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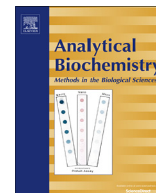

Validation of high-throughput single cell analysis methodology

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ABSTRACT

High-throughput quantitative polymerase chain reaction (qPCR) approaches enable profiling of multiple genes in single cells, bringing new insights to complex biological processes and offering opportunities for single cell-based monitoring of cancer cells and stem cell-based therapies. However, workflows with well-defined sources of variation are required for clinical diagnostics and testing of tissue-engineered products. In a study of neural stem cell lines, we investigated the performance of lysis, reverse transcription (RT), preamplification (PA), and nanofluidic qPCR steps at the single cell level in terms of efficiency, precision, and limit of detection. We compared protocols using a separate lysis buffer with cell capture directly in RT–PA reagent. The two methods were found to have similar lysis efficiencies, whereas the direct RT–PA approach showed improved precision. Digital PCR was used to relate preamplified template copy numbers to C_q values and reveal where low-quality signals may affect the analysis. We investigated the impact of calibration and data normalization strategies as a means of minimizing the impact of inter-experimental variation on gene expression values and found that both approaches can improve data comparability. This study provides validation and guidance for the application of high-throughput qPCR workflows for gene expression profiling of single cells.

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Introduction

Single cell analysis has yielded new insights into biological phenomena that are driven by cell populations composed of different cell types, such as in colon cancer [1], or cells responding heterogeneously to a given stimulus, such as T-cell responses to vaccines [2]. In addition to messenger RNA (mRNA)[1] expression profiling using reverse transcription (RT)–quantitative polymerase chain reaction (qPCR), microarray profiling, and RNA–Seq [3–5], methodologies to study genetic variation [6,7], DNA methylation [8], and protein and metabolite levels [9] have also been developed. As single cell analysis moves from being a research tool closer to applications in clinical diagnostics [10,11] and regenerative medicine [12,13], the molecular assays used to screen for disease- and tissue-related traits require stringent validation in order to meet criteria for approval as in vitro diagnostic tests [14] as well as means for ongoing standardization and quality control (QC). Measurement performance characteristics such as linearity, precision, and limit of detection, as well as appropriate controls, are all important aspects of clinical diagnostic tests or assays to assess the quality and consistency of tissue-engineered and stem cell products [15].

RT–qPCR is a key methodology for accurate and precise measurement of mRNA biomarkers and is already used for clinical monitoring and disease stratification [16,17]. A key development enabling high-throughput expression analysis of single cells is the use of microfluidic qPCR arrays that combine screening of hundreds of gene targets with the quantitative accuracy and dynamic range offered by RT–qPCR [18]; this has been applied to research in a wide range of fields, including cancer development, neurology, and stem cell biology [1,19,20]. The impact of RT, preamplification (PA), and qPCR steps on assay precision has been characterized for single cell RT–qPCR using standard microliter volume qPCR

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[1] *Abbreviations used:* mRNA, messenger RNA; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; QC, quality control; PA, preamplification; dPCR, digital PCR; LOD, limit of detection; 4-OHT, 4-hydroxy-tamoxifen; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CE, cell equivalents; EDTA, ethylenediaminetetraacetic acid; B2M, β-2-microglobulin; PPIA, peptidylprolyl isomerase A; RPLP0, large ribosomal protein 0; C_q, quantification cycle; NCC, no cell control; NTC, no template control; RT–minus, reverse transcriptase–negative; cDNA, complementary DNA; RQ, relative quantity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; DCX, doublecortin; GATA6, GATA binding protein 6; GFAP, glial fibrillary acidic protein; MAPT, microtubule-associated protein tau; MASH1, mammalian achaete–scute complex homolog 1; NCAM1, neural cell adhesion molecule 1; NEFL, neurofilament, light polypeptide; NES, nestin; NGN1, neurogenin 1; SOX2, SRY (sex determining region Y)–box 2; GOI, gene of interest; RG, reference gene; SD, standard deviation; CV, coefficient of variation; LCM, laser capture microscopy; gDNA, genomic DNA; MIQE, minimum information for publication of quantitative real-time PCR experiments; FD, fold difference.

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instruments [21,22]; however, to our knowledge, sources of technical variability within the entire workflow of high-throughput single cell analysis have not been investigated.

We previously investigated the accuracy, linearity, and precision of high-throughput nanofluidic qPCR platforms for gene expression biomarker analysis with conventional real-time platforms [23] as well as the performance of different RT–qPCR and PA protocols for single cell analysis [24]. Following on from this work, in the current study we investigate the precision of RT–PA and nanofluidic qPCR for a high-throughput single cell analysis approach. We investigate differences between alternative protocols with a separate lysis and DNase step compared with capture of the single cell directly in RT–PA buffer. We also demonstrate how digital PCR (dPCR) can be used for validation of assay performance characteristics such as limit of detection (LOD).

We draw on a model relevant to the fields of stem cell biology and regenerative medicine by applying the validation approaches to the measurement of single cells from two human neural stem cell lines: CTX0E03 and CTX0E16. Both cell lines were generated from stem cells from the same donor; however, CTX0E03 cells have been shown to be effective in a rat model of ischemic stroke [25] and are now in clinical trials for treatment of stroke-related disability [26], whereas CTX0E16 cells do not show clinical efficacy. We use data from a comparison study of the two cell lines at the single cell level (further results of which will be published elsewhere) to assess the impact of different mRNA quantification strategies, including calibration by a standard curve, reference gene, and global normalization.

Materials and methods

Cell culture

Sister neural stem cell lines CTX0E03 and CTX0E16 were provided by ReNeuron (Guildford, UK). The cell lines were established from somatic stem cells in the cortical neuroepithelium of the same donor and immortalized with the c-mycER^{TAM} gene, which is conditionally expressed in the presence of 4-hydroxy-tamoxifen (4-OHT) and enables cell expansion. In the absence of 4-OHT and growth factors (see below), cells undergo differentiation into neural cell types [25].

CTX0E03 cells (passage 9) and CTX0E16 cells (passage 16) were maintained in laminin-coated flasks (Sigma) in RMM medium [Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco) containing 0.03% human serum albumin (VWR), 100 μg/ml human apo-transferrin (Sigma), 16.2 μg/ml putrescine dihydrochloride (Sigma), 5 μg/ml insulin (Sigma), 60 ng/ml progesterone (Sigma), 2 mM L-glutamine (Sigma), 40 ng/ml sodium selenite (Sigma), 10 ng/ml basic fibroblast growth factor (Peprotech), 20 ng/ml epidermal growth factor (Peprotech), and 100 mM 4-OHT (Sigma)] at 37 °C and 5% CO_2.

To prepare bulk RNA from CTX cells under proliferating and differentiated conditions, a time course experiment was performed. CTX cells were revived from liquid nitrogen into six T75 flasks and maintained in RMM medium (20 ml of medium per flask with growth factors and 4-OHT as above) with medium renewal every 2 to 3 days. When the cells reached 80% confluence, one flask of cells (representing the basal condition) was rinsed with 10 ml of phosphate-buffered saline (PBS) without calcium or magnesium (PAA, part no. H15-002) and cells were dissociated with 4 ml of TrypZean, a recombinant form of trypsin that is free from animal source contaminants (Lonza), for 5 min at 37 °C. TrypZean was neutralized by the addition of 8 ml of Trit inhibitor (DMEM/F12 medium containing 0.044% human serum albumin (VWR), 0.55 mg/ml trypsin inhibitor (Sigma), and 0.25 units of Benzonase (Merck)), and the cells were counted using an automated cell counter (Vi-Cell XL, Beckman Coulter). The cells were centrifuged at 1500 rpm in an Eppendorf 5702 benchtop centrifuge, and the pooled cells were adjusted to a concentration of 1×10^6 cells/ml with PBS. The cells were recounted as before to ensure that the correct cell density had been achieved. The cell suspension was processed according to Section 'RNA isolation and preparation of reference RNA' in order to prepare cell pellets and cell lysates. The remaining flasks were maintained in culture as before until the cells reached confluency. At this point, an additional flask was harvested as above, and this represented "day 0" cells. The remaining flasks were washed with 10 ml of PBS per flask, and the medium was changed to differentiation medium (RMM medium without growth factors or 4-OHT) with medium renewal every 2 to 3 days. Further flasks were harvested for RNA isolation every 7 days (7, 14, 21, and 28 days post-confluence and growth factor withdrawal).

Laser capture microscopy

CTX0E03 or CTX0E16 cells were revived from liquid nitrogen into laminin-coated T75 flasks containing RMM medium (above) and maintained in culture for 3 days. After this time, the cells were rinsed 1× with PBS (PAA) and dissociated with TrypZean (as in Section 'Cell culture'). Cell density was assayed (as in Section 'Cell culture'), and 2×10^5 cells were transferred to laminin-coated PET laser dissection microscope slides (Zeiss) and allowed to adhere for 1 h at 37 °C and 5% CO_2. After this time, the medium was replaced with 1 ml of fresh RMM medium and the cells were placed at 37 °C and 5% CO_2 for 18 h. The medium was removed and replaced with 1 ml of RMM medium containing 4 μM calcein AM (Invitrogen). Cells were incubated at 37 °C and 5% CO_2 for 1 h to allow fluorescence to develop. The medium was removed from the cells, and the cells were rinsed once with 1 ml of PBS before fixing in 95% ethanol (Fisher)/5% acetic acid (Sigma) at −20 °C. Following 10 min of incubation at room temperature, the fixative was removed and the slides were air-dried before laser dissection using a Zeiss PALM Laser Capture Microscope. Cells were selected on the basis of displaying calcein fluorescence (excitation 488 nm/emission 516 nm) as an indicator of viability. (Only live cells convert nonfluorescent calcein AM to fluorescent calcein through the action of intracellular esterases.)

After cell selection, but prior to cutting, 15 μl of Cells Direct lysis buffer or RT–PA mix (see Sections 'Single cell processing and RT–PA (single cells captured in lysis buffer) and Single cell processing and RT–PA (single cells captured in RT–PA buffer)') was added to wells of a 96-well capture plate (Zeiss) and individual cells were dissected and catapulted into wells containing the mix. The collection plate was placed on top of a 96-well plate, and the samples were transferred to the plate by centrifuging for 1 min at 1000 rpm in a Jouan CR412l centrifuge. Plates were then sealed using a silicone sealing mat (Web Scientific) and placed on ice until heating (lysis buffer) or RT–PA. Cells captured in lysis buffer were heated at 75 °C for 15 min (PTC 225 Tetrad PCR System, MJ Research) prior to freezing at −80 °C. Cells captured in RT–PA buffer were processed according to Section 'Single cell processing and RT–PA (single cells captured in RT–PA buffer)'.

RNA isolation and preparation of reference RNA

For isolation of purified RNA from CTX0E03 or CTX0E16 cell lines, cells were cultured as in Section 'Cell culture'. Following cell detachment, cells were resuspended in PBS at a concentration of 10^6 cells/ml. Aliquots (1 ml) were prepared, and the cells were pelleted by centrifugation at 13,500 rpm in an MSE MicroCentaur benchtop centrifuge for 2 min. The supernatant was removed, and cell pellets were stored at −80 °C until required. Alternatively,

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