0 µl of RT/RNase block enzyme mixture

4. Gently mix the reaction without creating bubbles (**do not vortex**), then distribute the mixture to the experimental reaction tubes.

5. Add 1 µl of SideStep cell lysate. Lysates should be diluted in nuclease-free PCR-grade water to achieve the desired cell equivalents/µl just prior to addition. Do not exceed 100 cell equivalents or 1 µl of undiluted lysate per 25 µl reaction.

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

7. Centrifuge the reaction briefly.

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

Two-Step Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[b]	95°C
40	15 seconds	95°C
	1 minute[c]	60°C

[a] A protocol for three-step cycling is provided in the *Protocol* section.

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

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