Application Note

A workflow combining high-accuracy cell sorting with multiplex digital PCR for mitochondrial and genomic target copy number analysis

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Introduction

Copy number variations

Copy number variations (CNVs), or copy number alterations (CNAs), are structural changes in the genome. CNVs, including insertions, deletions, duplications, translocations, and inversions, lead to the gain or loss of copy numbers of a region, ranging from a few hundred base pairs up to whole chromosomes. CNVs are either inherited or the results of de novo somatic mutations.

Responsible for up to 10–20% variation in the genome, CNVs are a source of natural genetic diversity, as well as biological dysfunction in humans. CNVs often result in disruption of gene function, dosage imbalances and positional effects, which are associated with complex diseases and traits such as cancer, obesity, and neurodegenerative and autoimmune diseases.
The quantitative analyses of CNVs at disease-associated loci provide insights into molecular mechanisms of diseases and offer potential for the discovery of novel biomarkers.

Similar to genomic DNA copy number (gDNA CN) alterations, changes in mitochondrial DNA copy number (mtDNA CN) in blood or tissue have been linked to diseases. This is not a surprise, given the important role mitochondria play in cellular homeostasis. In contrast to fixed copy numbers of genomic DNA (healthy diploid state), mtDNA copy numbers fluctuate and vary between individuals, as well as between different tissues, cells and even among mitochondria within the same cell.
Drastic changes in the copy number of mtDNA can lead to mitochondrial dysfunction and disease formation. Therefore, changes in mtDNA CN can be used as a proxy for screening metabolic diseases, cancer, neurodegeneration, and aging-related diseases.

Digital PCR (dPCR) provides a highly sensitive and accurate detection of absolute quantities of mitochondrial and genomic targets within samples. ▷

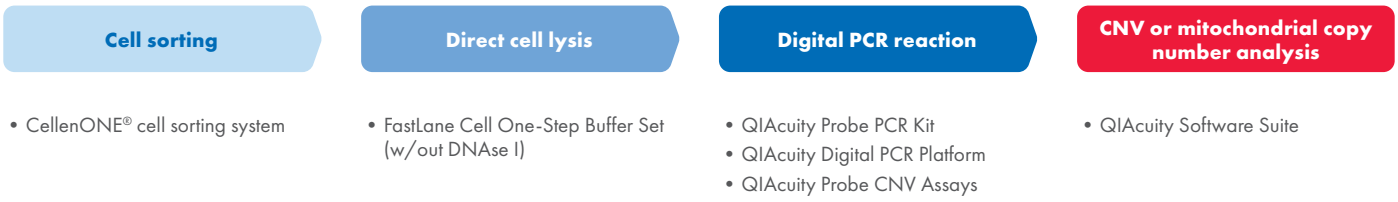

Figure 1. Simple workflow. From cell sorting to gDNA CN or mtDNA CN analyses.

Changes in copy numbers of target regions can be studied down to a single cell using dPCR at a high-resolution level.

Here, we present a workflow that combines two technologies, cellenONE® and QIAcuity® Digital PCR, which accelerate and streamline high-throughput analyses of target copy numbers in cultured cells. The workflow starts with detecting and sorting defined populations of cells as well as individual cells using cellenONE, followed by multiplexing dPCR on the QIAcuity platform. Copy number variations of target regions are then analyzed using the QIAcuity Software Suite, providing an intuitive and fast interpretation of results.

Single-cell sorting using cellenONE technology

cellenONE is a single-cell isolation and nanoliter dispensing instrument, developed by Cellenion (Lyon, France). Combining precision low-volume dispensing technology and advanced image processing, this open platform provides real-time and high-accuracy single-cell isolation (up to 100% single-cell accuracy). The instrument's dispensing speed reaches 96 single cells isolated 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, and 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 allows high recovery of 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. This makes cellenONE an ideal platform for processing clinical samples and rare cells. The instrument's high precision axis system enables efficient deposition of 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

In these experiments, different 384 MTP layouts with a specific number of cells isolated per well (ranging from one to 100 cells per well) were defined in the cellenONE X1 software. For both cell samples (HEK293 and HeLa cells), 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 pre-filled with lysis buffer.

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 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.

Plate preparation

Target 384 MTPs were pre-filled with 3 µL/well of lysis buffer (FastLane Cell One-Step Buffer Set, cat. no. 216413 without DNase I) and pre-filled plates were kept on ice until processing in the cellenONE. 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 384 MTP layouts were designed and set in the cellenONE X1 software before cell sorting. One 384 MTP layout was designed with 384 single cells, one cell per well, a second 384 MTP layout was designed with different numbers of cells per well (Figure 3).

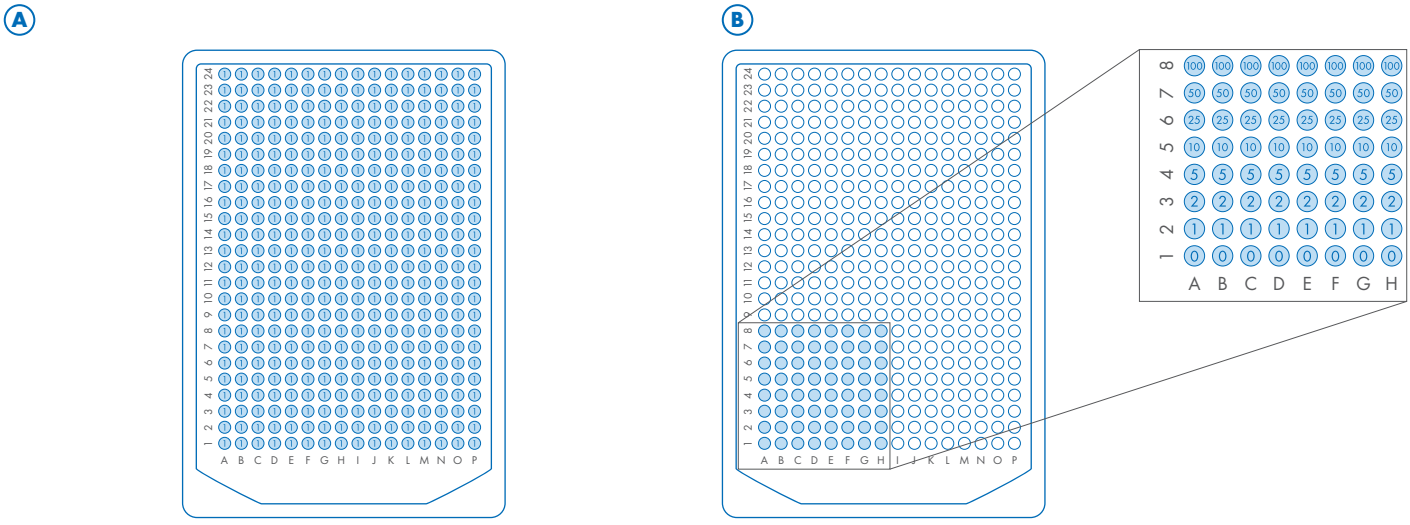

Figure 3. Representation of the two different 384 MTP layouts set in the cellenONE X1 for cell isolation. A 384 MTP layout for isolation of one single cell per well. **B** 384 MTP layout for isolation of different numbers of cells per well.

Configuration of the instrument and isolation of single cells using the cellenONE technology

Prior to cell isolation, small aliquots of the different cell samples 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 and HeLa cells into wells of three 384 MTPs according to the layouts defined above (Figure 3). An example of an isolated HeLa cell is shown below (Figure 4A).

After cell isolation, plates were centrifuged for 2 min at 2000 x g at 4°C to be ready for generation of cell lysates.

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
HeLa	20.0	35.5	1.71

Successful single-cell isolation using cellenONE

Once all cells were isolated, cellenREPORTs with parameters and images of every isolated cell, were generated for each of the 384 MTP. The diameter and elongation parameters of the isolated single cells were within the defined range (Table 2 and Figure 4B).

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
HeLa	20.0–32.0	23.6	1.16–1.87

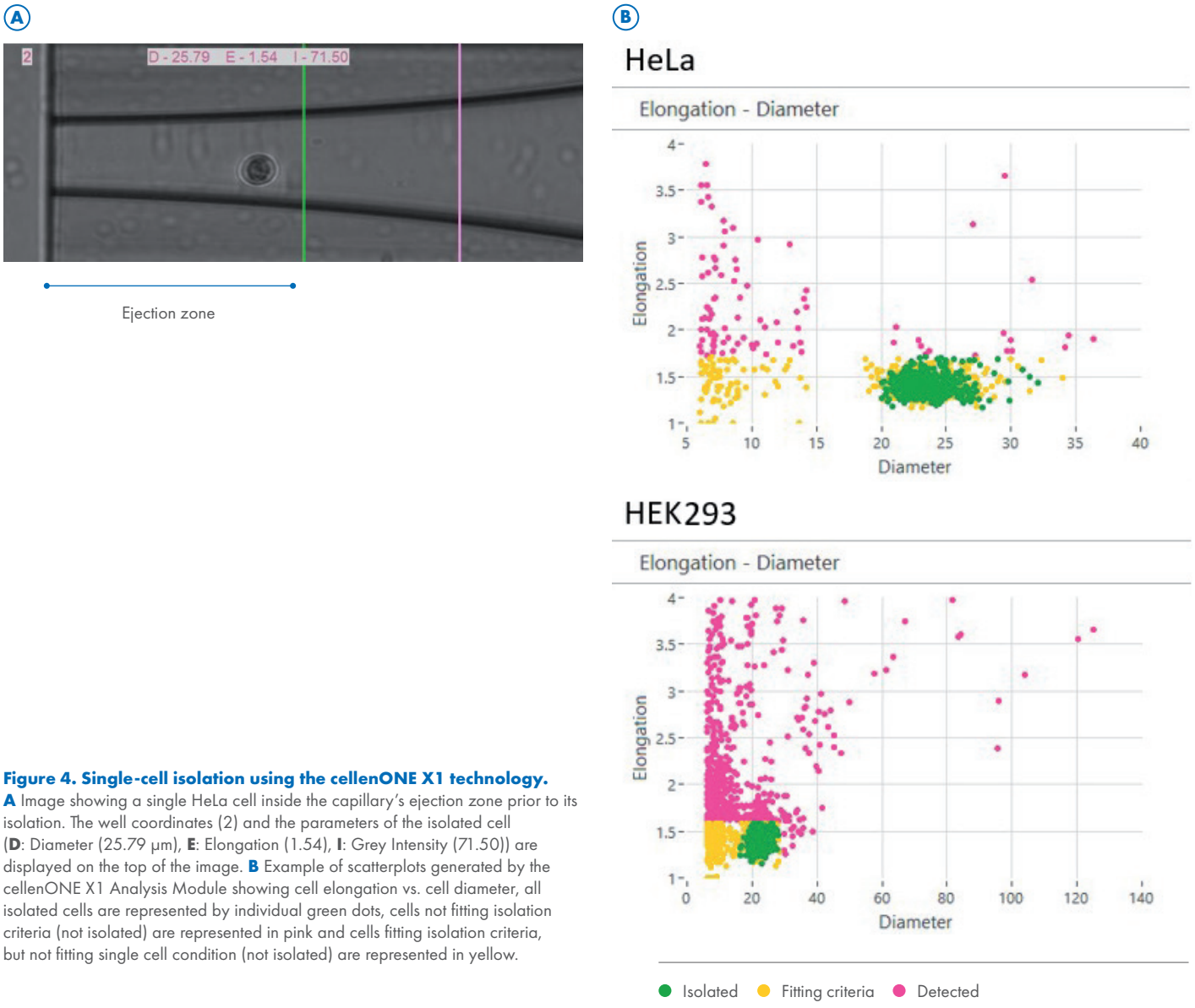

Figure 4. Single-cell isolation using the cellenONE X1 technology. **A** Image showing a single HeLa cell inside the capillary's ejection zone prior to its isolation. The well coordinates (2) and the parameters of the isolated cell (**D**: Diameter (25.79 µm), **E**: Elongation (1.54), **I**: Grey Intensity (71.50)) are displayed on the top of the image. **B** Example of scatterplots generated by the cellenONE X1 Analysis Module showing cell elongation vs. cell diameter, all isolated cells are represented by individual green dots, cells not fitting isolation criteria (not isolated) are represented in pink and cells fitting isolation criteria, but not fitting single cell condition (not isolated) are represented in yellow.

Direct cell lysis and copy number analysis using the QIAcuity Digital PCR System

Generation of cell lysates

DNA lysates from cells were prepared using FastLane Cell One-Step Buffer Set without adding DNase I. Cells were directly isolated into the wells of PCR plates containing 3 µL FastLane Lysis Buffer (without DNase I). Plates were then incubated for 5 min at ambient temperature and then heated to 75°C for 5 min (QInstrument ColdPlate Slim version). Plates were subsequently stored at -80°C until further processing.

Digital PCR

Digital PCR reactions were set up according to the standard QIAcuity dPCR Probe CNV Assays Quick Start Protocol (HB-3060). Total amount of cell lysates (3 µL) containing various amounts of cells (Figure 3, 1-100 cells/well) were directly taken into the dPCR reaction mix for each condition. QIAcuity dPCR Probe CNV Assays for RPP30 (cat. no. DCR0000181) and SPIN4 (cat. no. DCC0000464) and TaqMan probe-based assays were used for multiplex detection of target copy number levels at gDNA and mtDNA levels, respectively.

Digital PCR reaction mixes were then pipetted into the wells of QIAcuity 8.5k or 26k Nanoplates (cat. no. 250011 or cat. no. 250031). Nanoplates were sealed and placed in a QIAcuity Digital PCR instrument according to the instrument's user manual (QIAcuity Application Guide – QIAGEN).

The standard QIAcuity dPCR Probe CNV Assay cycling program was selected. Results were analysed using the QIAcuity Software Suite (Suite 2.0.20, and 2.1.7.187).

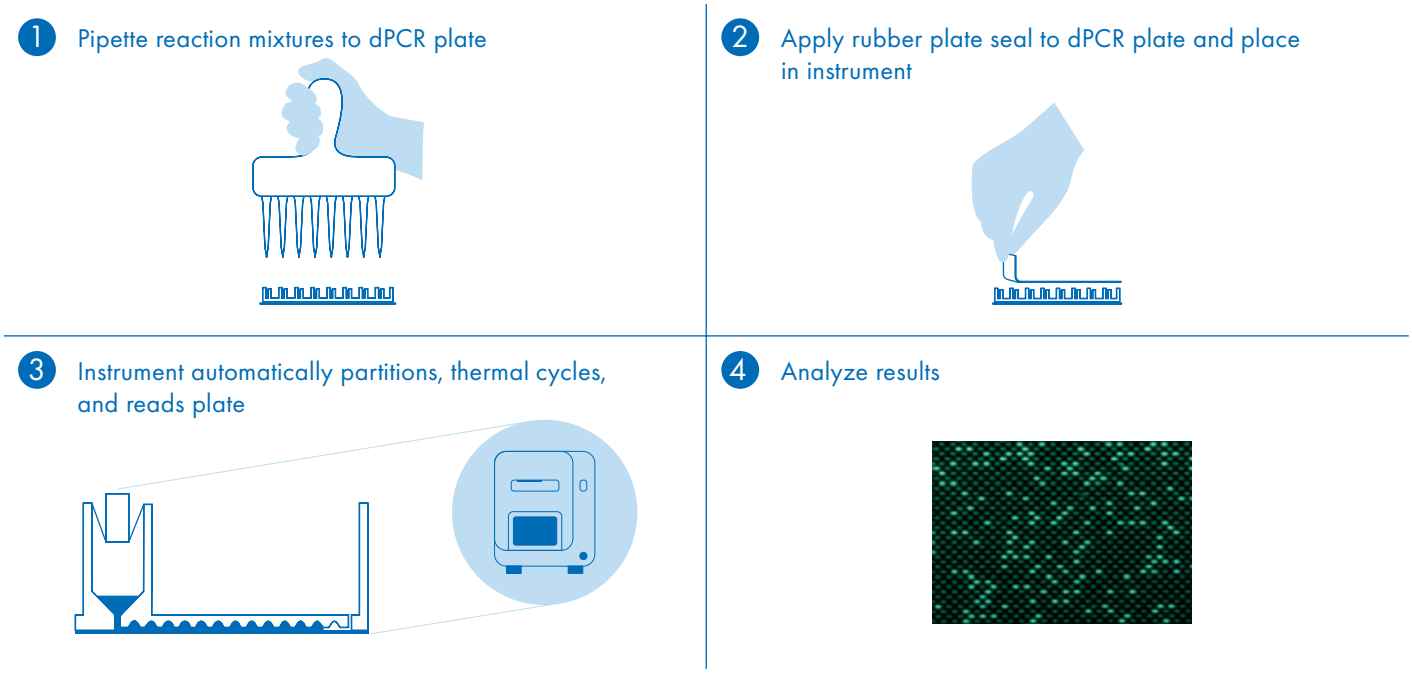

Figure 5. Setup of dPCR reactions. Four simple steps from sample to insight.

Results

Highly accurate and precise CNV analyses of various genomic and mitochondrial DNA targets in sorted cells

We used the outlined cellenONE-QIAcuity workflow to analyze CN of various gDNA and mtDNA target assays in HEK293 or HeLa cells. Cell sorting prior to cell lysis provides an exact number of cells loaded into dPCR reactions. The high accuracy of cell sorting, efficient lysate preparation and highly sensitive dPCR detection allows for optimal CN analysis from single cells to cell populations. Thanks to the multiplexing capacity of the QIAcuity platform, multiple targets can also be analyzed simultaneously within single cells.

A major challenge associated with multiplexing CN analysis is that different targets can have different copy numbers. While many gDNA targets are present in 2 copies in a healthy diploid genome, mtDNA targets can have much higher CN than gDNA targets within the same cells. Simultaneous detection of such a variable CN range requires high sensitivity and a high dynamic range of detection. Due to the partitioning of lysates and end-point PCR, the dPCR system from QIAGEN provides sensitive detection of absolute copies of low abundant copy targets as well as high copy targets at single cell level at a high resolution (Figure 6).

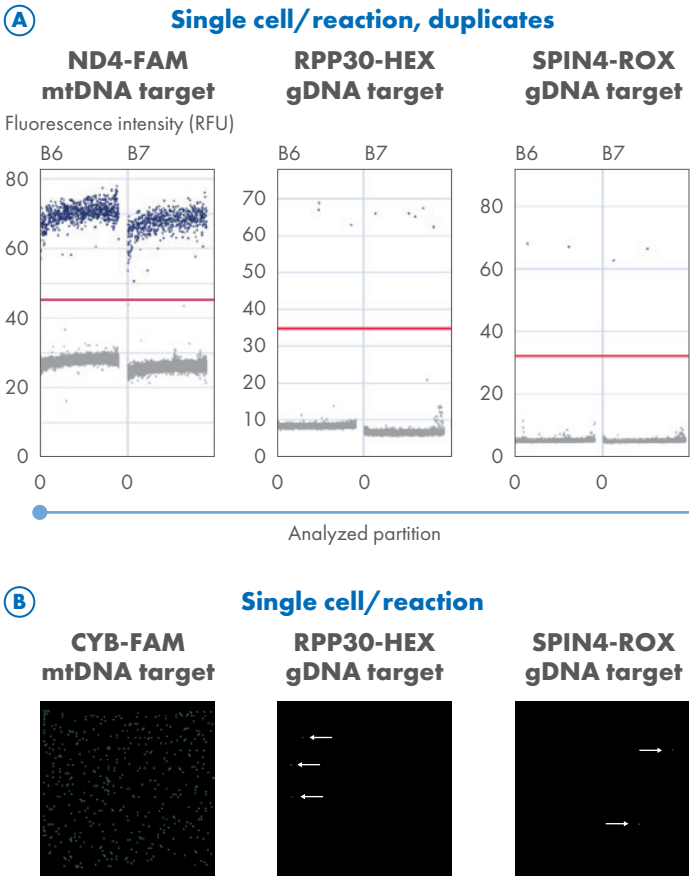

Figure 6. Genomic DNA and mtDNA targets show different copy numbers at the single-cell level of HEK293 cells. CN differences can be visualized at a high resolution using 1D scatterplots or signal maps in the QIAcuity Software Suite. Results are obtained using **A** ND4-FAM, RPP30-HEX (cat. no. DCR0000181) and SPIN4-ROX (cat. no. DCC0000464) assays, **B** CYB-FAM, RPP30-HEX and SPIN4-ROX in single multiplexing dPCR reactions loaded into 8.5k Nanoplates. SPIN4 (Chr. X) and RPP30 (Chr. 10) assays target gDNA and are expected to be present in 2 copies/genome in healthy wild-type cells. ND4 and CYB assays target mtDNA and are expected to be present in high copy numbers.

In addition, the high dynamic range of the QIAcuity allows for accurate readout of CN changes for high copy targets at increasing cell loading density (Figure 7). Therefore, CN changes in various targets with low, medium and high CN can be detected within single multiplexing reactions in a highly accurate and reproducible manner.

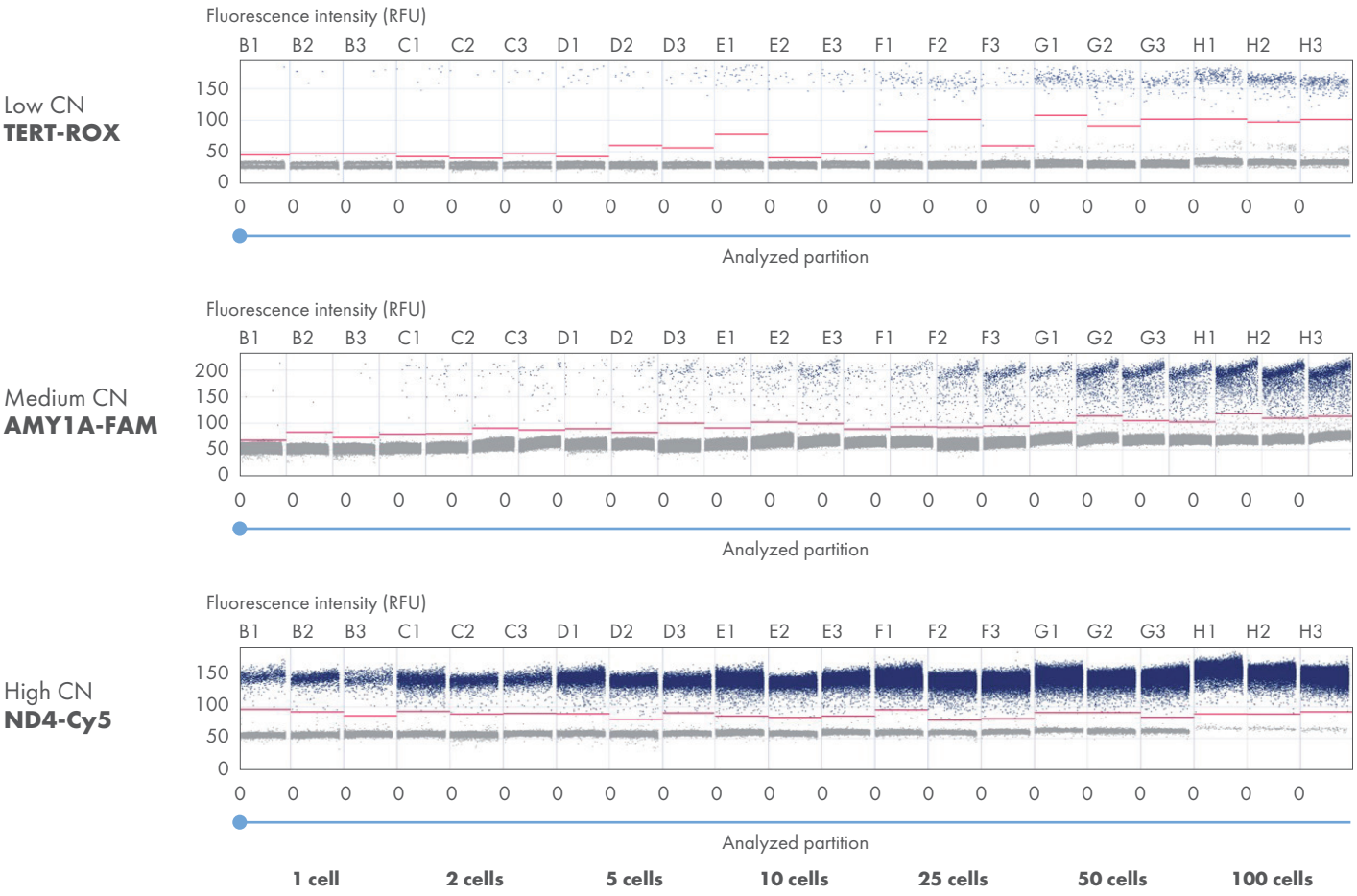

Figure 7. Simultaneous quantification of low, medium and high CN targets in HEK293 cell lysates. Cell lysates with increasing cell densities ranging from 1 to 100 cells per reaction were loaded into reactions. Triplicates are shown for each loading condition. Threshold was set automatically by the QIAcuity Software Suite. Results were obtained using AMY1A-FAM, TERT-ROX and ND4-Cy5 assays in multiplexing dPCR reactions loaded into 26k Nanoplates. TERT (cat. no. DCR0000186) (Chr.5, low CN) and AMY1A (Chr.11, medium CN) are gDNA targets whereas ND4 (high CN) is a mtDNA target.

Furthermore, differences in CN of mtDNA or gDNA targets in different cell types can be analyzed in a high-throughput manner using a combined cellenONE-QIAcuity workflow (Figure 8). Apart from studying cell populations, single-cell heterogeneity can also be examined for mtDNA CN in an absolute, high-throughput manner using this workflow (Figure 9).

Overall, this simple yet efficient workflow combining cell sorting with CN analysis on the QIAcuity platform delivers highly sensitive, reproducible, and linear quantification of genomic or mitochondrial DNA target copy numbers in cell lysates in a high-throughput manner.

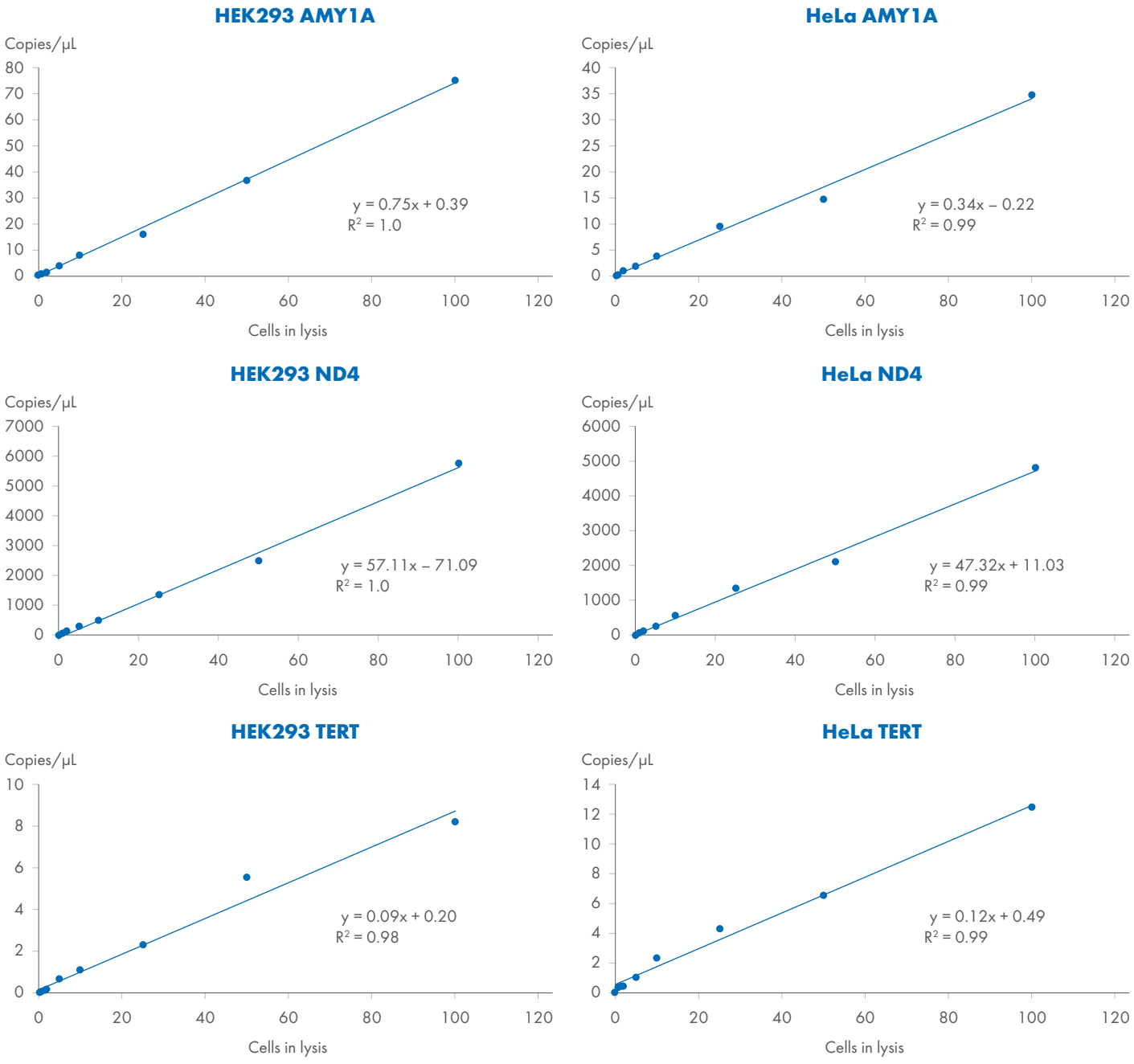

Figure 8. High linearity over a broad dynamic range. Linearity of detection is shown for CN of gDNA and mtDNA targets at increasing numbers of HEK293 or HeLa cells per reaction. AMY1A-FAM, TERT-ROX and ND4-Cy5 assays are used for multiplex dPCR reactions loaded into 26k Nanoplates. Results represent average concentration [copies/µL] obtained from triplicates. R^2>0.98 for all conditions.

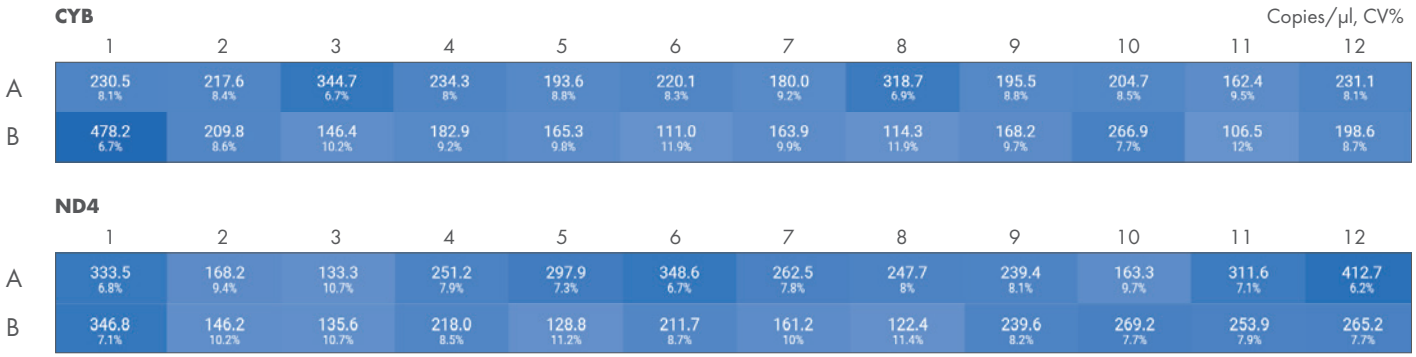

Figure 9. CN analysis of mtDNA. Assays targeting CYB and ND4 show heterogeneity of mtDNA copies in single cells. Each well (A1-A12 and B1-B12) contains a single cell. A) HEK293, B) HeLa single-cell lysates were loaded onto 8.5k Nanoplates and tested using CYB-FAM or ND4-FAM assays.

Conclusion

With the combination of both the cellenONE and QIAcuity technology, we achieved high-throughput absolute quantification of genomic and mitochondrial targets at the 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 the probe-based chemistry from QIAGEN allowed multiplexing of targets in a single dPCR reaction with no or minimal optimization. Overall, this simple yet efficient workflow combining cell sorting with copy number analysis delivered highly sensitive, reproducible, and linear quantification of target genomic or mitochondrial copy numbers in cell lysates.

Benefits of the combined cellenONE-QIAcuity dPCR workflow:

- Fast and accurate cell sorting ensures exact number of cells used in downstream reactions.
- Elimination of DNA purification significantly reduces hands-on time and increases DNA recovery.
- Probe-based detection allows for multiplexing of up to five targets in a single dPCR reaction with minimal optimization on the QIAcuity instrument.
- Accurate absolute DNA quantification by dPCR enables analysis of low abundance targets as well as multi-copy targets at single-cell level.

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