Application Note

A workflow combining high-accuracy cell sorting with digital PCR for analysis of miRNAs in defined cell pools and single cells

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Introduction

Analysis of miRNAs in defined cell pools and on the single-cell level

Compared to traditional methods of analyzing cell populations in bulk, single-cell analyses allow researchers to uncover new information about biological processes, such as the physiological and pathological processes of an individual cell.

The most recent miRBase release lists 2654 annotated human miRNAs. miRNAs are important non-coding, well-conserved, post-transcriptional regulators with great potential as diagnostic or prognostic biomarkers. miRNA expression profiles offer insights into cellular mechanisms and cellular states, such as human cancers. Each individual cell is a unique microenvironment. Hence, miRNA regulation is expected to vary from cell to cell. However, analysis of miRNAs is mainly based on bulk expression data from large populations of cells. Single-cell analyses have focused mainly on protein-coding RNAs, despite increasing evidence that non-coding RNAs are actively involved in cell function and specialization.

Commonly used PCR, quantitative PCR (qPCR), or next-generation sequencing (NGS)-based methods allow for single-cell analysis, but sometimes lack the sensitivity required to detect the target of interest.

Other factors affect the specificity and accuracy of miRNA quantification in single cells, including single-cell isolation, efficient lysis, RNA extraction and reverse transcription (RT).

Digital PCR (dPCR), on the other hand, provides highly accurate and precise detection and can be used to quantify miRNAs at a single cell or cell-pool level with a well-defined number of cells. dPCR, when combined with a high-accuracy single-cell sorting instrument such as the cellenONE®, solves two major challenges: high sensitivity of detection and accuracy of single-cell isolation.

Here, we present a highly efficient, high-throughput workflow to accurately analyze miRNAs in well-defined individual cells and populations of cells (Figure 1).

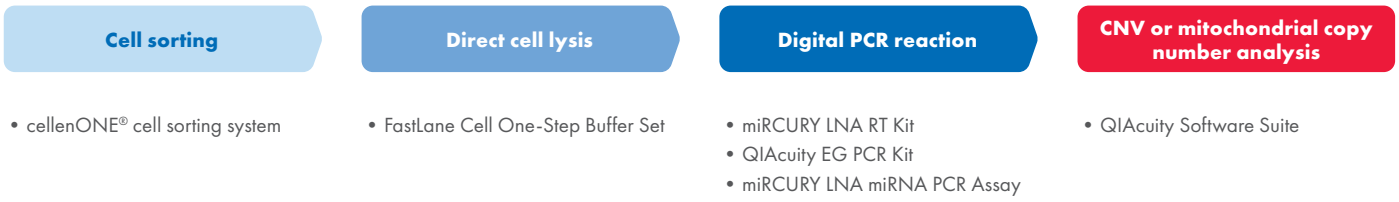

Figure 1. A streamlined single cell miRNA analysis workflow. From cell sorting to miRNA analyses.

Material and Methods

Single cell sorting using cellenONE technology

cellenONE (Figure 2) is a single-cell isolation and nanoliter dispensing instrument developed by Cellenion (Lyon, France). By combining precision low-volume dispensing technology and advanced image processing, this open platform provides real-time and highly accurate single-cell isolation (up to 100% single-cell accuracy). The dispensing speed of the instrument reaches 96 isolated single cells in less than 3 minutes. cellenONE applications range from cell line development to single-cell transcriptomics, genomics and proteomics analyses.

Using gentle acoustic waves for droplet generation, cellenONE preserves outstanding cell viability for all cloning applications and maintains protein expressions for omics applications. High-resolution optics allow isolation of any cell type (mammalian cells, bacteria, fungi other microorganisms). Cells can be sorted by the instrument based on their size, shape and fluorescence markers (four channels).

Working in aspirate/dispense mode with no dead volumes, cellenONE efficiently recovers a wide range of samples, from cell suspensions containing just a couple of microliters and a few dozen cells to much larger samples containing thousands of cells. With a high precision axis system, the instrument enables efficient deposition of single cells into a range of substrates, such as 96, 384 or 1536 microtiter plates (MTPs) and various nanowell substrates.

Experimental design

In these experiments, different 384 MTP layouts with a specific number of cells per well (ranging from 1 cell to 300 cells per well) were defined in the cellenONE X1 software. For HEK293 cell samples, only cell diameter and elongation parameters were used to precisely isolate a defined number of single cells in each well. Cells were isolated directly into wells prefilled with lysis buffer as described below.

Figure 2. cellenONE X1 system enables sorting of defined cell pools and single cells.

Cell culture, resuspension and sample preparation

HEK293 cells were passaged two days before isolation and cultured under standard conditions (DMEM/F12 with 10% FBS and Penicillin, Streptomycin, Amphotericin-B at 37°C with 5% CO_2). Prior to isolation, cells were washed twice with PBS, detached from their culture plates (0.5 mL trypsin for 1 min at 37°C), centrifuged (250 x g for 5 min at 4°C) and resuspended in PBS (400 cells/µL). The cell suspension was stored on ice and diluted to 200 cells/µL in degassed PBS immediately before processing.

Preparation of plates and layout

Target 384 MTPs were pre-filled with 10 µL/well of lysis buffer (FastLane Cell One-Step Buffer Set, cat. no. 216413) and pre-filled plates were kept on ice until processing in the cellenONE. Plates were then transferred onto cellenONE's target holder, pre-cooled to 4°C, for single-cell detection and isolation.

A specific 384 MTP layout with different numbers of HEK293 cells ranging from 300 cells to a single cell per well (in triplicates) was designed and set in the cellenONE X1 software prior to cell sorting (Figure 3A).

Configuration of the cell sorter and isolation of single cells/defined cell pools using the cellenONE technology

Prior to cell isolation, small aliquots of HEK293 cells were processed to define optimal isolation parameters for each sample as shown in Table 1.

Once configured, the cellenONE X1 system was then used to isolate HEK293 cells into wells of a 384 MTP according to the defined layout (Figure 3A).

After all cells were isolated, a cellenREPORT compiling all parameters and images of every isolated cell was generated for the 384 MTP. The diameter and elongation parameters of the isolated single cells were within the defined range (Table 2).

Table 1. Optimal single-cell isolation parameters

Cell sample	Minimum diameter (µm)	Maximum diameter (µm)	Maximum elongation (µm)
HEK293	16.1	29.0	1.59

Table 2. Parameters of isolated single cells

Cell sample	Diameter range (µm)	Mean diameter (µm)	Elongation range (µm)
HEK293	16.3–28.1	22.0	1.15–1.59

Direct cell lysis and miRNA analysis using the QIAcuity Digital PCR System

Generation of cell lysates and cDNA synthesis

After cell isolation, plates containing the sorted cells were centrifugated for 2 min at 2000 x *g* at 4°C. RNA lysates from cells were prepared using the FastLane Cell One-Step Buffer Set (cat. no. 216413) in which the cells were sorted. The cells were incubated for 5 min at room temperature within the cell lysis buffer followed by an incubation step of 5 min at 75°C to inactivate the lysis buffer. cDNA synthesis was performed immediately afterwards (Figure 3B).

Cell lysates were directly used for subsequent cDNA synthesis using the miRCURY LNA RT kit (cat. no. 339340). The cDNA reaction mix for 1 reaction was composed of 10 µL cell lysate, 4 µL 5x miRCURY RT SYBR Green reaction buffer, 2 µL 10x miRCURY enzyme mix and 4 µL nuclease-free water. The 384 MTP was sealed, mixed thoroughly and spun down before incubation. The cDNA synthesis was performed at 42°C for 1 hour followed by an incubation at 95°C for 5 min. The plate containing the cDNA was stored at -80°C until further processing.

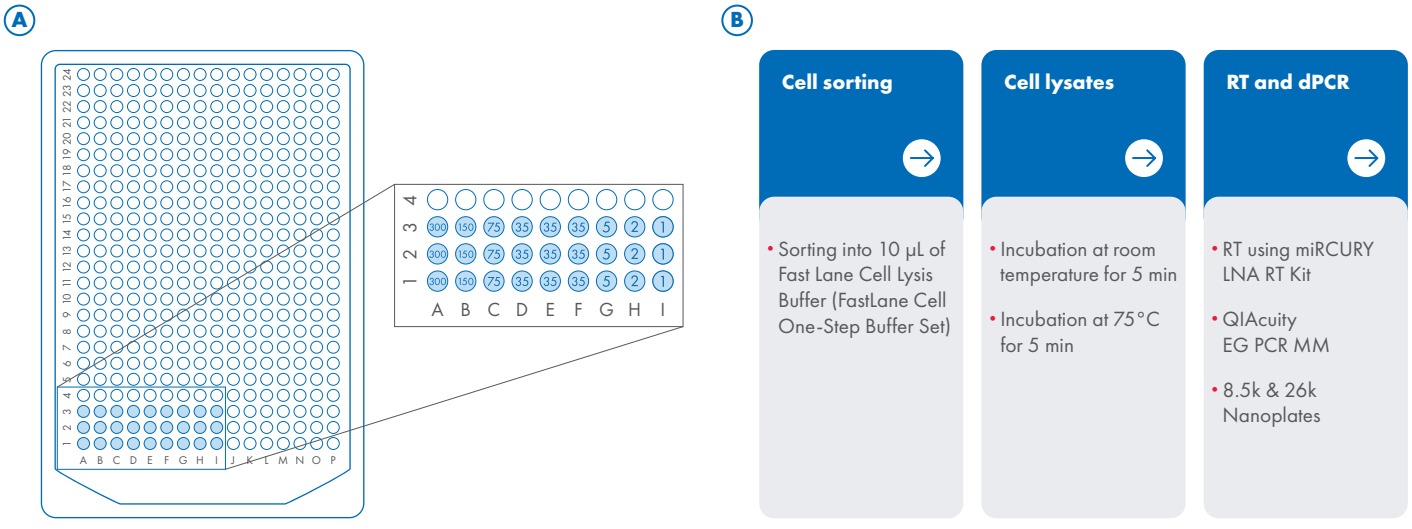

Figure 3. Plate layout for cell isolation in the cellenONE X1 system and downstream processing of cells including miRNA quantification via dPCR. A Cells were isolated in pools containing a range of cells from 300 cells/well down to a single cell per well. Three wells per cell number were filled. **B** Cells were lysed after sorting and used without further purification for cDNA synthesis and quantification of miRNAs via dPCR.

Digital PCR

dPCR reactions were set up as follows: miRCURY LNA miRNA PCR assays for hsa-miR-10a-5p and hsa-miR-10b-5p (cat. no. 339306, GeneGlobe ID YP00204778, YP00205637) were used for quantification of miRNAs in various cell lysates with a final concentration of 1x. The cDNA was diluted 7.5x in water before use as template in a dPCR reaction. The QIAcuity EG PCR mix (ca. no. 250111) was used in the dPCR with a final concentration of 1x. No additional water was added to the reaction. dPCR reaction mixes were prepared in pre-plates before being transferred either to QIAcuity 8.5k or 26k Nanoplates (cat. nos. 250021, 250001). Nanoplates were sealed and placed in a QIAcuity Digital PCR instrument according to the instrument's user manual (QIAcuity Application Guide – QIAGEN). Cycling program was set according to the recommendation in the handbook (HB-2947). Results were analysed using QIAcuity Software Suite (Suite 2.0.20).

Results

High-sensitivity detection of absolute miRNA copies in defined cell pools and at single cell level

We used the outlined workflow (Figure 1, Figure 3) to quantify miRNAs in cell pools with significantly reduced complexity (up to 300 cells per sort) or single cells. Cell sorting prior to cell lysis provides exact cell numbers loaded into a reverse transcription and dPCR reaction. The high accuracy of cell sorting, efficient lysate preparation and highly precise dPCR quantification allow for optimal miRNA analyses from single cells to cell populations.

The expression levels of hsa-miR-10a-5p and hsa-miR-10b-5p targets were analyzed in HEK293 cells. As shown in Figure 4 and Figure 5, both miRNAs could be detected with high linearity from cell pools containing 300 cells down to single cells. This workflow allows for the analysis of miRNAs derived from different cell lines and cell pool sizes in a high-throughput manner.

The unprecedented precision of the QIAcuity dPCR allows the detection of small differences in miRNA expression. The broad dynamic range of dPCR enables the analysis of multiple miRNAs using the same cell lysate without further dilutions (Figure 5A). The high quality of cell sorting, lysate preparation, RT and dPCR enables optimal analysis of cells in a wide linear range (Figure 4, Figure 5). Low-abundant miRNAs within cells can also be accurately detected due to the low limits of detection in dPCR.

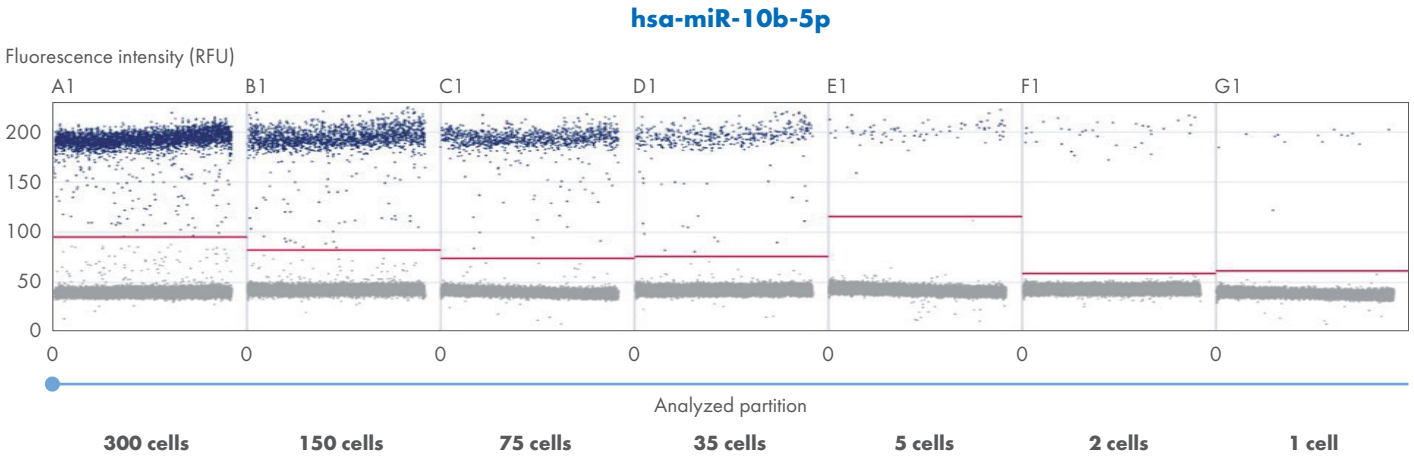

Figure 4. Analysis of miRNA quantification in different cell sorts. Different cell densities were sorted and lysed, and the miRNAs reverse transcribed and quantified via dPCR on 26k Nanoplates. The miRCURY LNA miRNA PCR assay targeting hsa-miR-10b-5p was used for quantification.

In addition, due to the partitioning of lysates and endpoint PCR, dPCR enables sensitive detection of absolute copies of miRNAs at the single-cell level. Variability in miRNA levels in individual cells can also be studied in an absolute, high-throughput manner using this workflow. Moreover, the elimination of the RNA purification step significantly reduced hands-on-time.

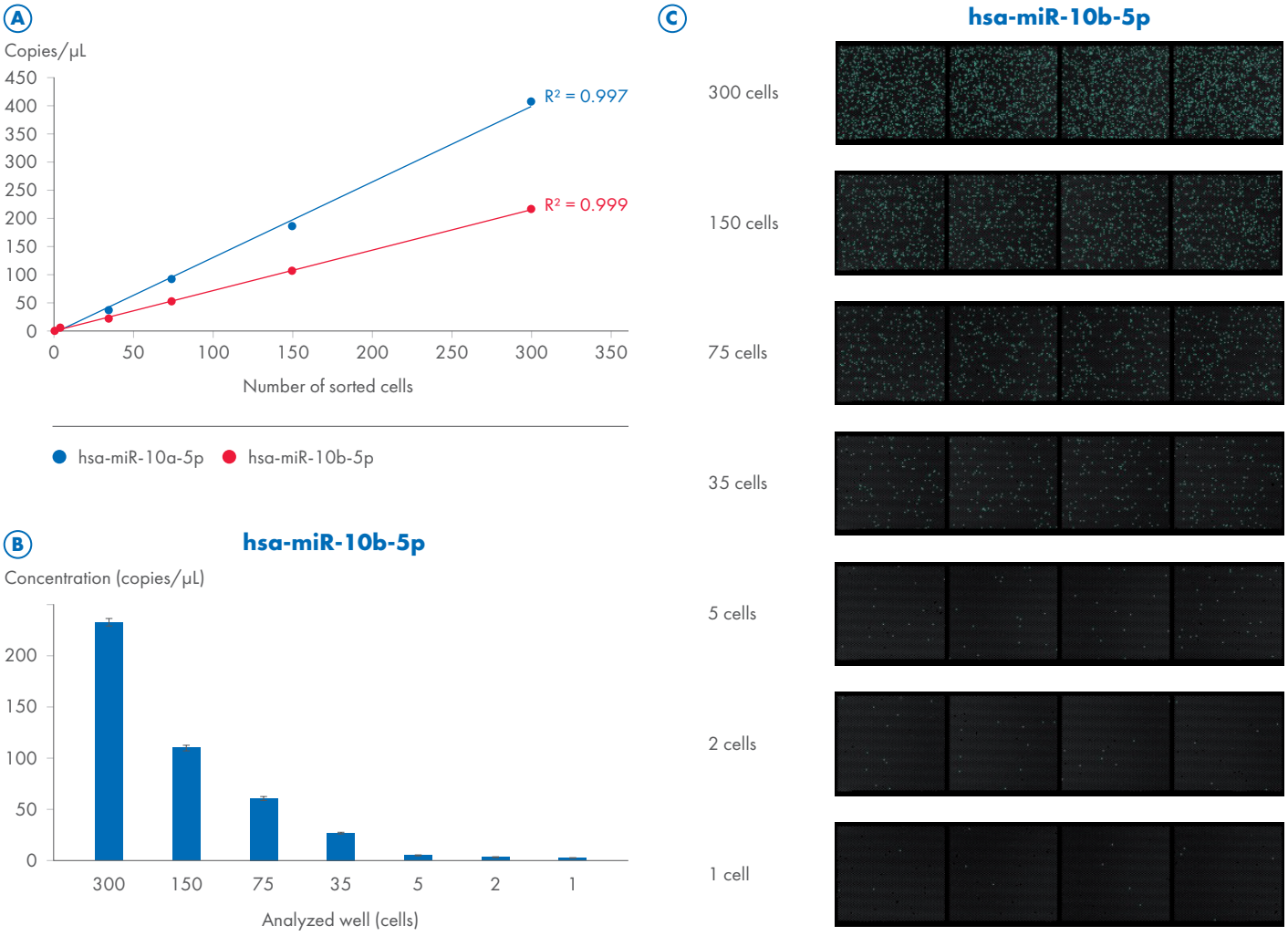

Figure 5. High sensitivity analysis of miRNAs over a broad range of HEK293 cell input.
A,B High linearity of miRNA expression over a range of 300 sorted cells down to a single cell. **C** Signalmap of different cell inputs showing positive partitions (green dots).

Conclusion

With the combination of both cellenONE and QIAcuity technology, we achieved high-throughput absolute quantification of miRNAs in well-defined cell pools and on single-cell level. cellenONE's single-cell isolation platform enabled fast, real-time and 100% accurate single-cell isolation. This allowed us to use the exact number of intact cells for subsequent dPCR reactions. Moreover, using FastLane lysis buffers for lysate generation significantly reduced hands-on time and QIAcuity's EG-based chemistry allowed for miRNA analyses without major optimization.

Overall, this simple, yet efficient workflow combining cell sorting with miRNA quantification delivered high-sensitive, reproducible and linear quantification from cell lysates as RT and PCR input.

Benefits of the combined cellenONE-QIAcuity dPCR workflow:

- Fast and accurate cell sorting ensures the exact number of cells taken into reactions.
- Elimination of RNA purification significantly reduces hands-on time and guarantees full recovery of miRNAs within cells.
- miRNA analysis at single-cell level. High-abundant and low-abundant miRNAs can be analyzed in cell pools and single cells.

References

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2. Isakova A, Neff N, Quake SR. Single-cell quantification of a broad RNA spectrum reveals unique noncoding patterns associated with cell types and states. *PNAS*. 2021; 51:118.
3. Tang F, Hajkova P, Barton SC, Lao K, Surani MA, MicroRNA expression profiling of single whole embryonic stem cells, *Nucleic Acids Research*. 2006; 34(2):e9.
4. Ho V, et al. Single cell quantification of microRNA from small numbers of non-invasively sampled primary human cells. *Commun Biol*. 2023; 6:458.

Ordering Information

Product	Contents	Cat. no.
FastLane Cell Probe Kit*	FastLane Cell One-Step Buffer Set, 2x QuantiTect Probe RT-PCR Master Mix, and QuantiTect RT Mix	216413
miRCURY LNA RT Kit	For 8–64 cDNA synthesis reactions: 5x RT SYBR Green Reaction Buffer, 5x RT Probe Reaction Buffer, 10x RT Enzyme Mix, UniSp6, RNA Spike-in template, RNase-Free Water	339340
miRCURY LNA miRNA PCR Assay	Contains forward and reverse primers for 200 SYBR® Green-based, real-time qPCR reactions, 166 EvaGreen-based digital PCR reactions for Nanoplate 8.5k or 50 EvaGreen-based digital PCR reactions for Nanoplate 26k	339306
QIAcuity EG PCR Kit	1 ml 3x concentrated QIAcuity EvaGreen Mastermix, 1 x 1.9 ml Water	250111
QIAcuity Nanoplate 8.5k 96-well	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
QIAcuity Nanoplate 26k 24-well	10 QIAcuity Nanoplate 26k 24-well, 11 Nanoplate Seals	250001
QIAcuity Eight Platform System	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts.	911052

* FastLane Cell One-Step Buffer Set is not sold separately. It can be purchased as part of any of the FastLane Cell RT-PCR Kits.

For more information on QIAcuity digital PCR products, visit: **www.qiagen.com/applications/digita-pcr/products**

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