Comparison of reverse transcription–quantitative polymerase chain reaction methods and platforms for single cell gene expression analysis

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Single cell gene expression analysis can provide insights into development and disease progression by profiling individual cellular responses as opposed to reporting the global average of a population. Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) is the “gold standard” for the quantification of gene expression levels; however, the technical performance of kits and platforms aimed at single cell analysis has not been fully defined in terms of sensitivity and assay comparability. We compared three kits using purification columns (PicoPure) or direct lysis (CellsDirect and Cells-to-CT) combined with a one- or two-step RT-qPCR approach using dilutions of cells and RNA standards to the single cell level. Single cell-level messenger RNA (mRNA) analysis was possible using all three methods, although the precision, linearity, and effect of lysis buffer and cell background differed depending on the approach used. The impact of using a microfluidic qPCR platform versus a standard instrument was investigated for potential variability introduced by preamplification of template or scaling down of the qPCR to nanoliter volumes using laser-dissected single cell samples. The two approaches were found to be comparable. These studies show that accurate gene expression analysis is achievable at the single cell level and highlight the importance of well-validated experimental procedures for low-level mRNA analysis.

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For the majority of gene expression studies, a population mean is taken as a measure of specific gene expression levels. Although this method is suitable for many studies, it does not yield information on the differences in expression between cells within a heterogeneous population or account for transcriptional responses occurring in “transcriptional bursts” [1,2]. Hence, single cell gene expression analysis may yield greater insights into cell biology, and this approach has many applications in the fields of diagnostics [3], embryology [4], stem cell biology, and tissue engineering [5].

Global analysis of single cell transcriptomes is possible using DNA microarray [6] and, more recently, RNA-seq technologies [7,8]. However, reverse transcription–quantitative polymerase chain reaction (RT-qPCR) is the “gold standard” for quantitative measurement of transcript abundance in single cells [9] and the most commonly used approach in research laboratories [10]. The MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines for reporting of RT-qPCR experiments [11] call for thorough characterization of RT-qPCR assays and upstream sample processing steps such as cell isolation and lysis that may affect RT-qPCR performance. Validation of the quantitative accuracy of single cell RT-qPCR is important due to the low levels of messenger RNA (mRNA) levels in each sample [12], whereas technical validation is less straightforward due to the heterogeneous nature of the samples and limited scope for technical replicates [13].

There are currently a variety of techniques available to isolate single cells, including micromanipulation, laser dissection microscopy, and FACS (fluorescence-activated cell sorting) [14,15]. Whichever single cell isolation method is employed, it is vital to employ lysis conditions that ensure complete cell lysis [16]. There are several lysis methods in use—some that enable the use of the lysate directly in RT-qPCR [10,13] and others that involve cell lysis and subsequent purification of the total RNA prior to RT-qPCR [17]. For all of these procedures, there is a need to understand any bias that could be introduced due to incomplete lysis, differing elution yields from purification steps, and potential inhibition of the RT-qPCR by the presence of lysis components.

In addition to stringent cell isolation and lysis conditions at low mRNA levels, special attention must be given to factors that can lead to unreliable RT-qPCR data. Several studies have shown that...
at low complementary DNA (cDNA) levels, inhibition of real-time PCR by reverse transcriptase can be significant [18,19]. In addition, obtaining reliable data from copy numbers below $10^2$ per qPCR has been shown to be problematic and the technical error associated with both the RT and the PCR steps can become more significant as the template level decreases [13].

Alongside reagent kits applicable to single cell gene expression analysis, microfluidic PCR platforms such as the BioMark (Fluidigm) [20] and OpenArray (Life Technologies) [21] facilitate thousands of assays to be performed in parallel. This technology has been applied to single cell analysis using both digital PCR (dPCR) [22] and qPCR approaches. The latter enables the analysis of tens to hundreds of gene targets in single cells to be performed [5,23,24]. Preamplification of template is required for these high-throughput approaches at the single cell level, although PCR-based preamplification has been suggested to introduce amplification bias [25]. Although the BioMark platform has been characterized for larger inputs of template RNA [26], the effect of preamplification and the scaling down of reaction volumes to the nanoliter scale has not been investigated for the low-level mRNA quantities found in a single cell. Recent advances in microfluidic approaches have also applied miniaturization not only to qPCR but also to the cell capture, lysis, and RT steps of single cell analysis. Microfluidic chip- and droplet-based technologies enable effective concentration of the mRNA transcripts found in a single cell [27–29].

In this study, we compared three commercially available RT–qPCR kits designed for low-level mRNA analysis (CellsDirect, Cells-to-CT, and PicoPure RT–qPCR kit) for their suitability for single cell gene expression analysis. The CellsDirect and Cells-to-CT kits are designed for direct input of the cell lysate in the RT–qPCR, whereas the PicoPure/Message Sensor kit combination involves purification of total RNA prior to the RT–qPCR. Dilutions of single cells to the single cell level were used to test all technical factors contributing to the qPCR measurement, namely efficiency of lysis/extraction, RT, and qPCR steps. In addition, an RNA standard (ERCC-84) from the set of controls developed by the External Quality Assessment Scheme (EQAS) of the Centre for Genomic Standards (CGS) was employed to measure the sensitivity of RT–qPCR and the effect of components such as lysis buffer on the reactions.

One of the kits (CellsDirect) was chosen to compare differences between performing analysis using a standard qPCR platform and the BioMark instrument based on its sensitivity for low-level mRNA quantification and suitability for high-throughput approaches. Adaptation of the method to include a preamplification step prior to qPCR, which is required for single cell analysis on the BioMark platform, was compared with one-step RT–qPCR using a dilution series of ERCC-84 RNA standard in a background corresponding to a single cell equivalent (CE). Laser-dissected single cells were collected to assess the impact of microliter versus nanoliter qPCR volume on the analysis of an endogenous transcript.

**Materials and methods**

**Cell culture**

Human foreskin fibroblast-1 (HFF-1) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, ATCC cell line SCRC-1041, LGC Standards, UK) containing 10% fetal calf serum (FCS, PAA Laboratories, Austria) incubated at 37 °C in a humidified atmosphere of 5% CO₂.

**qPCR assays**

Predesigned TaqMan qPCR assays were supplied by Applied Biosystems (Foster City, CA, USA) as a 20× premix containing both primers and FAM–nonfluorescent quencher (NFQ) probe (see Supplementary Table 1 in supplementary material). The efficiencies of these assays were characterized with a dilution series of HFF-1 cDNA (see Supplementary Methods and Supplementary Table 2 in supplementary material). Primers and hydrolysis probe (FAM–TAMRA) to ERCC-84 (GenBank accession: DQ883682) were used as described previously [26] and supplied by Sigma–Aldrich (Poole, UK). The final concentrations of each primer and probe for all assays were 900 and 250 nM, respectively. RT–qPCR and qPCR were performed in MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems).

**Comparison of CellsDirect, Cells-to-CT, and PicoPure RT–qPCR kits using a cell dilution series**

For cell dilutions, the number of HFF-1 cells was determined by averaging five counts using a hemocytometer, and the cell suspension was then diluted in phosphate-buffered saline (PBS) by serial dilution to $10^5$, $10^4$, $10^3$, $10^2$, and 1 cell(s)/µl ($n = 3$). Lysates were prepared and RT–qPCR was performed using the CellsDirect (Invitrogen, Paisley, UK), Cells-to-CT (Applied Biosystems), and PicoPure (Arcturus/Life Technologies/Message Sensor (Ambion) kits according to the manufacturers’ instructions as described below. For all three kits, 20% of the original lysate was used per RT–qPCR (CellsDirect and Message Sensor) or per qPCR (Cells-to-CT) ($n = 1$ RT–qPCR or qPCR per gene assay). Real-time PCR was performed on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). A DNA standard curve composed of a 10-fold serial dilution of GAPDH or B2M DNA standards (see “Preparation of DNA standards” section below) from $10^6$ to 10 copies ($n = 3$) was performed on each qPCR plate to quantify the number of cDNA copies produced by each RT reaction. In this and other experiments, raw data from the 7900HT system were processed using SDS (Sequence Detection System) software (version 2.3) with automatic baseline setting and manual threshold setting. Cₙ (quantification cycle) values were analyzed further in Microsoft Excel 2003.

**CellsDirect**

CellsDirect lysis buffer was prepared by mixing 10 parts resuspension buffer with 1 part lysis enhancer. Then 1 µl of cell suspension was added to 23 µl of lysis buffer and incubated on ice for 10 min, followed by cell lysis by incubation at 75 °C for 10 min. Lysates were frozen at −80 °C. Then 1 µl of 50 mM magnesium sulfate solution (provided with the kit) was added to the 24 µl of cell lysate prior to RT–qPCR. One-step RT–qPCR was performed with assays specific to B2M or GAPDH using 5 µl of cell lysate in 20-µl reaction volumes. Thermal cycling conditions were as follows: RT (42 °C for 15 min), followed by reverse transcriptase inactivation (95 °C for 2 min), followed by 40 cycles of PCR (95 °C for 15 s, 60 °C for 60 s).

**Cells-to-CT**

Cell lysates were prepared by the addition of 1 µl of cell suspension to 15.4 µl of lysis solution and mixed by pipetting, followed by a 5-min incubation step at room temperature. Stop solution (1.6 µl) was added to stop the lysis reaction, followed by a 2-min incubation at room temperature prior to freezing at −80 °C. Two-step RT–qPCR was performed with separate RT ($n = 1$) and qPCR ($n = 1$) steps. RT reactions were performed with 9 µl of cell lysate in 20-µl reaction volumes incubated at 37 °C for 60 minutes followed by heat inactivation for 5 minutes at 95 °C. qPCR was performed with 8 µl of RT reaction and TaqMan assays to B2M and
GAPDH in 20-µl reaction volumes with thermal cycling conditions of 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. (Note: The proportions of lysete in the RT reaction and cDNA in the qPCR are within the manufacturer’s recommendations of a maximum of 45% for both stages.)

**PicoPure**

Cell suspension (1 µl) mixed in 100 µl of extraction buffer was incubated for 30 min at 42 °C, followed by centrifugation (3000g, 2 min) to separate cell debris, and the supernatant was then frozen at –80 °C. RNA purification was performed by mixing the sample 1:1 with 70% ethanol, followed by wash steps using wash buffer 1 (100 µl) and wash buffer 2 (2 × 100 µl) and elution of the RNA with 30 µl of elution buffer.

**Message Sensor**

One-step RT–qPCR was performed using 6 µl of RNA eluted from the PicoPure columns in a 20-µl reaction volume containing assays to B2M or GAPDH, Platinum Taq DNA polymerase, and ROX (Invitrogen) in addition to the components of the Message Sensor RT kit (10× RT buffer, dNTP mix, RNasin, and RT). Thermal cycling conditions were as follows: RT (50 °C for 15 min), followed by reverse transcriptase inactivation (95 °C for 5 min), followed by 40 cycles of PCR (95 °C for 15 s, 60 °C for 60 s).


**Preparation of DNA standards**

GAPDH, B2M, and ERCC-84 DNA standards were prepared to quantify cDNA copies and estimate RT efficiencies of the RT–qPCR kits tested. DNA standards were produced by two-step RT–PCR and DNA standards were produced by two-step RT–PCR

**Preparation of IVT RNA standards**

In vitro transcribed (IVT) ERCC-84 RNA was produced from ERCC-84 plasmid DNA (courtesy of Marc Salit, NIST, USA). ERCC-84 plasmid DNA was cleaved into a single linear strand using BamHI restriction endonuclease enzyme (New England Biolabs, Hitchin, UK). RNA was in vitro transcribed from the linearized plasmid using a MEGAscript T7 kit (Ambion) followed by DNase treatment and cleanup using RNasey columns (Qiagen, Hilden, Germany). RNA concentration and length were measured using the Nanodrop 1000 spectrophotometer (ThermoScientific) and 2100 Bioanalyzer (Agilent) systems, respectively.

**Preparation of DNA standards**

GAPDH, B2M, and ERCC-84 DNA standards were prepared to quantify cDNA copies and estimate RT efficiencies of the RT–qPCR kits tested. DNA standards were produced by two-step RT–PCR from 200 ng of HFF-1 total RNA (GAPDH and B2M) or 200 ng of IVT ERCC-84 RNA. Primer pairs were designed using NCBI primer–BLAST to produce amplicons of similar length to the B2M and GAPDH RNA transcripts and were obtained from Sigma–Aldrich: GAPDH forward, 5'-AGGCTCCCGCTGGCTCTCT-3' (PCR product size: 993 bp); B2M forward, 5'-TAAGTGAGGCGTGCCGTGCT-3', and reverse, 5'-AACGATATACCCACTAGCCCTT-3' (PCR product size: 930 bp). The ERCC-84 primers used were as follows: forward, 5'-GCC AAT TGT CTG GGG CTT CGT T-3', and reverse, 5'-CCC CAC CCT GCA ACT GAG AAG-3' (PCR product size: 970 bp).

**cDNA synthesis for preparation of DNA standards**

First-strand synthesis was performed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) using the reverse primer (200 nM final concentration) for each gene in 20-µl reactions. RT incubation conditions were as follows: 48 °C for 30 min, followed by 95 °C for 5 min.

**PCR for preparation of DNA standards**

PCR was performed using the HotStar Taq Plus kit (Qiagen) in 50-µl reaction volumes containing 150 nM of the respective primer pairs. For the GAPDH and B2M DNA standards, two rounds of PCR were performed: The first PCR round was performed using 1 µl of cDNA, and the second round was performed using 50 ng of purified PCR product from the first round of PCR. For ERCC-84, one round of PCR was performed with 1 µl of cDNA. The PCR conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 10 min. After each PCR, products were purified using the QIAquick PCR purification kit (Qiagen). Quantity was determined using the NanoDrop 1000 spectrophotometer, and expected product length was confirmed using the 2100 Bioanalyzer.

**Investigating RT–qPCR precision and potential inhibitors**

To assess RT–qPCR precision for different levels of transcript abundance, serial dilutions of ERCC-84 RNA standard to 10⁴, 10³, and 10² copies were prepared in lysis buffer specific to the CellsDirect or Cells-to-CT kit, ensuring that the same proportion of lysis buffer was present in each RT–qPCR and the same as that used previously for the cell dilutions. In parallel with the aim of investigating the effect of lysis buffer on RT–qPCR, RT–qPCRs were also performed with the ERCC-84 serial dilution in the absence of lysis buffer. For testing of qPCR inhibition by reverse transcriptase, ERCC-84 DNA standards were diluted to 10⁴, 10³, and 10² copies in lysis buffer and RT–qPCR was performed in the presence and absence of the reverse transcriptase enzyme (this was substituted with nuclease-free water in the latter case). To investigate the impact of cell background, 10² copies of ERCC-84 RNA standard were spiked into lysis buffer containing variable concentrations of cell lysate equivalent to 200, 20, 2, 0.2, and 0 cells per qPCR. The cell lysate was prepared by dilution of the 10⁴ cell sample lysate, with 1 µl of the diluted cell lysate being used in place of 1 µl of lysis buffer. Dilutions prepared in the CellsDirect kit lysis buffer were heated at 75 °C for 10 min before use in RT–qPCR, in line with preparing cell lysates with this kit. For the one-step CellsDirect kit, three replicate RT–qPCR assays were performed for each RNA sample, whereas for the two-step Cells-to-CT kit, two 20-µl RT reactions were performed and pooled, followed by three replicate qPCR assays.

**Comparison of one-step RT–qPCR with preamplification of template**

Dilutions of ERCC-84 RNA standards to 10⁴, 10³, 10², and 10¹ copies were prepared in CellsDirect lysis buffer containing pooled HFF-1 RNA equivalent to that of a single cell per 8 µl. Six replicate one-step RT–qPCR or RT–preamplification reactions (one-step RT–PCR with 18 cycles of PCR) were performed for each dilution. One-step RT–qPCR was performed according to the CellsDirect protocol. RT–preamplification was also performed using the CellsDirect kit according to a protocol adapted from the BioMark Advanced Development Protocol 5, with 20-µl sample RT–preamplification master mixes being prepared using 8 µl of lysate, 2 × RT–PCR buffer, 0.2 µl of SuperScript III RT/Taq polymerase enzyme mix, and 20 × ERCC-84 gene-specific assay (final concentration 0.05×). The volume was made to 20 µl using nuclease-free water. RT–preamplification was performed on the GeneAmp PCR system 9700 (Applied Biosystems) under the following conditions: 50 °C for 15 min, followed by 95 °C for 2 min, followed by 18 cycles of 95 °C for 15 s and 60 °C for 4 min. Reactions were diluted 1:5 in Tris–EDTA (ethylene-diaminetetraacetic acid) (pH 8.0, Fluka, 100 µl final volume) and stored at –20 °C. Preamplified cDNA samples were analyzed by qPCR using the 7900HT real-time PCR cycler with incubations of 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s, using TaqMan Universal PCR Master Mix (Applied Biosystems) and 2.25 µl of preamplified sample in a 20-µl final volume. Triplicate qPCRs were performed for each preamplified sample.
Laser capture microdissection of HFF-1 cells

HFF-1 cells were revived from cryopreservation in liquid nitrogen by rapid thawing at 37 °C for 2 min and seeded in one T-175 flask (Corning). After 24 h, the cells were washed once in PBS and enzymatically detached with trypsin (Sigma), counted, and seeded onto laser microdissection membrane slides (Molecular Machines and Industries, Glattbrugg, Switzerland) at a density of 30,000 cells/mm². Cells were allowed to adhere overnight in culture medium (DMEM + 10% fetal bovine serum [FBS]) at 37 °C in a saturated humidified atmosphere with 5% CO2. After 24 h, the cells were incubated with 2 mM calcein–AM (Invitrogen) for 30 min, washed once in PBS, briefly fixed in absolute ethanol at room temperature for a few seconds, and air-dried. Dry slides were stored at 4 °C prior to laser microdissection. Then 100 HFF-1 cells were captured in 20 μl of CellsDirect lysis buffer in the lid of a 0.2-ml nuclease-free PCR tube using a Zeiss Axio Observer laser capture microscope using calcein fluorescence (488 nM excitation wavelength) to identify single cells. The cells were lysed by heating at 75 °C for 15 min.

Comparison of BioMark and 7900HT qPCR systems

A serial dilution of a pool of 100 laser-captured HFF-1 single cells was used to evaluate the BioMark real-time PCR system with the 7900HT. The lysate was diluted 1:1 with CellsDirect lysis buffer before performing a 5-fold serial dilution to 22.5, 4.5, and 0.9 CE per RT–preamplification reaction. Duplicate RT–preamplification reactions were performed in a total volume of 22 μl containing a mix of 11 TaqMan assays (0.05 μl) (see ERCC-84 and assays listed in Supplementary Table 1) and 0.2 μl of SuperScript III/Platinum Taq enzyme mix with cycling conditions as in the “Comparison of one-step RT–qPCR with preamplification of template” section above. Preamplified samples were analyzed using the 7900HT and BioMark qPCR platforms. qPCR measurement of GAPDH was performed on the 7900HT as described in that section with duplicate qPCRs per preamplified sample.

The same samples were analyzed for the expression of GAPDH, B2M, collagen-type VI alpha 1 (COL6A1), mannose-6-phosphate receptor binding protein 1 (M6PRBP1), tumor necrosis factor receptor superfamily member 12A (TNFR12A), and colony-stimulating factor 3 (granulocyte) (CSF3) on the BioMark platform. Assays were prepared for loading onto BioMark 48.48 Dynamic Arrays by diluting each 20 μl assay 1:1 with Assay Loading Reagent (Fluidigm). For sample loading, a premix consisting of 10 parts TaqMan Universal Mastermix (Applied Biosystems):1 part Sample Loading Reagent (Fluidigm) was prepared, and 2.75 μl of this premix was mixed vigorously with 2.25 μl of preamplified cDNA by vortexing and centrifuging to pool contents. Then 5 μl of each assay or each sample mix was loaded per assay or sample inlet well, respectively. Nine replicate qPCRs were performed for each preamplified sample consisting of three replicate assay inlets and three replicate sample inlets. Thermal cycling conditions were identical to those described for the 7900HT. Ct data were generated using BioMark Real-Time PCR Analysis software (version 2.1.1) with global automatic threshold and linear baseline correction settings and a default quality threshold of 0.65. Data were exported for processing using Microsoft Excel 2003.

Statistical analysis

Linear regression analysis of \( \log_{10}(\text{mean cDNA copy number}) \) versus \( \log_{10}(\text{cell number}) \) (see Fig. 1 in Results) and of \( C_t \) versus \( \log_{2}(\text{RNA/DNA copy number}) \) (see Figs. 2 and 4 in Results) were

\[ y = 1.23x + 1.69 \quad R^2 = 0.972 \]  
\[ y = 1.15x + 1.66 \quad R^2 = 0.994 \]  
\[ y = 1.22x + 0.78 \quad R^2 = 0.999 \]  
\[ y = 1.10x + 2.22 \quad R^2 = 0.990 \]  
\[ y = 1.14x + 2.08 \quad R^2 = 0.987 \]
performed using Microsoft Excel 2003, with statistical analysis performed using GraphPad Prism (version 5a). Efficiency of PCR-amplification of DNA template was calculated from the slope ($S$) of $C_q$ versus log$_2$(DNA copy number) using the equation:

$$\text{PCR efficiency} (\%) = 100 \times \frac{2^{-\frac{1}{S}} - 1}{C_0}$$

The theoretical doubling of DNA template with each PCR cycle corresponds to a PCR
Results

Comparison of RT–qPCR kits using a cell dilution series

To assess the impact of scaling down cell lysis, RT, and qPCRs to the single cell range, we assessed the limits of detection of two reference genes (GAPDH and B2M) with three commercially available RT–qPCR kits (CellsDirect, Cells-to-CT, and PicoPure/Message Sensor), designed for the analysis of small numbers of cells, using a 10-fold cell dilution of fibroblasts from 10^3 cells to 1 cell (see Materials and Methods). Intact cells at each of the respective cell numbers were used to ensure that the data obtained reflected the entire process from cell lysis to Cq value. The results obtained for the cell dilution series are shown in Fig. 1. Linear regression was performed by logarithmic plots of cDNA copy numbers of GAPDH or B2M against the number of cells to gauge the linearity and proportionality of the response across the measured range, as indicated by R^2 and slope (ideal slope = 1.0), respectively. Copy numbers of each cDNA were measured against a DNA standard curve of GAPDH or B2M PCR product (see Materials and Methods) to estimate the gross efficiency of lysis and RT steps, which is indicated by the intercept of the linear regression equation (Fig. 1).

GAPDH and B2M transcripts were detected over the entire cell dilution series for the CellsDirect and PicoPure/Message Sensor kits for all three single cell samples, whereas the Cells-to-CT kit gave an undetermined reading at the single cell level for B2M. With the CellsDirect kit, the slopes of target copy number plotted against log2 cell number (1.23 for GAPDH and 1.25 for B2M vs. the theoretical slope of 1) indicate the least proportional response of the three approaches (Fig. 1A). The Cells-to-CT kit showed a more linear response than the CellsDirect kit (n = 2). Pre-amplified sample was analyzed for GAPDH expression in microtiter or nanoliter qPCRs using the 7900HT (A) or BioMark (B) platform. Data points represent mean Cq values for each pre-amplified sample (qPCR replicates: n = 9, BioMark); n = 2, 7900HT). Slopes and R^2 values are displayed from linear regression of Cq values against log2 CE.

Comparison of one-step RT–qPCR with preamplification using the CellsDirect kit. A 10-fold dilution series of ERCC-84 RNA standard from 10^4 to 10 copies was prepared in cell background equivalent to a single HFF-1 cell and six replicate RT–qPCR (open diamonds) or RT–preamplification reactions (open triangles) were performed. Three replicate qPCRs per preamplified sample were performed. Data points (n = 6) represent individual RT–qPCR or mean qPCR values (n = 3) from each RT–preamplification reaction. Slopes and R^2 values are displayed from correlation of Cq values against log2(RNA copy number). Arrows indicate delta Cq (ΔCq ± standard error between non- and preamplified sample series based on regression analysis.

Fig. 3. Comparison of one-step RT–qPCR with preamplification using the CellsDirect kit. A 10-fold dilution series of ERCC-84 RNA standard from 10^4 to 10 copies was prepared in cell background equivalent to a single HFF-1 cell and six replicate RT–qPCR (open diamonds) or RT–preamplification reactions (open triangles) were performed. Three replicate qPCRs per preamplified sample were performed. Data points (n = 6) represent individual RT–qPCR or mean qPCR values (n = 3) from each RT–preamplification reaction. Slopes and R^2 values are displayed from correlation of Cq values against log2(RNA copy number). Arrows indicate delta Cq (ΔCq ± standard error between non- and preamplified sample series based on regression analysis.

Fig. 4. Comparison of nanoliter- with microliter-scale qPCRs using a dilution series of laser-captured single cells. Lysate from a pool of 50 laser-captured HFF-1 single cells was diluted to the equivalent of 22.5, 4.5, and 0.9 cells and preamplified using the CellsDirect kit (n = 2). Pre-amplified sample was analyzed for GAPDH expression in microtiter or nanoliter qPCRs using the 7900HT (A) or BioMark (B) platform. Data points represent mean Cq values for each pre-amplified sample (qPCR replicates: n = 9, BioMark); n = 2, 7900HT). Slopes and R^2 values are displayed from linear regression of Cq values against log2 CE.
approaches, suggesting that this methodology gave more proportional and accurate results.

**Precision of RT–qPCR kits at single cell level**

The performance of all three kits was assessed in terms of the number of successful RT–qPCR repeats and the variation between replicate measurements (SD $C_q$) observed across a dilution series of ERCC-84 RNA standard from $10^4$ to $10^0$ copies (Table 1). For the CellsDirect and PicoPure/Message Sensor kits, all of the RT–qPCR repeats across the range gave a positive $C_q$ value, whereas for the Cells-to-CT kit, the 100% success rate was achieved upward of $10^5$ copies. Overall, the precision of the CellsDirect kit was higher than that of the other two approaches, with SD $C_q$ below 0.25 for all three levels of RNA concentration (Table 1).

**Investigating potential inhibitors of RT–qPCR**

The results of the cell dilution series with the CellsDirect and Cells-to-CT kits suggested possible inhibition of these two direct lysis kits by reaction components such as lysis buffer, reverse transcriptase and cell background. To address this, RT–qPCR was performed using RNA or DNA standards (see Materials and Methods), varying these three parameters. A 10-fold serial dilution of exogenous ERCC-84 RNA ("RNA standard") was used to investigate potential inhibition of the RT–qPCR process by the presence of lysis buffer (Fig. 2A and B). Results were analyzed by linear regression of $C_q$ values versus log$_2$(RNA copy number), rather than the usual convention for qPCR data of plotting $C_q$ versus log$_2$(DNA copy number/concentration) so that the slope reflects the linearity of both the RT and qPCRs (vs. the ideal slope of ~1.0), as opposed to the efficiency of the qPCR assay alone. Investigation of the effect of lysis buffer on the CellsDirect kit revealed a significant decrease in $C_q$ values of RT–qPCR in the presence of lysis buffer compared with the RNA template in water (~1.6 cycles, $P < 0.0001$), showing a beneficial effect of the lysis buffer in the RT–qPCR (Fig. 2A). For the Cells-to-CT kit, mean $C_q$ values of ERCC-84 RNA template in lysis buffer were significantly higher than those in a water background (two-way ANOVA, $P < 0.0001$), particularly for $10^4$ copies of RNA standard ($P < 0.0001$) (Fig. 2B). ERCC-84 RT–qPCR results using the Cells-to-CT kit in the presence of lysis buffer were also less linear than in the absence of this component ($R^2 = 0.946$ vs. 0.989) (Fig. 2B).

A dilution series of ERCC-84 DNA standard was performed to measure the qPCR efficiency of the kits and possible inhibition of the qPCR step by the reverse transcriptase enzyme (Fig. 2C and D). Because the CellsDirect kit is a one-step kit with the reverse transcriptase and Taq polymerase combined, it was not possible to investigate the effect of the reverse transcriptase in isolation; however, the slope of the DNA standard dilution series (in lysis buffer) (Fig. 2C) suggested an acceptable qPCR efficiency (a slope of ~1.1 equates to efficiency of PCR amplification of 87%; see Materials and Methods). For the Cells-to-CT kit, investigation of the effect of RT enzyme on qPCR efficiency revealed significantly lower $C_q$ values ($P < 0.0001$) and improved linearity in the absence of reverse transcriptase ($R^2 = 0.986$ vs. 0.979) (Fig. 2D).

The effect of cell background on RT–qPCR was analyzed by measuring a fixed number of ERCC RNA copies in the presence of no background or 0.2, 2, 20, or 200 CEs (Fig. 2E and F). For the CellsDirect kit, this analysis showed that no significant differences in $C_q$ values were observed in the presence of varying cell background levels (Fig. 2E). In the results obtained by the Cells-to-CT kit, the addition of cell background equivalent to 200 cells led to an improvement in performance, with a reduction in mean $C_q$ of 4 cycles compared with smaller quantities of cell background ($P < 0.005$) (Fig. 2F).

**Evaluation of BioMark qPCR platform**

Having characterized three different RT–qPCR methodologies suitable for analysis of several genes in single cells, the suitability of a recently developed high-throughput qPCR platform for multi-target single cell analysis was also evaluated. Single cell analysis using BioMark microfluidic real-time PCR arrays ("dynamic arrays") requires preamplification of the sample to generate sufficient material for the analysis of multiple genes. Therefore, initially the use of preamplification prior to qPCR was investigated using ERCC-84 RNA standards and compared with a one-step RT–qPCR approach, using the 7900HT system in both cases (Fig. 3). For these studies, the CellsDirect kit was used for both RT–qPCR and RT–preamplification because this kit showed good linearity and high precision at low copy numbers (Fig. 2 and Table 1). In addition, this is a one-step RT–qPCR kit that is amenable to high-throughput procedures.

Initially, the propensity for preamplification to introduce amplification bias of individual genes within a complex background based on their abundance was investigated using a 10-fold serial dilution of exogenous ERCC-84 RNA standard from $10^4$ to $10^0$ copies in a single cell background equivalent. Preamplification of template using the CellsDirect kit with 18 cycles of PCR followed by quantification by qPCR was compared with one-step RT–qPCR (Fig. 3). Both methods displayed good precision and linearity, with $R^2$ values greater than 0.99 and slopes of ~1.02 and ~1.03 (vs. an ideal slope of 1.0) for the one-step RT–qPCR and preamplification approaches, respectively. Regression analysis confirmed that there was no significant difference between the slopes of the two data series and indicated a consistent decrease in $C_q$ values across the copy number range (mean $C_q$ of 12.93, standard error of 0.29) for the preamplified samples compared with the nonamplified samples (Fig. 3).

Following the analysis of preamplification methodology on the 7900HT qPCR platform, qPCR performance of the nanofluidic BioMark Dynamic Arrays was compared with that of the standard microliter volume qPCR platform based on GAPDH measurements in a 5-fold dilution series from a pool of 100 laser capture microdissection (LCM)-collected single HFF-1 cells diluted to 22.5, 4.5, and 0.9 CEs ($n = 2$) (Fig. 4). GAPDH expression was detectable in 0.9 CEs using both microliter- and nanoliter-volume qPCR platforms (Fig. 4A and B, respectively). Linear regression analysis of the

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**Table 1**

<table>
<thead>
<tr>
<th>RNA copies</th>
<th>Cells Direct</th>
<th>Cells-to-CT</th>
<th>Message Sensor</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^4$</td>
<td>√</td>
<td>0.24, N/A</td>
<td>0.73</td>
</tr>
<tr>
<td>$10^3$</td>
<td>√</td>
<td>0.04, 0.34</td>
<td>0.23</td>
</tr>
<tr>
<td>$10^2$</td>
<td>√</td>
<td>0.12, 0.39</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Note. SD, standard deviation. Triplicate RT–qPCRs were performed with different copy numbers of ERCC-84 RNA standard, and the numbers of positive reactions and SDs between replicate $C_q$ values were measured for each kit.
Discussion

In this study, we have compared three RT–qPCR kits (CellsDirect, Cells-to-CT, and a combination of the PicoPure extraction kit with the Message Sensor RT–qPCR kit) for analysis of gene expression at the single cell level. The sensitivity, linearity, and reaction efficiencies of the kits were investigated using a cell dilution series and RNA dilution series (Figs. 1 and 2). This approach ensured that the entire process from cell lysis to quantification is assessed, namely lysis/extraction, RT, and qPCR steps; hence, any biases or inhibition imposed from the various stages of process would affect the final Cq value. It should be noted that whereas the efficiency of qPCR is normally modeled by linear regression of Cq values versus log_{10} (DNA concentration), with a slope of ~3.3 indicating the ideal doubling of template with each PCR cycle (efficiency = 2.0) [31], the efficiencies of the nonexponential lysis and RT reactions are described as the yield of cDNA molecules (% efficiency for known RNA input) based on quantification of cDNA using a DNA standard curve [13,32]. RT efficiency may be influenced by multiple factors, including transcript copy number as well as total input per reaction [32]. Therefore, the slope of linear regression relating reaction input in terms of cell numbers to measured cDNA copies (Fig. 1) or RNA copies to Cq values (Figs. 2–4) reflects any biases in the multiple reaction stages, with significant deviation from the ideal slope of 1.0 likely to affect the accuracy of gene expression quantification, whereas the intercept of the regression is more informative regarding the efficiency of cell lysis and RT.

The cell dilution series (Fig. 1) demonstrated that all three kits assessed were capable of mRNA detection at the single cell level. The precision of the kits in terms of transcript copy number was investigated using dilutions of an IVT RNA standard (ERCC-84) (Table 1); the CellsDirect and PicoPure/Message Sensor kits again showed comparable sensitivity, detecting 10^2 copies, with the CellsDirect kit demonstrating higher precision at this level of transcript abundance, whereas consistent detection (100% positive) was observed for the Cells-to-CT approach at 10^3 copies and above. Thus, the CellsDirect and Cells-to-CT kits might not be ideal for high-throughput procedures. The CellsDirect kit compared with the CellsDirect kit (where lysate was stored at −80°C) showed significant inhibition at the single cell level of 45% and RT reaction (40%) in the RT and qPCRs, respectively, for the Cells-to-CT kit compared with the CellsDirect kit (where lysate formed 25% of the RT–qPCR) may have contributed to the inhibitory effects observed for the former method. This is an important factor to consider when developing a workflow for single cell analysis because larger volumes of lysis buffer for cell capture will necessitate a higher proportion of lysis reactions in the RT–qPCR.

In summary, the above results based on cell and RNA dilutions suggest that the Cells-to-CT kit may perform optimally with a higher cell number/total RNA level than that found in a single cell. In comparison, the PicoPure/Message Sensor kit would be suitable for single cell gene expression studies with a relatively small sample size. However, due to the requirement for the purification step, this kit combination might not be ideal for high-throughput procedures. The CellsDirect kit also performed well at low cell and RNA copy numbers and did not demonstrate inhibition due to lysis buffer or RT components (Fig. 2), suggesting its suitability for single cell analysis.

It has been suggested that to minimize technical noise associated with single cell RT–qPCR, the amount of RT product in the qPCR should be maximized and technical replicates should be avoided [13]. However, the number of genes that could be assessed would be low because the lysate could be split into only a few RT–qPCRs before quantification would become unreliable. Methods for sample preamplification using specific target amplification overcome this limitation. The emergence of microfluidic PCR platforms and arrays, such as the BioMark system, also facilitates multigene analysis alongside high sample throughput. The BioMark platform has previously been shown to produce accurate RT–qPCR results across a range of target copy numbers, but not with total quantities of template corresponding to the single cell level [26,37]. Single cell analysis using this platform increases the number of gene targets; however, it also necessitates preamplification of template RNA or cDNA prior to qPCR, which could introduce bias due to
different levels of transcript abundance [25]; hence, this aspect was evaluated using dilutions of exogenous RNA standard in a constant cell background equivalent to a single cell. These investigations showed no detectable copy number bias for ERCC-84 within the range from 10,000 to 10 copies, as indicated by good proportionality observed in the slopes of both dilution series and consistent $\Delta C_{q}$ values across the copy number range with and without preamplification (Fig. 3). Interestingly, the variation between replicates for low copy number is similar for both approaches (Fig. 3), indicating that this could stem from the RT step, which is known to add significant variability for low copy numbers of RNA and using single cell templates [12,38].

The impact of scaling down qPCRs from standard microliter volumes to the nanoliter range was investigated for low cell number applications by comparing the performance of the BioMark arrays with that of the 7900HT in the quantification of GAPDH present in a dilution series generated from laser-captured single cells (Fig. 4). GAPDH was detected at the single cell level on both platforms, and the linearity of the RNA dilution series was also comparable, indicating that there is no reduction in sensitivity or accuracy of single cell RT–qPCR analysis by miniaturization of the qPCR.

Conclusions

Our study highlights that differences in the precision and linearity of RT–qPCR methodologies can have considerable impact on gene expression measurements of a single cell. Inhibitory effects due to reaction components such as lysis buffer and RT reagents can reduce the sensitivity of detection by RT–qPCR. Assessment of technical aspects associated with the recently developed BioMark qPCR platform indicates the suitability of this high-throughput approach for single cell analysis and highlights the importance of using external RNA standards and reference materials for validation of methodologies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.05.010.

References