Accurate and Reproducible RT-qPCR Gene Expression Analysis on Cell Culture Lysates Using the SingleShot™ SYBR® Green Kit

Tech Note

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Abstract

Analysis of gene expression in cell cultures is frequently limited by the number of samples that can be processed using traditional real-time PCR workflows. Here we evaluate Bio-Rad's SingleShot™ SYBR® Green Kit, which eliminates the time-consuming cell harvesting and RNA isolation steps and permits use of crude cell lysates as input samples for cDNA synthesis. We find that the SingleShot Kit produces accurate, highly reproducible gene expression results with superior clearance of genomic DNA (gDNA).

Introduction

Gene expression profiling in cultured cells using reverse transcription quantitative PCR (RT-qPCR) is commonly used to assess the effects of treatments such as RNA interference (RNAi) and drug compound administration. The standard workflow for gene expression analysis in cell cultures is a multistep process that requires harvesting of cells, isolation of RNA, removal of contaminating DNA, cDNA synthesis, and finally qPCR, where cell harvesting and RNA isolation are the rate-limiting steps. The throughput of harvesting procedures and classical RNA extraction methods, such as phenol/chloroform extraction and ethanol precipitation or column-based solid-phase extraction, is limited by time-consuming and laborious protocols, which preclude the evaluation of large numbers of samples in a single experiment.

The increasing need for high-throughput gene expression analysis for applications such as genome-wide RNAi and compound library screening has led to an elegant method that permits gene expression analysis of 96- and 384-well cell culture samples without the need for cell harvesting and RNA purification. Instead crude cell lysates are used as input for reverse transcription, significantly increasing throughput and making the workflow amenable to automation.

We previously performed an extensive characterization of this method by evaluating the *Power* SYBR® Green Cells-to-C_T Kit (Life Technologies Corporation) (Van Peer et al. 2012). Comparison with the hitherto gold standard workflow involving cell harvesting and RNA purification demonstrated good accuracy and superior sensitivity. Here, we evaluate Bio-Rad's SingleShot™ SYBR® Green Kit, by comparing it with the Cells-to-C_T Kit, and show that it produces accurate expression results with high reproducibility and with superior clearance of contaminating gDNA. In addition, we show that both kits also enable quantification of nuclear RNA.

Methods

Cell Culture

Neuroblastoma cell lines (SH-EP, SK-N-AS, NGP, IMR-32) were cultured in 96-well culture plates at a density of 10,000 cells/well. Cells were grown in RPMI 1640 (Invitrogen Corporation) supplemented with fetal calf serum (10%), L-glutamine (1%), penicillin/streptomycin (1%), kanamycin (1%), and HEPES buffer (25 mM). Cell lysis and cDNA synthesis using the SingleShot™ SYBR® Green Kit and Cells-to-C_T Kit were carried out 48 hr after seeding.

SingleShot™ SYBR® Green Kit

The SingleShot Kit was used according to the manufacturer's instructions. In brief, cells were washed with 125 µl of Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and subsequently lysed with 50 µl of SingleShot Cell Lysis Buffer containing DNase. Lysis reactions were incubated 5 min at room temperature followed by 5 min at 75°C. cDNA synthesis was carried out on 4 µl of cell lysate in a total volume of 20 µl (20%) using the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR that is supplied with the SingleShot™ SYBR® Green Kit (both from Bio-Rad).

Power SYBR® Green Cells-to-C_T Kit

Cell lysis and reverse transcription were carried out using the *Power* SYBR® Green Cells-to-C_T Kit according to the manufacturer's instructions. cDNA synthesis was carried out on 10 µl of cell lysate in a total volume of 50 µl (20%) using Cells-to-C_T reagents.

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Quantitative PCR

Quantitative PCR gene expression measurements were performed and reported according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al. 2009). Reactions were performed in 384-well plates using a CFX384™ Real-Time PCR Detection System and contained 2.5 µl SsoAdvanced™ Universal SYBR® Green Supermix, included in the SingleShot Kit (all from Bio-Rad), 1.25 pmol of both forward and reverse primer, and 2 µl of 4x diluted SingleShot Kit– or Cells-to-C_T Kit–generated cDNA in a total volume of 5 µl. All reactions were performed in duplicate and quantification cycle (Cq) values were averaged. Normalized relative expression levels were calculated using Biogazelle's qbase+ Software version 2.6 (www.qbaseplus.com) (Hellemans et al. 2007). Normalization was performed using three stably expressed neuroblastoma reference genes (*HPRT1*, *TBP*, and *YWHAZ*) validated using the geNorm module of qbase+ Software. All qPCR assays were extensively validated in silico using the RTPrimerDB qPCR assay evaluation pipeline (www.rtprimerdb.org) (Lefever et al. 2009) as well as empirically validated, checking both primer efficiency and specificity.

Results

Accuracy

We assessed the ability of the SingleShot™ SYBR® Green Kit to accurately quantify gene expression by comparing it with the previously validated Cells-to-C_T Kit (Van Peer et al. 2012). cDNA from four neuroblastoma cell lines, two bearing amplification of the *MYCN* transcription factor gene (NGP and IMR-32) and two without *MYCN* amplification (SH-EP, SK-N-AS), was prepared using either the SingleShot Kit or the Cells-to-C_T Kit. Relative expression levels of ten genes of interest (*DKK3*, *INHBA*, *PLAT*, *RGS4*, *MYC*, *MTHFD2*, *MYCN*, *TGFBI*, *PMP22*, *NTRK2*) known to be differentially expressed in cells with and without *MYCN* amplification (Vermeulen et al. 2009) were quantified. This resulted in a wide coverage of expression levels, which is desirable for robust correlation analysis. High and significant correlations were observed between Cq values (Pearson r = 0.99, P <0.05) and $\log_{10}$ normalized relative expression levels (Pearson r = 0.98, P <0.05) obtained with the two methods (Figure 1A), suggesting that the SingleShot™ SYBR® Green Kit accurately quantifies gene expression. Results were confirmed in an independent experiment (data not shown).

Reproducibility

To test the reproducibility of the SingleShot™ SYBR® Green Kit, expression of the ten target genes was compared in duplicate lysates from four neuroblastoma cell line cultures. High and significant correlations were observed between Cq values (Pearson r = 0.99, P <0.05) and $\log_{10}$ normalized relative expression levels (Pearson r = 0.97, P <0.05) from lysis replicates (Figure 1B). Results were confirmed in an independent experiment (data not shown).

Effectiveness of Genomic DNA Removal

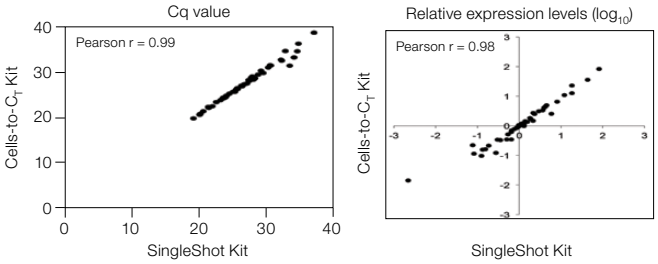
Accurate gene expression quantification requires efficient removal of gDNA from cell lysates because qPCR assays do not always distinguish between cDNA and gDNA. The effectiveness of the DNase treatment included in the SingleShot Kit was evaluated and compared with that included in the Cells-to-C_T Kit. Duplicate SingleShot Kit and Cells-to-C_T Kit lysates were prepared both in the presence and the absence of DNase for four neuroblastoma cell lines. Equal fractions of crude cell lysate were used as input for qPCR using four gDNA-specific assays (*NEUROD1*, *XRCC3*, *PLAT*, *MTHFD2*).

The SingleShot Kit DNase treatment protocol appeared to be more effective than the Cells-to-C_T protocol. Absence of a DNA-specific signal was observed for the majority of sample-assay combinations (24 out of 32). For the remaining combinations, a larger shift in quantification cycle upon DNase treatment (5.8 cycles on average) was observed than for the Cells-to-C_T Kit (4.3 cycles on average), which indicates almost complete removal of gDNA (Figure 1C). Results were confirmed in an independent experiment (data not shown).

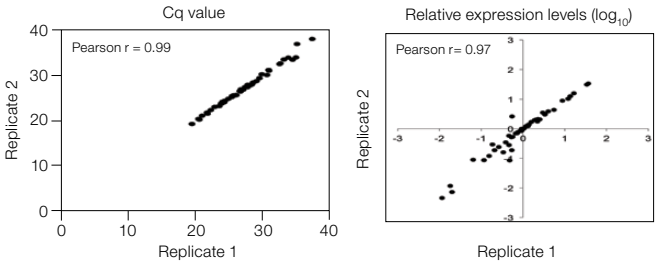
Quantification of Nuclear Localized RNA

Given the increasing interest in the role of nuclear RNA molecules in physiological and disease processes, we tested the SingleShot Kit's ability to quantify nuclear RNA. To this end we measured expression levels of three widely studied long noncoding RNAs (lncRNAs) with known nuclear localization (*HOTAIR*, *MALAT1*, and *NEAT1*). High and significant correlations were observed when comparing lncRNA expression results from the SingleShot Kit and the Cells-to-C_T Kit for both Cq values (Pearson r = 0.99, P <0.05) and $\log_{10}$ relative expression levels (Pearson r = 0.99, P <0.05) (Figure 1D). Results were confirmed in an independent experiment (data not shown).

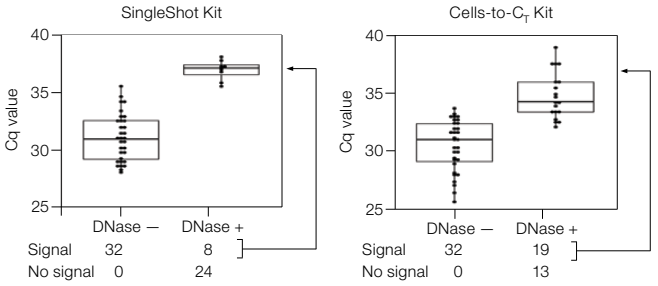
A. Accuracy



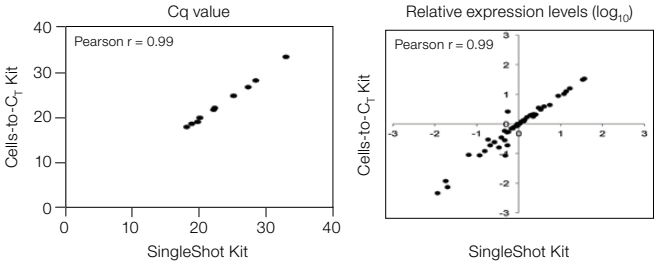
B. Reproducibility



C. DNase Effectiveness



D. Quantification of Nuclear Localized RNAs



Evaluation of the SingleShot™ SYBR® Green Kit. A, assessment of the accuracy of the SingleShot Kit by comparison of Cq values and $\log_{10}$ relative expression levels obtained with the SingleShot Kit and Cells-to-C_T Kit; **B**, assessment of SingleShot Kit reproducibility by comparison of Cq values and $\log_{10}$ relative expression levels of SingleShot lysis replicates; **C**, comparison of the effectiveness of DNase treatment of the SingleShot Kit and the Cells-to-C_T Kit protocols; **D**, assessment of the ability of the SingleShot Kit to quantify nuclear localized RNAs by comparison of Cq values and $\log_{10}$ relative expression levels of lncRNAs obtained with the SingleShot Kit and Cells-to-C_T Kit. Cq, quantification cycle.

Conclusions

The low throughput of the cell harvesting and RNA purification steps of classic RT-qPCR workflows has long hampered cell culture studies that require large sample sizes. Methods that permit the use of crude cell lysates instead of purified RNA as input for reverse transcription offer a fast and straightforward alternative that significantly increases sample processing throughput. The compatibility of this method with 96- and 384-well cell cultures also enables substantial downscaling of reactions, lowering reagent usage and overall experiment cost.

Here, we evaluated Bio-Rad's SingleShot™ SYBR® Green Kit and demonstrate its high accuracy and reproducibility, benchmarking it with the previously validated *Power* SYBR® Green Cells-to-C_T Kit from Life Technologies. Accurate gene expression results were reproducibly obtained over a wide range of expression levels for various genes and in multiple cell lines. In addition to mRNAs, nuclear localized lncRNAs could be quantified. We also observed largely improved clearance of gDNA from lysates compared with the Cells-to-C_T Kit. The SingleShot™ SYBR® Green Kit is thus an excellent alternative to traditional, lower-throughput real-time PCR workflows.

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