TECHNICAL NOTE

Single-Cell Gene Expression Profiling using TaqMan® Gene Expression Assays with the C_1™ Single-Cell Auto Prep System

PURPOSE

This application note evaluates the use of the TaqMan Gene Expression Assays for Specific Target Amplification (STA) with the C_1 Single-Cell Auto Prep System. Please refer to the protocol entitled, *"Using the C_1™ Single-Cell Auto Prep System to Capture Cells from Cell Culture and Perform Preamplification Using TaqMan® Assays"* (PN 100-6117) for more information.

INTRODUCTION

When quantifying mRNA, real-time PCR can be performed as either a one-step reaction, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single step, or as a two-step reaction, in which reverse transcription and PCR amplification occur in separate tubes (Wong et al. 2005). This application note compares the C_1 Single-Cell Auto Prep System using a two-step method to a conventional one-step, in-plate reverse transcription (RT) and STA PCR method to evaluate single-cell gene expression using TaqMan assays. The Ambion® Single Cell-to-C_t™ and the CellsDirect™ protocols were used to perform RT and targeted preamplification to prepare 48 single-cell cDNA libraries for real-time PCR analysis.

Many of the challenges in a one-step single-cell gene expression workflow stem from three important issues:

1. **Verification:** Inability to individualize and confirm single, viable cells prior to experimentation and analysis
2. **Reproducibility:** Intra-assay variability stemming from multiple, liquid-handling steps
3. **Limit of Detection:** Lack of sensitivity with one-step methods to detect low abundant transcripts due to RNA degradation (Wong et al. 2005).

It is widely understood that a two-step method offers significant advantages, including better sensitivity and reproducibility compared to one-step workflow; however, it is significantly more expensive in a tube or plate-based format. The C_1 System is an automated cell preparation solution that offers several technical and economic advantages. It standardizes the isolation, verification, lysis, and preparation of 96 single, living cells in parallel. It complements cellular enrichment techniques, such as Fluorescence Activated Cell Sorting (FACS) and allows researchers to visually confirm the capture and viability of target cells after enrichment (Figure 1A). The C_1 System eliminates the complicated liquid handling and process of preamplification methods such as STA, minimizing manual manipulation of cell samples and therefore the variability between experiments. Due to the nanoliter reaction scale, the two-step RT-PCR method implemented on the C_1 System offers significant cost saving on a per reaction basis.

The C_1 System utilizes an Integrated Fluidic Circuit (IFC) to enable capture and visualization of single cells using a confocal microscope. In addition, automated scripts enable fluorescent viability/cytotoxicity staining to verify viability upstream of analysis, thereby

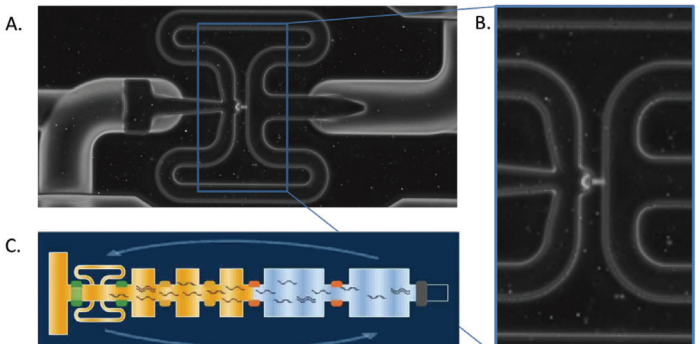

Figure 1. C_1 Integrated Fluidic Circuit (IFC) capture site and multi-step reaction architecture. The C_1 IFC contains 96 single-cell capture sites and utilizes multiple compartments to perform multi-step reactions and to mix reagents without manual manipulation. **A.** This panel shows a single BJ fibroblast cell isolated in a capture site on the IFC. **B.** Inset panel displays enlarged image of cell capture site. **C.** Diagram of the IFC architecture downstream of the capture site, illustrating the individual chambers that carry out the step-wise chemistry required for STA. After the cell is lysed the cellular contents flow into the first chamber. Next, RT reagents flow into the first two chambers. Afterward, preamplification reagent flows through the chamber expanding the reaction volume and the reaction is thermal cycled across all five chambers. Finally, harvest reagent pushes the entire contents of all wells out to the harvest inlet. After a simple dilution step, real-time PCR is performed on the BioMark™ HD System.

eliminating variability in the analysis downstream of the experiment. After capture and staining, the C_1 IFC employs multi-step reaction architecture (Figure 1B) to enable fully automated multi-step reaction chemistry. The C_1 Single-Cell Auto Prep System has incorporated automated thermal and pneumatic controls within a closed environment to standardize the addition, mixing, and thermal cycling of all reagents without requiring manual intervention.

TECHNICAL BACKGROUND

Studies of single-cell eukaryotic gene expression have revealed that transcription occurs in pulses (Chubb et al. 2006; Livak et al. 2013) resulting in significant stochastic variation in transcript quantities from cell-to-cell (Sanchez-Freire et al. 2012). High-throughput, cost-effective methods are required in order to collect enough data from a sufficient number of cells to characterize the variation inherent in single-cell gene expression; performing such a study using conventional real-time PCR in plates would be cost prohibitive due to the large volume of PCR master mix required (Livak et al. 2013). The C_1 Single-Cell Auto Prep System in tandem with the BioMark HD System provides a complete automated workflow to isolate single cells and perform high-throughput real-time PCR, enabling precise assessment of cell-to-cell transcriptional heterogeneity. This workflow enables an automated real-time PCR protocol that provides a cost effective means to evaluate more individual cells and greater sensitivity to detect less abundant transcripts. This two-step protocol was developed specifically for single-cell gene expression analysis.

EXPERIMENT

Performance Evaluation of TaqMan Gene Expression Assays on the C_1 System

This experiment evaluates the sensitivity and reproducibility to detect 48 transcripts in the BJ fibroblast cell line over a broad dynamic range using two different RT-STA workflows. The Ambion Single Cell-to-C_t two-step protocol was carried out on the C_1 PreAmp IFC for cells with an average diameter of 17-25 µm (PN 100-5758) and compared to the CellsDirect one-step protocol for RT and STA performed on cells pre-sorted by FACS in a 96-well plate. The sensitivity of each method was determined by comparing the C_t values for each transcript using TaqMan Gene Expression assays for transcripts that varied in abundance. TaqMan assays were chosen to interrogate a broad range of transcripts according to previous empirically determined RNA copy number. Analyses comparing the C_t values for the assays were performed on the single-cell transcriptional profiles to examine the efficacy of each method.

Materials and Methods

A T-75 flask of BJ fibroblasts were grown to 80% confluence, trypsinized and split into two equal fractions for experimentation. One fraction was used to evaluate the TaqMan Gene Expression Assays with the Single Cell-to-C_t protocol on the C_1 System. The remaining cells were sorted by FACS into a 96-well PCR plate for analyses with the CellsDirect workflow. For the Single Cell-to-C_t C_1 protocol, the RT and preamplification steps were performed using 48 pooled TaqMan assays in conjunction with the Ambion Single Cell-to-C_t kit (Life Technologies, PN 100-4904 B1 and Protocol C, *Fluidigm® Real-Time PCR Analysis Software User Guide*, PN 68000088).

The Single Cell-to-C_t protocol has been adapted for the C_1 System, whereby the RT and preamplification steps are carried out in succession (on a nanoliter scale) in accordance with the manufacturer's protocol offering greater sensitivity to detect low abundant transcripts. For the C_1 workflow, the C_1 PreAmp IFC was used to isolate individual BJ fibroblast cells on the C_1 System from a prepared suspension of cells (200 cells/µl). Single-cell capture and viability were verified on the C_1 IFC using the Live/Dead® Viability/Cytotoxicity Kit for Mammalian Cells (Life Technologies, PN L3224) by on-chip staining and visualization with fluorescence microscopy prior to lysis.

BJ fibroblasts were also sorted by FACS and processed using the CellsDirect protocol with the same 48 pooled TaqMan assays used in the C_1 workflow. The cells were sorted based on viability using the same Live/Dead Viability/Cytotoxicity Kit stated above (Life Technologies, PN L3224) to obtain live cells for analyses. The RT-STA cDNA amplicons from both the C_1 and CellsDirect experiments were diluted according to manufacturer's instructions, and loaded onto a single Gene Expression Dynamic Array™ 96.96 IFC (PN 68000130) for real-time PCR analysis on the BioMark HD System. Additionally, no-template control (NTC) and tube controls (containing 24 BJ fibroblast cells isolated by a dilution series from a count obtained with a hemocytometer or FACS) were performed for both the C_1 and FACS sorted experiments. The comparison of the ensemble averages for each single-cell experiment was compared to their respective tube controls (Figure 2).

RESULTS

The heat map of C_t values in Figure 3 illustrates the gradient of transcript levels from low to highly expressed genes from the real-time PCR analysis of the TaqMan assays performed using either the two-step Single Cell-to-C_t or one-step CellsDirect workflows. The real-time PCR result from the cells prepared with the C_1 protocol exhibited a shift in C_t of three to nine cycles (Figure 4) across a broad range of transcript levels demonstrating greater sensitivity over the CellsDirect workflow performed in plate. In addition, the single cells run on the C_1 System and CellsDirect in tube format captured 48/48 and 34/48 of the transcripts respectively. Of the low abundance transcripts the CellsDirect workflow in-tube format identified 4/16 transcripts, whereas all 16 were identified with the C_1 System. Tube controls were performed whereby the bulk analysis of cells was compared to the ensemble average of the single cell experiments. The correlation between tube (24 cells) and the ensemble single-cell data for Single Cell-to-C_t and CellsDirect methods were R^2 = 0.96 and 0.85 respectively (Figure 2).

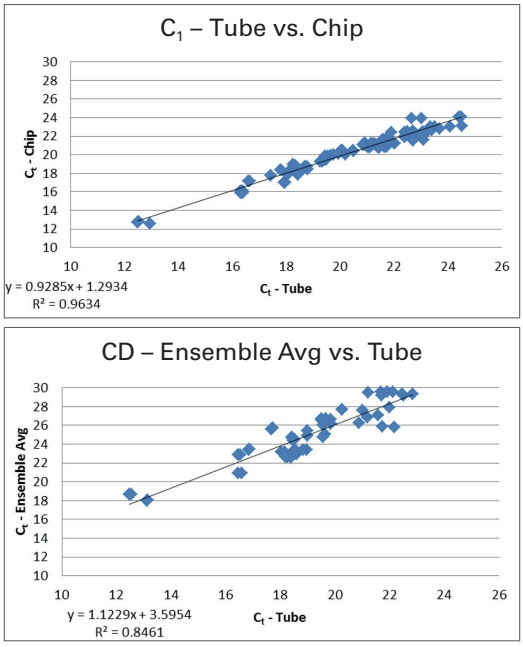

Figure 2. Ensemble vs. Chip Comparison. The correlation of the ensemble average of single cells vs. tube was determined for each method (C_1 and Single Cell-to-C_t and in-plate and CellsDirect) by comparing the ensemble average of C_t values of the single cells with a tube control of 24 cells.

CONCLUSION

TaqMan® Gene Expression Assays demonstrated improved single-cell transcript sensitivity with the C_1 System combined with the two-step Single Cell-to-C_t workflow on an automated platform at the nanoliter scale. The adaptation of TaqMan Assays to a fully integrated microfluidics workflow minimizes reagent usage and enables a cost effective real-time PCR protocol and reproducible analysis of gene expression at the single-cell level. With the combined ability to verify viable cell capture and implement the highly sensitive two-step RT-STA protocol with TaqMan Assays, the C_1 System enables a comprehensive methodology for single-cell gene expression. Moreover, the high-throughput capabilities of the C_1 System combined with the microfluidic-based single-cell gene expression workflow provides a robust platform for generating large data sets for statistically significant analyses with minimal intra-assay variation.

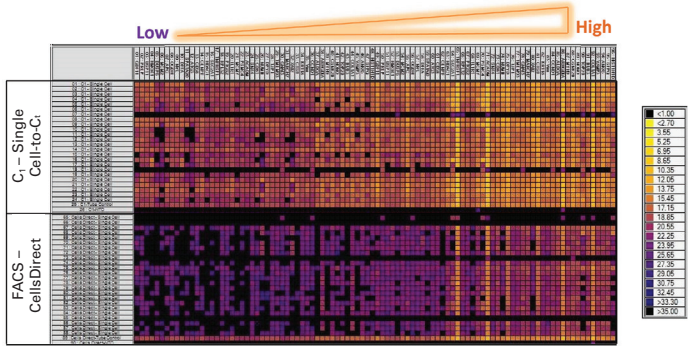

Figure 3. Heat map of C_t values for C_1/Single Cell-to-C_t method and in-plate/CellsDirect method over the same 48 TaqMan Assays. The transcripts are sorted from low to highly expressed genes (left to right).

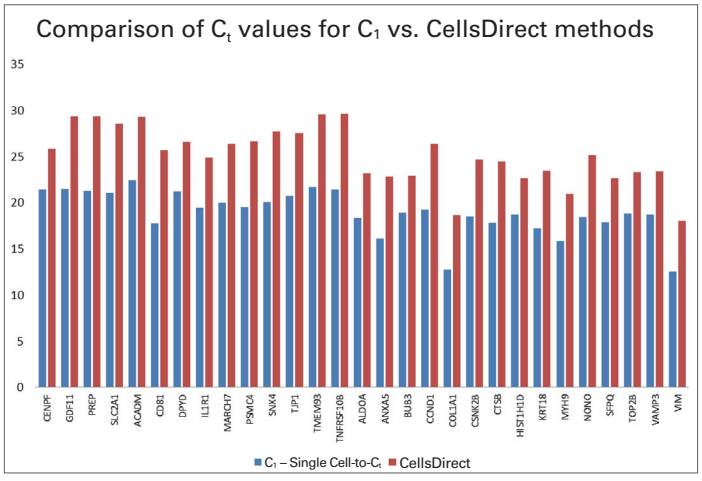

Figure 4. Comparison of C_1/Single Cell-to-C_t to in-plate/CellsDirect methods using TaqMan assays. The Ensemble C_t values are compared between the Single Cell-to-C_t workflow performed on the C_1 System and the CellsDirect workflow performed in a 96-well plate. This plot illustrates the difference in C_t observed in Figure 3, whereby a shift of three to nine cycles is observed between the two methods indicating a clear sensitivity advantage using the C_1 method.

REFERENCES

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