TECHNICAL NOTE

Single-Cell MicroRNA Expression Profiling with the C_1™ Single-Cell Auto Prep System

INTRODUCTION

MicroRNAs (miRNAs) are ubiquitous small, non-coding molecules that can have a significant impact on cell biology and disease. miRNAs regulate messenger RNA (mRNA) expression by either preventing mRNA translation or promoting degradation of mRNA (Bonifacio et al. 2010). miRNAs regulate thousands of human protein-coding genes in differentiation, development, and cell cycle management and in many diseases such as cancer and cardiovascular disease. Research in disease models has shown that even a single miRNA can regulate multiple mRNAs across biological systems.

Current miRNA research focuses on the impact of differential miRNA expression on the regulation of mRNA. Over 1000 miRNAs have been characterized and determined to be differentially expressed by cell or tissue type. Studying miRNA expression at the single-cell level removes much of the ambiguity by allowing researchers to measure variable expression between cells of the same sample type and ascertain cell sub-populations. However, conventional single-cell methods, such as tube-based real-time PCR or microarrays, require manual isolation, handling, and interrogation for hundreds of miRNAs. These manual workflows are tedious, inflexible, and prone to error.

Because a single miRNA has the ability to regulate many biological systems and has context-specific expression, it can be challenging to distinguish true biological heterogeneity from technical variability. This technical note explains how the C_1 Single-Cell Auto Prep System simplifies the isolation and preparation of individual cells to reliably detect miRNA expression signatures.

TECHNICAL BACKGROUND

This technical note describes an automated method to prepare microRNA libraries from live, single cells using the C_1™ Single-Cell Auto Prep System and TaqMan® Megaplex™ assays for targeted miRNA expression profiling on the BioMark™ HD System. In less than 14 hours, 96 single cells were isolated, stained, visually verified, lysed, reverse transcribed, preamplified, and interrogated for miRNA expression. With the C_1 System workflow, single-cell miRNA libraries containing over 370 miRNA species were prepared for downstream analysis on the BioMark HD System. Using this workflow, human induced pluripotent stem cells (iPSCs) and neural progenitor cells (NPCs) were assessed for pluripotency while BJ fibroblasts were profiled for senescent miRNA expression across several passages.

MATERIALS AND METHODS

Cell lysis, reverse transcription (RT), and preamplification using the Specific Target Amplification (STA) method were performed 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 of MicroRNA Species with TaqMan® Assays* (PN 100-6667) for more detailed information.

Cell culture of iPSCs, NPCs, and BJ Fibroblasts was performed using standard culturing conditions. Cells were grown to 80% confluence in a T-75 flask prior to experimentation. Human iPSCs were routinely passaged with 0.5 µM EDTA and maintained with Essential 8™ medium (Life Technologies) on Matrigel™ (1:80 DPBS). iPSCs were freshly dissociated into single-cell suspension using Accutase for 5–7 minutes at 37 ˚C. Human NPCs were differentiated from iPSCs in chemically defined conditions using small molecules LDN-193189 and SB-431542 for dual-SMAD inhibition, as well as the hedgehog-inhibitor cyclopamine (Chambers et al. 2009). Induction was confirmed by immune-staining for PAX6, an NPC- specific marker.

The C_1 Integrated Fluidic Circuit (IFC) was used to isolate individual cells from a prepared suspension of cells (200 cells/uL). Two types of IFCs were implemented, the C_1 PreAmp IFC for medium cells (P/N 100-5479) was used to isolate iPSCs and NPCs, while the C_1 IFC for large cells (PN 100-5758) was used for BJ fibroblasts. Single-cell capture and viability were verified by staining on the C_1 IFC with either the Live/Dead® Viability/Cytotoxicity

Kit for Mammalian Cells (Life Technologies, PN L-3224) or StainAlive™ TRA-1-60 Antibody (DyLight™ 488, Stemgent) and CellTracker™ Orange CMRA (Life Technologies). Single cells were visualized by microscopy prior to lysis on the C_1 System.

After capture and visualization, the C_1 System automatically lysed the individualized cells and performed reverse transcription and preamplification for 377 miRNAs per cell. miRNAs were reverse transcribed using the TaqMan® Megaplex™ Human miRNA RT Pool A (Life Technologies) and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies). Preamplification of reverse-transcribed miRNA species was performed using the Ambion® Single Cell-to-C_t™ kit (Life Technologies).

From a Megaplex RT cDNA yield of 377 miRNA species, 96 specific miRNAs were interrogated for expression analysis on the BioMark HD System. Control and targeted miRNA expression assays were selected to screen each cell type. Positive tube controls and no template controls (NTC) were prepared and processed in each IFC. Targeted miRNA expression analysis was performed using a gene expression Dynamic Array™ 96.96 IFC (PN 68000130) and the BioMark HD System for real-time PCR. For more detailed information on BioMark System methodology, refer to the *Real-Time PCR Analysis Software User Guide* (PN 68000088).

Data analysis was performed using the SINGuLAR™ Analysis Toolset, an R-script package for single-cell analysis. The SINGuLAR toolset was used for principal component analysis (PCA) and violin plots to identify cell populations.

PCA reduces the dimensionality of correlated variables, such as co-expressed miRNAs, through orthogonal linear transformation into principal components (PC). PCA can reveal simplified models of structure in the expression data. The violin plots demonstrate the conversion of C_t values to the log2 expression values on the Y-Axis. Cluster density (number of single cells) is displayed on the X-axis.

RESULTS

Occupancy rates of BJ Fibroblasts and iPSCs

Single cells were isolated by the C_1 System and verified by microscopy. Cells captured on the C_1 IFC were characterized for pluripotency and viability prior to RT-PCR and analyses. Single BJ Fibroblasts occupy 88 of the 96 capture sites (Appendix B). BJ Fibroblasts were stained with the Live/Dead Viability/Cytotoxicity Kit, which consists of Calcein-AM and ethidium homodimer to assess viability (Figure 1). Single iPSCs occupy 83 of the 96 capture sites (Appendix B). To simultaneously determine pluripotency and viability, iPSCs were dual stained with TRA-1-60 Antibody and CellTracker™ Orange (Figure 1). Refer to the technical note, *Automated Cell Staining of Induced Pluripotent Stem Cells on the C_1 Single-Cell Auto Prep System* (PN 100-6857) for more detailed information.

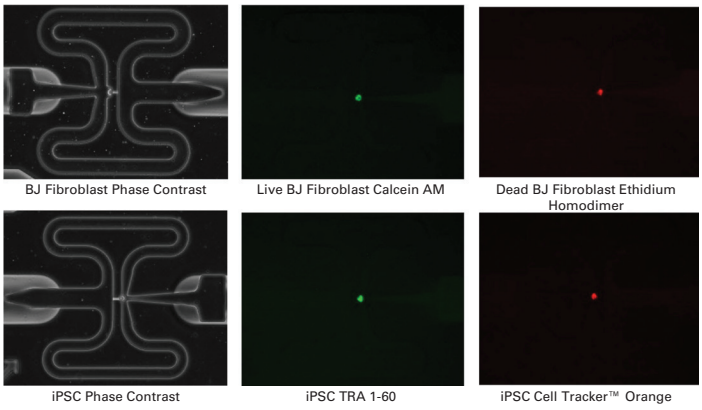

Figure 1. **Single-cell capture and staining.** Single BJ Fibroblasts are captured and confirmed by phase contrast microscopy followed by viability assessment using Live/Dead® Viability/Cytotoxicity Kit. Single iPSCs are captured and confirmed by phase contrast microscopy followed by pluripotency (TRA-1-60) and viability (CellTracker Orange) assessment.

Role of miRNA expression analysis to characterize cellular differentiation and reprogramming

miRNA expression profiles of each cell type (BJ Fibroblasts, iPSCs, and NPCs) demonstrate up- and down- regulation of a variety of lineage-specific miRNAs. Moreover, heterogeneity of miRNA expression is discerned within sub-populations of cells, which may be indicative of miRNA turnover or pluripotent cell state.

For many systems, miRNA expression analysis can be used to monitor pathway-specific regulation of pluripotent state and differentiation. In heterogeneous cell populations, monitoring miRNAs at a single-cell level provides a more definitive picture of pathway regulation. Figure 3 illustrates the miRNA heterogeneity observed within single iPSCs. Additionally, as shown in Figure 2, PCA of iPSC and NPC miRNA expression profiles reveals two distinct populations (Appendix A for C_t values).

iPSC induction efficiency is enhanced by modulating miRNA levels (Li et al. 2011). The presence or absence of certain miRNAs can indicate a pluripotent state. Further analysis of iPSCs with an extended panel of assays shows a variety of regulatory miRNAs known to characterize a variety of developmental transitions (Figure 3), and published analyses of miRNAs in iPSCs reveal many expression profiles consistent with pluripotent state. Liu et al. (2013) demonstrate that miR-452 directly target and suppress multiple stemness regulators, including Bmi-1, LEF1, and TCF4, resulting in cells with reduced stem-like traits.

Consistent with published results, iPSCs do not express

miR-155, which is expressed in the latest stages of differentiation and is not present in pluripotent cells (Kamata et al. 2010). Interestingly, among the most highly expressed miRNAs is miR-372 (Li et al. 2009), which is typically observed in embryonic stem cells.

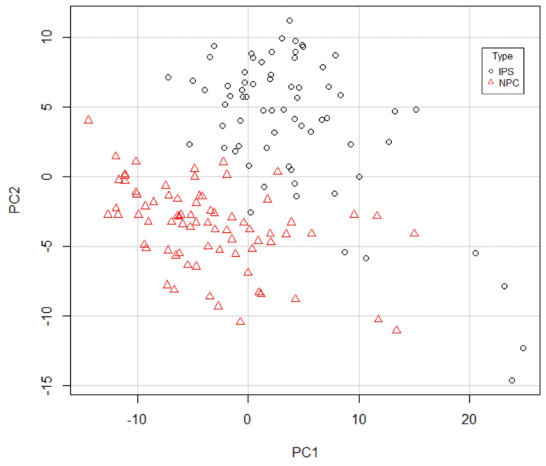

Figure 2. PCA comparing miRNAs expressed in single iPSCs and their NPC progeny. There is clear separation of iPSCs and their derived NPC progeny, demonstrating the differences between cell types in their global miRNA profile.

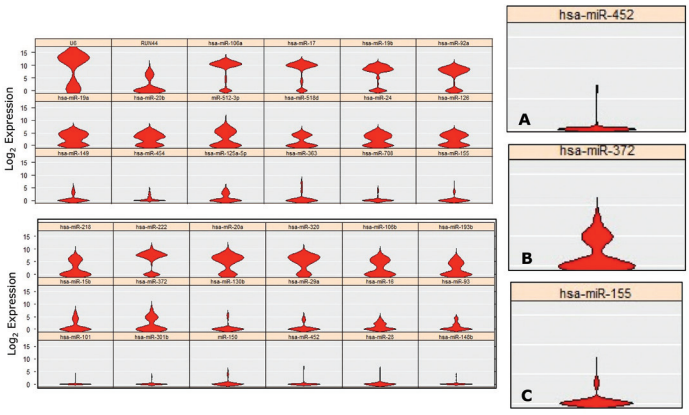

Figure 3. Violin plots of miRNA expression in iPSC populations. The miRNA expression profiles of individual cells demonstrate the variability of abundance and population heterogeneity. Canonical miRNAs indicative of different stages of iPSC development are highlighted in A. miR-452, B. miR-372, and C. miR155 panels. The presence of miR-372 and absence of miR-452 and miR-155 in the expression profile are indicative of mature iPSCs as described in the literature.

Comparison of BJ Fibroblasts from Passage 24 vs. Passage 7

Defective synthesis of canonical miRNAs results in cellular senescence in primary fibroblasts, identifying global miRNA disruption as a senescence trigger (Gómez-Cabello D, et al 2013). This finding illustrates the significance of implementing global approaches to study miRNAs and their effect on senescence. In particular, senescence is marked by down-regulation of miR-155, miR-17, and miR-106a in human foreskin fibroblasts (Bonifacio LN, and Jarstfer MB, 2010).

To examine the effects of cell culture passage on up- and down- regulation of miRNA expression, PCA analysis was performed on passaged BJ fibroblasts. Cells between passages 7 and 24 form distinct clusters due to changes in miRNA expression (Figure 4A). Typically observed in senescent cells, miR-106b, miR-143, and miR-34a are up-regulated in Passage 24. When compared, miRNA expression levels in Passage 13 to Passage 24 are indistinguishable by PCA analysis (Figure 4B). While miRNA expression at close passages does not demonstrate senescence, subpopulations and unique miRNA expression patterns are observed in single cells (Figure 4B and 4D).

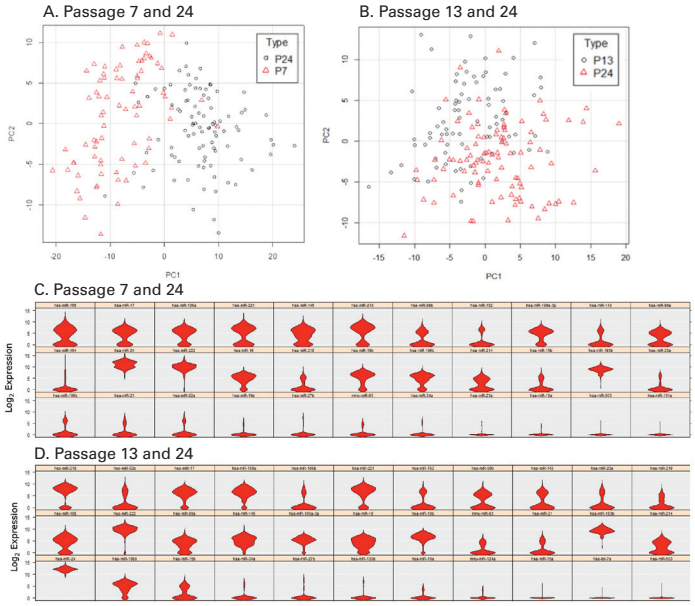

Figure 4. PCA analysis of miRNA expression of BJ fibroblasts at varying passage number. A. Passages 7 and 24 display two clusters illustrating differing miRNA expression profiles in senescent passage 24. B. Passages 13 and 24 are compared and undistinguishable based on miRNA expression profile. C. Violin plots of passages 7 and 24 bimodal subpopulations. D. Although clusters cannot be distinguished by PCA, violin plots of passages 13 and 24 illustrate clonal subpopulations.

CONCLUSION

Single-cell analysis for miRNA expression reveals heterogeneity across cell populations. The miRNA expression profiles and relative C_t values are significantly different between iPSCs and their NPC progeny. Violin plots of miRNA expression in iPSCs (Figure 3) show biomodal expression of miRNAs known to characterize turnover and differentiation. This identification of heterogeneity is lost in bulk analyses and confounds interpretation of miRNA expression profiles within cell populations.

The C_1 System provides automated processing and interrogation of single-cells to differentiate miRNA expression and observe senescence in cell culture passaging. C_1 Single-Cell miRNA Expression Profiling is a simple and sensitive workflow to achieve single-cell resolution, rapid data acquisition, and assay flexibility for real-time PCR analysis.

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miR	iPSC (C_t)	NPC (C_t)	miR	iPSC (C_t)	NPC (C_t)
hsa-miR-101	24	25	hsa-miR-19b	12	12
hsa-miR-106a	11	12	hsa-miR-20a	14	14
hsa-miR-106b	16	17	hsa-miR-20b	17	17
hsa-miR-130b	22	20	hsa-miR-21	22	22
hsa-miR-145	29	28	hsa-miR-210	23	18
hsa-miR-148a	23	25	hsa-miR-218	19	16
hsa-miR-152	–	23	hsa-miR-221	19	19
hsa-miR-155	23	22	hsa-miR-222	15	17
hsa-miR-15a	–	26	hsa-miR-23b	23	–
hsa-miR-15b	19	18	hsa-miR-24	17	14
hsa-miR-16	20	18	hsa-miR-27b	–	22
hsa-miR-17	12	12	hsa-miR-301b	27	22
hsa-miR-181a	25	25	hsa-miR-34a	–	21
hsa-miR-183	30	–	hsa-miR-371-3p	29	–
hsa-miR-18a	23	23	hsa-miR-372	20	–
hsa-miR-193b	17	17	hsa-miR-454	23	21
hsa-miR-199a-3p	26	–	hsa-miR-542-3p	27	–
hsa-miR-19a	17	18	hsa-miR-9	23	22
hsa-miR-99b	21	20	hsa-miR-92a	15	15
mmu-miR-124a	23	24	hsa-miR-99a	–	24
mmu-miR-93	19	20	–	–	–

Appendix A. Corresponding miRNA and C_t Values for the expression profiles comparing iPSCs to their NPC progeny (see Figure 2).

Cell Type	Stain	Number of Single Cells	% Cell Occupancy
iPSC	• TRA-1-60 • CellTracker Orange	83	86
NPC	• TRA-1-60 • CellTracker Orange	94	98
BJ Fibroblast	• Calcein AM/ Ethidium Homodimer	88	92

Appendix B. iPSC and NPC were dual stained on chip for surface antibody and viability. Percent occupancy refers to the number of single cells isolated in an individual capture site out of the 96 available capture sites.

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Corporate Headquarters
7000 Shoreline Court, Suite 100
South San Francisco, CA 94080 USA
Toll-free: 1.866.FLUIDLINE | Fax: 650.871.7152
www.fluidigm.com

Sales
North America | +1 650 266 6170 | info-us@fluidigm.com
Europe/EMEA | +33 1 60 92 42 40 | info-europe@fluidigm.com
Japan | +81 3 3662 2150 | info-japan@fluidigm.com
China (including Hong Kong) | +86 21 3255 8368 | info-china@fluidigm.com
Asia | +1 650.266.6170 | info-asia@fluidigm.com
Latin America | +1 650 266 6170 | info-latinamerica@fluidigm.com