SideStep II Cell Lysis Analysis Kit

Instruction Manual

Catalog #400916

Revision C.0

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SideStep II Cell Lysis Analysis Kit

Contents

SideStep II Cell Lysis Analysis Kit

MATERIALS PROVIDED

Catalog #400916

Materials Provided	Concentration	Quantity[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
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

[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.

STORAGE CONDITIONS

All components: Store 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 DNase I and QPCR normalization primer sets at –20°C.

ADDITIONAL MATERIALS REQUIRED

PBS, cold (see *Preparation of Media and Reagents*)
RNase- and DNase-free H_2O
Microcentrifuge tubes

Revision C.0

INTRODUCTION

The SideStep II Cell Lysis Analysis Kit allows you to go from mammalian cells to QPCR or QRT-PCR experiments without any nucleic acid purification steps. In addition to our SideStep Lysis and Stabilization Buffer, the kit provides reagents to facilitate downstream QRT-PCR applications, including RNase-free DNase and the QPCR Normalization Primer Sets.

SideStep II Cell Lysis Analysis Kit Overview

SideStep Lysis and Stabilization Buffer

Quantitative reverse transcription PCR (QRT-PCR) is an important technique for studying mRNA levels in biological samples. 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 QPCR or 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 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 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 cell lysis analysis 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

Applications

SideStep technology may facilitate any application where nucleic acids are analyzed from cultured mammalian cells. The technology is particularly well suited for applications involving the analysis of RNA or DNA levels in a large number of samples. Examples include the following applications:

- siRNA knockdown detection
- miRNA detection
- mRNA profiling in cell differentiation or drug treatment experiments
- screening of compounds for effects on target mRNA levels
- time-course experiments
- screening of samples for the presence/absence of DNA or RNA target

For your convenience, we offer the SideStep buffer in combination with a variety of downstream analysis kits. See the table below for available combinations.

SideStep Kits for Specific Analysis Applications

Application	Product	Catalog #
QRT-PCR	SideStep II QRT-PCR Master Mix, 1-Step	400917
	SideStep II SYBR® Green QRT-PCR Master Mix, 2-Step	400909
	SideStep II QRT-PCR Master Mix, 2-step (for probe detection)	400918
QPCR	SideStep SYBR® Green QPCR Master Mix	400904
mRNA Purification directly from cell lysates	SideStep mRNA Enrichment Kit	400902
cDNA Synthesis directly from cell lysates	SideStep II QPCR cDNA Synthesis Kit	400908

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.

QPCR and QRT-PCR Assay Considerations

DNase Treatment

Treating SideStep lysates with DNase may yield better results in certain downstream applications. For two-step QRT-PCR experiments using SYBR® Green for detection, treatment of SideStep lysates with DNase is highly recommended. For QRT-PCR protocols that use a probe-based detection method, DNase treatment is not necessary if primers are designed to avoid amplification of genomic DNA, as described in the *Primer Design* section below.

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

See *Appendix I* for a complete DNase treatment protocol.

Primer Design

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

For QRT-PCR experiments, primers can 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 I*. If SideStep lysates will be used in QRT-PCR assays with SYBR Green detection, DNase treatment is strongly recommended regardless of primer design.

Probe and Protocol Selection

SideStep lysates may be analyzed using linear probes (e.g. TaqMan® probes) in either one-step or two-step QRT-PCR protocols. For SYBR Green detection in QRT-PCR, use a two-step protocol and treat lysates with DNase prior to cDNA synthesis.

Optimizing QPCR and QRT-PCR Assays

Prior to performing a large-scale experiment or screen using QPCR or QRT-PCR analysis of lysates, the assay should be optimized for the specific target of interest and the specific primer/probe system using purified RNA. Important optimization parameters include primer and probe 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).

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 II* 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.

PROTOCOL

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. When analyzing a small number of cells in downstream applications such as QRT-PCR, 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 QPCR or 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 ≤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.

   Note *If your experimental design requires DNase treatment of the lysate, refer to* Appendix I *for a protocol.*

9. Process the lysate using the appropriate application 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. For QRT-PCR applications, see *QRT-PCR Guidelines*, below, for lysate dilution and processing guidelines.

QRT-PCR Guidelines

One-Step QRT-PCR

Note ***One-step QRT-PCR analysis of SideStep lysates should be performed using probe-based detection methods only.*** *For SYBR Green dye-based detection, perform two-step QRT-PCR experiments.*

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.

Two-Step QRT-PCR

DNase Treatment

For two-step QRT-PCR using a nonspecific double-stranded DNA binding dye, such as SYBR Green, for detection, treatment of SideStep lysates with DNase prior to cDNA synthesis is strongly recommended. For two-step protocols with probe-based detection, DNase treatment is not necessary if the primers are designed to avoid amplification of genomic DNA. If you intend to normalize your QRT-PCR data to genomic DNA content using the QPCR normalization primers, reserve an untreated portion of any DNase-treated lysate sample for use in those QPCR reactions.

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 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. Use nuclease-free water to dilute the SideStep lysate 1:100 for a final concentration of 100 cell equivalents/µl, and then immediately add 1 µl of the diluted lysate to a 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.*

APPENDIX I: 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. Additionally, when using lysates in a two-step QRT-PCR protocol with SYBR Green for detection, DNase treatment is recommended regardless of primer design.

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 *Protocol* 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 II: QPCR WITH THE 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 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 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 *Protocol* 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 HT/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 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 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 normalization 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 primers 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:

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

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.[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 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 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, 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

Observations	Suggestions
No or low amounts of PCR products detected after QPCR or QRT-PCR	Optimize the QPCR or QRT-PCR assay. Important optimization parameters include primer design, primer concentration, probe design and probe concentration.
	For best results, design primers that produce amplicons <150 bp in length.
	Increase the number of PCR cycles.
	Determine the optimal primer annealing temperature for the PCR cycling program.
	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.
	The addition of too many cell-equivalents to the PCR or cDNA synthesis reaction may be inhibitory. Prepare the lysates at $\leq 10^4$ cells/µl, and refer to the *QRT-PCR Guidelines* section for the upper limits of lysate addition.
	Verify that the QRT-PCR protocol successfully amplifies the target RNA from a positive control sample, such as Agilent's QPCR Human Reference RNA (catalog #750500).
Unexpected PCR products	Optimize primer design and the relative primer concentrations.
	Alternatively-spliced forms of the transcript may be present. Redesign primers to another part of the mRNA.
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. If using SYBR Green for product detection, treat the lysate with DNase I prior to cDNA synthesis (see *Appendix I* for a protocol).
All three sets of 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.

ENDNOTES

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