Application Note

A workflow combining high-accuracy cell sorting with multiplex digital PCR for single-cell gene expression analysis

Julius Albers[2], Sandra Ruiz[1], Andreas Missel[2], Guilhelm Tourniaire[1], Afif Abdel Nour[2] and Özlem Karalay[2]

[1] Cellenion, Lyon, France

[2] QIAGEN GmbH, Hilden, Germany

Introduction

Understanding gene expression at the single-cell level allows us to capture individual cell heterogeneity rather than the average output of a cell population. Transcriptome analysis using popular PCR- and NGS-based techniques often lack the sensitivity required to detect low-abundance single-cell targets.

Digital PCR (dPCR) is emerging as the go-to method for applications warranting high sensitivity. Through absolute quantification of RNA targets, subtle changes in the expression of target genes can be studied down to a single-cell level.

Here we present a workflow that combines high-accuracy single-cell isolation with nanoplate-based dPCR for high-throughput analysis of gene expression in cultured cells. A defined multi-cell population or individual cells are detected and sorted using the cellenONE® cell-sorting system (Cellenion, Lyon, France) followed by rapid RNA extraction and multiplex one-step reverse transcription PCR on the QIAcuity® Digital PCR System (RT-dPCR). Finally, gene expression is quantified using the fast and intuitive QIAcuity Software Suite (Figure 1).

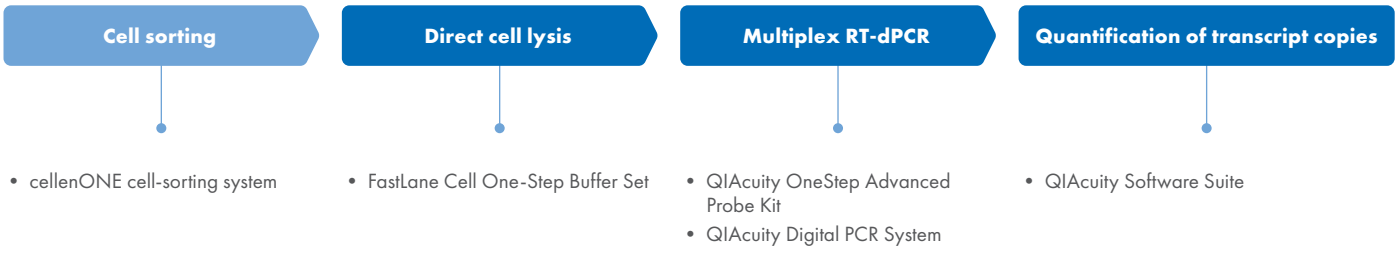

Figure 1. A streamlined single-cell gene expression analysis workflow

Single-cell sorting using cellenONE technology

cellenONE X1 system:
The single-cell isolation instrument combines precision low-volume dispensing with advanced image processing to sort a wide range of cells (mammalian cells, bacteria, fungi and other microorganisms) based on their size, shape and fluorescence markers (4 channels). It works in aspirate/dispense mode with no dead volume to enable high recovery of various samples, including clinical ▷

samples and rare cells. The gentle generation of drops, thanks to its piezo-acoustic technology, ensures the integrity of the cells and maintains intact transcript expressions for omics applications. Furthermore, its high precision axis system deposits single cells into a range of substrates such as 96, 384 or 1536 microtiter plates (MTP) and various nanowell substrates (Figure 2).

Figure 2. cellenONE X1 system.

Experimental design:

Two cell lines – HEK293 and HeLa – were used in the study. Different 384 MTP layouts with a specific number of cells per well (ranging from 1–100 cells per well) were defined in the cellenONE X1 software. For both cell samples, cell diameter and elongation parameters were defined to precisely isolate a specific number of single cells in each well.

Cell culture, resuspension and sample preparation:

HEK293 and HeLa 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 in 5% CO_2). Before isolation, cells were washed twice with PBS, detached from their culture plates (0.5 ml trypsin for 1 minute at 37°C), centrifuged (250 x g for 5 minutes 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.

Plate preparation:

Target 384 MTPs were pre-filled with 10 µl/well of Fast Lane Cell One-Step Lysis Buffer (available as part of FastLane Cell Probe Kit, Cat. No. 216413) and kept on

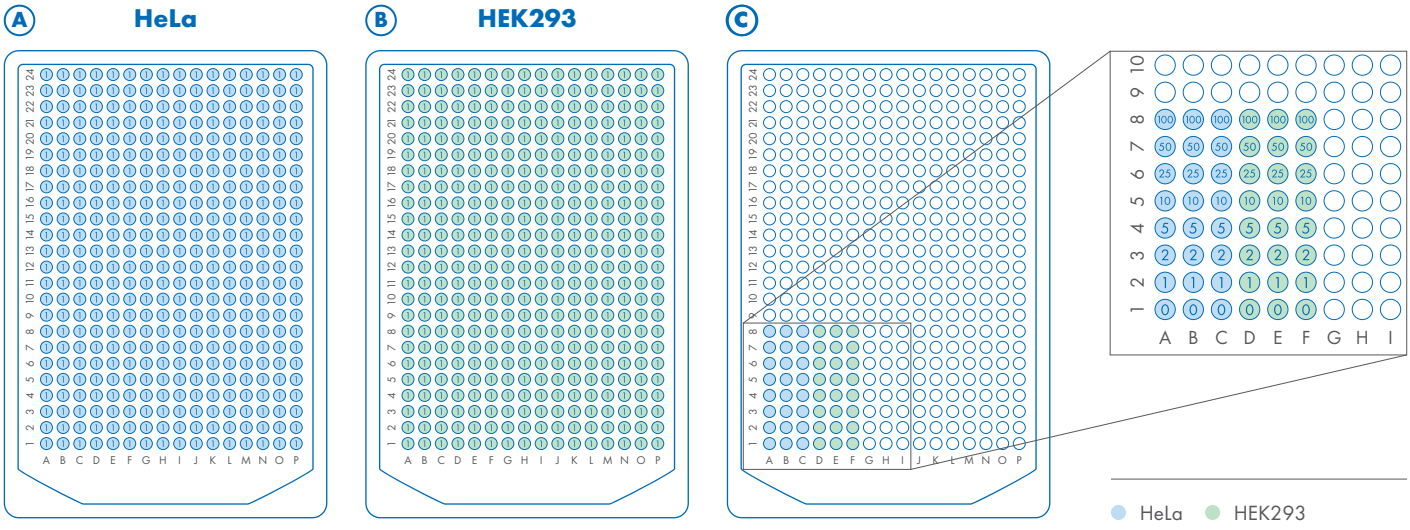

Figure 3. Representation of the three different layouts for cell isolation in the cellenONE X1 system. A 384 MTP layout for isolation of one single HeLa cell per well; **B** 384 MTP layout for isolation of one single HEK293 cell per well; **C** 384 MTP layout for isolation of different numbers of HeLa and HEK293 cells per well.

ice until processing. Pre-filled plates were then transferred onto cellenONE's target holder (pre-cooled to 4°C) for single-cell detection and isolation.

Plate layout:

Different layouts were designed and set in the cellenONE X1 software before cell sorting as shown in Figure 3.

Configuration of the instrument and single-cell isolation:

Before isolation, small aliquots of the two cell samples were processed to define optimal isolation parameters for each, as shown in Table 1. Once configured, the cellenONE X1 system was used to isolate HEK293 and HeLa cells into wells of three 384 MTPs according to the layouts defined in Figure 3.

Table 1. Optimal single-cell isolation parameters

Cell sample	Minimum diameter (µm)	Maximum diameter (µm)	Maximum elongation (µm)
HEK293	15	50	2.0
HeLa	15	32	1.8

Successful isolation of single cells using the cellenONE technology

Single cells were successfully isolated. Figure 4 A shows an example of an isolated HEK293 cell. Once all cells were isolated, cellenREPORTs, compiling all parameters and images of every isolated cell, were generated for each 384 MTP. The diameter and elongation parameters of the isolated single cells were within the defined range (Table 2 and Figure 4 B).

Table 2. Parameters of isolated single cells

Cell sample	Diameter range (µm)	Mean diameter (µm)	Elongation range (µm)
HEK293	15–34	22	1.18–2.0
HeLa	15–30	21	1.14–1.8

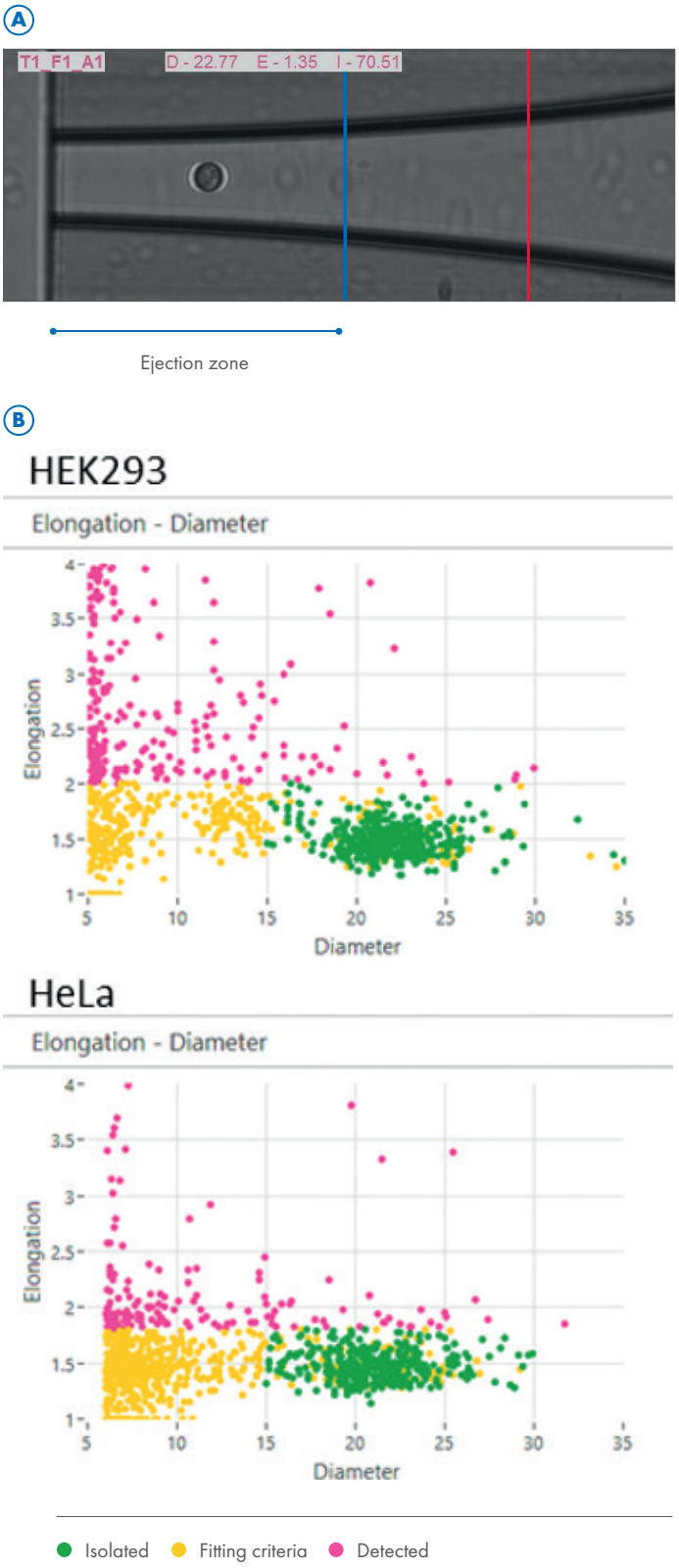

Figure 4. Single cells isolated using the cellenONE X1 technology. **A** Image showing a single HEK293 cell inside the capillary's ejection zone before its isolation. The well coordinates (A1) and the parameters of the isolated cell (**D**: Diameter (22.77 µm), **E** Elongation (1.35), **I** Grey Intensity (70.51)) are displayed on the top of the image. **B** HEK293 and HeLa isolation scatterplots generated by the cellenONE X1 Analysis Module showing cell elongation vs. cell diameter; all isolated cells are represented by individual green dots.

Direct cell lysis and single-cell gene expression analysis using QIAcuity OneStep Advanced Probe PCR Kit on the QIAcuity Digital PCR System

Generation of cell lysates:

Cell RNA lysates were prepared using Fast Lane Cell One-Step Buffer Set (available as part of FastLane Cell Probe Kit, Cat. No. 216413). In addition to lysing cells, the buffers stabilize cellular RNA and genomic DNA, eliminating the need for RNA purification. Cells were directly isolated into the wells of PCR plates containing 10 µl Fast Lane Lysis Buffer. Plates were then incubated for 5 minutes at ambient temperature and then heated to 75°C for 5 minutes on a thermoblock (QINSTRUMENTS ColdPlate Slim version). Plates were subsequently frozen at −80°C and stored until further processing.

RT-dPCR:

The reactions were set up according to standard QIAcuity OneStep Advanced Probe Kit Quick Start Protocol. 2 µl of cell lysates were directly taken into the RT-dPCR reaction mix for each condition. TaqMan probe-based assays were used for multiplex detection of target gene expression levels. RT-dPCR reaction mixes were then pipetted into the wells of QIAcuity 8.5k Nanoplates. The Nanoplates were sealed and placed in a QIAcuity Digital PCR instrument (Series 0; R&D version) according to the instrument's user manual. Standard QIAcuity One Step RT-dPCR cycling program was selected. Results were analyzed using QIAcuity Software Suite (Suite 2.0.20, CSW 2.0.0.144).

High-sensitive detection of absolute transcript copies at the single-cell level

Using the workflow outlined in Figure 1, we checked the transcript levels of various targets in HEK293 and HeLa cells. As shown in Figure 5, the differences in gene expression levels of a target in different cell lines can easily

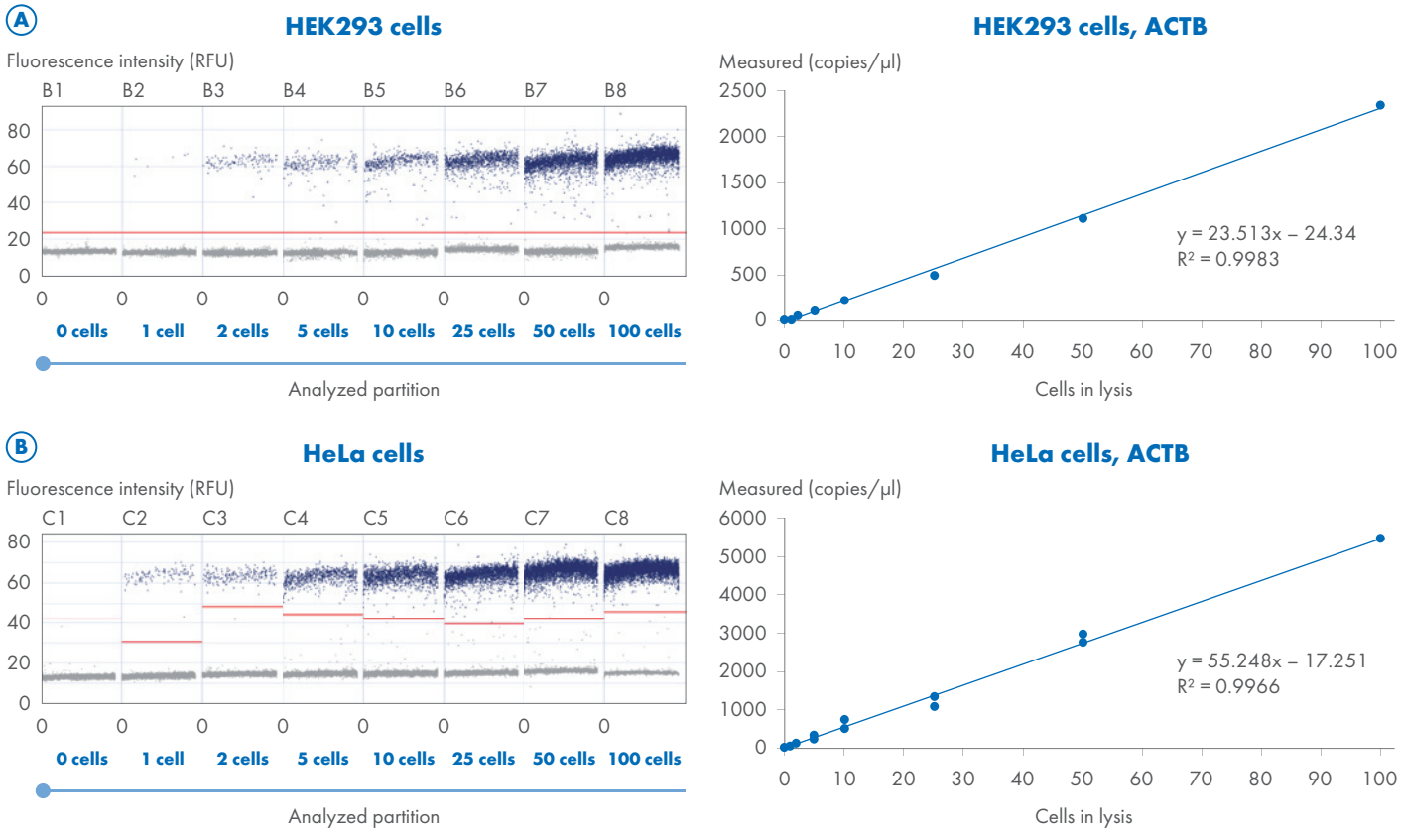

Figure 5. Analysis of gene expression levels in different cell lines at different cell densities. Results are obtained using ACTB-FAM assay in RT-dPCR for both cell lines.

be studied in a high-throughput manner. Cell sorting before cell lysis allows an exact number of cells to be used for RT-dPCR reactions. The high quality of cell sorting, lysate preparation and RT-dPCR enables an optimal analysis of cells in a wide linear range (Figure 5).

Due to the multiplexing capacity of the QIAcuity platform, multiple targets can be simultaneously analyzed (Figure 6). Transcripts with low abundance within cells can also be accurately detected, thanks to the low limits of detection in dPCR. In addition, due to the partitioning of lysates and endpoint PCR, dPCR enables sensitive detection of absolute copies of transcripts at the single-cell level (Figure 7). Variability in gene expression levels of targets from one individual cell to another can also be studied in an absolute, high-throughput manner using this workflow (Figure 8).

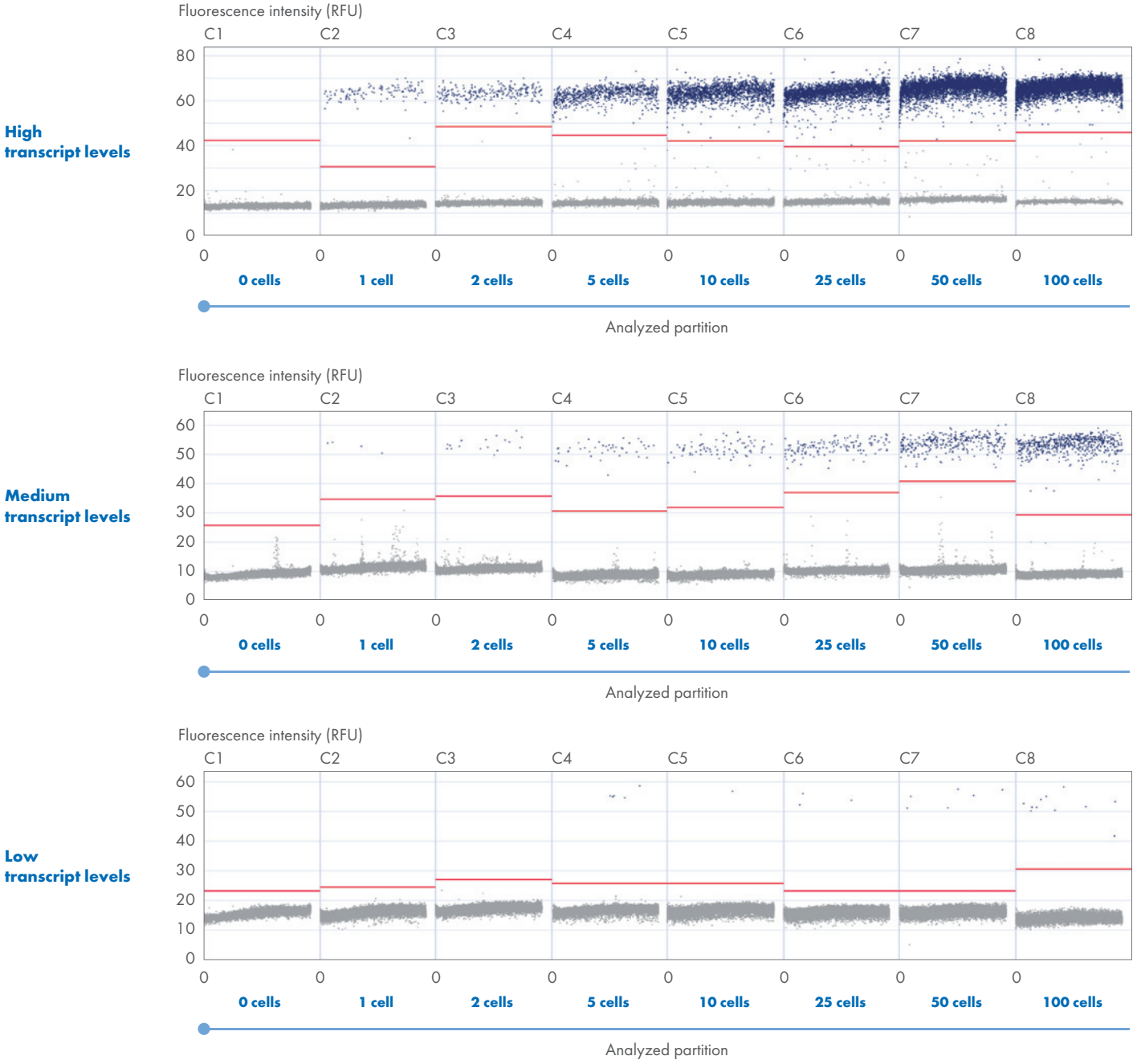

Figure 6. Simultaneous quantification of low-, medium- and high-abundance targets in cell lysates in multiplex RT-dPCR reactions. Cell lysates with increasing cell densities were loaded. ACTB-FAM, MYC-ROX and KDR-HEX assays were used for multiplexing.

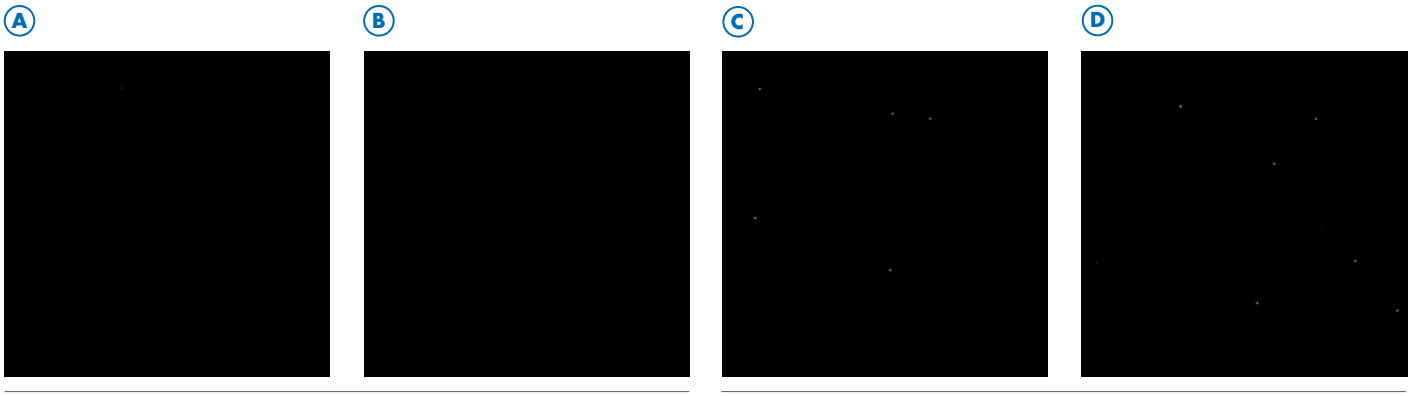

Figure 7. High sensitivity analysis of gene expression levels in single cells. 5 and 6 copies of target ACTB transcripts were detected in single HEK293 cells (seen as green dots) using ACTB-FAM assay in 1-Step RT-dPCR (**C** and **D**). No transcripts were detected in NTCs (**A** and **B**).

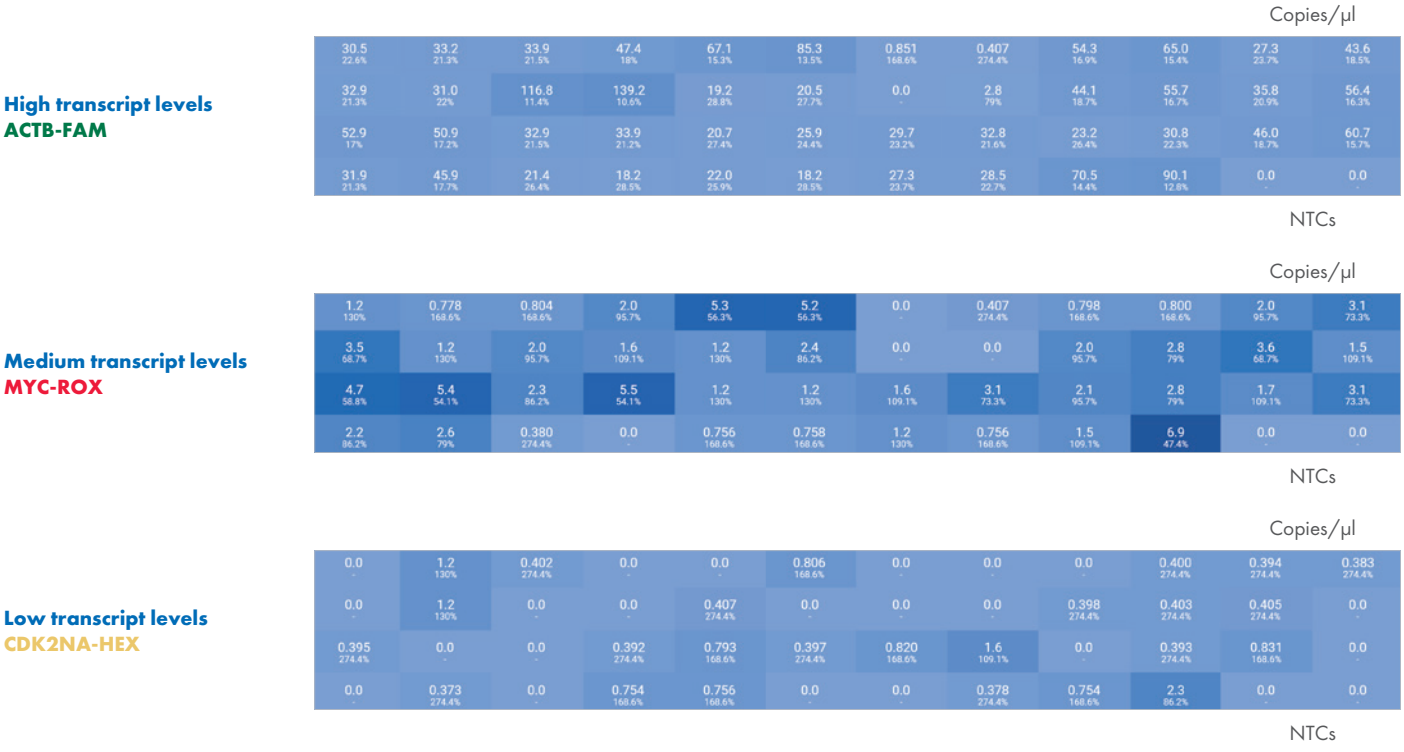

Figure 8. Multiplex analysis of gene expression levels in single cells. Single-cell lysates and NTCs were loaded onto 8.5k Nanoplates and tested using ACTB-FAM, MYC-ROX and CDK2NA-HEX assays.

Conclusion

With the cellenONE-QIAcuity workflow, we achieved high-throughput absolute quantification of low-abundance targets at the single-cell level. cellenONE's single-cell isolation platform enabled real-time and 100% accurate single-cell isolation without compromising viability and transcript expression. This allowed us to use the exact number of intact cells for RT-dPCR reactions. Moreover, the elimination of the RNA purification step significantly reduced hands-on time, and QIAcuity's probe-based chemistry allowed the multiplexing of up to five targets in a one-step RT-dPCR format with no or minimal optimization. Overall, this simple yet efficient workflow combining cell sorting with absolute gene expression analysis delivered high-sensitive, reproducible and linear quantification of transcript levels in cell lysates.

Ordering Information

Product	Contents	Cat. no.
FastLane Cell Probe Kit (200)	FastLane Cell One-Step Buffer Set*, 2x QuantiTect Probe RT-PCR Master Mix, and QuantiTect RT Mix	216413
QIAcuity Nanoplate 8.5k 96-well (10)	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
QIAcuity OneStep Advanced Probe Kit (1 ml)	1 ml OneStep Advanced Probe Master Mix (4x), 45 µl OneStep RT Mix (100x), 1 ml Enhancer GC, 20 µl QN Internal Control RNA, 2 x 1.9 ml RNase-free water; for 100 reactions in Nanoplate 26K and 333 reactions in Nanoplate 8.5K	250131
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 up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

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