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(54) Title: HIGH-ACCURACY QUANTIFICATION OF NUCLEIC ACIDS FROM PRE-DEFINED AMOUNTS OF CELLS USING PLATE-BASED DIGITIAL PCR

(57) Abstract: The present invention relates to a method of quantifying the amount of at least one nucleic acid target, the method comprising: a) Providing a defined number of cells, wherein the defined number of cells is at least one cell; and b) Lysing the at least one cell, thereby releasing RNA and DNA; and c) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA; and/or performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate; and d) Quantifying the amount of the at least one nucleic acid target.

HIGH-ACCURACY QUANTIFICATION OF NUCLEIC ACIDS FROM PRE-DEFINED AMOUNTS OF CELLS USING PLATE-BASED DIGITIAL PCR

FIELD OF INVENTION

The present invention is in the field of molecular biology, in particular in the field of targeted nucleic acid analysis. More specifically, the invention is in the field of mRNA and miRNA expression analysis as well as targeted genomic and mitochondrial copy number variation analysis, wherein a specific amount of cells is isolated and nucleic acids are quantified using plate-based digital PCR.

BACKGROUND

Single cell analyses allow researchers to uncover new information and gain novel insights into biological processes of an individual cell in comparison to traditional methods analyzing cell populations in bulk. Thus, individual cell heterogeneity can be analyzed rather than the average output of the cell population.

Drastic cell to cell variation can be observed especially at the level of transcriptome even though cells are identical on a genetic and morphological level. Capturing this transcriptomic heterogeneity is particularly important for disease and drug development studies where the differences in biological response to drugs from individual cells versus from tissues might provide additional insights into the disease progression or prevention. Intricate transcriptional networks can be outlined to generate improved drug response models. In addition to disease and drug screening therapies, single cell transcript analyses can be applied to *e.g.* aging, stem cell, gene and cell therapies, prenatal screening, organoid studies, as well as infectious disease studies among others.

Capturing cell heterogeneity is also important for detection of naturally occurring mutations, chromosomal rearrangements or copy number variation (CNV; or copy number alterations (CNA)) events within cells. CNVs are structural changes in genome (such as deletions, insertions, duplications,

translocations, and inversions) that lead to gain or loss of copy numbers of a region, ranging from a few base pairs to a few hundred base pairs up to whole chromosomes. CNVs are either inherited or the results of de novo somatic changes. 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, shortening of chromosomal ends and positional effects, which are associated with complex diseases and traits such as cancer, obesity, aging and neurodegenerative and autoimmune diseases. The quantitative analyses of CNVs at disease-associated loci, therefore, 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, not surprisingly given the important role mitochondria play in cellular homeostasis. In contrast to fixed copy number of genomic DNA (healthy diploid state), drastic cell to cell variation can be observed at the level of mitochondrial DNA (mtDNA), even though cells are identical in genetic identity and morphology. mtDNA copy numbers fluctuate and vary between individuals, as well as between different tissues, cells and even among mitochondria within the same cell. Individual cells can carry hundreds to hundred thousand copies of mtDNA. However, the drastic changes in copy number of mtDNA can lead to mitochondrial dysfunction and disease formation. Therefore, similar to genomic CNVs, capturing the copy number heterogeneity in mtDNA is important for disease and drug development studies and can be used as a proxy for screening metabolic diseases, cancer, prenatal testing, neurodegeneration, and aging-related diseases.

Moreover, single cell CNV analyses can be applied to gene and cell therapies, CAR-T therapies, intentional or unintentional (off-target effects during gene editing) gene alterations induced by viral vectors or CRISPR modifications, organoid studies, quantification of organelles as well as infectious disease studies.

Additionally, non-coding RNAs are actively involved in cell function and specialization. miRNAs, for example, are important non-coding, well conserved, post-transcriptional regulators with a great potential as diagnostic or prognostic biomarkers. The most recent miRbase release lists 2654 annotated human miRNAs. Drastic cell to cell variation can be observed not only on mRNA transcript level, but also on (small) non-coding RNA (such as miRNAs) transcript level, even if cells are identical on a genetic and morphological level. Furthermore, each individual cell provides a unique microenvironment. miRNA expression profiles give insights on cellular states (*e.g.*, human cancers) and cellular mechanisms. miRNAs are known to regulate the expression of up to 1/3 of the encoded human

genes and with that to influence a wide range of basic cellular functions. miRNA profiling in single cells or small cell pools is particularly important when looking at expressions with high variability among cells or whenever a very limited number of cells is available (*e.g.*, early embryos). Additionally, in the biomarker research field it is particularly important to identify and to analyze the expression of specific miRNA molecules in rare cell subsets that may not be identified in complex cell pools. Analysis of single cell/small cell pools miRNA levels involve 5 main steps: 1) isolation of individual cells, 2) cell lysis, thereby releasing miRNA, which may be stabilized by the lysis buffer, 4) polyadenylation and reverse transcription of miRNAs, and 5) quantitation of miRNA levels (optionally with pre-amplification for low abundant miRNAs). Quick and efficient extraction of miRNA from single cells and its stabilization after cell lysis is needed to preserve the natural "miRNome" of cells and reduce alterations in the output due to technical issues.

Single cell reverse transcription quantitative polymerase chain reaction (scRT-qPCR) and single cell RNA-sequencing (scRNA-seq) and their technological variations, are to date the two main approaches for single cell RNA analyses. Analogously, qPCR and single cell DNA-sequencing (scDNA-seq) are the two main approaches for single cell genome/mitochondrial DNA analysis.

Currently, quantitative real-time PCR (qPCR) is a widely used method for RNA and DNA target quantification. qPCR methods provide quick and cost efficient analyses of one or more nucleic acid targets of interest, however, their sensitivity and specificity for target detection heavily depends on template quantity and quality, PCR conditions, as well as presence of PCR inhibitors within reactions. As reviewed in Quan et al. (Quan et al., 2018, MDPI, "dPCR: A Technology Review"), quantitative real-time PCR is based on conventional PCR. In conventional PCR, target DNA (which may also be reverse transcribed RNA) is amplified in multiple cycles, wherein at the end of each of a 100% efficient PCR cycle the number of target DNA molecules is doubled (exponential amplification). Hence, 2^n copies can theoretically be produced after n cycles. However, in practice, the PCR reagents will be depleted at one point and amplification products will self-anneal, resulting in reduced amplification efficiency until a plateau is reached and the amplification process saturates. At the end of a conventional PCR reaction, the amplified products can be analyzed using for example, but not limited to, agarose gel electrophoresis (end-point measurement). The specificity of a conventional PCR relies on sequence hybridization. The sensitivity of a conventional PCR depends on enzyme-based amplification (Quan et al., 2018, MDPI, "dPCR: A Technology Review").

As mentioned above, quantitative real-time PCR is based on conventional PCR, but the amount of amplified PCR products are measured after each amplification cycle using a fluorescent readout. A typical real-time PCR amplification plot shows a sigmoidal-shaped curve (on a linear scale) and includes

a baseline phase, followed by an exponential phase that reaches a plateau via a linear phase. The most efficient phase of amplification is represented by the exponential phase and the amount of amplified PCR products doubles with each cycle (at an amplification efficiency of 100%). A relative quantification of a target to a calibrator is enabled by real-time PCR. The "absolute" amount of target sequence in a qPCR reaction is measured relative to a standard curve, which is generated from a sample of known quantity or copy number. This method implies that the amplification efficiencies of the standards and the sample are equivalent. Differences in PCR efficiencies can significantly affect the accuracy of the quantification (Quan et al., 2018, MDPI, "dPCR: A Technology Review").

For quantification by qPCR a standard curve is needed to calculate the target nucleic acid amount of the sample. Usually the standard curve is prepared by a serial dilution by the user. The preparation of the standard curve is critical for a precise target quantification. Depending on the user's skills and quality of the used equipment for standard curve preparation, the process of cDNA or DNA dilution and preparation of the DNA standard curve itself can be very tedious, laborious and error prone, which can lead to wrong quantification results.

In contrast to qPCR, single cell sequencing provides global RNA analysis or DNA analysis in a high-throughput fashion from thousands of individual cells but might miss rare targets, especially in low amounts of starting material, due to low sequencing coverage and high technical noise. Additional difficulty comes into play in analysis and interpretation of large data sets obtained using scRNA-seq/scDNA-seq.

Another approach to quantify DNA (including also reverse transcribed RNA) makes use of a digital polymerase chain reaction (dPCR; also abbreviated as e.g. digital PCR, DigitalPCR, or dePCR), which is a refinement of other polymerase chain reaction methods such as qPCR.

Digital polymerase chain reaction (dPCR) enables the absolute quantification of target nucleic acids present in a sample and alleviates the shortcomings of qPCR. Unlike qPCR, dPCR does not rely on calibration curves for sample quantification. Hence, it avoids the pitfalls associated with variations in reaction efficiencies. dPCR is a method of absolute nucleic acid quantification that hinges on the detection of end-point fluorescent signals and the enumeration of binomial events, i.e. absence or presence of fluorescence in a partition. In dPCR, the sample is first partitioned into many independent PCR sub-reactions such that each partition contains either a few, one or no target sequences. Several different methods can be used for sample partitioning, including microwell plates, microfluidic Nanoplates, capillaries, oil emulsion, *e.g.* water-in-oil-droplets, and arrays of miniaturized chambers with nucleic acid binding surfaces.

Digital PCR (dPCR) offers certain advantages over qPCR and scRNA-seq/scDNA-seq. In dPCR, the sample is distributed into thousands of nanopartitions, and each target molecule moves into partitions in a random fashion. This effectively increases the effective concentration of each target molecule within the PCR reaction, in contrast to traditional PCR where PCR reaction takes place in a bulk sample. End-point PCR takes place in each of the dPCR partitions independently, tolerating contaminants or PCR inhibitors that might be present in samples. dPCR detects signals coming from individual partitions and calculates absolute amounts of target molecules using Poisson statistics, eliminating the need for standard curves. Due to these features, dPCR offers quantification of nucleic acid targets of interest at much higher sensitivity and specificity, independent of PCR efficiency. Multiplexing analysis of targets of interest allows to focus on specific targets, rather than the bulk transcriptome or genome, for example. In addition, dPCR removes the challenges associated with bioinformatic analysis and interpretation of data, enabling researchers obtain *e.g.* gene expression profiles or target copy numbers from cells in a fast, efficient and budget friendly manner from isolation to target quantification. Therefore, the use of digital PCR provides unmatched sensitivity and specificity of quantification for targeted single-cell or population-based nucleic acid expression or copy number analysis. It can be used to quantify subtle changes in amounts of rare targets at a single cell level.

An accurate method for quantifying nucleic acid targets of single cells to achieve highly accurate targeted RNA expression data, or genomic DNA target copy numbers, or mitochondrial DNA target copy numbers, and which is particularly suitable for the analysis of low-abundant targets, is therefore of great interest. Additionally, there is a need for a method to encounter the drawbacks of the currently available methods.

SUMMARY OF THE INVENTION

Digital PCR, when combined with a high accuracy single cell sorting or cell isolation instrument, solves two major problems: high sensitivity of detection and accuracy of single cell isolation. The present invention provides highly efficient and high-throughput methods for quantification of nucleic acid target amounts in well-defined individual cells or population of cells.

The present invention provides for a method of quantification of nucleic acid target amounts in well-defined individual cells or population of cells. The nucleic acid landscape of single cells or a population

of cells at a specific time point is captured and targets of interest are analyzed. The quick transfer of cells into lysis buffer reduces artefacts, because cells have less time to adapt to stressors or environmental changes. Furthermore, DNA and RNA loss is eliminated, because no nucleic acid purification step is present. Instead, reverse transcription and digital PCR (or if no RNA targets are analyzed, only digital PCR) are performed on the nucleic acids of interest or the nucleic acid targets of interest. Through the combination of accurate cell sorting/isolation methods, the quick lysis of cells, and plate-based digital PCR as a very accurate quantification method, even lowly abundant targets can be quantified accurately. The results can be compared to the results of other (single) cells.

Hence, the present invention provides for a method of quantifying the amount of at least one nucleic acid target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell; and
b) Lysing the at least one cell, thereby releasing RNA and DNA; and
c) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA; and/or
performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate; and
d) Quantifying the amount of the at least one nucleic acid target.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a method of quantifying the amount of at least one nucleic acid target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell; and
b) Lysing the at least one cell, thereby releasing RNA and DNA; and
c) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital

polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA; and/or

performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate; and

d) Quantifying the amount of the at least one nucleic acid target.

The present invention also provides for a method of quantifying the amount of at least one ribonucleic acid (RNA) target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;
b) Lysing the at least one cell, thereby releasing RNA, wherein the released RNA comprises at least one RNA target;
c) Optionally performing a DNA digestion step using one or more DNA nucleases to remove cellular DNA, wherein the one or more DNA nucleases are inactivated prior to the performance of a reverse transcription reaction;
d) Performing a reverse transcription reaction on the released RNA comprising the at least one RNA target, thereby generating complementary DNA (cDNA);
e) Performing a digital polymerase chain reaction (dPCR) on the at least one reverse transcribed RNA target within the cDNA, wherein the dPCR is performed in a plate;
f) Quantifying the amount of the at least one RNA target.

The present invention also provides for a method of quantifying the amount of at least one coding RNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;
b) Lysing the at least one cell, thereby releasing RNA, wherein the released RNA comprises coding RNAs comprising at least one coding RNA target;
c) Optionally performing a DNA digestion step using one or more DNA nucleases to remove cellular DNA, wherein the one or more DNA nucleases are inactivated prior to the performance of a reverse transcription reaction;
d) Performing a reverse transcription reaction on the released RNA comprising coding RNA comprising at least one coding RNA target, thereby generating complementary DNA (cDNA);

e) Performing a digital polymerase chain reaction (dPCR) on the at least one reverse transcribed coding RNA target within the cDNA, wherein the dPCR is performed in a plate;

f) Quantifying the amount of the at least one coding RNA target.

The present invention also provides for a method of quantifying the amount of at least one non-coding RNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;

b) Lysing the at least one cell, thereby releasing RNA, wherein the released RNA comprises non-coding RNAs comprising at least one non-coding RNA target;

c) Optionally performing a DNA digestion step using one or more DNA nucleases to remove cellular DNA, wherein the one or more DNA nucleases are inactivated prior to the performance of a reverse transcription reaction;

d) Performing a reverse transcription reaction on the released RNA comprising non-coding RNA comprising at least one non-coding RNA target, thereby generating complementary DNA (cDNA);

e) Performing a digital polymerase chain reaction (dPCR) on the at least one reverse transcribed non-coding RNA target within the cDNA, wherein the dPCR is performed in a plate;

f) Quantifying the amount of the at least one non-coding RNA target.

The present invention also provides for a method of quantifying the amount of at least one small non-coding RNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;

b) Lysing the at least one cell, thereby releasing RNA, wherein the released RNA comprises small non-coding RNAs comprising at least one small non-coding RNA target;

c) Optionally performing a DNA digestion step using one or more DNA nucleases to remove cellular DNA, wherein the one or more DNA nucleases are inactivated prior to the performance of a reverse transcription reaction;

d) Performing a reverse transcription reaction on the released RNA comprising small non-coding RNA comprising at least one small non-coding RNA target, thereby generating complementary DNA (cDNA);

e) Performing a digital polymerase chain reaction (dPCR) on the at least one reverse transcribed small non-coding RNA target within the cDNA, wherein the dPCR is performed in a plate;

f) Quantifying the amount of the at least one small non-coding RNA target.

The present invention also provides for a method of quantifying the amount of at least one desoxyribonucleic acid (DNA) target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;

b) Lysing the at least one cell, thereby releasing DNA;

c) Performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate;

d) Quantifying the amount of the at least one DNA target.

The present invention also provides for a method of quantifying the amount of at least one genomic DNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;

b) Lysing the at least one cell, thereby releasing genomic DNA;

c) Performing a digital polymerase chain reaction (dPCR) on at least one genomic DNA target within the released genomic DNA, wherein the dPCR is performed in a plate;

d) Quantifying the copy number of the at least one genomic DNA target.

The present invention also provides for a method of quantifying the amount of at least one mitochondrial DNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;

b) Lysing the at least one cell, thereby releasing mitochondrial DNA;

c) Performing a digital polymerase chain reaction (dPCR) on at least one mitochondrial DNA target within the released mitochondrial DNA, wherein the dPCR is performed in a plate;
d) Quantifying the amount of the at least one mitochondrial DNA target.

The present invention also provides for a method of quantifying the amount of at least one genomic DNA target and at least one mitochondrial DNA target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell;
b) Lysing the at least one cell, thereby releasing genomic DNA and mitochondrial DNA;
c) Performing a digital polymerase chain reaction (dPCR) on at least one genomic DNA target within the released genomic DNA and on at least one mitochondrial DNA target within the released mitochondrial DNA, wherein the dPCR is performed in a plate;
d) Quantifying the amount of the at least one genomic DNA target and of the at least one mitochondrial DNA target.

dPCR is performed in a plate. Additionally, in one embodiment of all methods of the invention, one or both of the steps of i) lysis, and ii) reverse transcription (if applicable) are independently of each other performed in one or more different plates or in one or more different reaction vessels (referring herein to a Falcon tube or Eppendorf tube or similar vessels known in the art).

In another embodiment of all methods of the invention, lysis, reverse transcription (if applicable) and dPCR are performed in the same plate.

The present invention also provides for a method of quantifying the amount of at least one nucleic acid target, the method comprising:

a) Providing a defined number of cells, wherein the defined number of cells is at least one cell,
b) Lysing the at least one cell, thereby releasing RNA and DNA,
c) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA, wherein the dPCR is performed in a plate; and/or

performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate, and

d) Quantifying the amount of the at least one nucleic acid target.

In one embodiment, step b) and c) above are performed in the same plate.

In one embodiment, the reverse transcription reaction in step c) above is also performed in a plate, preferably the same plate in which the subsequent dPCR is performed.

In an alternative aspect, the cells according to the present invention are not put into lysis buffer, but are alternatively loaded onto plates and lysed by heat (thermal lysis). Hence, if Nanoplates are used, a defined number of cells can be loaded into the wells. The intact cells are then distributed into the partitions and are lysed thereafter through thermal lysis. The released nucleic acids can then be subjected to reverse transcription, if required, and dPCR.

Therefore, the present invention also provides for a method of quantifying the amount of at least one nucleic acid target, the method comprising:

a) Providing a defined number of cells onto a plate, wherein the defined number of cells is at least one cell,

b) Lysing the at least one cell by thermal lysis, thereby releasing RNA and/or DNA,

c) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA, wherein the dPCR is performed in a plate; and/or
performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate, and

d) Quantifying the amount of the at least one nucleic acid target.

The present invention also provides for an *in vitro* method of assessing the risk of a subject to develop cancer or a disease, wherein the cancer or disease is associated with deregulated expression of at least one coding RNA target, or with deregulated expression of at least one non-coding RNA target, or with at least one genomic or mitochondrial structural variation, preferably a copy number variation (CNV). With the quantification methods according to the invention, the amount of at least one nucleic acid target of interest can be determined. Since in certain embodiments, this amount may serve as a prognostic marker known in the art, the risk of the subject to develop cancer or a disease can be assessed.

The present invention also provides for an *in vitro* method of diagnosing a subject of having cancer or a disease, wherein the cancer or disease is associated with deregulated expression of at least one coding RNA target, or with deregulated expression of at least one non-coding RNA target, or with at least one genomic or mitochondrial structural variation, preferably a copy number variation (CNV). With the quantification methods according to the invention, the amount of at least one nucleic acid target of interest can be determined. Since in certain embodiment, this amount is a biomarker marker for cancer or a particular disease and known in the art, the subject can be diagnosed of cancer or the particular disease.

The present invention also provides for an *in vitro* method of monitoring the progression of cancer or a disease in a subject, wherein the cancer or disease is associated with deregulated expression of at least one coding RNA target, or with deregulated expression of at least one non-coding RNA target, or with at least one genomic or mitochondrial structural variation, preferably a copy number variation (CNV). With the quantification methods according to the invention, the amount of at least one nucleic acid target of interest can be determined. Since in certain embodiments, this amount may serve as a prognostic marker known in the art, the progression of cancer or a disease in a subject can be monitored.

The present invention also provides for an *in vitro* method of monitoring a subjects' response to a therapy of a cancer or a disease, wherein the cancer or disease is associated with deregulated expression of at least one coding RNA target, or with deregulated expression of at least one non-coding RNA target, or with at least one genomic or mitochondrial structural variation, preferably a copy number variation (CNV). With the quantification methods according to the invention, the amount of at least one nucleic acid target of interest can be determined. Since in certain embodiments, this amount may serve as a prognostic marker known in the art, the subject's response to a therapy of the cancer or the disease can be monitored.

In these regards, the following literature discloses examples of dysregulated RNA or DNA targets, which are associated with cancer or other diseases. Farazi et al. ("miRNAs in human cancer", 2011, Journal of Pathology, DOI: 10.1002/path.2806) discloses several miRNAs associated with cancer and also discloses the abundance of several miRNAs in different cancers. Similarly, in Condrat et al. ("miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis", 2020, Cells, DOI: 10.3390/cells9020276) miRNAs and their association with different cancers and other diseases are reviewed. The review article also elucidates on the topic of miRNAs as biomarkers in diseases, including their role in diagnosis and prognosis. Henriksen et al. ("Single Cell Analysis Identifies the miRNA Expression Profile of a Subpopulation of Muscle Precursor Cells Unique to Humans With Type

2 Diabetes", 2018, Front. Physiol., DOI: 10.3389/fphys.2018.00883) could show that the miRNA expression profile of a subpopulation of muscle precursor cells is associated with type 2 diabetes in humans. Rai et al. ("Analysis of mitochondrial DNA copy number variation in blood and tissue samples of metastatic breast cancer patients (A pilot study)", 2021, Biochemistry and Biophysics Reports, DOI: 10.1016/j.bbrep.2021.100931) discloses different mitochondrial DNA copy numbers in breast cancer tissue as compared to non-tumor breast tissue. Fazzini et al. ("Association of mitochondrial DNA copy number with metabolic syndrome and type 2 diabetes in 14 176 individuals", 2021, J Intern Med, DOI: 10.1111/joim.13242) could show an association of mitochondrial DNA copy number with metabolic syndrome and type 2 diabetes. Reznik et al. ("Mitochondrial DNA copy number variation across human cancers", 2016, eLIFE, DOI: 10.7554/eLife.10769.001) disclose that there is mitochondrial DNA copy number variation across different human cancers. In Shlien and Malkin ("Copy number variations and cancer", 2009, Genome Medicine, DOI: 10.1186/gm62), the role of DNA copy number variations in cancer is reviewed. Wong et al. ("A Comprehensive Analysis of Common Copy-Number Variations in the Human Genome", 2006, Am J Hum Genet., DOI: 10.1086/510560) discloses the association of specific CNVs with some diseases, such as sense-related diseases, and cancer. Andhale and Shrivastava ("Huntington's Disease: A Clinical Review", 2022, Cureus, DOI: 10.7759/cureus.28484) is a review about Huntington's disease. This disease is an autosomal dominantly inherited condition, which is caused by a CAG trinucleotide repeat expansion in the huntingtin gene on chromosome 4. Ding et al. ("Evaluating the molecule-based prediction of clinical drug responses in cancer", 2016, Bioinformatics, DOI: 10.1093/bioinformatics/btw344) could show that expression levels of certain mRNAs or miRNAs in specific cancer types can serve as predictors for drug responses.

The present invention also provides for kits for performing the methods according to the invention. Such kits may comprise appropriate buffers, primers, probes and other components needed to perform the methods according to the invention.

"A genomic DNA target" as used herein, generally refers to a region on genomic DNA. In a preferred embodiment, "a genomic DNA target" refers to a region on genomic DNA, that harbors or is suspected to harbor a copy number variation (CNV). CNVs are structural changes in the genome (such as deletions, insertions, duplications, translocations, and inversions) that lead to gain or loss of copy numbers of a region, ranging from few hundred base pairs up to whole chromosomes. CNVs are either inherited or the results of de novo somatic changes. 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, shortening of chromosomal ends and positional effects, which are associated with complex diseases and traits such as, but not limited

to, cancer, obesity, aging, and neurodegenerative and autoimmune diseases. Therefore, 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.

Hence, in certain embodiments, the copy number variation of the region on the genomic DNA may be associated with increased disease risk, or increased risk of disease progression, or increased risk of cancer development, or increased risk of cancer progression, or increased risk for obesity.

“A mitochondrial DNA target” as used herein, generally refers to a region on mitochondrial DNA. In a preferred embodiment, “a mitochondrial DNA target” refers to a region on mitochondrial DNA, that harbors or is suspected to harbor a copy number variation (CNV). Similar to genomic CNVs, changes in mitochondrial DNA copy number (mtDNA CN) in blood or tissue have been linked to diseases not surprisingly given the important role mitochondria plays in cellular homeostasis. In contrast to fixed copy number of genomic DNA (healthy diploid state), drastic cell to cell variation can be observed at the level of mitochondrial DNA (mtDNA) even though cells are identical in genetic identity and morphology. mtDNA copy numbers fluctuate and vary between individuals, as well as between different tissues, cells and even among mitochondria within same cell. Individual cells can carry hundreds to hundred thousand copies of mtDNA. However, the drastic changes in copy number of mtDNA can lead to mitochondrial dysfunction and disease formation. Therefore, similar to genomic CNVs, capturing the copy number heterogeneity in mtDNA is important for disease and drug development studies and can be used as a proxy for screening metabolic diseases, cancer, prenatal testing, neurodegeneration, and aging-related diseases.

"An RNA target” as used herein, refers to any type or subset of RNA that is analyzed/quantified according to the methods of the invention. RNA targets therefore comprise, among others, coding and non-coding RNA targets. RNA targets need to be reverse transcribed into cDNA prior to analysis and dPCR is then performed on at least one reverse transcribed RNA target within the cDNA.

“A coding RNA target” as used herein, refers to peptide/protein-coding RNA targets. Thus, the term refers to messenger RNAs (mRNAs), for example, but not limited to, ß-actin (ACTB) mRNA.

With respect to coding RNA targets, approximately 360,000 mRNA molecules are present in a single mammalian cell. Some mRNAs comprise 3% of the mRNA pool whereas others account for less than 0.1%. These rare or low-copy mRNAs may even have a copy number of as low as 0 or 1 molecules per cell.

"A non-coding RNA target" as used herein, refers to RNA targets, that do not encode peptides/proteins. However, they may have specialized functions. According to Li and Chen ("Small and Long Non-Coding RNAs: Novel Targets in Perspective Cancer Therapy", 2015, Curr Genomics), those functions include, but are not limited to, regulation of transcription and translation, or functions in protein scaffolding for example. Non-coding RNAs comprise long non-coding RNAs (>= 200 nt) and small non-coding RNAs (< 200 nt). Examples for long non-coding RNA targets comprise long intergenic non-coding RNAs (lincRNAs), natural antisense transcripts (NATs), transcribed ultraconserved regions (T-UCRs) and non-coding pseudogenes. Examples for small non-coding RNA targets comprise microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs).

If not further specified, "DNA target" as used herein, refers to genomic and mitochondrial DNA targets. Depending on the context, this may either mean that it refers to both or to either of them. For example, if a sentence reads "performing a dPCR on at least one DNA target (...)", then it is meant that if dPCR is performed on one DNA target, this target may either be a genomic or a mitochondrial DNA target. If dPCR, in this example, is performed on two DNA targets, however, then each of these two DNA targets can be independently selected from genomic DNA targets and mitochondrial DNA targets. The same principle applies to more than two DNA targets. The same general principle also applies to "RNA targets". If it is referred to "nucleic acid targets" (or simply "targets") herein, this means that each one of these nucleic acid targets can be independently selected from the various DNA targets and RNA targets described herein.

Hence, in one embodiment, each of the at least one nucleic acid targets is independently selected from a coding RNA target; a non-coding RNA target; a genomic DNA target; and a mitochondrial DNA target. Preferably, the coding RNA target is an mRNA; the non-coding RNA target is a miRNA; the genomic DNA target is a region on genomic DNA, that harbors or is suspected to harbor a copy number variation (CNV); and the mitochondrial DNA target is a region on mitochondrial DNA, that harbors or is suspected to harbor a CNV.

In one embodiment, each of the at least one nucleic acid targets is independently selected from an mRNA; a miRNA; a region on genomic DNA, that harbors or is suspected to harbor a copy number variation (CNV); and a region on mitochondrial DNA, that harbors or is suspected to harbor a CNV.

In one embodiment, the genomic structural variation is a copy number variation.

In one embodiment, the mitochondrial structural variation is a copy number variation.

In one embodiment, the small non-coding RNA is a miRNA.

The term “at least one” as used herein, refers to 1, 2, 3, or more. For example, at least one target is meant to include 1, 2, 3, or more targets, respectively. Similarly, “at least two” as used herein refers to 2, 3, 4 or more. For example, at least two targets is meant to include 2, 3, 4 or more targets, respectively.

The term “accuracy” as used herein refers to a measure of trueness. Accuracy is how close a given set of measurements are to their true value. It is a description of only systematic errors, a measure of statistical bias of a given measure of central tendency. Low accuracy causes a difference between a result and a true value.

“Accuracy” should not be confounded with “precision”, which defines how close the measurements are to each other. In other words, precision is a description of random errors, a measure of statistical variability.

According to the methods of the invention, the sample from which a defined number of cells is provided comprises eukaryotic-, human-, animal-, plant-, bacterial-, archaeal-, oomycete-, viral-, and/or fungal cells.

Preferably, the sample from which a defined number of cells is provided is a eukaryotic sample. Most preferably, the sample from which a defined number of cells is provided is a human sample.

In one embodiment, the sample from which a defined number of cells is provided may be a male or a female sample. Alternatively, the sample may comprise a mixture of male and female cells. Thus, according to another embodiment, the sample comprises one or more additional cells originating from a different individual.

Hence, in one embodiment, the at least one cell originates from a eukaryotic sample, preferably a human sample. In one embodiment, the human sample originates from a subject having cancer or being suspected of having cancer. In another embodiment, the human sample originates from a subject having or suspected of having a disease.

In one embodiment, the sample from which a defined number of cells is provided originates from a subject having cancer or being suspected of having cancer. In another embodiment, the sample from which a defined number of cells is provided originates from a subject having or suspected of having a disease.

In certain embodiments, the cancer or the disease is associated with deregulated expression of at least one coding RNA target. The amount of the at least one coding RNA target can be quantified by the methods according to the present invention.

In certain other embodiments, the cancer or the disease is associated with deregulated expression of at least one non-coding RNA target, preferably at least one small non-coding RNA target. The amount of the at least one non-coding RNA target can be quantified by the methods according to the present invention.

In again certain other embodiments, the cancer or the disease is associated with at least one genomic or mitochondrial structural variation, preferably a copy number variation (CNV). The amount of the at least one genomic or mitochondrial structural variation can be quantified by the methods according to the present invention, namely by quantifying the amount of at least one genomic DNA target and/or the amount of at least one mitochondrial DNA target.

(Single cell-) CNV analyses can be applied to gene and cell therapies, CAR-T therapies, intentional gene alterations induced by viral vectors or CRISPR modifications, organoid studies, quantification of organelles, as well as infectious disease studies.

In one embodiment, the sample from which a defined number of cells is provided may originate from one of the following sample or tissue types comprising whole blood, blood fractions, plasma, serum, tumor cells, body fluids, oral specimen, oral fluids, saliva, sputum, swab, urine, human biotic tissue, clothing samples containing biological material, vaginal swabs, sperm, skin or wound swabs or other samples containing biological material or other parts of the human body upon availability for isolation of nucleic acids. As used herein the terms “oral fluids” and “body fluids” refer to fluids that are excreted or secreted from the buccal cavity and from the body, respectively, from which nucleic acids can be isolated. As non-limiting examples, oral and body fluids may comprise saliva, sputum, swab, and urine.

In one embodiment, the sample from which a defined number of cells is provided comprises cells or cell types or cell lines or a mixture of different, i.e. at least two, cells or cell types or cell lines in (a cell) suspension. In one embodiment, the cells or cell types or cell lines comprise genetically modified cells, cell types, or cell lines. In one embodiment, the cells or cell types or cell lines comprise genetically unmodified cells, cell types, or cell lines. In one embodiment, the cells or cell types or cell lines comprise genetically modified cells, cell types, or cell lines and unmodified cells, cell types, or cell lines.

If necessary, the preparation of the cells from the sample prior to the step of providing a defined number of cells can occur by appropriate and applicable techniques known in the art, for example, but not limited to, by trypsinization of cell culture cells, if applicable.

An exact, defined number of cells can be obtained by various methods. In one embodiment, two, three, four, five, or more cells are isolated or sorted and thereby provided. In a preferred embodiment, a single cell is provided.

In a preferred embodiment of the methods of the invention, the defined number of cells is provided by a method selected from the group comprising or consisting of fluorescence-activated cell sorting, micromanipulation, microfluidics, immunopanning, magnet-activated cell sorting, and laser microdissectioning. In a more preferred embodiment of the methods of the invention, the defined number of cells is at least one cell and this at least one cell is provided by a method selected from the group comprising or consisting of fluorescence-activated cell sorting, micromanipulation, microfluidics, immunopanning, magnet-activated cell sorting, and laser microdissectioning.

Fluorescence-activated cell sorting (FACS) is a commonly used technique to isolate specific cells of interest, either as single cells or as a pool of cells of interest. The cells of interest are usually, but not necessarily, fluorescently labelled and flow through a flow cytometer. A laser excites the fluorophore. The emitted light is detected and the cells of interest can be separated from other cells and used for subsequent steps. Cells can also be isolated based on their size and/or their light scattering profile.

Micromanipulation can occur manually, *i.e.* a skilled person uses a microscope and a micropipette to pick up individual cells, or mechanically, *i.e.* using a robot or the like, which picks up individual cells based on, for example, but not limited to, visual inputs.

Microfluidics enable the isolation of cells of interest, while using small volumes of fluids and small amounts of a sample. Cells can be isolated based on labelling or based on the cell-intrinsic properties, such as, but not limited to, size, shape, density, deformability, electric polarizability/impedance, and other hydrodynamic properties. Individual cells can be separated through traps, valves, or droplets, for example, although other ways of separating them, which are known in the art, are also encompassed within the methods of the invention.

Immunopanning is used to isolate cells based on their binding to immobilized antibodies. Specifically, those antibodies bind to surface antigens on the cells of interest. Unbound cells are washed away and the antibody-bound cells of interest can be retrieved.

In magnet-activated cell sorting (MACS) cells of interest bind to antibodies which in turn are bound to magnetic beads. Specifically, these antibodies target an antigen present on the cells of interest. A magnetic field is then applied, which attracts the magnetic beads, while the unbound cells pass by. The bead-bound cells of interest can then be eluted and used for subsequent steps. Alternatively, the cells of interest are the ones which do not bind to the beads, while the unwanted cells are bound by them. In this case, the cells passing by need to be collected, while the bead-bound cells can be discarded.

Laser microdissection (LMD), also called laser capture microdissection (LCM), can be used to isolate cells of interest from a tissue sample by applying a laser beam to cut a desired area which was previously marked using a microscope. Single cells or a plurality of cells can be obtained using this method.

In addition to the above, similar cell isolation methods known in the art, which enable the isolation of an exact number of cells, e.g. single cells, two cells, three cells, four cells, five cells and more than five cells, are also encompassed. An example is the cellenONE® X1 technology. It is based on an image (brightfield and/or 4-channel fluorescence)-based single cell selection and isolation method. As low as 1 µl of sample comprising just a few cells can be processed using this technology, and the generated drops can have a volume of 150-600 pL.

Preferably, the diameter, elongation parameters, and/or any targetable structure, such as, but not limited to, size, shape, granularity, proteins or peptides, and/or nucleic acids, in or on the cell or cells of interest are known, or information thereon can be received while performing the methods of the invention. These parameters or structures can be used for the isolation of the cell or cells of interest.

In a preferred embodiment, the provided at least one cell is directly isolated into lysis buffer. Thereby, the RNA landscape of the cells is preserved as good as possible. Cells adapt their transcriptome very quickly to changes in their environment or stressors or the like. Furthermore, ribonucleases present in cells or in the environment can quickly degrade RNA. Therefore, RNA is relatively unstable and the faster the cells of interest are lysed, the less artefacts or variations in the RNA landscape will arise, which is an important requirement for single cell RNA analyses. All kinds of RNAs present in cells can in principle be subject to analysis within the respective methods of the invention, for example, but not limited to, mRNAs and/or miRNAs can be analyzed. In one embodiment, cell lysis and all subsequent steps of the methods of the invention, i.e. reverse transcription (if RNA is analyzed) and dPCR, are performed in the same plate. The advantage of using one plate for all steps from lysis to dPCR is that no material is lost through pipetting and purification steps and the procedure is time-efficient as less pipetting steps are required. In addition, risk of contamination during handling is minimized.

Furthermore, the cells, which are analyzed, are intact prior to their loading on the plate and hence, if loaded on Nanoplates, their nucleic acid content is distributed onto the partitions of the Nanoplate.

In one embodiment, lysis of the at least one cell releases both, RNA and DNA. In this embodiment, DNA targets and/or RNA targets can be analyzed.

"Lysing the at least one cell, thereby releasing RNA" as used herein, means that at least RNA is released. The release of further types of nucleic acids, such as genomic DNA or mitochondrial DNA, is optional if they do not comprise targets of interest.

"Lysing the at least one cell, thereby releasing DNA" as used herein, means that at least genomic and/or mitochondrial DNA is released. The release of further types of nucleic acids, such as RNA, is optional if they do not comprise targets of interest.

"Lysing the at least one cell, thereby releasing mitochondrial DNA" as used herein, means that at least mitochondrial DNA is released. The release of further types of nucleic acids, such as genomic DNA or RNA, is optional if they do not comprise targets of interest. "Lysing the at least one cell, thereby releasing genomic DNA" as used herein, means that at least genomic DNA is released. The release of further types of nucleic acids, such as mitochondrial DNA or RNA, is optional if they do not comprise targets of interest.

In cases where a direct isolation into lysis buffer may not be possible, for example, but not limited to, certain isolation device- or sorting device-dependent requirements, or requirements of the kits used, cells may be isolated into phosphate-buffered saline or a different buffered solution or into appropriate medium and are quickly lysed afterwards. In one preferred embodiment, the lysed cell(s) is/are directly used for subsequent steps, e.g. for a reverse transcription reaction and a dPCR reaction, or for a dPCR reaction if no RNA is analyzed. However, the methods according to the invention also allow for a storage of the lysate before the subsequent steps of the methods according to the invention are performed. Hence, in another embodiment, the lysate is stored, for example at approx. 4°C, or -20°C or -80°C, until it is used in subsequent steps of the methods of the invention.

In a preferred embodiment of the methods according to the invention, the lysis buffer comprises components that stabilize RNA and/or DNA. In an alternative embodiment, the components that stabilize RNA and/or DNA may be provided in the solution comprising the cells to be sorted or isolated, or in the final solution of cells used directly prior to lysis. In another alternative embodiment, the components that stabilize RNA and/or DNA may be provided shortly after cell lysis.

Similar as with the quick lysis of cells described above, RNA stabilization after or during cell lysis helps to preserve the natural RNA landscape of the cells. Currently, commercial kits are available for i) preparation of RNA lysates from cells directly by using one or more buffers, ii) stabilization of cellular RNA, and optionally iii) elimination of contaminating DNA. These kits eliminate the need for an RNA purification step and accelerate and streamline RNA sample preparation from cells in a high-throughput and reproducible manner. They also positively affect the accuracy of the present invention. Thus, the methods according to the present invention which relate to the analysis of RNA targets, do not require an additional RNA extraction step after cell lysis. Instead, the lysate, or a fraction of it, can directly be used for subsequent steps. Since no additional RNA purification step is required, this additionally speeds up the procedure and avoids loss of RNA, in particular lowly abundant RNAs, that might otherwise get lost during the additional purification step.

Similarly, commercial kits are available for i) preparation of lysates from cells directly by using one or more buffers, and optionally ii) stabilization of cellular RNA and optionally DNA. These kits eliminate the need for an RNA and/or DNA purification step and accelerate and streamline sample preparation from cells in a high-throughput and reproducible manner. They also positively affect the accuracy of the present invention. Thus, the methods according to the present invention do not require an additional RNA and/or DNA extraction step after cell lysis. Instead, the lysate, or a fraction of it, can directly be used for subsequent steps. Since no additional RNA and/or DNA purification step is required, this additionally speeds up the procedure and avoids loss of nucleic acids, in particular lowly abundant nucleic acid targets, that might otherwise get lost during the additional purification step.

In a preferred embodiment of the methods according to the invention which relate to the analysis of DNA targets, the lysis buffer does not comprise DNA nucleases, such as, but not limited to, DNase I. One or more DNA nucleases may, however, be added after lysis and prior to a reverse transcription reaction, if only RNA targets are analyzed. In fact, if RNA targets, but no DNA targets are analyzed, the addition of one or more DNA nucleases is preferred in order to eliminate the risk of detecting genomic DNA instead of complementary DNA (cDNA) in subsequent steps according to the methods of the invention. Standard DNA nuclease concentrations known in the art can be used. The one or more DNA nucleases are then inactivated prior to the reverse transcription step. Inactivation may occur by using a DNA nuclease inhibitor, or by a DNA nuclease-resistant aptamer targeting the DNA nuclease, or by chelating the ions required for DNA nuclease activity, or by RNA purification, or by DNA nuclease precipitation. Preferably, DNA nuclease inactivation is performed by heat inactivation, for example, but not limited to, incubation at 75°C for 5 minutes.

Hence, in one embodiment, a DNA digestion step using one or more DNA nucleases to remove cellular DNA is performed after lysis and prior to a reverse transcription reaction, wherein the one or more DNA nucleases are inactivated prior to the performance of a reverse transcription reaction.

The lysis buffer may also comprise a proteinase, such as Proteinase K. If Proteinase K is present in the lysate, the sample is heat-inactivated prior to the subsequent steps. Heat inactivation protocols for Proteinase K are known in the art, one non-limiting example being incubation for 5-10 min at 75°C.

Hence, in one embodiment, the lysate is heat-inactivated prior to the subsequent steps.

In another embodiment, the lysate is not heat-inactivated prior to the subsequent steps.

Either 100%, or 90%, or 80%, or 70%, or 60%, or 50%, or 40%, or 30%, or 20%, or 10%, or 1%, or between 100% and 1%, or between 100% and 10%, or between 100% and 20%, or between 90% and 1%, or between 90% and 10%, or between 90% and 20%, or between 100% and 80%, or between 20% and 1%, or between 70% and 30% of the lysate is used for the subsequent steps according to the methods of the invention. The amount of lysate used can vary depending on the subsequent steps, the starting material amount and the experimental conditions. For example, lysates comprising a high number of cells might need or benefit from using less lysate input for downstream steps.

The direct use of the lysate in the subsequent steps according to the methods of the invention without prior purification of DNA and/or RNA has the advantage that no nucleic acids are lost during purification. As a consequence, even very lowly abundant targets (*e.g.* RNA targets present in as low as 1 copy per cell) can accurately be detected and quantified in absolute amounts using the methods according to the invention.

In one embodiment, lysis is performed in a reaction vessel. In an alternative embodiment, lysis is performed in a plate, which is different from the plate in which the reverse transcription reaction is performed. Alternatively or additionally, lysis is performed in a plate, which is different from the plate in which the dPCR is performed. In another embodiment, lysis is performed in the same plate in which the reverse transcription reaction, if performed, and the dPCR are performed.

In one embodiment, for dPCR, or for reverse transcription directly followed by dPCR, the dPCR reaction mixture or the RT-dPCR reaction mixture, respectively, which comprises the lysate or fractions thereof, is distributed into a plurality of partitions prior to the (reverse transcription and) amplification step, and the (reverse transcription and) the dPCR is/are performed in each of said plurality of partitions.

In a preferred embodiment of the methods according to the invention, which relates to the analysis of RNA targets, a reverse transcription reaction is directly performed on the released RNA of the lysate, or on a fraction of the released RNA of the lysate (*e.g.* on targets of interest). Specifically, the reverse transcription reaction is performed on the released RNA comprising at least one RNA target. In one embodiment, random primers, or target-specific primers, or oligo(dT) primers may be used for reverse transcription. Reverse transcription generates complementary DNA (cDNA). In one embodiment, the reverse transcription reaction is performed in a reaction vessel. In an alternative embodiment, the reverse transcription reaction is performed in a plate, which is different from the plate in which dPCR is performed later on. In another embodiment, the reverse transcription reaction is performed in the same plate in which dPCR is performed later on. The cDNA, or a fraction thereof, is used as a template for the subsequent dPCR reaction.

In one preferred embodiment, the cDNA is directly used for dPCR. However, since cDNA is relatively stable as compared to RNA, the methods according to the invention also allows for a storage of the cDNA before the subsequent steps of the methods according to the invention are performed. Hence, in another embodiment, the cDNA is stored until a dPCR reaction is performed on the cDNA or a fraction thereof. cDNA storage may occur at approx. 4°C, or -20°C or -80°C.

Either 100%, or 90%, or 80%, or 70%, or 60%, or 50%, or 40%, or 30%, or 20%, or 10%, or 1%, or between 100% and 1%, or between 100% and 10%, or between 100% and 20%, or between 90% and 1%, or between 90% and 10%, or between 90% and 20%, or between 100% and 80%, or between 20% and 1%, or between 70% and 30% of the cDNA is used for the subsequent steps according to the methods of the invention. The amount of cDNA used can vary depending on the subsequent steps, the starting material amount and the experimental conditions. For example, cDNA from a high number of cells might need or benefit from using less cDNA input for downstream steps.

In one embodiment, the dPCR is performed on at least one reverse transcribed RNA target within the cDNA. In this embodiment, the dPCR may be performed simultaneously on one, two, three, four, or five reverse transcribed RNA targets within the cDNA. Each of the at least one targets may be independently selected from the group comprising low-copy targets, medium-copy targets, and high-copy targets. Alternatively, each of the at least one targets may be independently selected from the group consisting of low-copy targets, medium-copy targets, and high-copy targets.

In another embodiment, the dPCR is performed on at least one DNA target within the released DNA. In this embodiment, the dPCR may be performed simultaneously on one, two, three, four, or five DNA targets within the released DNA. Each of the at least one targets may be independently selected from

the group comprising low-copy targets, medium-copy targets, and high-copy targets. Alternatively, each of the at least one targets may be independently selected from the group consisting of low-copy targets, medium-copy targets, and high-copy targets.

In yet another embodiment, the dPCR is performed on at least one reverse transcribed RNA target within the cDNA and on at least one DNA target within the released DNA. In this embodiment, the dPCR may be performed simultaneously on one, two, three, or four DNA targets within the released DNA, while the other targets are reverse transcribed RNA targets within the cDNA, wherein the dPCR is performed on a maximum of five nucleic acid targets in total. Each of the at least one targets may be independently selected from the group comprising low-copy targets, medium-copy targets, and high-copy targets. Alternatively, each of the at least one targets may be independently selected from the group consisting of low-copy targets, medium-copy targets, and high-copy targets.

In one embodiment, the dPCR is performed simultaneously on one target, or on two targets, or on three targets, or on four targets, or on five targets within the cDNA and/or the released DNA.

In one embodiment, each of the at least one targets according to the methods of the invention is independently selected from the group comprising a low-copy target, a medium-copy target, and a high-copy target; or comprising low-copy targets, medium-copy targets, and high-copy targets in cases of more than one target.

In one embodiment, each of the at least one targets according to the methods of the invention is independently selected from the group consisting of a low-copy target, a medium-copy target, and a high-copy target; or consisting of low-copy targets, medium-copy targets, and high-copy targets in cases of more than one target.

In addition to the lysed cell or cells of interest, the RT-dPCR reaction mixture (for RNA target analysis, or RNA and DNA target analysis), or the dPCR reaction mixture (for DNA target analysis), comprises a buffer and dNTPs in addition to the enzymes required.

As used herein, the term "dNTP" refers to deoxyribonucleoside triphosphates. Non-limiting examples of such dNTPs are dATP, dGTP, dCTP, dTTP, dUTP, which may also be present in the form of labelled derivatives, for instance comprising a fluorescent label, a radioactive label, or a biotin label. dNTPs with modified bases are also encompassed, wherein these bases are for example hypoxanthine, xanthine, 7-methylguanine, inosine, xanthinosine, 7-methylguanosine, 5,6-dihydrouracil, 5-methylcytosine, pseudouridine, dihydrouridine, or 5-methylcytidine. Furthermore, ddNTPs of the above-described molecules are encompassed in the present invention.

In one embodiment, the methods according to the invention are high-throughput methods.

Detection of the amplification products may comprise direct or indirect fluorescent and/or chemical labelling and/or polynucleotide-based and/or peptide- or protein-based labelling of the amplicon by a composition comprising one or more of the following: probes and/or guiding molecules and/or proteins and/or peptides and/or nucleic acids and/or derivatives of the aforementioned listed components. In one embodiment, the probes and/or the amplification products can be labelled with one or more fluorophores, quenchers, barcodes, indices, peptides, proteins, radioactive tags, biotin tags, and/or chemical moieties attached to them. In another embodiment or additionally, the probes and/or the amplification products can comprise modifications, such as base modifications, sugar modifications and/or backbone modifications. Probes may be RNA probes or DNA probes. Non-limiting examples for probes comprise Taqman probes, Scorpion probes, Molecular-Beacon probes, and/or Fluorescence Resonance Energy Transfer Probes.

Alternatively, intercalating dyes or other known DNA labelling or detection agents may be used instead of probes.

Preferably, quantification is based on the detection of the amplified at least one nucleic acid target by using at least one probe per analyzed target, wherein the at least one probe specifically binds to each of the amplification products per nucleic acid target. Hence, different probes are needed for different targets. Preferably, the at least one probe is fluorescently labelled.

In one embodiment, the at least one probe is at least one TaqMan probe.

In one embodiment, quantification is based on the detection of the amplified at least one reverse transcribed RNA target and/or of the amplified at least one DNA target by using at least one fluorescently-labelled probe per analyzed target, wherein the at least one probe specifically binds to each of the amplification products per target.

Fluorescence is usually detected using a high-powered detection module with fluorescence filter for all partitions per well. Several fluorescent dyes may be used, as long as the corresponding fluorescent channels can be separated from each other. Examples for dyes comprise, but are not limited to, HEX, VIC, JOE, FAM, EvaGreen®, TAMRA, ATTO 550, ROX, Texas Red, Cy5, and Cy5.5.

In addition to the cell isolation method and the lysate generation method, the technology employed for the nucleic acid quantification drastically affects the accuracy and robustness of the results.

After PCR, the concentration of the target nucleic acids is quantified with a statistically defined accuracy using Poisson's statistics, wherein partitions with either 0, 1, or more target sequences are each needed for the calculations. Interestingly, sample partitioning efficiently concentrates the target sequences within the isolated microreactors. This concentration effect reduces template competition and thus enables the detection of rare mutations in a background of wild-type sequences (Quan et al., 2018, MDPI, "dPCR: A Technology Review").

dPCR may also allow for a higher tolerance to inhibitors present in a sample, because there is no need to have an amplification efficiency per cycle of almost 100%, as required for qPCR. Instead, it is sufficient if at the end of the amplification reaction either a signal or no signal is detectable.

As for normal PCR, dPCR also carries out a single reaction within a sample, however the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts.

dPCR involves partitioning the PCR solution into at least a few hundred, but in most cases several thousand or tens of thousands or more of nano-liter sized partitions, where a separate PCR reaction takes place in each one. A dPCR solution is made similarly to a quantitative assay either using probes, e.g. fluorescence-quencher probes, or intercalating dyes, and a PCR master mix, which comprises DNA polymerase, dNTPs, MgCl2, and reaction buffers at optimal concentrations.

After multiple PCR amplification cycles, the samples are checked for fluorescence with a binary readout of "0" (absence) or "1" (presence). The fraction of fluorescing partitions is recorded. The partitioning of the sample allows one to estimate the number of different molecules by assuming that the molecule population follows the Poisson distribution, thus accounting for the possibility of multiple target molecules inhabiting a single partition.

In contrast to a qPCR reaction, a dPCR reaction is an endpoint PCR reaction. dPCR uses the number of fluorescence-positive partitions over the total to back-calculate the target concentration. In contrast to qPCR, calibration curves are not needed for sample quantification in dPCR. All in all, compared to qPCR, dPCR provides a more robust quantification, is less prone to inhibitors and is independent of a quantification standard.

The benefits of dPCR include increased precision through massive sample partitioning, which ensures reliable measurements of the desired target due to reproducibility. Also, dPCR is highly quantitative as it does not rely on relative fluorescence of the solution to determine the amount of amplified target nucleic acids.

According to the present invention, dPCR is performed in a plate. It offers a number of advantages over other dPCR methods, which comprise the use of microfluidic chips and discs, microarrays, microdroplets, or droplet crystals based on oil-water emulsions.

In contrast to the performance of a dPCR in a plate, as is the case in the present invention, droplet digital PCR (ddPCR) is a method of dPCR in which a for example 20 microliter sample reaction including assay primers and either Taqman probes or an intercalating dye, is divided into about 20,000 nanoliter-sized oil droplets through a water-oil emulsion technique, thermocycled to end point in a 96-well PCR plate, and fluorescence amplitude read for all droplets in a droplet flow cytometer. Each droplet needs to be measured individually, one by one.

In contrast, plate-based dPCR enables a faster readout, since all partitions of a well (sample) can be read out simultaneously. Further key advantages of performing dPCR in a plate, for example, but not limited to, a Nanoplate, include but are not limited to: i) user-friendly, familiar plates are easy to pipet just like for qPCR, which facilitates the application also for users that are less experienced or unexperienced in using microfluidics, ii) fixed partitions prevent variation in size and coalescence (which can occur in droplet-based dPCRs), thereby maximizing consistency in volume and number of partitions, iii) sealed (Nano)plates prevent well to well contamination, iv) there is an integrated quality control, meaning one can look at the plate image for single fluorescent signals from the individual positive partition counts, thus the user receives not only a table with counts, but also images, v) plates are amenable to front-end automation, minimizing hands-on steps, vi) higher volumes may be loaded into plates as compared to droplet dPCR, vii) higher scalability with respect to the loading volume and the number of partitions and wells.

Additionally, in droplet digital PCR, the time until the results are delivered usually takes longer than for plate-based digital PCR. Furthermore, the workflow and handling is more complex for droplet dPCR.

Furthermore, the chemistry used for droplet dPCR as compared to dPCR in plates is different. For droplet dPCR, a water-oil emulsion is required to enable the formation of droplets and the mix also needs to comprise agents that stabilize the droplets, so that they do not fall apart after being generated. Additionally, the mix may not comprise agents that interfere with the droplets. None of these specific chemistry requirements are necessary for plate-based dPCR, which uses a mechanical partitioning approach instead of a chemical one.

One consequence of the aforementioned features is that plate-based dPCR has the unprecedented technical effect of providing for a higher accuracy and precision than droplet-based dPCR, since each plate in which dPCR is performed, provides the same conditions (e.g. same partition number, size, and

volume) for each analysis and each sample. This is different from droplets, which are unstable and can vary in size, skewing the distribution of target molecules, see also Košir et al. ("Droplet volume variability as a critical factor for accuracy of absolute quantification using droplet digital PCR", Anal Bioanal Chem., 2017, DOI: 10.1007/s00216-017-0625-y) in this regard.

Additionally, plate-based PCR offers the advantage, that a higher and faster throughput can be achieved, since all partitions of a well can be read-out simultaneously.

Fundamentally, each partition that contains at least one molecule of a target of interest creates a signal, and no signal is produced in partitions in which no molecule of a target of interest is present. Hence, the number of positive and negative partitions can be used to calculate and quantify the concentration or amount of at least one nucleic acid target in at least one cell.

According to dMIQE Group and Huggett (dMIQE Group and Huggett, 2020, Clin Chem., "The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020"), two assumptions for dPCR to fit the Poisson distribution are that all partitions are of equal volume, and that target molecules are randomly distributed across partitions. In practice, this means that each partition has an equal chance of comprising target molecules. The number of target molecules present within positive partitions may be one, two, or more molecules, and it is currently impossible to determine how many molecules a given positive partition may comprise. However, the number of molecules in a negative partition is known. If all partitions are of equal volume, the mean concentration of target molecules per partition (λ) can be estimated from the probability that a partition is negative using the proportion of negative partitions and the Poisson distribution. This concentration is derived from the number of negative partitions (w) and the total number of partitions in the reaction (n):

$$\lambda = -\ln\left(\frac{w}{n}\right)$$

In the case when all partitions comprise the amplification target, *i.e.* all partitions have a positive signal and the dPCR experiment is saturated, a quantification is not possible.

Thus, for exemplary dPCR systems making use of 8500, 26000 or 22000 partitions per well, the upper dynamic range is in theory reached when 8499 (= 99,98%), 25999 (= 99,99%) or 21999 (= 99,99%) of the partitions, respectively, comprise at least one target molecule, *i.e.* a reverse transcribed RNA molecule from one RNA target. Accordingly, for the above mentioned dPCR systems, λ would be (rounded to the nearest integer):

$$\lambda_{8500} = -\ln\left(\frac{8500-8499}{8500}\right) = 9$$

$$\lambda_{26000} = -\ln\left(\frac{26000-25999}{26000}\right) = 10$$

$$\lambda_{22000} = -\ln\left(\frac{22000-21999}{22000}\right) = 10$$

The above λ values mark the statistical/theoretical limit of the dPCR method.

The minimum amount of molecules per analyzed input sample volume needed for one positive partition in a dPCR is one (= lowest detectable target concentration in the dPCR experiment).

In one embodiment, the quantification result of the at least one target is compared with the quantification result of said at least one target of at least one further cell. This at least one further cell also underwent the same steps according to the methods of the invention as the cell or cells the at least one further cell is compared to. The at least one further cell can originate from the same sample or from another sample. The at least one further cell can be the same or a different cell type. The at least one further cell can have the same or different properties.

In one embodiment, the quantification result of the at least one target is compared with the quantification result of said at least one target within the cDNA and/or the released DNA of at least one further cell. Thereby, insight can be gained on concentrations of targets of interest in single cells or in pre-defined numbers of cells, or cells exposed to different environments, or cells treated in a special way. In all these cases, the readout may not be compensable by lots of other cells that are present in an undefined cell pool.

Generally, a “plate” according to the invention is a microtiter plate known in the art. In one embodiment, the plate according to the invention is a Nanoplate. Nanoplates are microtiter plates. Different plates can be used for the different steps of the method, in particular for the lysis, the reverse transcription reaction step, and the dPCR step. Non-limiting examples include 96-well plates, 384-well plates, or Nanoplates.

In one embodiment, the plates according to the invention have at least 2 wells, or at least 6 wells, or at least 8 wells, or at least 12 wells, or at least 24 wells, or at least 48 wells, or at least 96 wells, or at least 192 wells, or at least 384 wells, or at least 768 wells, or at least 1536 wells, or between 6 and 1536 wells, or between 24 and 1536 wells, or between 96 and 1536 wells, or between 192 and 1536 wells, or between 384 and 1536 wells, or between 12 and 384 wells, or between 12 and 96 wells, or

between 12 and 48 wells, or between 24 and 384 wells, or between 24 and 96 wells, or between 24 and 48 wells, or between 8 and 96 wells, or between 2 and 384 wells, or 2 wells, or 6 wells, or 8 wells, or 12 wells, or 24 wells, or 48 wells, or 96 wells, or 192 wells, or 384 wells, or 768 wells, or 1536 wells.

In one embodiment, the plates according to the invention have no partitions per well, or at least 10 partitions/well, or at least 100 partitions/well, or at least 500 partitions/well, or at least 1000 partitions/well, or at least 2000 partitions/well, or at least 3000 partitions/well, or at least 4000 partitions/well, or at least 5000 partitions/well, or at least 6000 partitions/well, or at least 7000 partitions/well, or at least 8000 partitions/well, or at least 8500 partitions/well, or at least 9000 partitions/well, or at least 10000 partitions/well, or at least 12000 partitions/well, or at least 14000 partitions/well, or at least 15000 partitions/well, or at least 18000 partitions/well, or at least 20000 partitions/well, or at least 22000 partitions/well, or at least 25000 partitions/well, or at least 26000 partitions/well, or at least 30000 partitions/well, or at least 50000 partitions/well, or between 10 and 26000 partitions/well, or between 100 and 26000 partitions/well, or between 4000 and 26000 partitions/well, or between 8000 and 50000 partitions/well, or between 8000 and 30000 partitions/well, or between 8000 and 26000 partitions/well, or between 8000 and 25000 partitions/well, or between 8000 and 22000 partitions/well, or between 8000 and 20000 partitions/well, or between 8000 and 18000 partitions/well, or between 8000 and 15000 partitions/well, or between 8000 and 14000 partitions/well, or between 8000 and 12000 partitions/well, or between 10000 and 50000 partitions/well, or between 10000 and 30000 partitions/well, or between 10000 and 26000 partitions/well, or between 10000 and 25000 partitions/well, or between 10000 and 22000 partitions/well, or between 10000 and 20000 partitions/well, or between 10000 and 18000 partitions/well, or between 10000 and 15000 partitions/well, or between 10000 and 14000 partitions/well, or 10 partitions/well, or 100 partitions/well, or 500 partitions/well, or 1000 partitions/well, or 2000 partitions/well, or 3000 partitions/well, or 4000 partitions/well, or 5000 partitions/well, or 6000 partitions/well, or 7000 partitions/well, or 8000 partitions/well, or 8500 partitions/well, or 9000 partitions/well, or 10000 partitions/well, or 12000 partitions/well, or 14000 partitions/well, or 15000 partitions/well, or 18000 partitions/well, or 20000 partitions/well, or 22000 partitions/well, or 25000 partitions/well, or 26000 partitions/well, or 30000 partitions/well, or 50000 partitions/well.

Increasing the number of partitions increases sensitivity.

Any combination of the amount of wells and the amount of partitions/well disclosed above is encompassed within the scope of the present invention.

Generally, the plate in which dPCR is performed is a microtiter plate.

In one embodiment, the plates in which dPCR is performed have at least 2 wells, or at least 6 wells, or at least 8 wells, or at least 12 wells, or at least 24 wells, or at least 48 wells, or at least 96 wells, or at least 192 wells, or at least 384 wells, or at least 768 wells, or at least 1536 wells, or between 6 and 1536 wells, or between 24 and 1536 wells, or between 96 and 1536 wells, or between 192 and 1536 wells, or between 384 and 1536 wells, or between 12 and 384 wells, or between 12 and 96 wells, or between 12 and 48 wells, or between 24 and 384 wells, or between 24 and 96 wells, or between 24 and 48 wells, or between 8 and 96 wells, or between 2 and 384 wells, or 2 wells, or 6 wells, or 8 wells, or 12 wells, or 24 wells, or 48 wells, or 96 wells, or 192 wells, or 384 wells, or 768 wells, or 1536 wells.

In one embodiment, the plates in which dPCR is performed have at least 10 partitions/well, or at least 100 partitions/well, or at least 500 partitions/well, or at least 1000 partitions/well, or at least 2000 partitions/well, or at least 3000 partitions/well, or at least 4000 partitions/well, or at least 5000 partitions/well, or at least 6000 partitions/well, or at least 7000 partitions/well, or at least 8000 partitions/well, or at least 8500 partitions/well, or at least 9000 partitions/well, or at least 10000 partitions/well, or at least 12000 partitions/well, or at least 14000 partitions/well, or at least 15000 partitions/well, or at least 18000 partitions/well, or at least 20000 partitions/well, or at least 22000 partitions/well, or at least 25000 partitions/well, or at least 26000 partitions/well, or at least 30000 partitions/well, or at least 50000 partitions/well, or between 10 and 26000 partitions/well, or between 100 and 26000 partitions/well, or between 4000 and 26000 partitions/well, or between 8000 and 50000 partitions/well, or between 8000 and 30000 partitions/well, or between 8000 and 26000 partitions/well, or between 8000 and 25000 partitions/well, or between 8000 and 22000 partitions/well, or between 8000 and 20000 partitions/well, or between 8000 and 18000 partitions/well, or between 8000 and 15000 partitions/well, or between 8000 and 14000 partitions/well, or between 8000 and 12000 partitions/well, or between 10000 and 50000 partitions/well, or between 10000 and 30000 partitions/well, or between 10000 and 26000 partitions/well, or between 10000 and 25000 partitions/well, or between 10000 and 22000 partitions/well, or between 10000 and 20000 partitions/well, or between 10000 and 18000 partitions/well, or between 10000 and 15000 partitions/well, or between 10000 and 14000 partitions/well, or 10 partitions/well, or 100 partitions/well, or 500 partitions/well, or 1000 partitions/well, or 2000 partitions/well, or 3000 partitions/well, or 4000 partitions/well, or 5000 partitions/well, or 6000 partitions/well, or 7000 partitions/well, or 8000 partitions/well, or 8500 partitions/well, or 9000 partitions/well, or 10000 partitions/well, or 12000 partitions/well, or 14000 partitions/well, or 15000 partitions/well, or 18000 partitions/well, or 20000 partitions/well, or 22000

partitions/well, or 25000 partitions/well, or 26000 partitions/well, or 30000 partitions/well, or 50000 partitions/well.

Increasing the number of partitions increases sensitivity.

Any combination of the amount of wells and the amount of partitions/well disclosed above is encompassed within the scope of the present invention.

The plate choice (including the choice of the number of wells, the number of partitions/well, and the loading volume) may depend on, *inter alia*, the application, the desired throughput, and the sample availability. For rare events, plates with more partitions/well, such as 26000 partitions/well, may be beneficial. Similarly, for highly diluted targets in a sample, plates with more partitions/well, such as 26000 partitions/well, may also be beneficial. If a high throughput is desired or needed, plates with more wells, but potentially less partitions/well, might be better, for example a 96-well plate with 8500 partitions/well.

In one embodiment, the dPCR is performed in a Nanoplate. Additionally, reverse transcription and/or cell lysis may also be performed in a Nanoplate.

Nanoplates are 24-well- or 96-well plates, meaning that up to 24 or 96 different samples can be loaded, respectively. The 24-well Nanoplates either have approximately 8500 or 26000 partitions/well. The 96-well Nanoplate has approximately 8500 partitions/well. Typically, the sample input volume for Nanoplates with approx. 26000 partitions/well is 40 µl, and the sample volume for Nanoplates with approx. 8500 partitions/well is 12 µl.

There is not much flexibility with respect to the reaction volume for a given amount of partitions/well. If the volume is too small, the reaction may not be feasible, and if the volume is too large, this could, for example, affect downstream priming. A precise determination of the cycled sample volume is needed to quantify the amount of the at least one nucleic acid target in dPCR. In general, plates in which dPCR is performed, provide partitions of fixed sizes that enable an exact and reproducible concentration calculation. However, to compensate for even the slightest volume variations between different wells of a plate or between different plate batches, compensation factors can be used for concentration calculations.

One example of such a compensation factor is the volume precision factor (VPF) which is available for each plate, when the QIAcuity® Digital PCR System is used. The VPF is a set of factors for each well. It consists of 96 individual factors that can address the well-to-well variability and reduce variations between different molding forms resulting in batch-to-batch variability. This increases the precision of

concentration measurements in dPCR, particularly for sensitive applications such as analysis of rare nucleic acid targets.

To achieve higher accuracy, multiple wells can be grouped and analyzed as a single well (hyperwell). For the analysis, hyperwells can be treated as a single well but with more partitions. This may be helpful for rare event detection if the sample volume to be analyzed exceeds the volume that can be loaded into a single well. Alternatively or additionally, wells with more partitions can be used for rare event detection.

Further specifics of the results obtained in the methods according to the invention are detailed in the figure legends and examples.

EXAMPLES

The following examples serve to illustrate the invention. The amplified and detected loci shall not be regarded as limiting the scope of the invention, but are just exemplary loci that can be used within the scope of the present invention.

Example 1: Workflow for analysis of coding RNA targets (mRNAs)

The general workflow for this example is shown in figure 1. The cellenONE® cell sorting system was used to sort cells directly into lysis buffer. Reverse transcription and subsequent digital PCR were performed in a plate and at least one mRNA target was quantified. The detailed experimental procedure is described below.

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% CO2). 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:

Different 384-well microtiter plate (MTP) layouts with specific numbers 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.

Target 384-well 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 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.

Configuration of the instrument and cell isolation:

Prior to isolation, small aliquots of the two cell line samples were processed to define optimal isolation parameters (see figure 2A) for each cell line. Once configured, the cellenONE® X1 system was used to isolate HEK293 and HeLa cells into wells of three 384-well MTPs according to the designed layouts defined as shown in figure 2B.

Successful isolation of cells:

Cells, including single cells, were successfully isolated using the cellenONE® technology. Figure 3A 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-well MTP. The diameter and elongation parameters of the isolated cells were within the defined range (see figures 3B and 3C).

Direct cell lysis and single-cell gene expression analysis were performed using the FastLane Cell Probe Kit (Qiagen, cat. no. 216413) and the QIAcuity OneStep Advanced Probe PCR Kit (Qiagen, cat. no. 250131) on the QIAcuity Digital PCR System (Qiagen, cat. no. 911021).

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). For this, 47.6 µl Buffer FCPL were mixed with 2.4 µl gDNA Wipeout Buffer 2. 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 384-well MTP plates containing 10 µl of this 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, Cat. No. 2016-0111). Plates were subsequently frozen at −80°C and stored until further processing.

Reverse transcription digital PCR (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. 12 µl were the total volume of the reaction mixture. 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, the transcript levels of various targets in HEK293 and HeLa cells were analyzed. As shown in figures 4A (HEK293 cells) and 4B (HeLa cells), the differences in gene expression levels of a target in different cell lines can easily be studied in a high-throughput manner. Cell sorting followed by 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 (figures 4A and 4B). Additionally, multiple targets can be simultaneously analyzed (figure 5). Notably, transcripts with low abundance within cells can still be accurately detected, thanks to the low limits of detection in dPCR. Due to the partitioning of lysates and endpoint PCR, dPCR enables sensitive detection of absolute copies of transcripts at the single-cell level (figure 6). Variability

in gene expression levels of targets from one individual cell to another can also be studied in an absolute, high-throughput manner using the methods according to the invention (figure 7A-C).

The workflow of the methods of the invention therefore achieves high-throughput absolute quantification of low-abundance targets at the single-cell level. Real-time and 100% accurate single-cell isolation without compromising viability and transcript expression allows for the use of an exact number of intact cells for RT-dPCR reactions. Moreover, the elimination of the RNA purification step significantly reduces hands-on time. Additionally, multiplexing can be performed in a one-step RT-dPCR format.

Overall, this simple, yet efficient workflow combining isolation of a defined number of cells with absolute gene expression analysis delivers high-sensitive, reproducible and linear quantification of transcript levels in cell lysates.

Example 2: Workflow for analysis of DNA (genomic DNA and/or mtDNA)

The general workflow for this example is shown in figure 8. The cellenONE® cell-sorting system was used to sort cells directly into lysis buffer not comprising DNAseI. Digital PCR was performed in a plate, and CNVs or mitochondrial copy numbers were analyzed. The detailed experimental procedure is described below.

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% CO2). 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 (250g 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 Fast Lane Cell One-Step Lysis Buffer (available as part of FastLane Cell Probe Kit, Cat. No. 216413; without DNase I) and kept on 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 prior to cell sorting, as shown in figure 9. One 384 MTP layout was designed with 384 single cells, one cell per well, a second 384 MTP layout was designed with different number of cells per well (ranging from 1–100 cells per well).

Configuration of the instrument and single-cell isolation:

Prior to isolation, small aliquots of the different cell samples were processed to define optimal isolation parameters for each cell line, as shown in table 1:

Cell sample	Minimum diameter (µm)	Maximum diameter (µm)	Maximum elongation (µm)
HEK293	16.1	29	1.59
HeLa	20	35.5	1.71

For both cell samples (HEK293 and HeLa cells), only cell diameter and elongation parameters were defined to precisely isolate a specific number of cells into each well. 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 9. The actual parameters of the sorted cells are shown in table 2:

Cell sample	Diameter range (µm)	Mean diameter (µm)	Elongation range (µm)
HEK293	16.3-28.1	22.0	1.15-1.59
HeLa	20-32	23.6	1.16-1.87

Cells were isolated directly into Fast-Lane lysis buffer (see below).

Direct cell lysis and single-cell target copy number analysis using QIAcuity Probe PCR Kit on the QIAcuity Digital PCR System:

Generation of cell lysates:

DNA lysates from cells were prepared using Fast Lane Cell One Step Buffer Set without addition of DNAse I. Cells were directly isolated into the wells of 384-well MTP plates containing 3µl Fast Lane Lysis Buffer (without DNase I). Plates were then incubated 5 minutes at ambient temperature and then heated to 75°C for 5 minutes (QInstrument ColdPlate Slim; 2016-0111) for heat inactivation of the lysis buffer. Plates were subsequently stored frozen at -80°C until further processing.

Successful isolation of single cells:

An example of an isolated HeLa cell is shown in figure 10A. 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 10B).

Digital PCR:

dPCR reactions were set up according to standard QIAcuity dPCR Probe CNV Assays Quick Start Protocol (HB-3060). Total amount of cell lysates (3 µl) containing various amounts of cells were directly taken into the dPCR reaction mix for each condition. QIAcuity dPCR Probe CNV Assays and TaqMan probe-based assays were used for multiplex detection of target copy number levels at the gDNA and mtDNA levels, respectively. dPCR reaction mixes were then pipetted into the wells of QIAcuity 8.5k or 26k Nanoplates. The Nanoplates were sealed and placed in a QIAcuity Digital PCR instrument (Series 0) according to the instrument's user manual. Standard QIAcuity dPCR Probe CNV Assay cycling program was selected. Results were analyzed using QIAcuity Software Suite (Suite 2.0.20, and 2.1.7.187).

Highly sensitive analysis of copy number variations of genomic DNA (gDNA) and mitochondrial DNA (mtDNA) at both single-cell and population level:

The workflow outlined above (see also figure 8) was used to check copy numbers of various genomic and mtDNA targets in HEK293 or HeLa cells. Cell sorting prior to cell lysis provides 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 copy number analysis in single cells and in cell populations. In accordance with the methods of the invention, multiple targets in a cell can be analyzed

simultaneously in one reaction. A major challenge associated with multiplexing copy number analysis is that different targets can have different copy numbers. While many gDNA targets are present in two copies in healthy diploid genomes, mtDNA targets can have much higher copy numbers than gDNA targets within the same cells. Simultaneous detection of such variable copy number ranges requires not only high sensitivity but also high dynamic range of detection. Due to partitioning of lysates and end point PCR, dPCR provides sensitive detection of absolute copies of low copy targets as well as high copy targets at single cell level at a high resolution (figure 11A and B).

In addition, the high dynamic range of the methods according to the invention allows for an accurate readout of copy number changes for high copy targets at increasing cell loading density (figure 12). Therefore, copy number changes in various targets with low, medium and high copy number can be detected within single multiplexing reactions in a highly accurate and reproducible manner. Furthermore, differences in copy number of mtDNA or gDNA targets in different cell types can be analyzed in a high throughput manner using the methods according to the invention (figure 13). Apart from studying cell populations, single cell heterogeneity can also be examined for gDNA and mtDNA copy number in an absolute, high throughput manner using this workflow (figure 14; mtDNA single-cell heterogeneity shown).

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

The method according to the invention achieves high-throughput absolute quantification of genomic DNA targets and mitochondrial DNA targets even at the single-cell level. Fast, real-time and 100% accurate single-cell isolation enabled the use of an exact number of intact cells for dPCR reactions. Moreover, using a lysis buffer that stabilizes RNA and DNA and that eliminates the requirement to purify the nucleic acids of interest, significantly reduced hands-on time. Furthermore, the methods according to the invention allow the multiplexing of targets in a single dPCR reaction with no or minimal optimization. Overall, this simple yet efficient workflow combining cell sorting with dPCR and copy number analysis delivers highly sensitive, reproducible, and linear quantification of target genomic or mitochondrial copy numbers in cell lysates.

Example 3: Workflow for analysis of small non-coding RNA targets (*e.g.* miRNAs)

The general workflow for this example is shown in figure 15. The cellenONE® cell-sorting system was used to sort cells directly into lysis buffer. Reverse transcription and digital PCR were performed in a plate and target miRNAs were analyzed and quantified. The detailed experimental procedure is described below.

Cell culture, resuspension and sample preparation:

Immortalized 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 in 5% C02). 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 (250xg 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:

Using the FastLane Cell Probe Kit, 47.6 µl Buffer FCPL were mixed with 2.4 µl gDNA Wipeout Buffer 2. Target 384 MTPs were pre-filled with 10 µL/well of this Fast Lane Cell One-Step Lysis Buffer (available as part of FastLane Cell Probe Kit, Cat. No. 216413) and kept on 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.

Isolation of cells:

A specific 384 MTP layout with a defined number of cells ranging from 300 cells per well down to 1 cell per well was prepared in the cellenONE® X1 software (figure 16). Prior to isolation, a small aliquot of cells was processed to define optimal isolation parameters for HEK293 cells. Only cell diameter and elongation parameters were used to precisely isolate the specific number of cells into each well. Optimal parameters: 16.03 µm minimum diameter and 30 µm maximum diameter. 1.60 µm was used as maximum elongation parameter. Once configured, the cellenONE® X1 system was used to isolate HEK293 cells into wells of a 384 MTP according to the layout defined in figure 16.

Direct cell lysis and miRNA analysis using the QIAcuity EG PCR Kit on a QIAcuity dPCR 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 384-well MTP 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. Plates were subsequently frozen at - 80° C and stored until further processing.

miRNA poly-adenylation and reverse transcription using the miRCURY LNA RT Kit:

Cell lysates were directly used as input into a miRCURY LNA RT reaction. The reaction mix for one reaction was composed of 10 µL cell lysate, 4 µL 5x miRCURY RT SYBR Green reaction buffer, 2 µL miRCURY enzyme mix and 4 µL water. The reaction was mixed thoroughly and incubated for 60 minutes at 42°C. In this step, the poly-adenylation and reverse transcription of miRNAs took place. Afterwards, the reaction was incubated for 5 minutes at 95°C. The cDNA was stored at -20°C until further use.

miRNA quantification using dPCR:

Before setting up the dPCR reaction, the cDNA was diluted 7.5x in water. The QIAcuity EG PCR mix was used in the dPCR. The master mix as well as the miRCURY LNA miRNA PCR assays hsa-miR-10a-5p and hsa-miR-10b-5p were used in a final concentration of 1x. No additional water was added to the final reaction (66% of template in dPCR reaction). The reaction mixes were prepared in pre-plates before being transferred either to a 8.5k or a 26k Nanoplate. The plates were sealed and placed in a QIAcuity dPCR instrument according to the instrument's user manual. The miRCURY dPCR standard cycling according to the handbook was selected. Results were analyzed using the QIAcuity Software Suite (Suite 2.1.8).

High-sensitivity detection of absolute miRNA copies at single-cell level:

The expression levels of hsa-miR-10a-5p and hsa-miR-10b-5p targets in HEK293 cells were analyzed. As shown in figure 17 and figure 18A, 18B, and 18C, both miRNAs could be detected with a high linearity from cell pools containing 300 cells down to single cell level. The method according to the invention therefore allows for an analysis of miRNAs derived from different cell lines and for an analysis of different cell pool sizes in a high-throughput manner.

Fast, real-time and 100% accurate single-cell isolation according to the methods of the invention enables the use of an exact number of intact cells for RT and dPCR reactions. The high quality of cell isolation, lysate preparation, RT and dPCR enables an optimal analysis of cells in a wide linear range (figures 17 and 18A, 18B, and 18C). Low-abundant miRNAs within cells can also be accurately detected, due 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 miRNAs, even at the single-cell level. Variability in miRNA levels in individual cells can also be studied in an absolute, high-throughput manner using the methods according to the invention. Moreover, the elimination of the RNA purification step significantly reduces hands-on-time.

Overall, this simple yet efficient workflow combining accurate cell isolation into lysis buffer with absolute miRNA quantification delivers high-sensitive, reproducible and linear quantification from cell lysates used as input for RT and dPCR.

Example 4: Workflow for analysis of targets, wherein cell lysis and dPCR are performed within the same plate

In order to further increase time- and cost efficiency of the methods of the invention, intact cells may also directly be loaded onto a plate that includes lysis buffer and that is also used later on for dPCR. An exemplary workflow thereto is described hereinafter.

10 µl of CaseWork Go Lysis Buffer (Qiagen, Mat. No:1116186) is pre-loaded into a 24-well 26k dPCR plate. 2 µL of a prepared cell suspension is loaded into the preloaded lysis buffer (PBS, 10^4 - 1 cell(s) per well). The plate is sealed with a Airpore foil (Qiagen, Mat. No.:1017662) and incubated at room temperature for 10 min.

Optionally, i.e. if Proteinase K is present in the lysate, the sample is heat-inactivated after the previous step, for example for 5 min at 75°C. When Proteinase K is present, the plate could be closed, e.g. with an Airpore foil (Qiagen, Mat. No.:1017662), and incubated e.g. in an thermal incubator. The sheet

would prevent a) contamination and b) (lower) evaporation. In contrast to a qPCR cover foil, which could be used as well, condensation wouldn't be condensation on the foil.

Subsequently, the plate is unsealed and 28 µl of a previously prepared mastermix (see for example table 3 below) is added and mixed with the lysate by pipetting up and down (bubble formation is avoided). The plate is sealed with a Nanoplate Seal (Qiagen) and a dPCR is run. Exemplary cycling and imaging conditions are shown below in tables 4 and 5, respectively.

Mastermix (MM) Setup					
Reagent		**1x**	**final**	**1x**	**26x**
4x OS Probe MM		10.00	1x	10.00	260.00
100x	RT Mix	0.40	1x	0.40	10.40
	Primer	Var.	0.4µM	Var.	Var.
	Probe	Var.	0.2µM	Var.	Var.
40%	Trehalose (can be added for difficult targets)	5.00	5%	5.00	130.00
10U/µL	Restriction Enzyme	0.10	0.03U/µL	0.10	2.60
CaseWork GO Lysis Buffer		10.00		10.00	
Cells/gDNA		2.00		2.00	
Water		Var.		Var.	Var.
Total Vol		40		40µl	728µl
Vol. to transfer				40µl	28µl

Table 3: MM Setup suitable for one 24 Well 26k dPCR Plate

Cycling		
50°C	40:00	
95°C	05:00	
95°C	00:15	40x
60°C	00:30 – 01:00	

Table 4: dPCR Cycling

Imaging		
Channel	Exposure	Gain
GREEN	500	6
YELLOW	500	6

Table 5: dPCR Imaging

Three different experiments were performed using the aforementioned general workflow. These are described in more detail in sections 4.1-4.3 below.

Example 4.1: Use of 1 to 100000 cells

For this experiment, the following dPCR layout was used:

	dPCR Layout			
	1	**2**	**3**	
A	100000.00			**total cell count**
B	10000.00			
C	1000.00			
D	100.00			
E	10.00			
F	1.00			
G	gDNA			
H	NTC			
	MYC QF (13) + RPP30 (DCR0000181) Assay used			

The mastermix setup was as follows:

MM Setup					
Reagent		**1x**	**final**	**1x**	**26x**
4x OS Probe MM		10.00	1x	10.00	**260.00**
100x	RT Mix	0.40	1x	0.40	**10.40**
40x	Assaymix MYC FAM	1.00	1x	1.00	**26.00**
40x	Assaymix RPP30 HEX	1.00	1x	1.00	**26.00**
40%	Trehalose	5.00	5%	5.00	**130.00**
10U/µL	XbaI	0.10	0.03U/µL	0.10	**2.60**
CaseWork GO Lysis Buffer		10.00		**10.00**	
Cells/gDNA		2.00		**2.00**	
Water		10.50		10.50	**273.00**

Total Vol	40		40µl	**728µl**
Vol. to transfer			**40µl**	**28µl**

Assays			
Gene	**FWD Primer**	**REV Primer**	**Probe**
MYC QF			
RPP30	DPH181	DPH181	DPH181

Volume of template working solution [µL]	100	
Stock concentration [µM]	100	
	from oligo stock [µL]	final concentration
ProbeWT (100µM)	8.00	8µM
PrimerFOR (100µM)	16.00	16µM
PrimerREV (100µM)	16.00	16µM
Buffer TE	60.00	

The protocol was as follows:

gDNA	
final Conc. gDNA	2.00
Stock Conc. gDNA	289.30
Vol. Stock gDNA	6.9µl
Vol. NADB	993µl
final Vol	1000µl

5

Lysis Protocol	
preload 10µL CaseWork Go Lysis Buffer	
load 2µL Cell suspension	cells resuspended in 1.4mL PBS
seal dPCR Plate (Qiagen Airpore foil)	
10min, RT	
unseal dPCR Plate	

add 28µL of prepared MM	
mix by pippeting up and down (avoid bubble formation)	
seal dPCR Plate (Qiagen dPCR Foil)	

Conc. Cells in Stock	50000000.00	5000000.00	500000.00	50000.00	5000.00	500.00
Vol. Cell Stock	2µL	2µL	2µL	2µL	2µL	2µL
cells per reaction	100000.00	10000.00	1000.00	100.00	10.00	1.00
µL Lysis Buffer	10µL	10µL	10µL	10µL	10µL	10µL

The dPCR parameters were as follows:

Cycling		
50°C	40:00	
95°C	05:00	
95°C	00:15	40x
60°C	01:00	

Imaging		
Channel	Exposure	Gain
GREEN	500	6
YELLOW	500	6

5

Example 4.2: Use of 0.58 to 580 cells

For this experiment, the following dPCR layout was used:

	dPCR Layout			
	1	**2**	**3**	
A	580.00			**total cell count**
B	58.00			

C	5.80	
D	0.58	
E	gDNA	
F	NTC	
G		
H		
	MYC QF (13) + RPP30 (DCR0000181) Assay used	

The mastermix setup was as follows:

MM Setup					
Reagent		**1x**	**final**	**1x**	**22x**
4x OS Probe MM		10.00	1x	10.00	**220.00**
100x	RT Mix	0.40	1x	0.40	**8.80**
40x	Assaymix MYC FAM	1.00	1x	1.00	**22.00**
40x	Assaymix RPP30 HEX	1.00	1x	1.00	**22.00**
40%	Trehalose	5.00	5%	5.00	**110.00**
10U/µL	XbaI	0.10	0.03U/µL	0.10	**2.20**
CaseWork GO Lysis Buffer		10.00		**10.00**	
Cells/gDNA		2.00		**2.00**	
Water		10.50		10.50	**231.00**
Total Vol		40		40µl	**616µl**
Vol. to transfer				**40µl**	**28µl**

Assays			
Gene	**FWD Primer**	**REV Primer**	**Probe**
MYC QF			
RPP30	DPH181	DPH181	DPH181

Volume of template working solution [µL]	100	

Stock concentration [µM]	100	
	from oligo stock [µL]	final concentration
ProbeWT (100µM)	8.00	8µM
PrimerFOR (100µM)	16.00	16µM
PrimerREV (100µM)	16.00	16µM
Buffer TE	60.00	

The protocol was as follows:

gDNA	
final Conc. gDNA	2.00
Stock Conc. gDNA	289.30
Vol. Stock gDNA	6.9µl
Vol. NADB	993µl
final Vol	1000µl

Lysis Protocol
preload 10µL CaseWork Go Lysis Buffer
load 2µL Cell suspension
seal dPCR Plate (Qiagen Airpore foil)
10min, RT
unseal dPCR Plate
add 28µL of prepared MM
mix by pippeting up and down (avoid bubble formation)
seal dPCR Plate (Qiagen dPCR Foil)

Conc. Cells in Stock	290000.00	29000.00	2900.00	290.00
Vol. Cell Stock	2µL	2µL	2µL	2µL
cells per reaction	580.00	58.00	5.80	0.58
µL Lysis Buffer	10µL	10µL	10µL	10µL

The dPCR parameters were as follows:

Cycling		
50°C	40:00	
95°C	05:00	
95°C	00:15	40x
60°C	01:00	

Imaging		
Channel	Exposure	Gain
GREEN	500	6
YELLOW	500	6

Example 4.3: Use of 0.9 to 900 cells

For this experiment, the following dPCR layout was used:

	dPCR Layout			
	1	**2**	**3**	
A	900.00			**total cell count**
B	90.00			
C	9.00			
D	0.90			
E	gDNA			
F	NTC			
G				
H				
	MYC QF (13) + RPP30 (DCR0000181) Assay used			

The mastermix setup was as follows:

MM Setup				
Reagent	**1x**	**final**	**1x**	**22x**
4x OS Probe MM	10.00	1x	10.00	**220.00**

100x	RT Mix	0.40	1x	0.40	**8.80**
40x	Assaymix MYC FAM	1.00	1x	1.00	**22.00**
40x	Assaymix RPP30 HEX	1.00	1x	1.00	**22.00**
10U/µL	XbaI	0.10	0.03U/µL	0.10	**2.20**
CaseWork GO Lysis Buffer		10.00		**10.00**	
Cells/gDNA		2.00		**2.00**	
Water		15.50		15.50	**341.00**
Total Vol		40		40µl	**616µl**
Vol. to transfer				**40µl**	**28µl**

Assays			
Gene	**FWD Primer**	**REV Primer**	**Probe**
MYC QF			
RPP30	DPH181_FWD	DPH181_REV	DPH181_HEX

Volume of template working solution [µL]	100	
Stock concentration [µM]	100	
	from oligo stock [µL]	final concentration
ProbeWT (100µM)	8.00	8µM
PrimerFOR (100µM)	16.00	16µM
PrimerREV (100µM)	16.00	16µM
Buffer TE	60.00	

The protocol was as follows:

gDNA	
final Conc. gDNA	2.00
Stock Conc. gDNA	289.30
Vol. Stock gDNA	6.9µl
Vol. NADB	993µl

final Vol	1000µl

Lysis Protocol
preload 10µL CaseWork Go Lysis Buffer into dPCR Plate
load 2µL Cell suspension
seal dPCR Plate (Qiagen Airpore foil)
10min, RT
unseal dPCR Plate
add 28µL of prepared MM
mix by pippeting up and down (avoid bubble formation)
seal dPCR Plate (Qiagen dPCR Foil)

Conc. Cells in Stock	450000.00	45000.00	4500.00	450.00
Vol. Cell Stock	2µL	2µL	2µL	2µL
cells per reaction	900.00	90.00	9.00	0.90
µL Lysis Buffer	10µL	10µL	10µL	10µL

The dPCR parameters were as follows:

Cycling		
50°C	40:00	
95°C	05:00	
95°C	00:15	40x
60°C	01:00	

5

Imaging		
Channel	Exposure	Gain
GREEN	500	6
YELLOW	500	6

Fig. 19 is a quantification table depicting the results of the experiments of Examples 4.1 (Fig. 19A), 4.2 (Fig. 19B), and 4.3 (Fig. 19C).

Fig. 20 is a scatterplot depicting the results of the experiments of Examples 4.1 (Fig. 20A), 4.2 (Fig. 20B), and 4.3 (Fig. 20C).

Fig. 21 is a graph depicting the results of the experiments of all Examples 4.1-4.3.

FIGURE LEGENDS

Fig. 1: A streamlined (single-cell) gene expression analysis workflow.

Fig. 2: (Single-)cell isolation parameters and plate layout for (single-)cell isolation for the example cell lines HeLa and HEK293.

A) Optimal (single-)cell isolation parameters for HeLa and HEK293 cells.

B) Representation of three different layouts for cell isolation using the cellenONE® X1 system. (A) 384-well MTP layout for isolation of one single HeLa cell per well, (B) 384-well MTP layout for isolation of one single HEK293 cell per well, (C) 384-well MTP layout for isolation of different numbers of HeLa and HEK293 cells per well.

Fig. 3: 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 µm), I: Grey Intensity (70.51)) are displayed on the top of the image. On the right side of the cell, there are two vertical lines shown in the picture. They "delimit" the sedimentation zone, which is a safety zone. If this zone comprises at least one additional cell, then the cell in the ejection zone (herein indicated as the zone on the left of the sedimentation zone) will be discarded.

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 dots in the darkest shade of gray.

C) Parameters of the isolated (single) cells.

Fig. 4: Analysis of gene expression levels in different cell lines at different cell densities. Results are obtained using ACTB-FAM assay in RT-dPCR for the HEK293 and HeLa cell lines.

A) Analysis of gene expression levels in HEK293 cells. Left: each dot represents a positive partition; depicted is the fluorescence intensity of the positive partitions per well for different numbers of analyzed cells. Right: graph depicting the concentration of the analyzed target (shown as copies/µl) in dependence of the number of cells analyzed.

B) Analysis of gene expression levels in HeLa cells. Left: each dot represents a positive partition; depicted is the fluorescence intensity of the positive partitions per well for different numbers of analyzed cells. Right: graph depicting the concentration of the analyzed target (shown as copies/µl) in dependence of the number of cells analyzed.

Fig. 5: Simultaneous quantification of low-, medium- and high-abundancy targets in cell lysates in multiplex RT-dPCR reactions. HeLa cell lysates with increasing cell densities were loaded. ACTB-FAM, MYC-ROX and KDR-HEX assays were used for multiplexing. Each dot represents a positive partition. Depicted is the fluorescence intensity of the positive partitions per well for different numbers of analyzed cells.

Fig. 6: High sensitivity analysis of gene expression levels in single cells. Target ACTB transcripts were detected in single HEK293 cells (depicted as dots) using ACTB-FAM assay in 1-Step RT-dPCR (**C** and **D**, respectively). No transcripts were detected in non-template controls (NTCs) (**A** and **B**). The positive partitions are highlighted herein using white arrows pointing towards the fluorescent partitions. Each subfigure represents a well with 8500 partitions.

Fig. 7: Multiplex analysis of gene expression levels in single cells. Single-cell (HeLa) lysates and non-template controls (NTCs; for each plate, the last two fields of the last row are NTCs) were loaded onto 8.5k Nanoplates and tested using ACTB-FAM, MYC-ROX and CDK2NA-HEX assays.

A) ACTB-FAM assay.

B) MYC-ROX assay.

C) CDK2NA-HEX assay.

Fig. 8: A streamlined (single-cell) genomic CNV and/or mitochondrial copy number analysis workflow.

Fig. 9: Representation of the two plate layouts used for cell isolation via the cellenONE® X1 system. Left: 384-MTP layout for isolation of a single cell per well. Right: 384-MTP layout for isolation of different numbers of cells per well (0, 1, 2, 5, 10, 25, 50, or 100 cells per well), wherein a magnification for wells A1-8, B1-8, C1-8, D1-8, E1-8, F1-8, G1-8, and H1-8 is shown.

Fig. 10: 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 coordinate (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. On the right side of the cell, there are two vertical lines shown in the picture. They "delimit" the sedimentation zone, which is a safety zone. If this zone comprises at least one additional cell, then the cell in the ejection zone will be discarded.

B) Example of scatterplots generated by the cellenONE® X1 Analysis Module showing cell elongation vs. cell diameter. All isolated cells are represented by individual dots in the darkest shade of gray. Left: HeLa isolation scatterplot. Right: HEK293 isolation scatterplot.

Fig. 11: Genomic DNA (gDNA) and mitochondrial DNA (mtDNA) targets show different copy numbers at single cell level. Copy number differences can be visualized at a high resolution using 1D scatterplots or signal maps in QIAcuity Software Suite. Results are obtained using **A)** ND4-FAM, RPP30-HEX and

SPIN4-ROX assays, **B)** CYB-FAM, RPP30-HEX and SPIN4-ROX assays, in single multiplexing digital PCR 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.

A) 1D scatterplot, wherein the dots above the horizontal threshold line correspond to the partitions, which showed a positive fluorescence signal after amplification of the indicated target in single HEK293 cells. The well number is indicated on top.

B) Signal maps showing the positive partitions for the indicated assays in single HEK293 cells. The positive partitions are highlighted herein using white arrows pointing towards the fluorescent partitions.

Fig. 12: Simultaneous quantification of low, medium and high copy number targets in HEK293 cell lysates. Cell lysates with increasing cell densities were loaded into reactions. Triplicates are shown from each individual loading condition. Results were obtained using TERT-ROX, AMY1A-FAM and ND4-Cy5 assays in multiplexing digital PCR reactions loaded into 26k Nanoplates. TERT (Chr. 5, low copy number) and AMY1A (Chr. 11, medium copy number) are genomic DNA targets, whereas ND4 (high copy number) is a mitochondrial DNA target.

Fig. 13: Linearity of detection is shown for copy numbers of genomic DNA and mitochondrial DNA targets at increasing number of HEK293 or HeLa cells per reaction. AMY1A-FAM, TERT-ROX and ND4-Cy5 assays were used for multiplexing digital PCR reactions loaded into 26k Nanoplates. AMY1A (Chr. 11, medium copy number) and TERT (Chr. 5, low copy number) are genomic DNA targets, whereas ND4 (high copy number) is a mitochondrial DNA target. Results represent average copies/µl obtained from triplicates. $R^2 > 0.97$ for all conditions.

Fig. 14: Copy number analysis of mitochondrial DNA using assays targeting CYB and ND4 shows heterogeneity of mitochondrial DNA copies in single cells. Row "A": HEK293, and row "B": HeLa single-cell lysates were loaded onto 8.5k Nanoplates and tested using CYB-FAM or NB4-FAM assays.

Fig. 15: A streamlined (single-cell) miRNA analysis workflow.

Fig. 16: Plate layout for cell isolation using the cellenONE® X1 system. Cells were isolated in pools containing 300 cells/well, 150 cells/well, 75 cells/well, 35 cells/well, 5 cells/well, 2 cells/well, or a single cell per well. A magnification of wells A1-4, B1-4, C1-4, D1-4, E1-4, F1-4, G1-4, H1-4, and I1-4 is shown.

Fig. 17: Analysis of miRNA quantification for different numbers of sorted cells. Different cell densities were sorted and lysed, and the miRNAs were reverse transcribed and quantified via dPCR on 26k Nanoplates. The miRCURY LNA miRNA PCR assay targeting hsa-miR-10b-5p was used for quantification.

Fig. 18: High sensitivity analysis of miRNAs over a broad range of HEK293 cell input.

A) High linearity of miRNA expression over a range of 300 sorted cells down to a single cell. The miRCURY LNA miRNA PCR assays hsa-miR-10a-5p and hsa-miR-10b-5p were used. Quantified copies/µl were plotted against the cell number for each target.

B) High linearity of miRNA expression over a range of 300 sorted cells down to a single cell. The miRCURY LNA miRNA PCR assay hsa-miR-10b-5p was used. Quantified copies/µl were plotted against the cell number for each target.

C) Signal map of different cell number inputs showing positive partitions (fluorescent dots in the picture) for the different indicated cell numbers. The miRCURY LNA miRNA PCR assay hsa-miR-10b-5p was used.

Fig. 19: Quantification results of genomic DNA over a broad range of Jurkat cell and HeLa cell input.

A) Quantification results of the experiment outlined in Example 4.1. 1 – 100000 (1:10 dilutions) Jurkat cells were analyzed in triplicates and the amount of MYC and RPP30 copies/µl was quantified.

B) Quantification results of the experiment outlined in Example 4.2. 0.58 - 580 (1:10 dilutions) HeLa cells were analyzed in triplicates and the amount of MYC and RPP30 copies/µl was quantified.

C) Quantification results of the experiment outlined in Example 4.3. 0.9 - 900 (1:10 dilutions) HeLa cells were analyzed in triplicates and the amount of MYC and RPP30 copies/µl was quantified.

Fig. 20: Scatterplot showing fluorescence intensities measured for genomic DNA targets in Jurkat cell lysates and HeLa cell lysates.

A) Jurkat cell lysates with decreasing cell densities were loaded into reactions. Triplicates are shown from each individual loading condition. Results were obtained using MYC-FAM and RPP30-HEX assays in digital PCR reactions loaded into 26k Nanoplates. The experiment is described in Example 4.1.

B) HeLa cell lysates with decreasing cell densities were loaded into reactions. Triplicates are shown from each individual loading condition. Results were obtained using MYC-FAM and RPP30-HEX assays in digital PCR reactions loaded into 26k Nanoplates. The experiment is described in Example 4.2.

C) HeLa cell lysates with decreasing cell densities were loaded into reactions. Triplicates are shown from each individual loading condition. Results were obtained using MYC-FAM and RPP30-HEX assays in digital PCR reactions loaded into 26k Nanoplates. The experiment is described in Example 4.3.

Fig. 21: Graph depicting the quantification results shown in Fig. 19 for all experiments described in Examples 4.1-4.3.

CLAIMS

1. A method of quantifying the amount of at least one nucleic acid target, the method comprising:
    e) Providing a defined number of cells, wherein the defined number of cells is at least one cell; and
    f) Lysing the at least one cell, thereby releasing RNA and DNA; and
    g) Performing a reverse transcription reaction on the released RNA, thereby generating complementary DNA (cDNA), and further performing a digital polymerase chain reaction (dPCR) on at least one reverse transcribed RNA target within the cDNA; and/or
    performing a digital polymerase chain reaction (dPCR) on at least one DNA target within the released DNA, wherein the dPCR is performed in a plate; and
    h) Quantifying the amount of the at least one nucleic acid target.

2. The method according to claim 1, wherein the provided at least one cell is directly isolated into lysis buffer.

3. The method according to any of the preceding claims, wherein each of the at least one nucleic acid targets is independently selected from a coding RNA target; a non-coding RNA target; a genomic DNA target; and a mitochondrial DNA target.

4. The method according to claim 3, wherein the coding RNA target is an mRNA; the non-coding RNA target is a miRNA; the genomic DNA target is a region on genomic DNA, that harbors or is suspected to harbor a copy number variation (CNV); and the mitochondrial DNA target is a region on mitochondrial DNA, that harbors or is suspected to harbor a CNV.

5. The method according to any of the preceding claims, wherein the at least one cell is provided by a method selected from the group consisting of fluorescence-activated cell sorting, micromanipulation, microfluidics, immunopanning, magnet-activated cell sorting, and laser microdissectioning.

6. The method according to any of the preceding claims, wherein the dPCR is performed in a Nanoplate.

7. The method according to any of the preceding claims, wherein the dPCR is performed simultaneously on one target, or on two targets, or on three targets, or on four targets, or on five targets within the cDNA and/or the released DNA.

8. The method according to any of the preceding claims, wherein each of the at least one nucleic acid targets is independently selected from the group consisting of a low-copy target, a medium-copy target, and a high-copy target.

9. The method according to any of the preceding claims, wherein quantification is based on the detection of the amplified at least one nucleic acid target by using at least one probe per analyzed target, wherein the at least one probe specifically binds to each of the amplification products per nucleic acid target.

10. The method according to claim 9, wherein the at least one probe is fluorescently labelled.

11. The method according to claim 10, wherein the at least one probe is at least one TaqMan probe.

12. The method according to any of the preceding claims, wherein the quantification result of the at least one target is compared with the quantification result of said at least one target within the cDNA and/or the released DNA of at least one further cell.

13. The method according to any of the preceding claims, wherein the at least one cell originates from a eukaryotic sample, preferably a human sample.

14. The method according to claim 13, wherein the human sample originates from a subject having cancer or being suspected of having cancer.

15. The method according to claim 13, wherein the human sample originates from a subject having or suspected of having a disease.

FIGURES

Fig. 1:

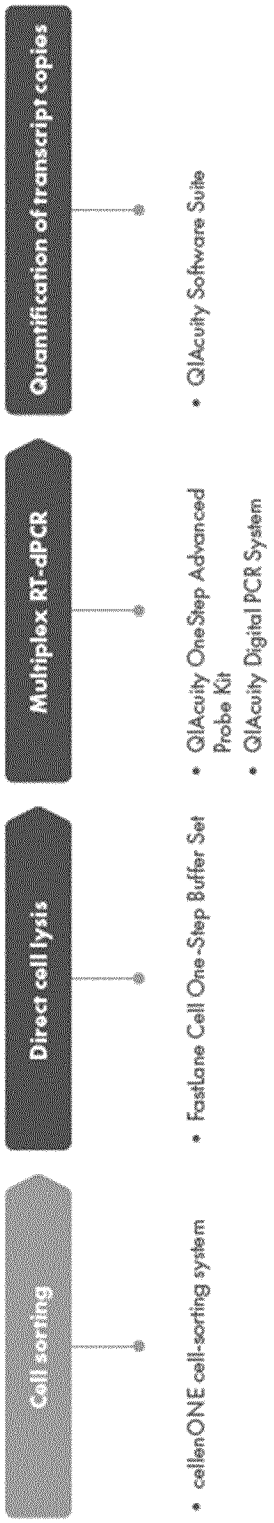


Fig. 2A):

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

Fig. 2B):

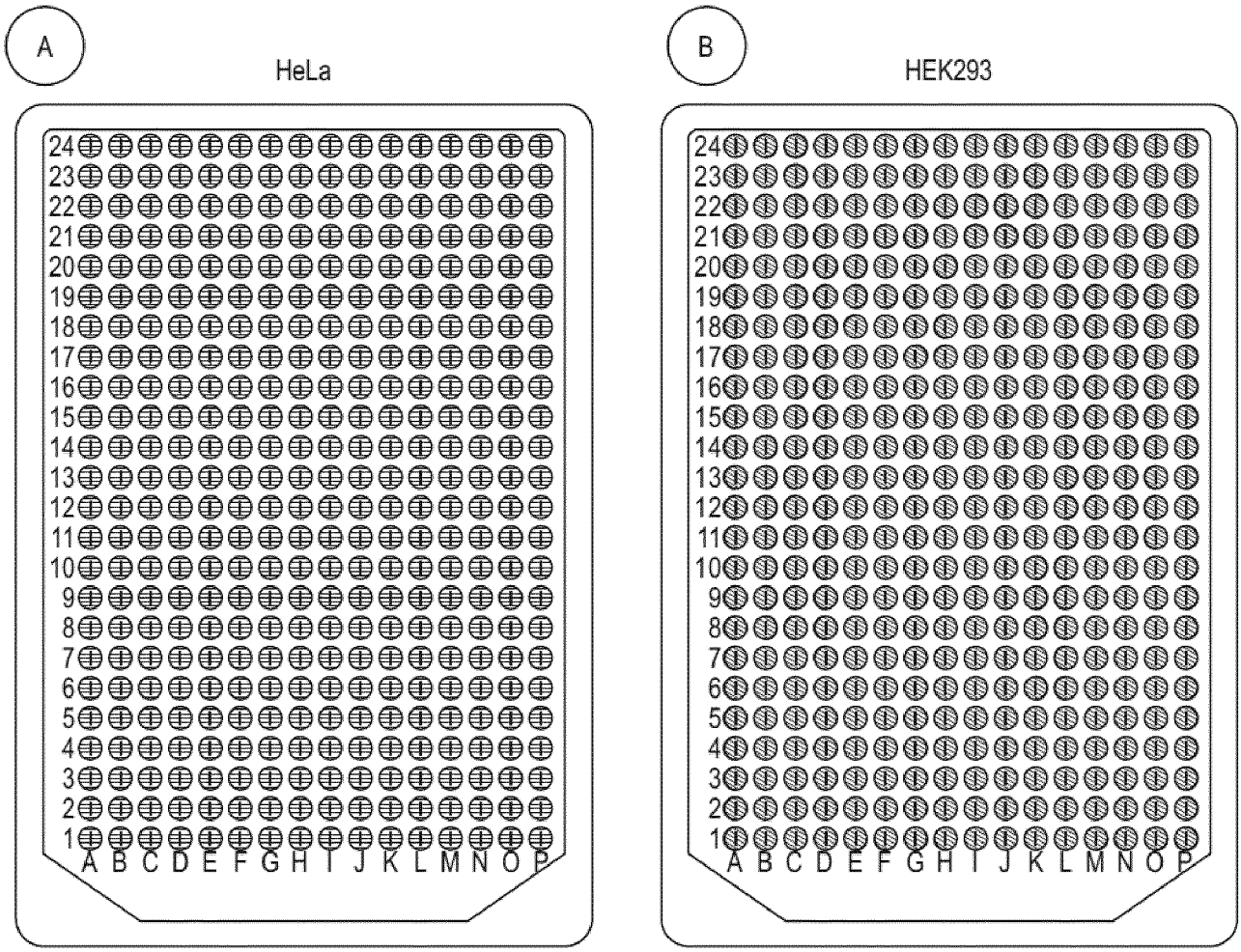


Fig. 2B) continued:

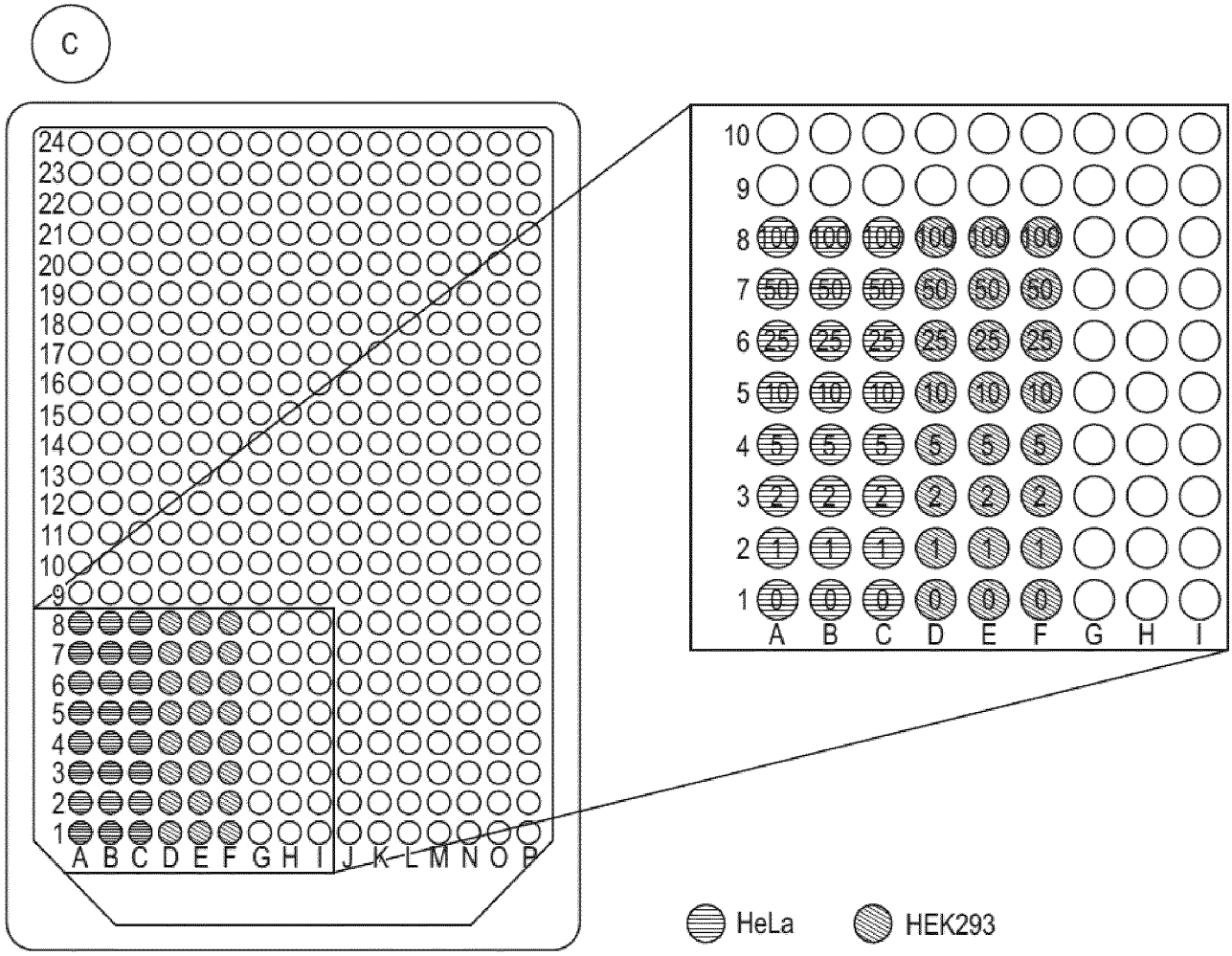


Fig. 3A):

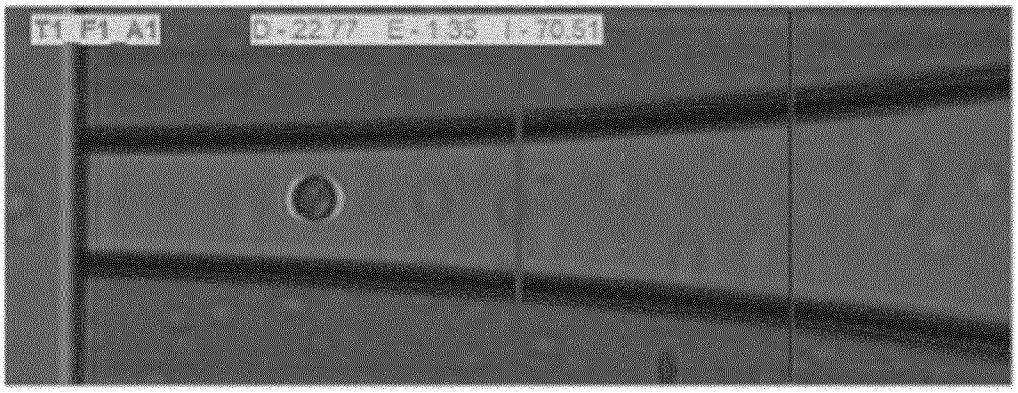

T1_F1_A1
D=22.77 E=1.35 I=70.51
Ejection zone

Fig. 3B):

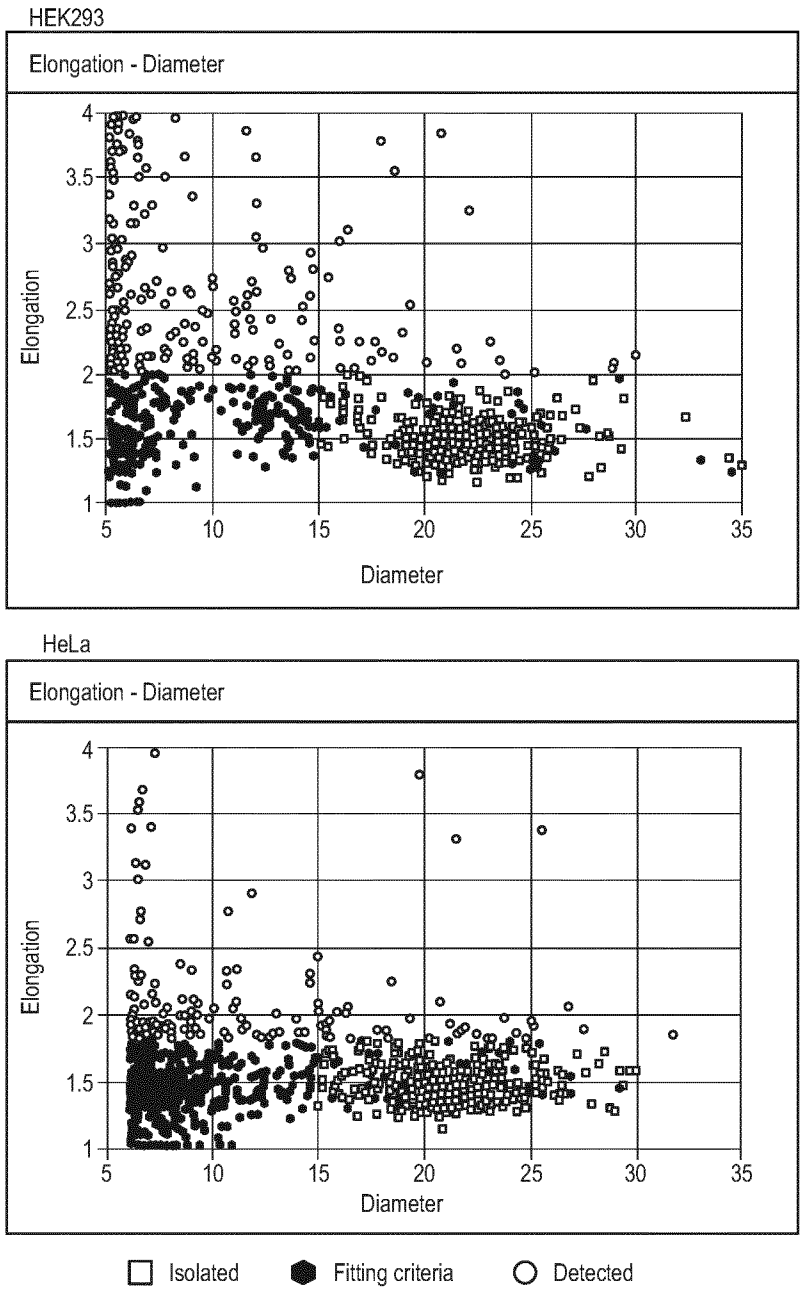

HEK293
Elongation - Diameter
Elongation
Diameter
HeLa
Elongation - Diameter
Elongation
Diameter
Isolated
Fitting criteria
Detected

Fig. 3C):

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

Fig. 4A):

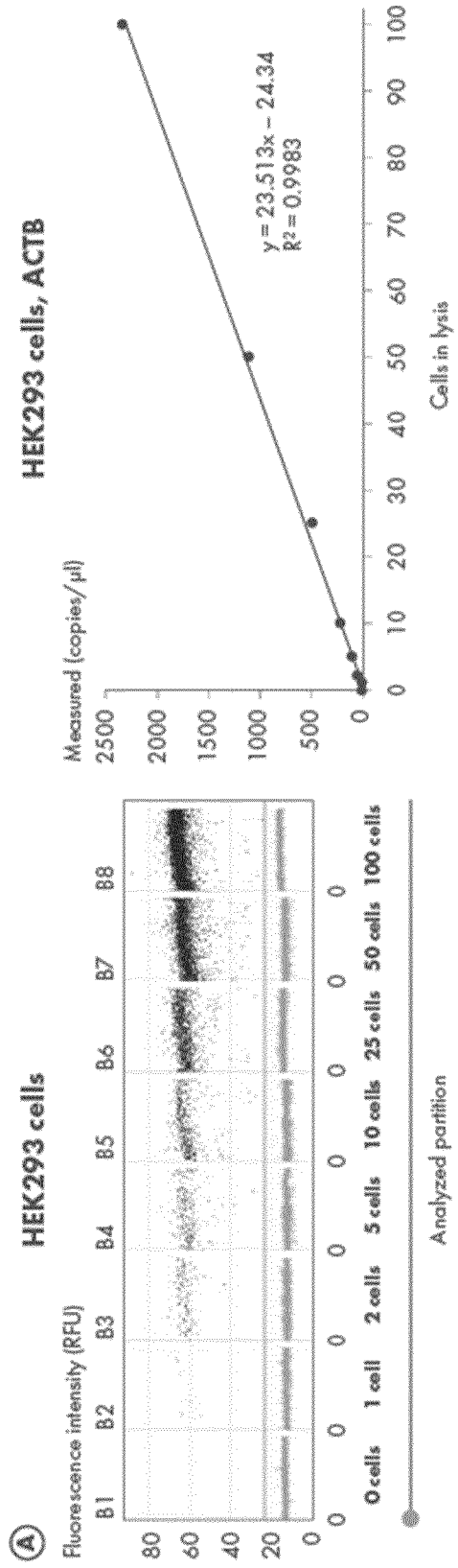

A
HEK293 cells
Fluorescence intensity (RFU)
B1 B2 B3 B4 B5 B6 B7 B8
80
60
40
20
0
0 0 0 0 0 0 0 0
0 cells 1 cell 2 cells 5 cells 10 cells 25 cells 50 cells 100 cells
Analyzed partition
HEK293 cells, ACTB
Measured (copies/µl)
2500
2000
1500
1000
500
0
y = 23.513x – 24.34
R² = 0.9983
0 10 20 30 40 50 60 70 80 90 100
Cells in lysis

Fig. 4B):

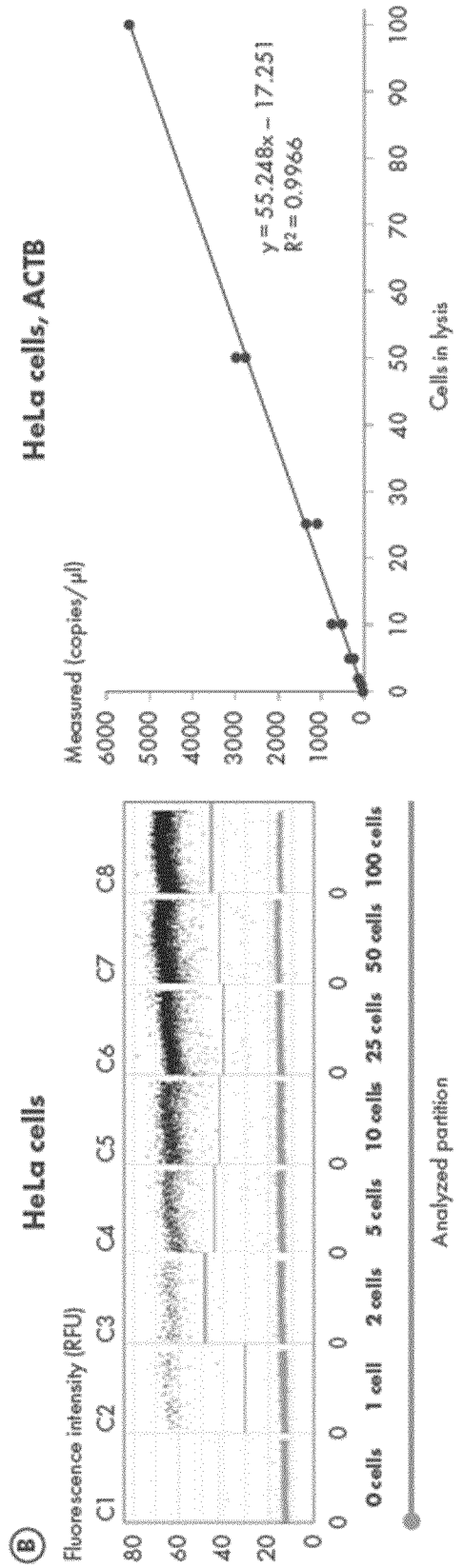

B
HeLa cells
Fluorescence intensity (RFU)
C1
C2
C3
C4
C5
C6
C7
C8
0 cells
1 cell
2 cells
5 cells
10 cells
25 cells
50 cells
100 cells
Analyzed partition
HeLa cells, ACTB
Measured (copies/µl)
y = 55.248x – 17.251
R² = 0.9966
Cells in lysis

Fig. 5:

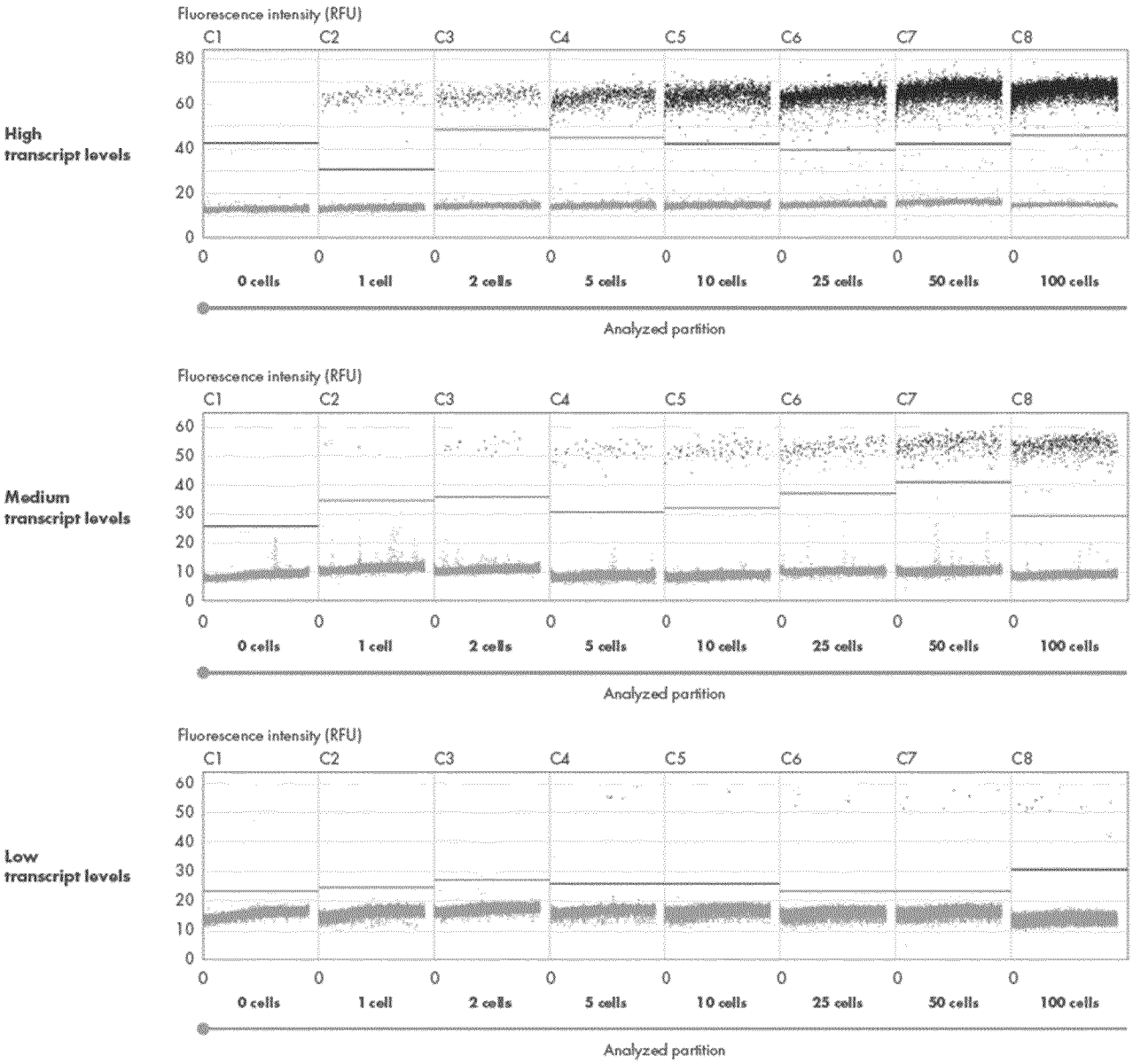
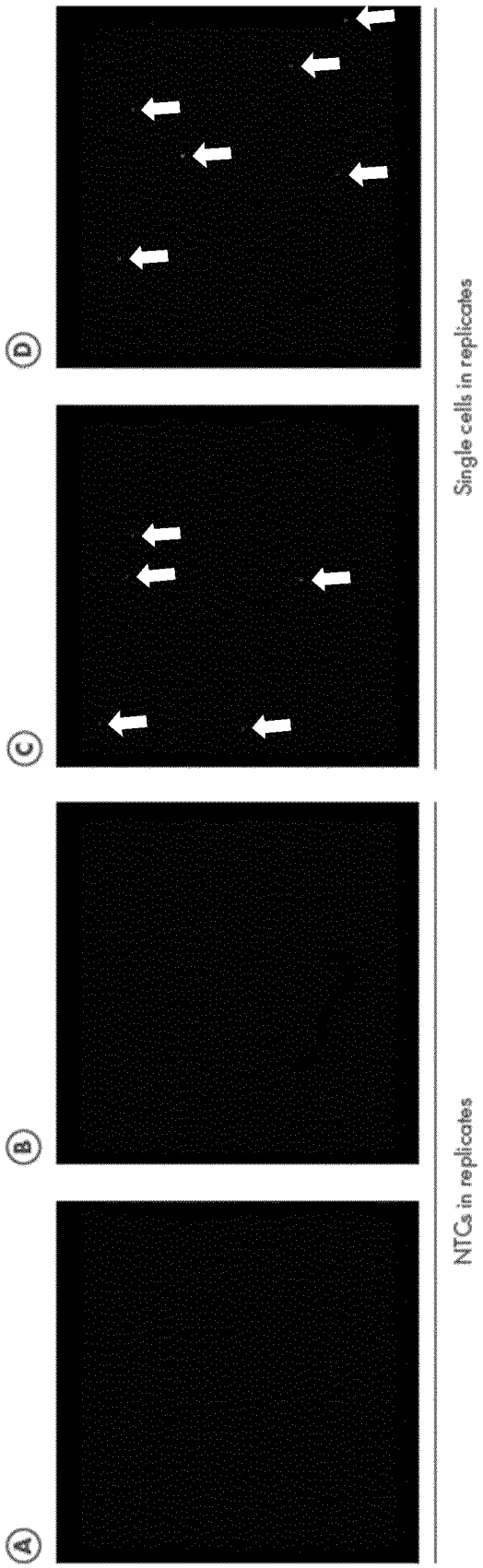

High transcript levels
Medium transcript levels
Low transcript levels
Fluorescence intensity (RFU)
C1
C2
C3
C4
C5
C6
C7
C8
0 cells
1 cell
2 cells
5 cells
10 cells
25 cells
50 cells
100 cells
Analyzed partition

Fig. 6:



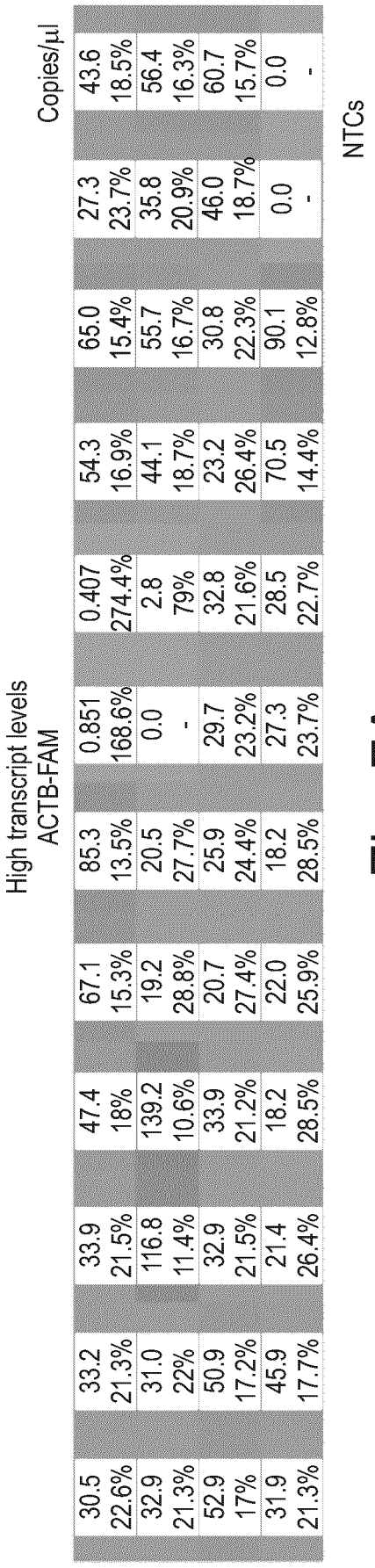

High transcript levels
ACTB-FAM
Copies/µl
NTCs
30.5
22.6%
33.2
21.3%
33.9
21.5%
47.4
18%
67.1
15.3%
85.3
13.5%
0.851
168.6%
0.407
274.4%
54.3
16.9%
65.0
15.4%
27.3
23.7%
43.6
18.5%
32.9
21.3%
31.0
22%
116.8
11.4%
139.2
10.6%
19.2
28.8%
20.5
27.7%
0.0
-
2.8
79%
44.1
18.7%
55.7
16.7%
35.8
20.9%
56.4
16.3%
52.9
17%
50.9
17.2%
32.9
21.5%
33.9
21.2%
20.7
27.4%
25.9
24.4%
29.7
23.2%
32.8
21.6%
23.2
26.4%
30.8
22.3%
46.0
18.7%
60.7
15.7%
31.9
21.3%
45.9
17.7%
21.4
26.4%
18.2
28.5%
22.0
25.9%
18.2
28.5%
27.3
23.7%
28.5
22.7%
70.5
14.4%
90.1
12.8%
0.0
-
0.0
-

Fig. 7A

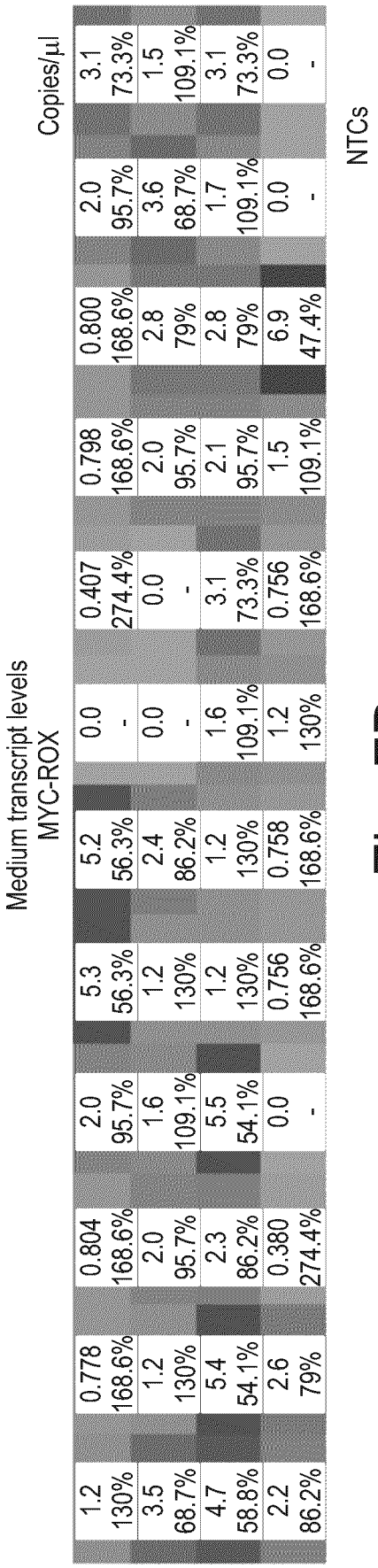

Medium transcript levels
MYC-ROX
Copies/µl
NTCs
1.2 130%
0.778 168.6%
0.804 168.6%
2.0 95.7%
5.3 56.3%
5.2 56.3%
0.0 -
0.407 274.4%
0.798 168.6%
0.800 168.6%
2.0 95.7%
3.1 73.3%
3.5 68.7%
1.2 130%
2.0 95.7%
1.6 109.1%
1.2 130%
2.4 86.2%
0.0 -
0.0 -
2.0 95.7%
2.8 79%
3.6 68.7%
1.5 109.1%
4.7 58.8%
5.4 54.1%
2.3 86.2%
5.5 54.1%
1.2 130%
1.2 130%
1.6 109.1%
3.1 73.3%
2.1 95.7%
2.8 79%
1.7 109.1%
3.1 73.3%
2.2 86.2%
2.6 79%
0.380 274.4%
0.0 -
0.756 168.6%
0.758 168.6%
1.2 130%
0.756 168.6%
1.5 109.1%
6.9 47.4%
0.0 -
0.0 -

Fig. 7B

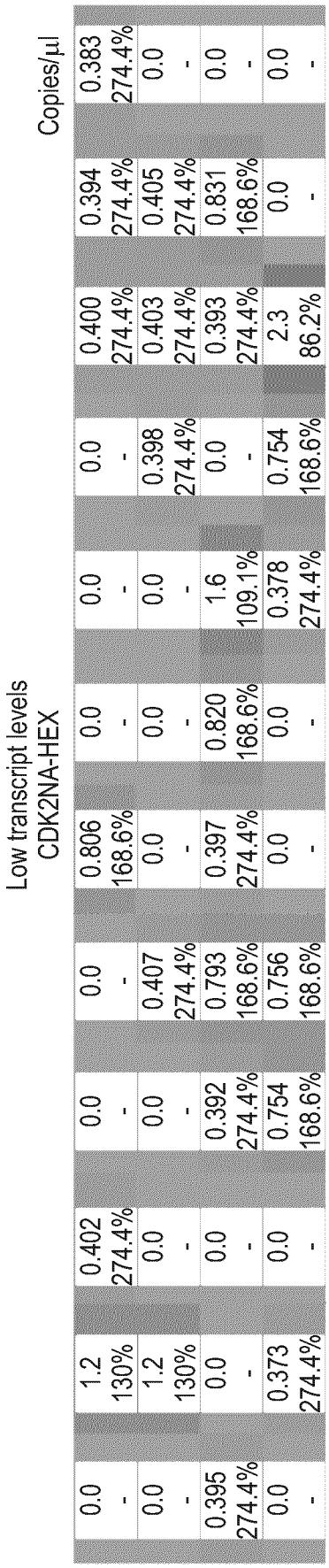
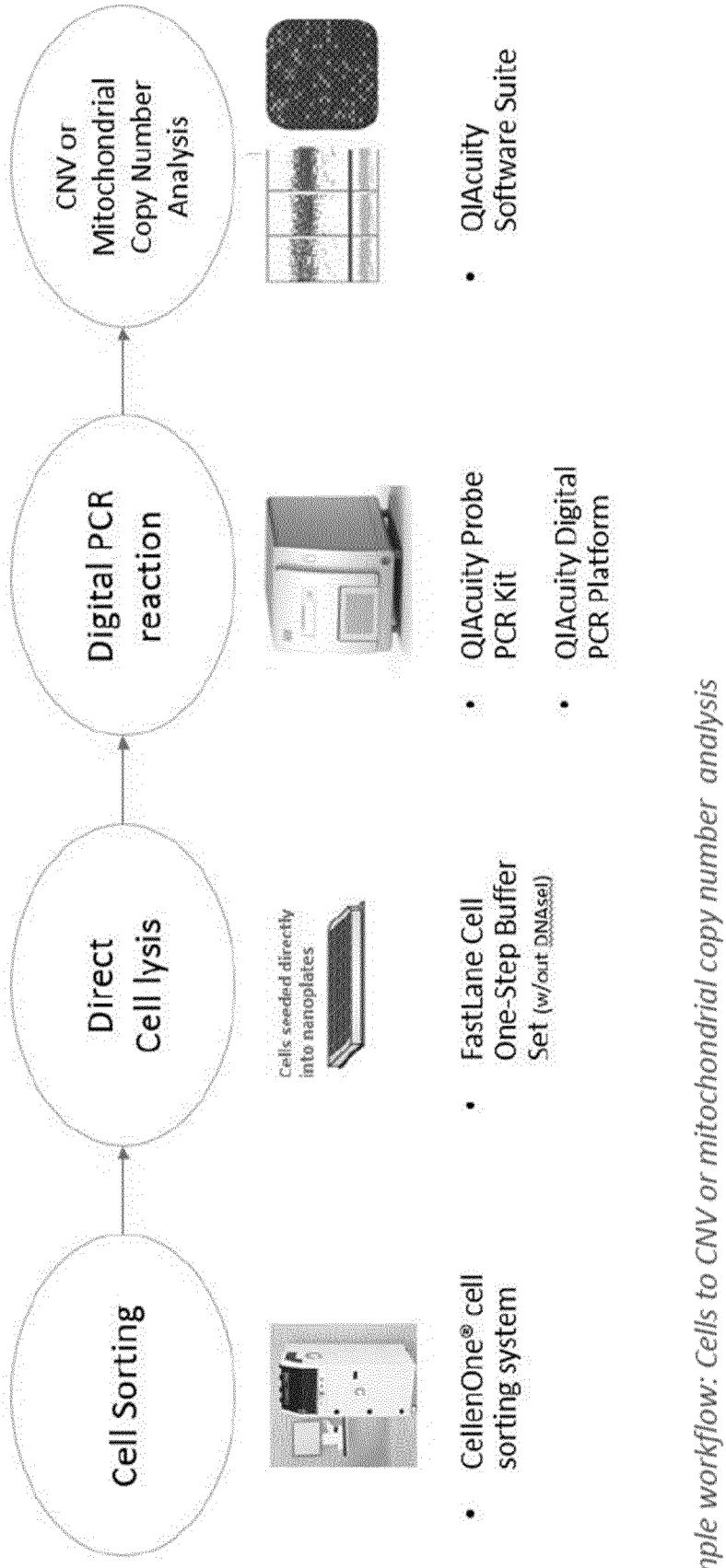

Low transcript levels
CDK2NA-HEX
Copies/µl
NTCs
0.0 - 0.395 274.4% 0.0 -
1.2 130% 1.2 130% 0.0 -
0.402 274.4% 0.0 - 0.0 - 0.373 274.4%
0.0 - 0.0 - 0.0 - 0.0 -
0.0 - 0.407 274.4% 0.793 168.6% 0.756 168.6%
0.806 168.6% 0.0 - 0.397 274.4% 0.0 -
0.0 - 0.0 - 0.820 168.6% 0.0 -
0.0 - 0.0 - 1.6 109.1% 0.378 274.4%
0.0 - 0.398 274.4% 0.0 - 0.754 168.6%
0.400 274.4% 0.403 274.4% 0.393 274.4% 2.3 86.2%
0.394 274.4% 0.405 274.4% 0.831 168.6% 0.0 -
0.383 274.4% 0.0 - 0.0 - 0.0 -

Fig. 7C

Fig. 8:



Simple workflow: Cells to CNV or mitochondrial copy number analysis

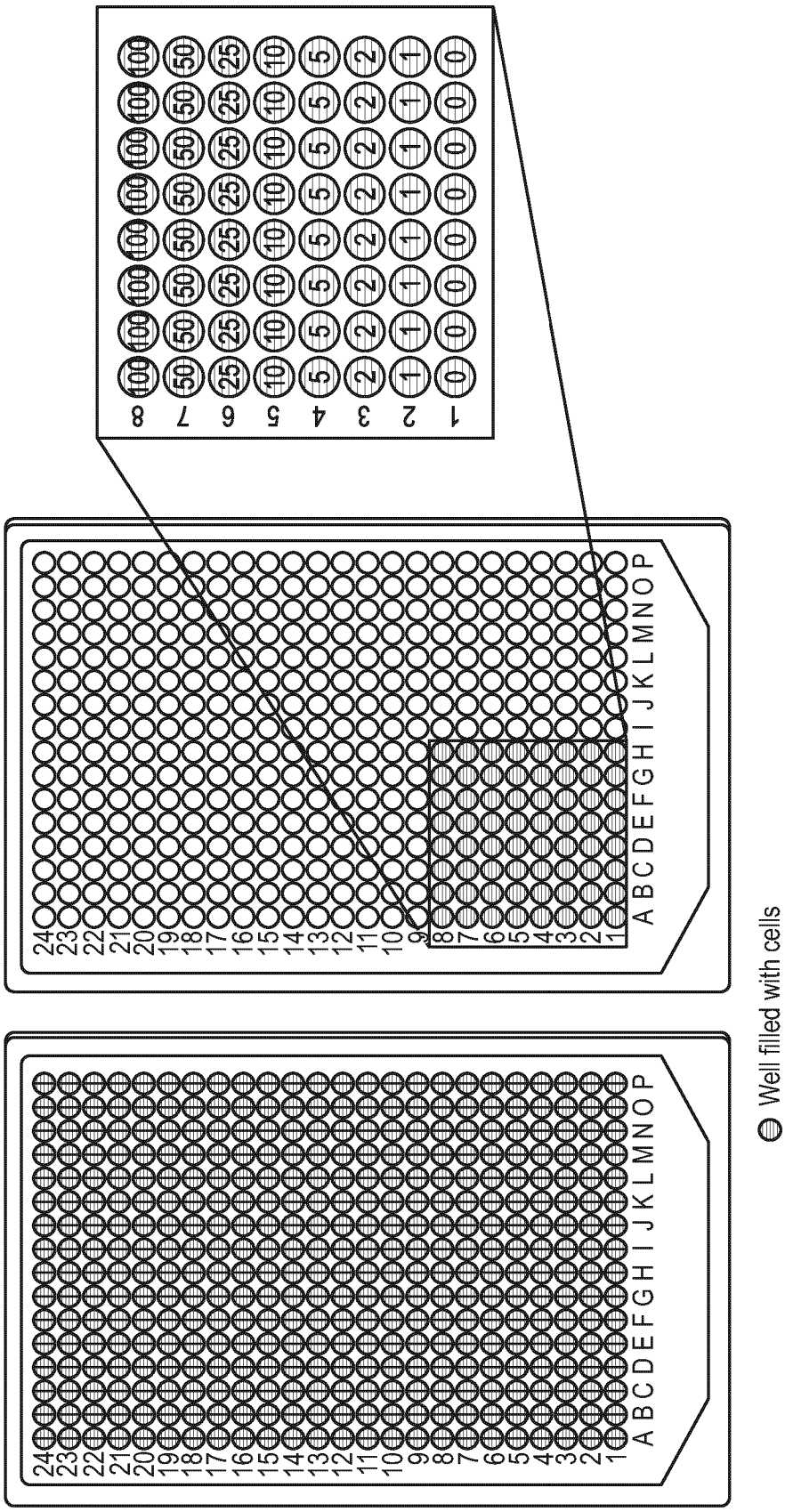
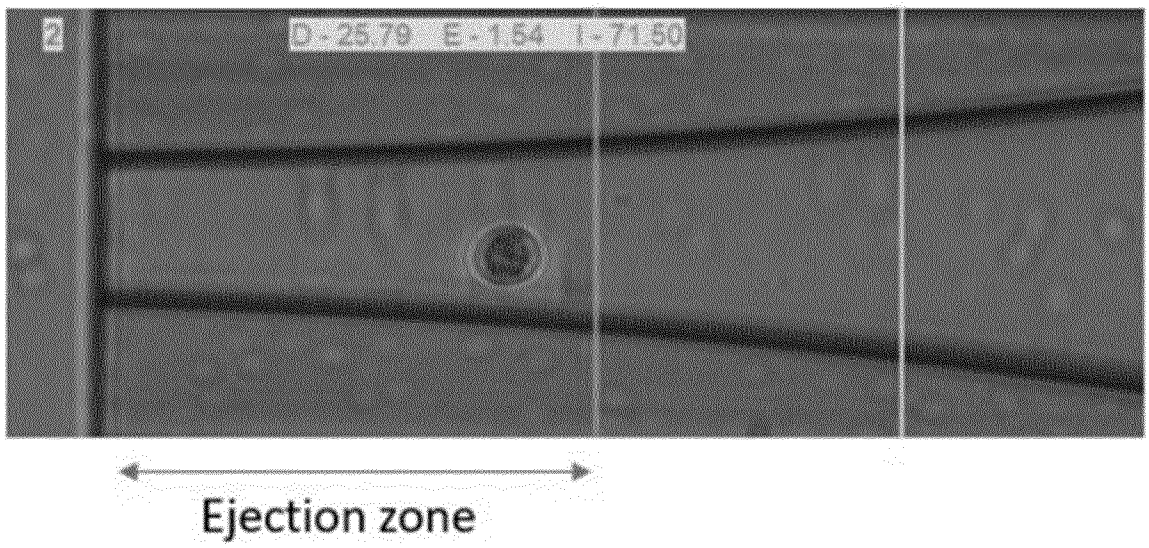

1 2 3 4 5 6 7 8
0 1 2 5 10 25 50 100
A B C D E F G H I J K L M N O P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Well filled with cells

Fig. 9

Fig. 10A):



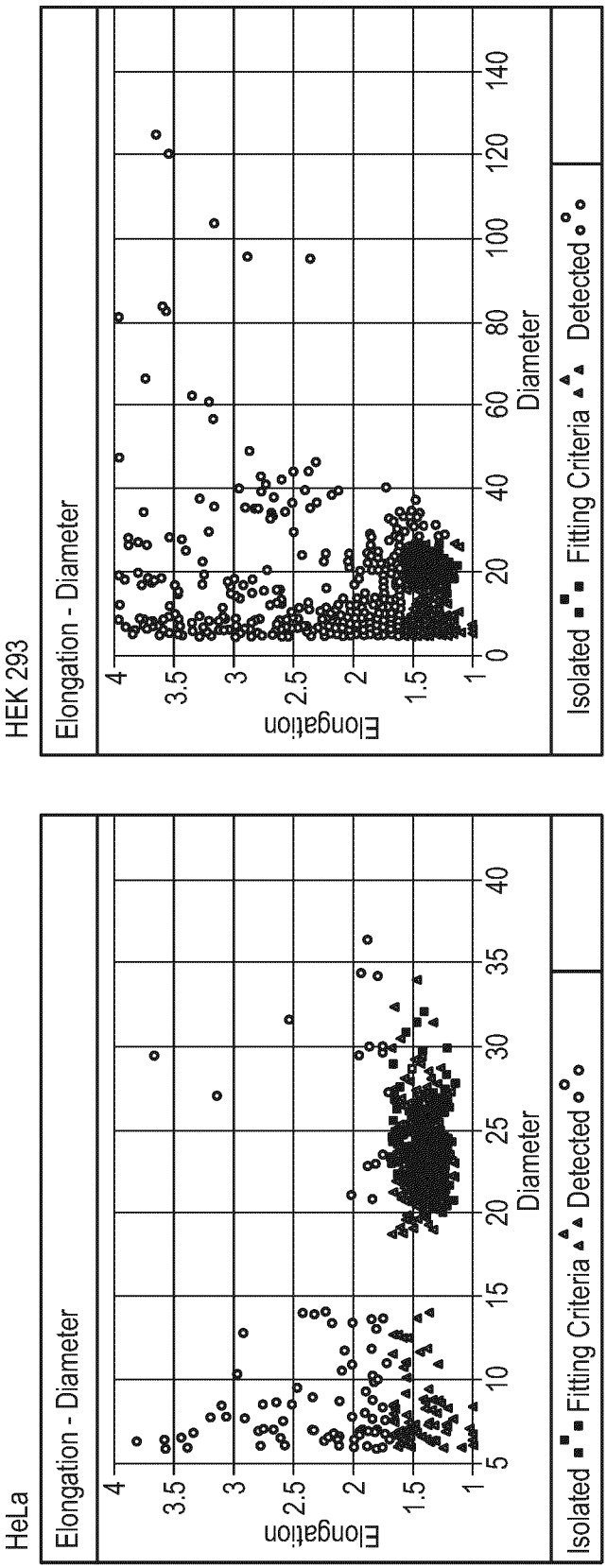

HEK 293
Elongation - Diameter
Elongation
Diameter
Isolated
Fitting Criteria
Detected
HeLa
Elongation - Diameter
Elongation
Diameter
Isolated
Fitting Criteria
Detected

Fig. 10B

Fig. 11A)

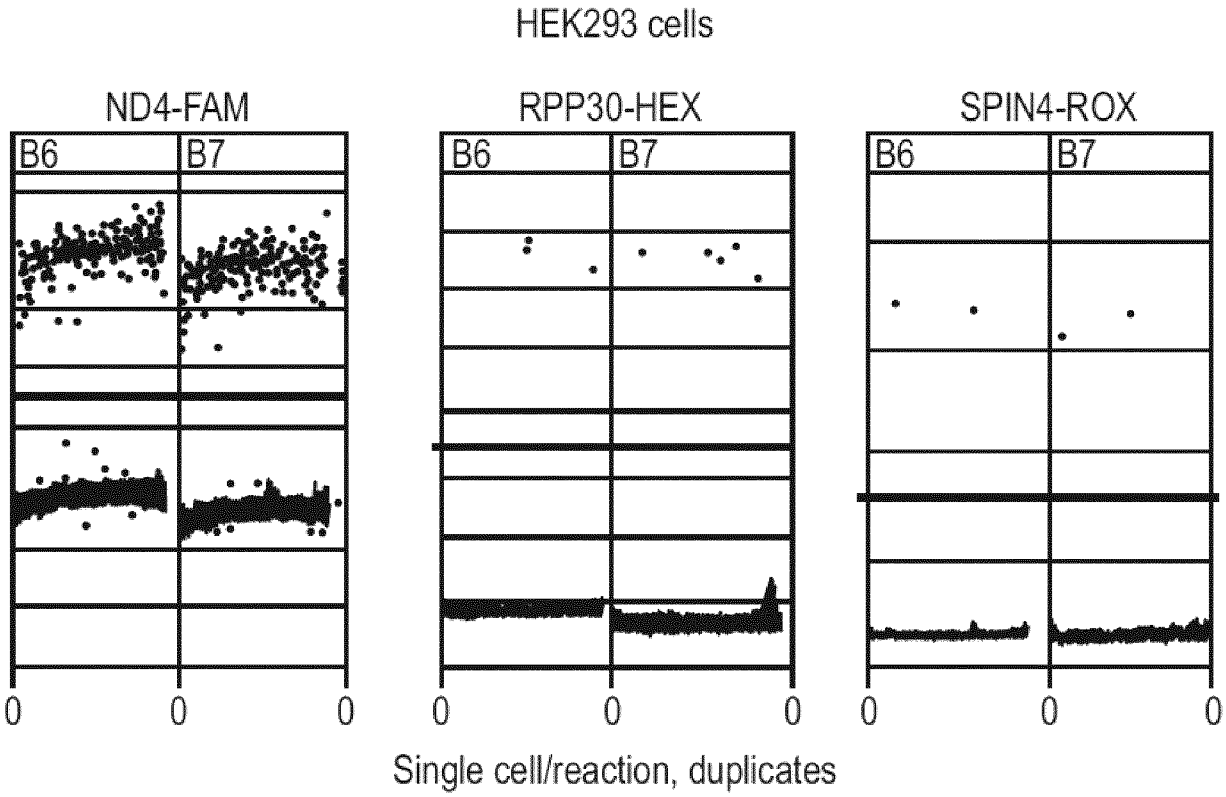


Fig. 11B)

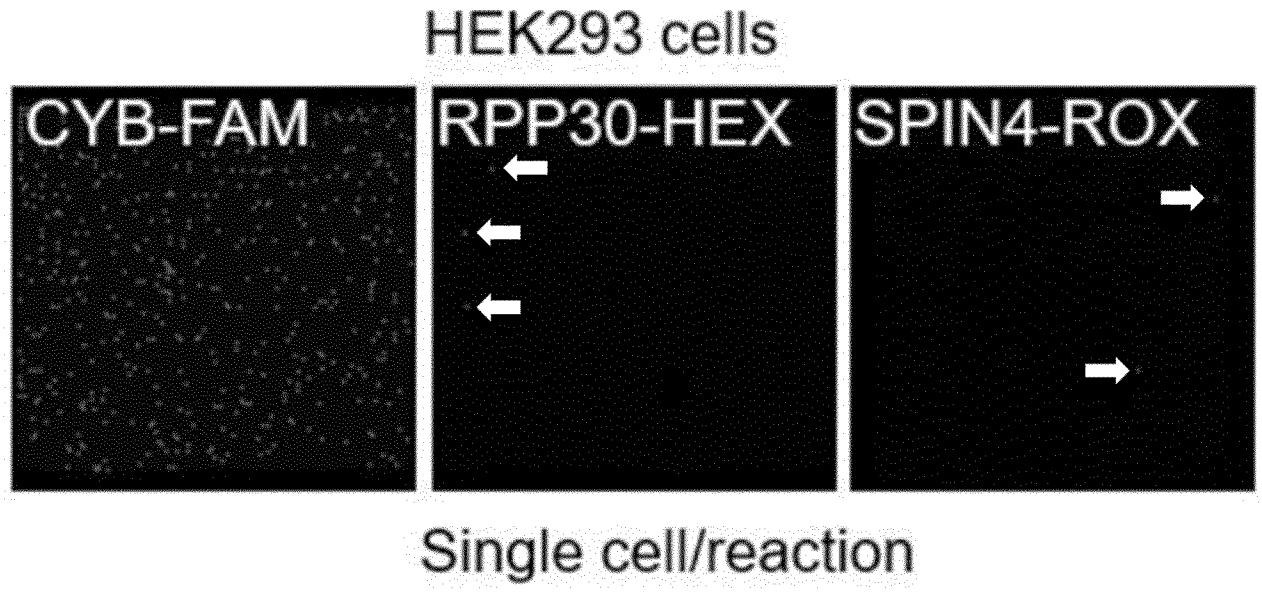


Fig. 12:

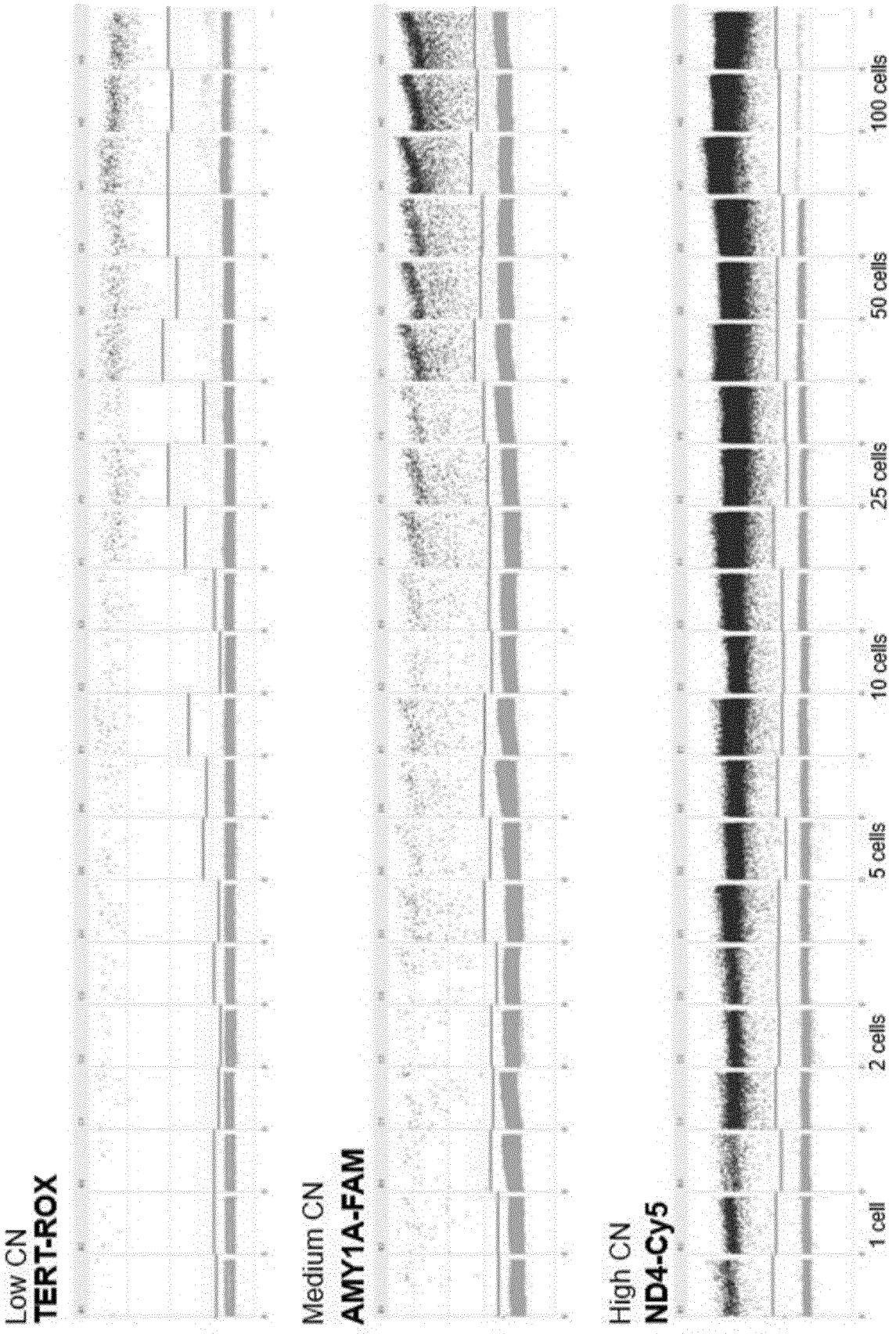

Low CN
TERT-ROX
Medium CN
AMY1A-FAM
High CN
ND4-Cy5
1 cell
2 cells
5 cells
10 cells
25 cells
50 cells
100 cells

Fig. 13:

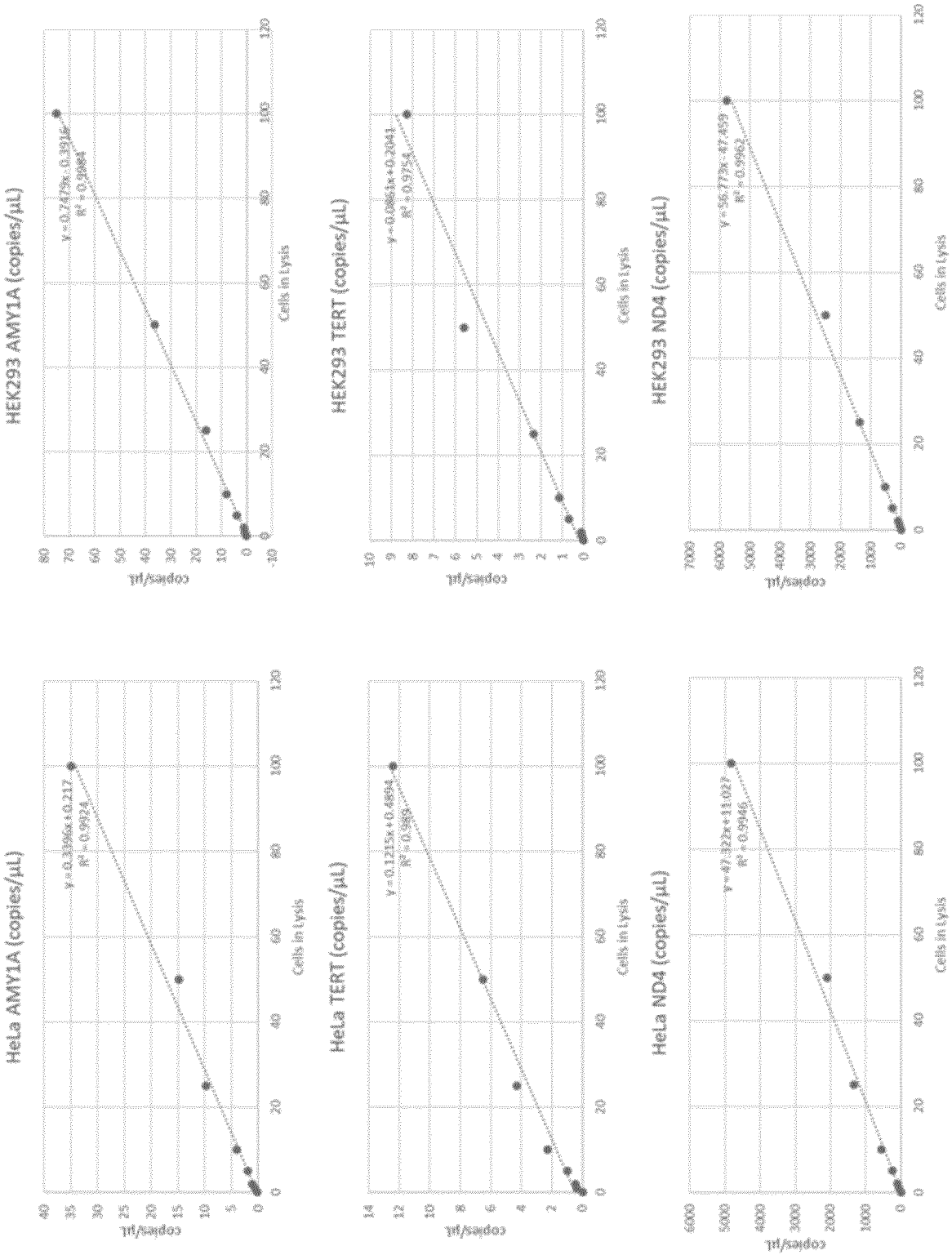

HEK293 AMY1A (copies/µL)
y = 0.7475x - 0.3914
R² = 0.9964
HEK293 TERT (copies/µL)
y = 0.0863x + 0.2041
R² = 0.9754
HEK293 ND4 (copies/µL)
y = 56.772x - 47.459
R² = 0.9962
HeLa AMY1A (copies/µL)
y = 0.3396x + 0.217
R² = 0.9924
HeLa TERT (copies/µL)
y = 0.1215x + 0.4894
R² = 0.9869
HeLa ND4 (copies/µL)
y = 47.822x + 11.027
R² = 0.9946
copies/µL
Cells in Lysis

Fig. 14:

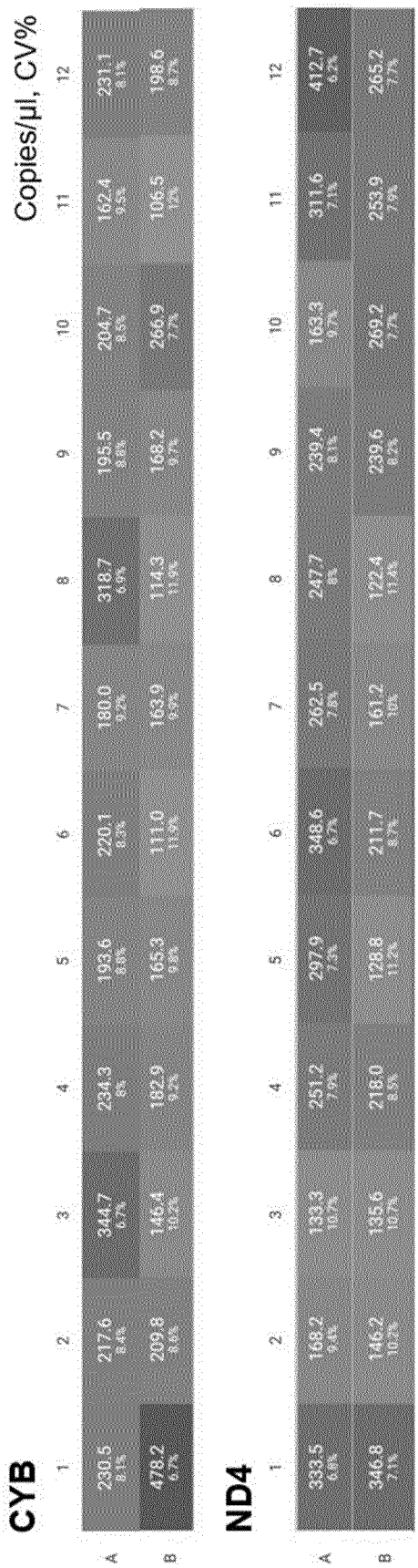

Copies/µl, CV%
CYB
1 2 3 4 5 6 7 8 9 10 11 12
A 230.5 8.1% 217.6 8.4% 344.7 6.7% 234.3 8% 193.6 8.6% 220.1 8.3% 180.0 9.2% 318.7 6.9% 195.5 8.8% 204.7 8.5% 162.4 9.5% 231.1 8.1%
B 478.2 6.7% 209.8 8.6% 146.4 10.2% 182.9 9.2% 165.3 9.8% 111.0 11.9% 163.9 9.5% 114.3 11.9% 168.2 9.7% 266.9 7.7% 106.5 12% 198.6 8.7%
ND4
1 2 3 4 5 6 7 8 9 10 11 12
A 333.5 6.8% 168.2 9.4% 133.3 10.7% 251.2 7.5% 297.9 7.3% 348.6 6.7% 262.5 7.6% 247.7 8% 239.4 8.1% 163.3 9.7% 311.6 7.1% 412.7 6.2%
B 346.8 7.1% 146.2 10.2% 135.6 10.7% 218.0 8.5% 128.8 11.2% 211.7 8.7% 161.2 10% 122.4 11.4% 239.6 8.2% 269.2 7.7% 253.9 7.9% 265.2 7.7%

Fig. 15:

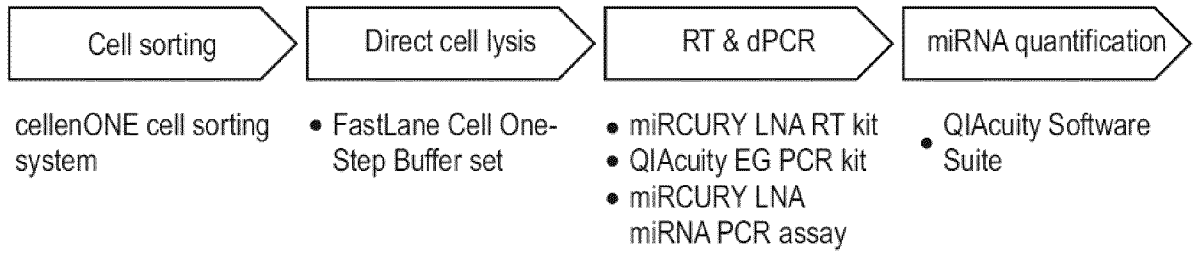

Cell sorting
Direct cell lysis
RT & dPCR
miRNA quantification
cellenONE cell sorting system
FastLane Cell One-Step Buffer set
miRCURY LNA RT kit
QIAcuity EG PCR kit
miRCURY LNA miRNA PCR assay
QIAcuity Software Suite

Fig. 16:

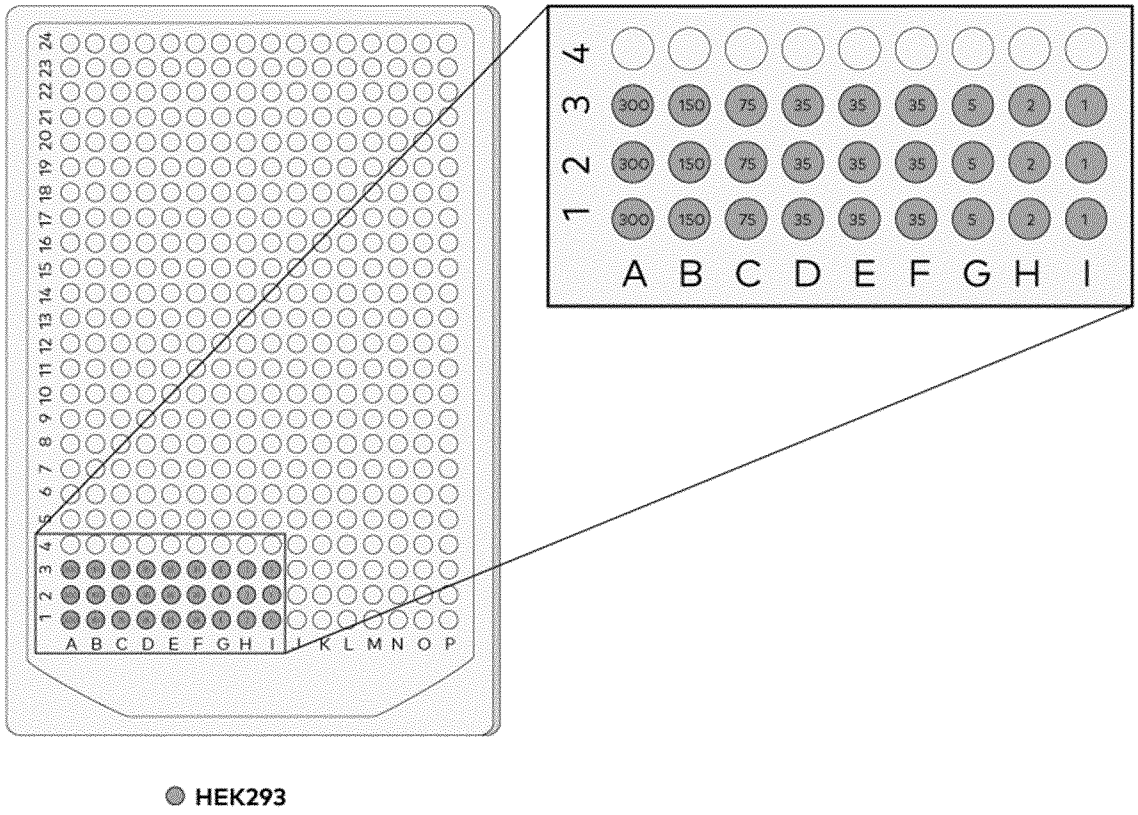

4
3 300 150 75 35 35 35 5 2 1
2 300 150 75 35 35 35 5 2 1
1 300 150 75 35 35 35 5 2 1
A B C D E F G H I
HEK293

Fig. 17:

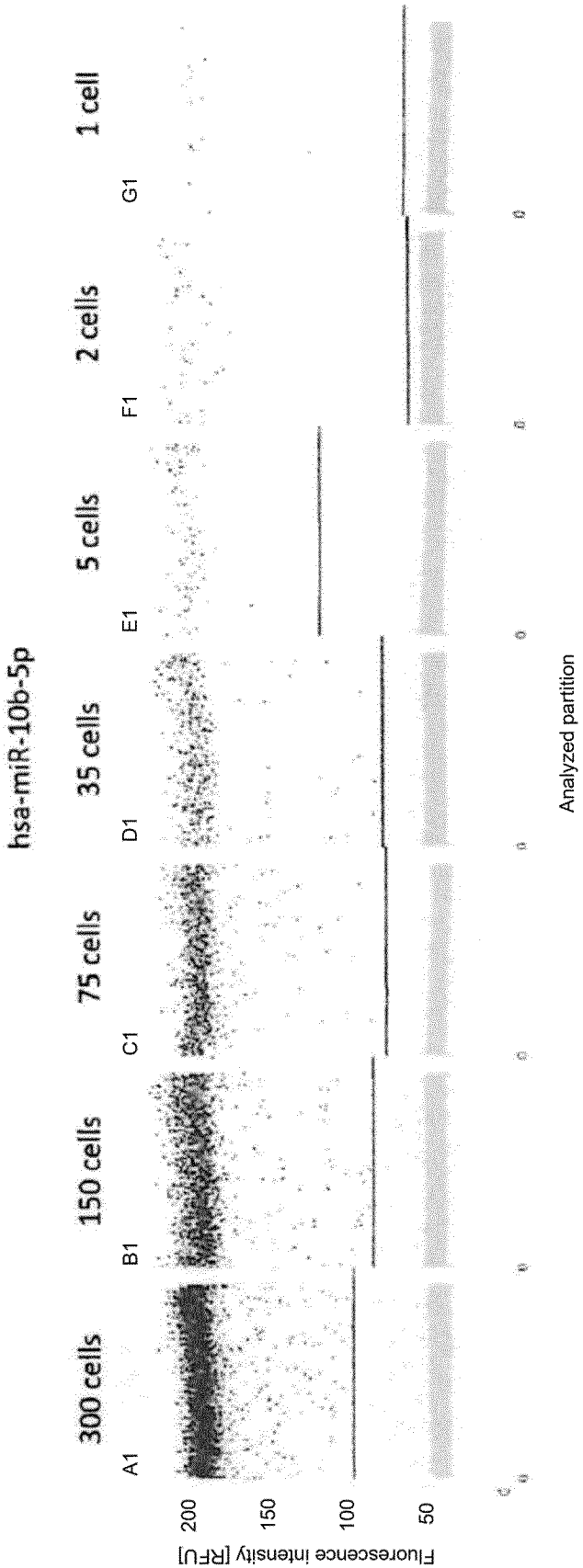

hsa-miR-10b-5p
300 cells
150 cells
75 cells
35 cells
5 cells
2 cells
1 cell
A1
B1
C1
D1
E1
F1
G1
200
150
100
50
Fluorescence intensity [RFU]
Analyzed partition

Fig. 18A):

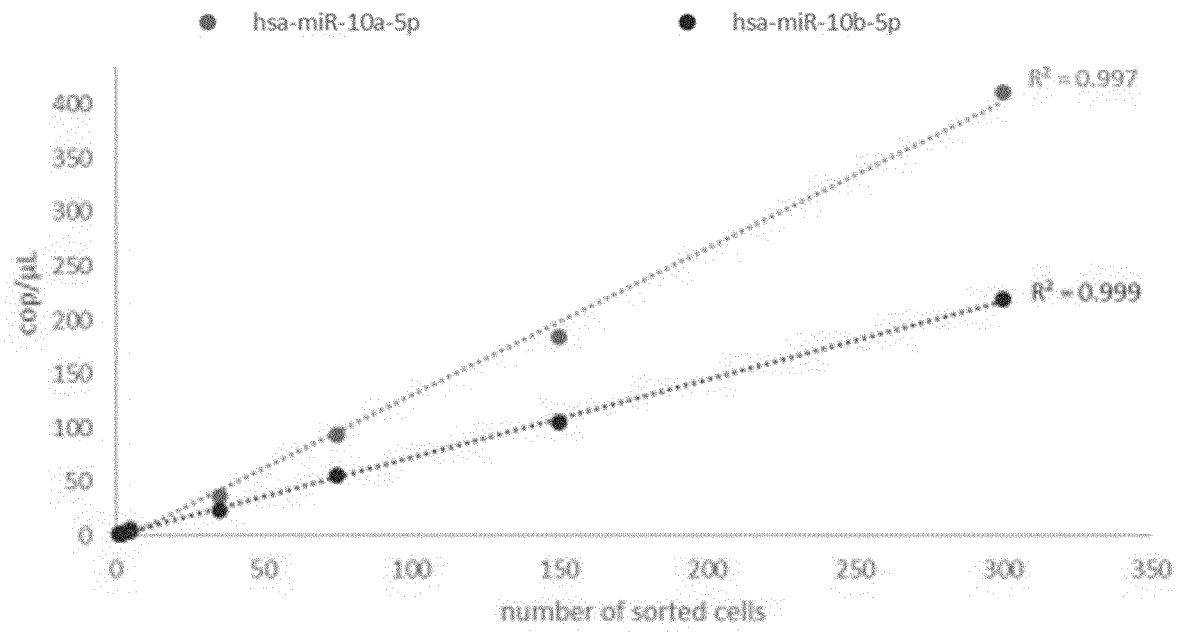


Fig. 18B):

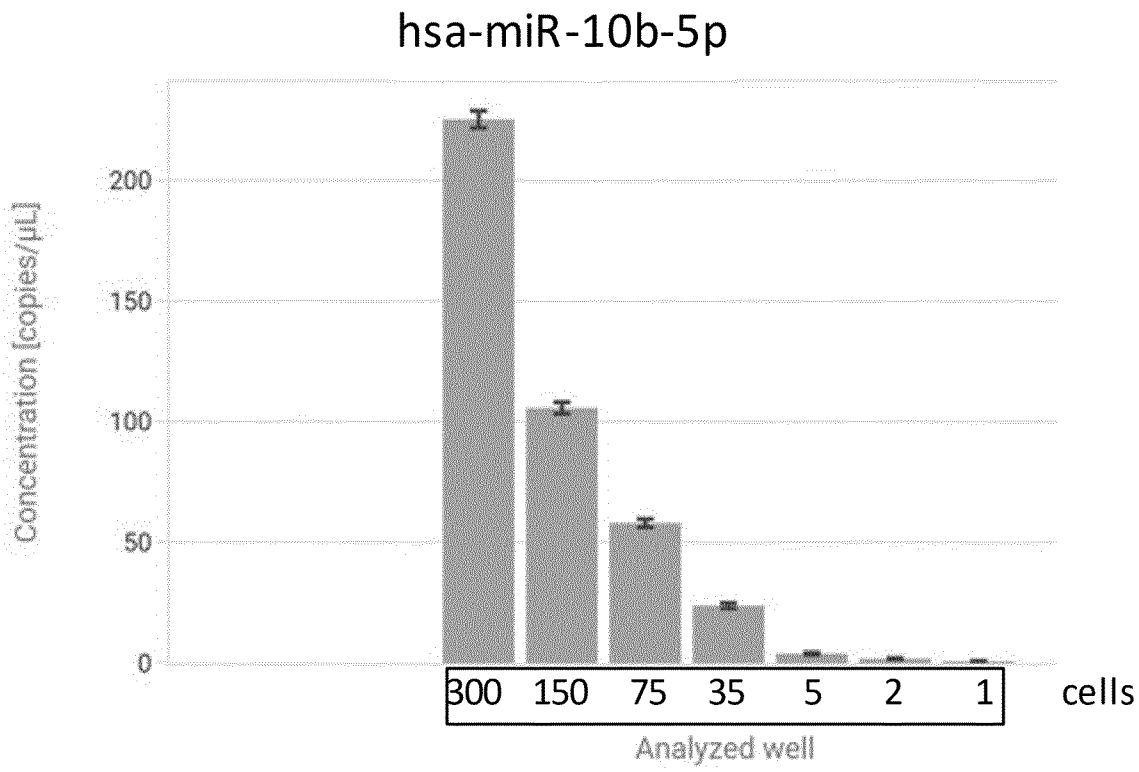


Fig. 18C)

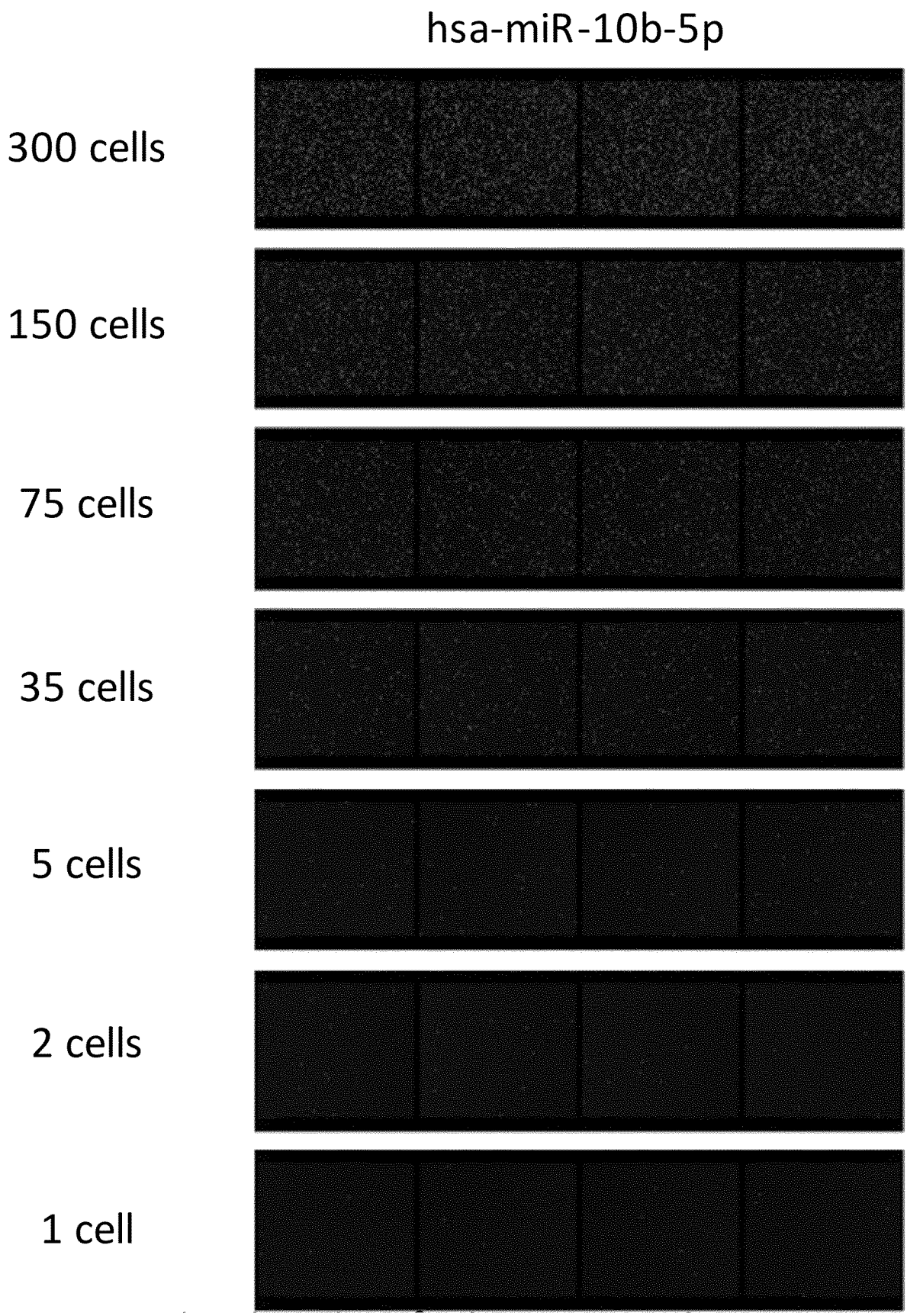

hsa-miR-10b-5p
300 cells
150 cells
75 cells
35 cells
5 cells
2 cells
1 cell

Fig. 19A):

Well	Sample/ NTC/ Control	Expected concentration in Sample	Target (Name)	Conc. [cp/µL] (dPCR reaction)	CI (95%) (dPCR reaction)	Conc. [cp/µL] (undiluted sample)
A1	100000.00		MYC	0.444	87.70%	8.89
A1	100000.00	100000.00	RPP30	0	-	0.00
A2	100000.00		MYC	93.55	4.70%	1871.10
A2	100000.00	100000.00	RPP30	1.519	36.40%	30.38
A3	100000.00		MYC	58.49	5.90%	1169.80
A3	100000.00	100000.00	RPP30	14.89	11.70%	297.80
B1	10000.00		MYC	1365.4	1.60%	27308.10
B1	10000.00	10000.00	RPP30	630.3	2.00%	12606.30
B2	10000.00		MYC	1410.4	1.60%	28208.10
B2	10000.00	10000.00	RPP30	687.9	2.00%	13757.90
B3	10000.00		MYC	1491.3	1.60%	29826.10
B3	10000.00	10000.00	RPP30	698.1	2.00%	13962.10
C1	1000.00		MYC	712.5	1.90%	14251.00
C1	1000.00	1000.00	RPP30	112.6	4.30%	2251.30
C2	1000.00		MYC	670	2.00%	13400.00
C2	1000.00	1000.00	RPP30	119.3	4.30%	2387.00
C3	1000.00		MYC	718.4	1.90%	14368.20
C3	1000.00	1000.00	RPP30	117.2	4.30%	2344.80
D1	100.00		MYC	66.21	5.60%	1324.10
D1	100.00	100.00	RPP30	13.6	12.30%	272.00
D2	100.00		MYC	70.52	5.60%	1410.30
D2	100.00	100.00	RPP30	13.36	12.70%	267.30
D3	100.00		MYC	71.5	5.50%	1430.00
D3	100.00	100.00	RPP30	14.44	12.10%	288.70
E1	10.00		MYC	2.042	31.80%	40.84
E1	10.00	10.00	RPP30	0.054	147.50%	1.07
E2	10.00		MYC	4.313	22.20%	86.27
E2	10.00	10.00	RPP30	1.381	39.20%	27.62
E3	10.00		MYC	3.941	22.90%	78.83
E3	10.00	10.00	RPP30	0.863	49.00%	17.26
F1	1.00		MYC	0.268	87.70%	5.35
F1	1.00	1.00	RPP30	0	-	0.00
F2	1.00		MYC	0.664	56.60%	13.27
F2	1.00	1.00	RPP30	0	-	0.00
F3	1.00		MYC	0.219	98.00%	4.38
F3	1.00	1.00	RPP30	0.11	119.00%	2.19
G1	gDNA		MYC	42.76	7.00%	855.10
G1	gDNA	gDNA	RPP30	18.73	10.50%	374.60
G2	gDNA		MYC	40.92	7.20%	818.30
G2	gDNA	gDNA	RPP30	15.31	11.70%	306.10
G3	gDNA		MYC	38.39	7.40%	767.70
G3	gDNA	gDNA	RPP30	16.76	11.10%	335.20
H1	NTC		MYC	0	-	-
H1	NTC	NTC	RPP30	0	-	-
H2	NTC		MYC	0	-	-
H2	NTC	NTC	RPP30	0	-	-
H3	NTC		MYC	0	-	-
H3	NTC	NTC	RPP30	0.053	147.50%	-

Fig. 19A

Patitions (Valid)	Partitions (Positive)	Partitions (Negative)	Threshold	Cell Type	Setup Sheet
14879	5	14874	38.72	Frozen Jurkat	Example 4.1
16156	0	16156	70.38	Frozen Jurkat	Example 4.1
25433	1724	23709	29.13	Frozen Jurkat	Example 4.1
25456	29	25427	44.88	Frozen Jurkat	Example 4.1
25347	1086	24261	29.13	Frozen Jurkat	Example 4.1
25351	281	25070	75.23	Frozen Jurkat	Example 4.1
25462	16303	9159	29.13	Frozen Jurkat	Example 4.1
25239	9496	15743	47.81	Frozen Jurkat	Example 4.1
25455	16348	9107	29.13	Frozen Jurkat	Example 4.1
25305	9977	15328	42.38	Frozen Jurkat	Example 4.1
25458	17000	8458	29.13	Frozen Jurkat	Example 4.1
25390	10232	15158	46.23	Frozen Jurkat	Example 4.1
25430	10419	15011	29.13	Frozen Jurkat	Example 4.1
25493	2037	23456	40.16	Frozen Jurkat	Example 4.1
25277	9607	15670	29.13	Frozen Jurkat	Example 4.1
25488	2081	23407	41.44	Frozen Jurkat	Example 4.1
25473	10422	15051	29.13	Frozen Jurkat	Example 4.1
25460	2095	23365	31.24	Frozen Jurkat	Example 4.1
25477	1213	24264	29.13	Frozen Jurkat	Example 4.1
25477	254	25223	22.8	Frozen Jurkat	Example 4.1
25480	1236	24244	29.13	Frozen Jurkat	Example 4.1
25480	239	25241	24.54	Frozen Jurkat	Example 4.1
25479	1281	24198	29.13	Frozen Jurkat	Example 4.1
25479	264	25215	27.09	Frozen Jurkat	Example 4.1
25467	38	25429	29.13	Frozen Jurkat	Example 4.1
25467	1	25466	28.31	Frozen Jurkat	Example 4.1
25468	78	25390	29.13	Frozen Jurkat	Example 4.1
25468	25	25443	29.33	Frozen Jurkat	Example 4.1
25467	73	25394	29.13	Frozen Jurkat	Example 4.1
25467	16	25451	46.16	Frozen Jurkat	Example 4.1
25454	5	25449	29.13	Frozen Jurkat	Example 4.1
25454	0	25454	34.68	Frozen Jurkat	Example 4.1
25385	12	25373	29.13	Frozen Jurkat	Example 4.1
25385	0	25385	38.51	Frozen Jurkat	Example 4.1
25471	4	25467	29.13	Frozen Jurkat	Example 4.1
25471	2	25469	29.58	Frozen Jurkat	Example 4.1
25471	792	24679	22.63	Frozen Jurkat	Example 4.1
25471	350	25121	18.13	Frozen Jurkat	Example 4.1
25449	739	24710	29.64	Frozen Jurkat	Example 4.1
25449	279	25170	31.24	Frozen Jurkat	Example 4.1
25471	709	24762	34.43	Frozen Jurkat	Example 4.1
25473	312	25161	21.04	Frozen Jurkat	Example 4.1
25447	0	25447	19.38	Frozen Jurkat	Example 4.1
25447	0	25447	37.23	Frozen Jurkat	Example 4.1
25324	0	25324	19.38	Frozen Jurkat	Example 4.1
25324	0	25324	29.58	Frozen Jurkat	Example 4.1
25446	0	25446	21.93	Frozen Jurkat	Example 4.1
25446	1	25445	24.48	Frozen Jurkat	Example 4.1

Fig. 19A (cont.)

Fig. 19B):

Well	Sample/ NTC/ Control	Expected concentration in Sample	Target (Name)	Conc. [cp/µL] (dPCR reaction)	CI (95%) (dPCR reaction)	Conc. [cp/µL] (undiluted sample)
A1	580.00		MYC	8.779	15.10%	175.60
A1	580.00	580.00	RPP30	3.286	24.70%	65.71
A2	580.00		MYC	9.571	14.50%	191.40
A2	580.00	580.00	RPP30	2.307	29.50%	46.14
A3	580.00		MYC	12.23	12.90%	244.70
A3	580.00	580.00	RPP30	3.047	25.70%	60.95
B1	58.00		MYC	2.678	27.40%	53.57
B1	58.00	58.00	RPP30	0.315	80.00%	6.30
B2	58.00		MYC	1.67	35.20%	33.41
B2	58.00	58.00	RPP30	0.054	147.50%	1.08
B3	58.00		MYC	2.235	30.20%	44.71
B3	58.00	58.00	RPP30	0.479	65.30%	9.57
C1	5.80		MYC	0.053	147.50%	1.06
C1	5.80	5.80	RPP30	0.053	147.50%	1.06
C2	5.80		MYC	0	-	0.00
C2	5.80	5.80	RPP30	0.055	147.50%	1.10
C3	5.80		MYC	0.107	119.00%	2.15
C3	5.80	5.80	RPP30	0	-	0.00
D1	0.58		MYC	0	-	0.00
D1	0.58	0.58	RPP30	0.053	147.50%	1.07
D2	0.58		MYC	0.111	119.00%	2.23
D2	0.58	0.58	RPP30	0	-	0.00
D3	0.58		MYC	0	-	0.00
D3	0.58	0.58	RPP30	0.054	147.50%	1.09
E1	gDNA		MYC	44.34	6.90%	886.90
E1	gDNA	gDNA	RPP30	0.537	62.00%	10.73
E2	gDNA		MYC	35.2	7.80%	703.90
E2	gDNA	gDNA	RPP30	0.332	80.00%	6.63
E3	gDNA		MYC	40.66	7.20%	813.20
E3	gDNA	gDNA	RPP30	0.701	54.40%	14.02
F1	NTC		MYC	0	-	-
F1	NTC	NTC	RPP30	0	-	-
F2	NTC		MYC	0	-	-
F2	NTC	NTC	RPP30	0	-	-
F3	NTC		MYC	0	-	-
F3	NTC	NTC	RPP30	0.055	147.50%	-

Fig. 19B

Partitions (Valid)	Partitions (Positive)	Partitions (Negative)	Threshold	Cell Type	Setup Sheet
25377	168	25209	28.96	Living HeLa	Example 4.2
25376	63	25313	37.44	Living HeLa	Example 4.2
25435	182	25253	28.96	Living HeLa	Example 4.2
25439	44	25395	37.44	Living HeLa	Example 4.2
25447	232	25215	28.96	Living HeLa	Example 4.2
25451	58	25393	37.44	Living HeLa	Example 4.2
25454	51	25403	28.96	Living HeLa	Example 4.2
25454	6	25448	37.44	Living HeLa	Example 4.2
25483	31	25452	28.96	Living HeLa	Example 4.2
25483	1	25482	37.44	Living HeLa	Example 4.2
25449	42	25407	28.96	Living HeLa	Example 4.2
25449	9	25440	37.44	Living HeLa	Example 4.2
25489	1	25488	28.96	Living HeLa	Example 4.2
25489	1	25488	37.44	Living HeLa	Example 4.2
25485	0	25485	28.96	Living HeLa	Example 4.2
25485	1	25484	37.44	Living HeLa	Example 4.2
25463	2	25461	28.96	Living HeLa	Example 4.2
25463	0	25463	37.44	Living HeLa	Example 4.2
25475	0	25475	28.96	Living HeLa	Example 4.2
25475	1	25474	37.44	Living HeLa	Example 4.2
25481	2	25479	28.96	Living HeLa	Example 4.2
25481	0	25481	37.44	Living HeLa	Example 4.2
25469	0	25469	28.96	Living HeLa	Example 4.2
25469	1	25468	37.44	Living HeLa	Example 4.2
25478	813	24665	28.96	Living HeLa	Example 4.2
25485	10	25475	37.44	Living HeLa	Example 4.2
25447	629	24818	28.96	Living HeLa	Example 4.2
25447	6	25441	37.44	Living HeLa	Example 4.2
25462	743	24719	28.96	Living HeLa	Example 4.2
25462	13	25449	37.44	Living HeLa	Example 4.2
25468	0	25468	28.96	Living HeLa	Example 4.2
25468	0	25468	37.44	Living HeLa	Example 4.2
25412	0	25412	28.96	Living HeLa	Example 4.2
25412	0	25412	37.44	Living HeLa	Example 4.2
25469	0	25469	28.96	Living HeLa	Example 4.2
25469	1	25468	37.44	Living HeLa	Example 4.2

Fig. 19B (cont.)

Fig. 19C):

Well	Sample/ NTC/ Control	Expected concentration in Sample	Target (Name)	Conc. [cp/µL] (dPCR reaction)	CI (95%) (dPCR reaction)	Conc. [cp/µL] (undiluted sample)
A1	900.00		MYC	20.43	9.90%	408.60
A1	900.00	900.00	RPP30	6.457	17.60%	129.10
A2	900.00		MYC	19.19	10.30%	383.80
A2	900.00	900.00	RPP30	5.559	19.00%	111.20
A3	900.00		MYC	22.16	9.60%	443.30
A3	900.00	900.00	RPP30	6.218	18.00%	124.40
B1	90.00		MYC	4.017	23.30%	80.34
B1	90.00	90.00	RPP30	0.622	59.10%	12.43
B2	90.00		MYC	4.588	21.30%	91.75
B2	90.00	90.00	RPP30	0.916	47.50%	18.33
B3	90.00		MYC	4.532	21.30%	90.65
B3	90.00	90.00	RPP30	0.532	62.00%	10.65
C1	9.00		MYC	0.319	80.00%	6.37
C1	9.00	9.00	RPP30	0.159	106.40%	3.19
C2	9.00		MYC	0.165	106.40%	3.30
C2	9.00	9.00	RPP30	0	-	0.00
C3	9.00		MYC	0.698	54.40%	13.95
C3	9.00	9.00	RPP30	0.054	147.50%	1.07
D1	0.90		MYC	0	-	0.00
D1	0.90	0.90	RPP30	0	-	0.00
D2	0.90		MYC	0	-	0.00
D2	0.90	0.90	RPP30	0	-	0.00
D3	0.90		MYC	0.218	98.00%	4.36
D3	0.90	0.90	RPP30	0	-	0.00
E1	gDNA		MYC	0	-	0.00
E1	gDNA	gDNA	RPP30	0	-	0.00
E2	gDNA		MYC	41.37	7.20%	827.50
E2	gDNA	gDNA	RPP30	0.497	65.30%	9.95
E3	gDNA		MYC	38.49	7.40%	769.70
E3	gDNA	gDNA	RPP30	4.283	22.10%	85.66
F1	NTC		MYC	0	-	-
F1	NTC	NTC	RPP30	0	-	-
F2	NTC		MYC	0	-	-
F2	NTC	NTC	RPP30	0	-	-
F3	NTC		MYC	0	-	-
F3	NTC	NTC	RPP30	0	-	-

Fig. 19C

Patitions (Valid)	Partitions (Positive)	Partitions (Negative)	Threshold	Cell Type	Setup Sheet
25430	390	25040	17.34	Living HeLa	Example 4.3
25444	124	25320	23.34	Living HeLa	Example 4.3
25465	364 106	25101	17.34	Living HeLa	Example 4.3
25467		25361	23.34	Living HeLa	Example 4.3
25400	418	24982	17.34	Living HeLa	Example 4.3
25407	118	25289	23.34	Living HeLa	Example 4.3
23640	71	23569	17.34	Living HeLa	Example 4.3
23640	11	23629	23.34	Living HeLa	Example 4.3
25466	85	25381	17.34	Living HeLa	Example 4.3
25466	17	25449	23.34	Living HeLa	Example 4.3
25424	85	25339	17.34	Living HeLa	Example 4.3
25428	10	25418	23.34	Living HeLa	Example 4.3
25461	6	25455	17.34	Living HeLa	Example 4.3
25461	3	25458	23.34	Living HeLa	Example 4.3
25465	3	25462	17.34	Living HeLa	Example 4.3
25465	0	25465	23.34	Living HeLa	Example 4.3
25451	13	25438	17.34	Living HeLa	Example 4.3
25451	1	25450	23.34	Living HeLa	Example 4.3
22711	0	22711	17.34	Living HeLa	Example 4.3
22711	0	22711	23.34	Living HeLa	Example 4.3
25463	0	25463	17.34	Living HeLa	Example 4.3
25463	0	25463	23.34	Living HeLa	Example 4.3
25458	4	25454	17.34	Living HeLa	Example 4.3
25458	0	25458	23.34	Living HeLa	Example 4.3
25454	0	25454	17.34	Living HeLa	Example 4.3
25454	0	25454	23.34	Living HeLa	Example 4.3
25454	738	24716	17.34	Living HeLa	Example 4.3
25454	9	25445	23.34	Living HeLa	Example 4.3
25361	701	24660	17.34	Living HeLa	Example 4.3
25364	79	25285	23.34	Living HeLa	Example 4.3
23583	0	23583	17.34	Living HeLa	Example 4.3
23583	0	23583	23.34	Living HeLa	Example 4.3
25194	0	25194	17.34	Living HeLa	Example 4.3
25194	0	25194	23.34	Living HeLa	Example 4.3
25461	0	25461	17.34	Living HeLa	Example 4.3
25461	0	25461	23.34	Living HeLa	Example 4.3

Fig. 19C (cont.)

Fig. 20A):

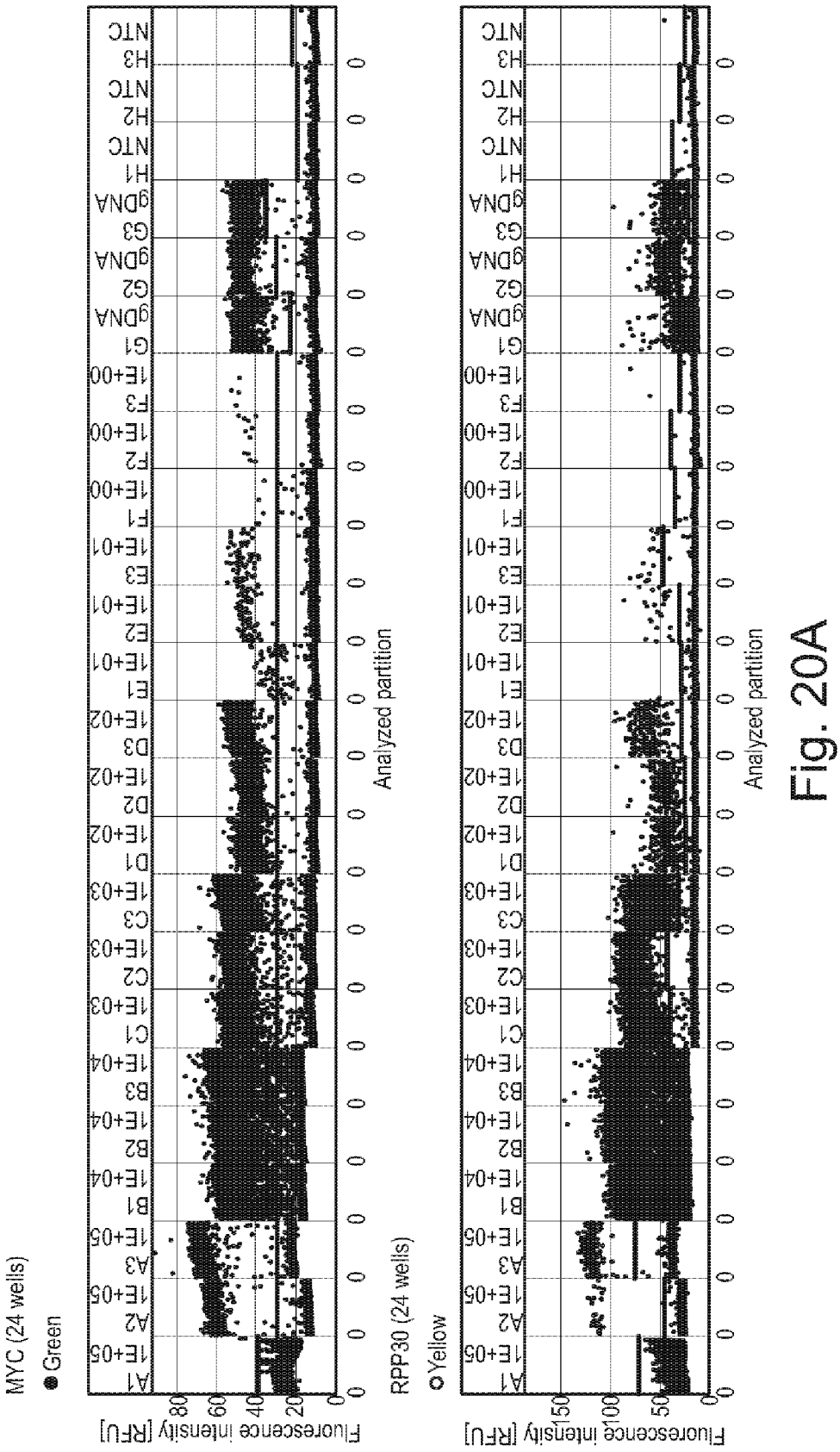


Fig. 20A

Fig. 20B):

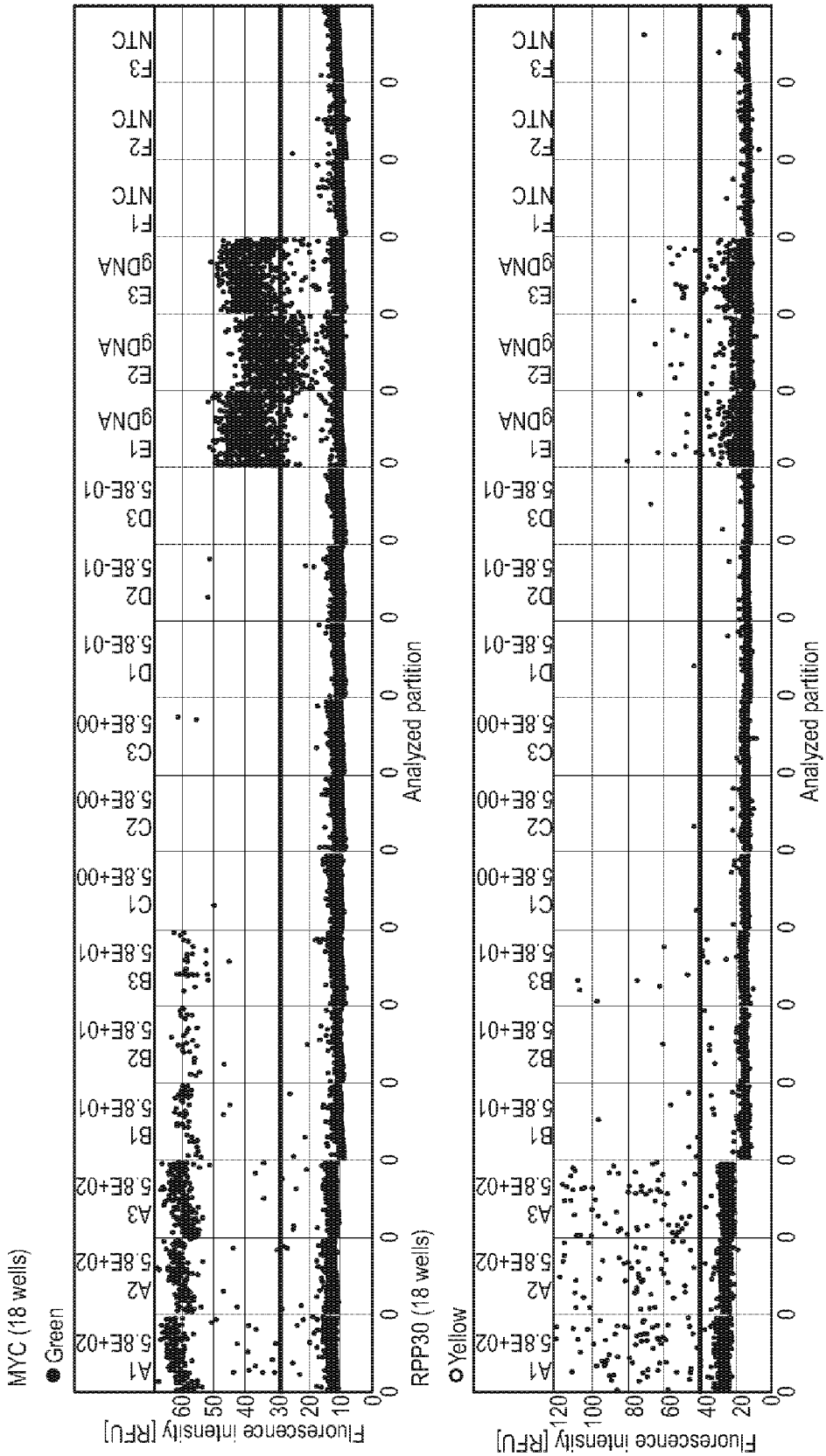


Fig. 20B

Fig. 20C):

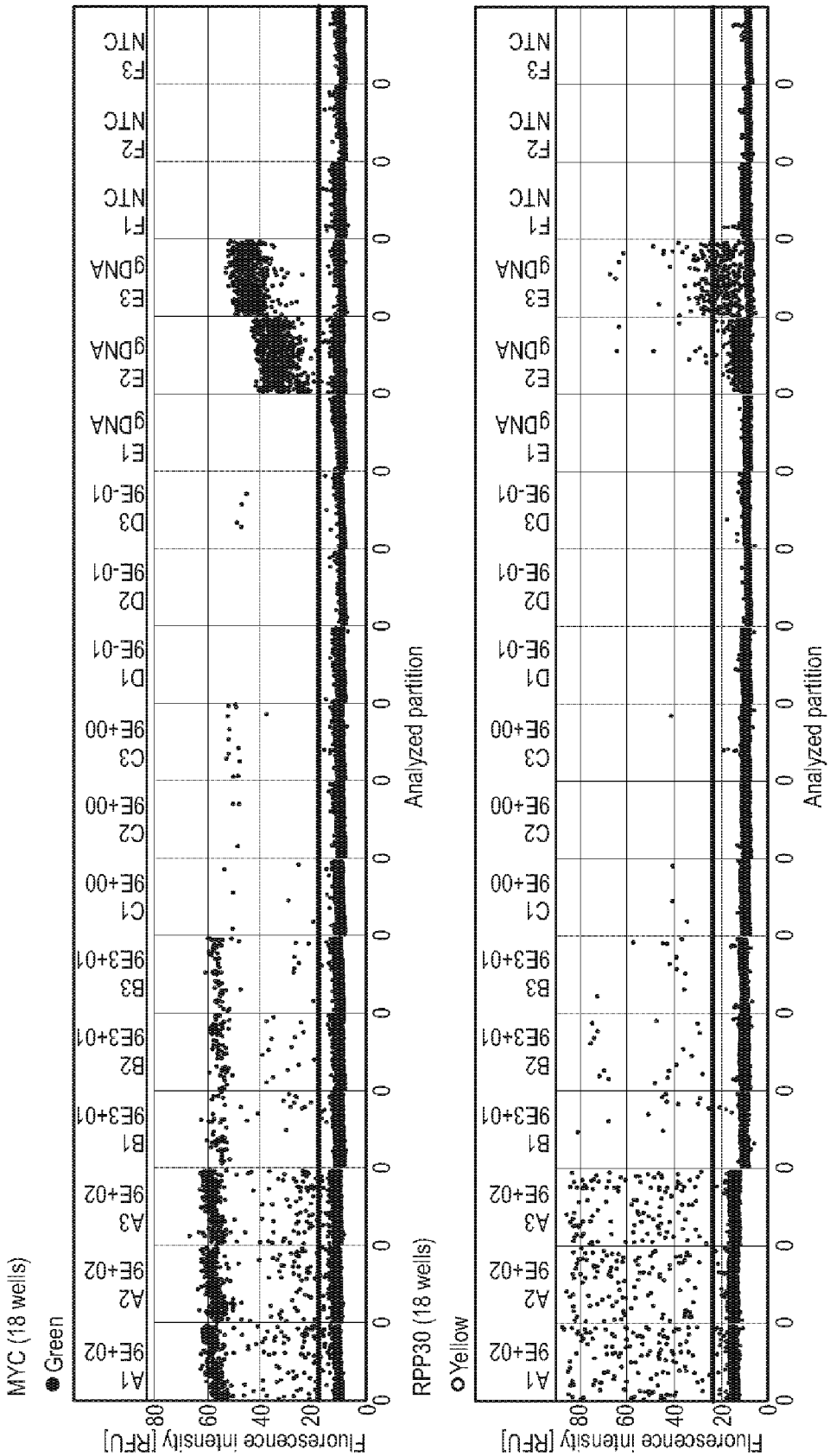
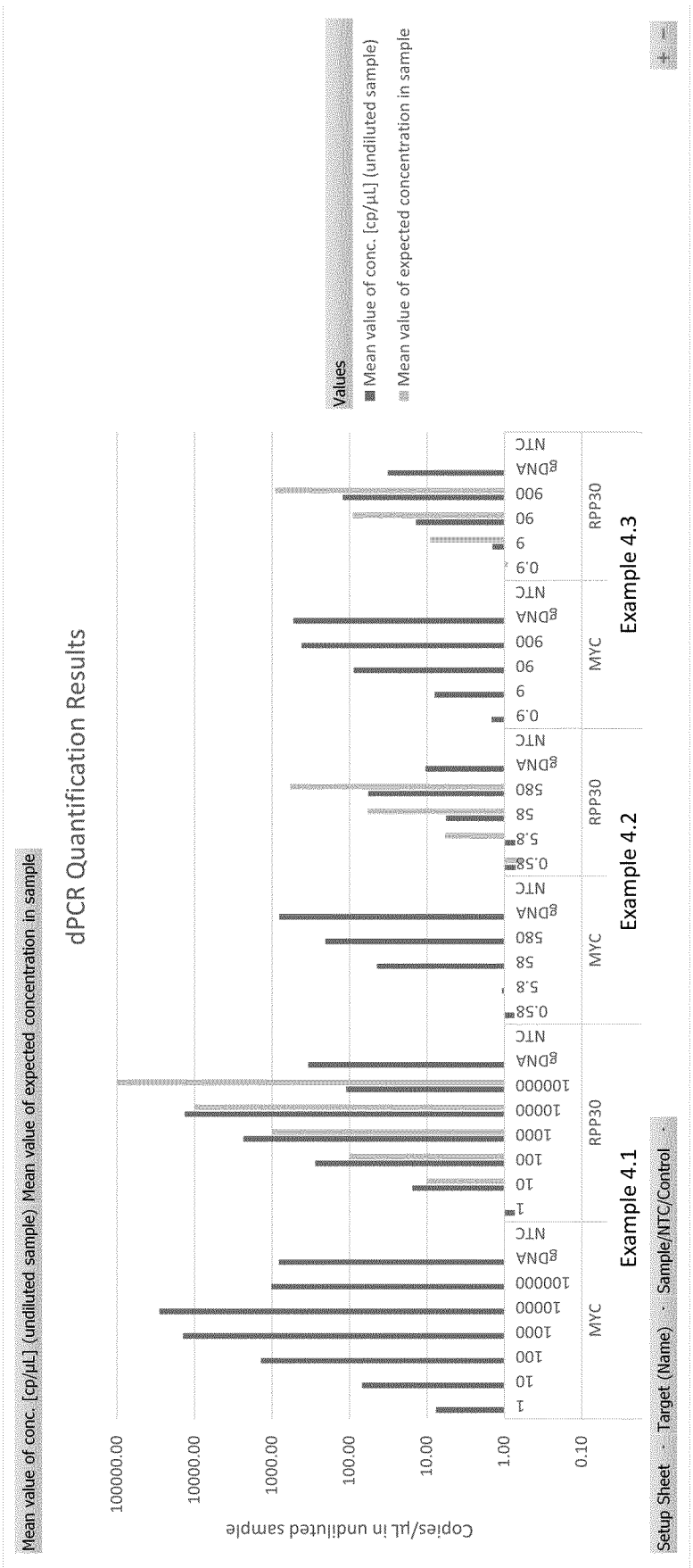


Fig. 20C

Fig. 21:


dPCR Quantification Results
Copies/µL in undiluted sample
100000.00
10000.00
1000.00
100.00
10.00
1.00
0.10
Values
Mean value of conc. [cp/µL] (undiluted sample)
Mean value of expected concentration in sample
Setup Sheet · Target (Name) · Sample/NTC/Control
MYC
RPP30
1
10
100
1000
10000
100000
gDNA
NTC
0.58
5.8
58
580
0.9
9
90
900
Example 4.1
Example 4.2
Example 4.3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/067922

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6851
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Albers Julius ET AL: "A workflow combining high-accuracy cell sorting with multiplex digital PCR for single-cell gene expression analysis", , 1 February 2023 (2023-02-01), XP093103069, Retrieved from the Internet: URL:https://www.qiagen.com/es/resources/resourcedetail?id=e1695b13-66bb-48b9-96bd-d04846fcaa71&lang=en [retrieved on 2023-11-17] page 3 - page 4; figures 4-6 -----	1-15
X	WO 2021/056651 A1 (SUZHOU INST BIOMEDICAL ENG & TECH CAS [CN]) 1 April 2021 (2021-04-01)	1,3-5, 7-11, 13-15
Y	page 4, line 2 - line 4; claims 1, 7 -----	2
	-/--	

[x] Further documents are listed in the continuation of Box C.
[x] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 August 2024	16/09/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Santagati, Fabio

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/067922

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 108 138 365 B (BGI SHENZHEN) 2 August 2022 (2022-08-02)	1,3-6, 13-15
Y	claims 1, 5	2

X	SPIESS A-N ET AL: "Gene expression analysis of single human spermatozoa: Approaches and caveats", JOURNAL FUR REPRODUKTIONSMEDIZIN UND ENDOKRINOLOGIE, KRAUSE UND PACHERNEGG GMBH, vol. 14, no. 5, 1 January 2017 (2017-01-01), page 239, XP009523545, ISSN: 1810-9292	1,3-5, 7-13
Y	the whole document	2

X	Kim J ET AL: "SINGLE-CELL REAL-TIME RT-PCR USING MICROWELL ASSEMBLY", , 31 December 2014 (2014-12-31), pages 887-889, XP093102041, Retrieved from the Internet: URL:https://www.rsc.org/images/loc/2014/PDFs/Papers/291_1003.pdf [retrieved on 2023-11-15]	1,3-5, 7-11, 13-15
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Y	DIEGO ADHEMAR JAITIN ET AL: "Supplementary Material: Massively Parallel Single-Cell RNA-Seq for Marker-Free Decomposition of Tissues into Cell Types", INTERNET CITATION, 14 February 2014 (2014-02-14), pages 1-35, XP002722806, ISSN: 0036-8075 Retrieved from the Internet: URL:http://www.sciencemag.org/content/suppl/2014/02/12/343.6172.776.DC1/Jaitin-SM.pdf [retrieved on 2014-04-04] page 2, paragraph 2	2

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/067922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2021056651 A1	01-04-2021	NONE	
CN 108138365 B	02-08-2022	CN 108138365 A	08-06-2018
		WO 2017084023 A1	26-05-2017