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(54) Title: METHOD FOR DIGITAL QUANTIFICATION OF A NUCLEIC ACID WITHOUT PHYSICALLY PARTITIONING THE REACTION MIXTURE

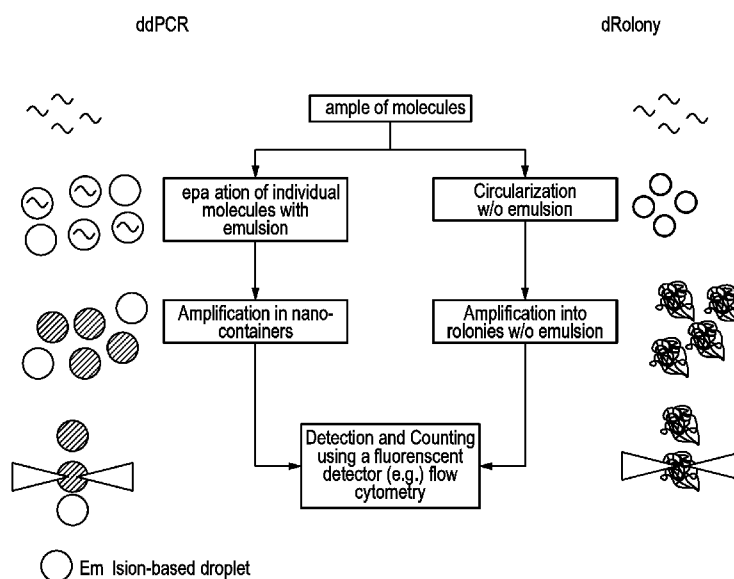


Fig. 1

(57) Abstract: The invention is directed to a method for digital quantification of individual sample molecules in vitro without physically partitioning the reaction mixture and to a kit adapted for carrying out said method.



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Method for digital quantification of a nucleic acid without physically partitioning the reaction mixture

RELATED APPLICATIONS

[0001] The present application claims priority to U.S. provisional patent application No. 62/794,304, filed on January 18, 2019, and European patent application No. 19 157 489.6, filed on February 15, 2019. The disclosures of the above-referenced applications are incorporated by reference in their entireties for all purposes.

[0002] The present invention relates to a method for digital quantification of individual sample molecules *in vitro* without physically partitioning the reaction mixture and to a kit adapted for carrying out said method.

FIELD OF THE INVENTION

[0003] The present invention relates to the field of molecular biology, more particularly to the quantification of nucleic acid molecules in a sample.

BACKGROUND OF THE INVENTION

[0004] Digital polymerase chain reaction (dPCR) is a biotechnological refinement of conventional polymerase chain reaction methods, that can be used to directly quantify and clonally amplify nucleic acids strands including DNA, cDNA or RNA. Traditional PCR carries out one reaction per single sample. dPCR carries out a single reaction within a reaction mixture physically separated into many partitions such that each partition contains ideally

one molecule at most. After multiple PCR amplification cycles, the partitions are checked for amplification with a binary readout of "0" or "1".

[0005] Droplet digital PCR (ddPCR), also referred to as emulsion PCR (ePCR), is the most common kind of dPCR. In ddPCR the PCR solution is divided into smaller reactions through a water oil emulsion technique, which are then made to run PCR individually. The PCR sample is partitioned into nanoliter-size samples and encapsulated into oil droplets. The oil droplets are made using a droplet generator that applies a vacuum to each of the wells. Approximately 20,000 oil droplets are made from each 20 μ L sample. In ddPCR the aqueous phase comprises one or more species of a polynucleotide templates, beads, enzymes, salts, buffers, and oligonucleotide primers, for amplifying said template.

[0006] ddPCR is useful for studying variations in gene sequences, such as copy number variants and point mutations and is used for molecular diagnostics applications.

[0007] ddPCR is described in a number of prior art documents such as WO 2012/149042 and WO 2014/149480.

[0008] Other kinds of dPCR technologies known in the art use micro well plates or chips (e.g. Formulatrix technology, also called chip-based dPCR), capillaries, and arrays of miniaturized chambers with nucleic acid binding surfaces to physically partition the reaction mixture or sample, respectively. Comparing to emulsion-based dPCR, Formulatrix technology has the capacity of high throughput, high multiplexing with 6-channel detection and fully automated workflow. However, it requires special plate for physical separation single molecule from individual samples. It is also limited by the number of partition chambers. For example, the current limitation of Formulatrix is 496 chambers for a single plate well.

[0009] However, afore-mentioned dPCR is limited by the process used to generate the large number of partitions required, which is an instrument-dependent process. As a consequence dPCR used in the art is characterized by high costs and requires high technical skills.

[0010] Against this background it is an object underlying the invention to provide a method for digital quantification of individual sample molecules where the above-described disadvantages of dPCR are avoided.

[0011] The present invention satisfies these and other needs.

SUMMARY OF THE INVENTION

[0012] The present invention provides a method for digital quantification of individual sample molecules *in vitro* without physically partitioning the reaction mixture, comprising the following steps:

- a. Amplification of individual sample templates in a reaction mixture to form individual continuous DNA strand particles each consisting of multiple concatemers of the original template;
- b. Labeling of the continuous DNA strands with a detectable marker;
- c. Quantification by counting individual labeled continuous DNA strands.

[0013] The inventors have developed a new method involving dPCR-like technology, however avoiding a physically partitioning of the reaction mixture such as the need of water oil emulsion technique or micro well chips and the disadvantages associated therewith. Therefore the new method is strictly delimited against dPCR used so far, and, in particular, against droplet digital PCR (ddPCR).

[0014] "Digital quantification" as used herein refers to a process that quantifies nucleic acids copy numbers without the use of standard curves by means of a digital amplification method, such as digital PCR (dPCR). Therefore, "digital quantification" allows an absolute quantification of nucleic acids copy number.

[0015] "Sample molecules" or "individual sample templates" as used herein refers to any kind of nuclei acid molecules contained in a sample to be analyzed which can be subjected to dPCR, such as single-stranded or double-stranded DNA and/or RNA.

[0016] "Without physically partitioning the reaction mixture" as used herein means that the amplification processes on the individual sample templates are carried out within the reaction mixture without providing physical barriers separating an individual sample template

amplification from another, e.g. by means of aqueous droplets dispersed in oil, or by means of a micro well chamber. In the method according to the invention the individual amplification processes on the individual sample templates run in the same reaction mixture in parallel without requiring the creation of physically separated reaction partitions.

[0017] "Reaction mixture" as used herein refers to the solution constituting the reaction environment. A reaction mixture according to the invention includes any solution known in the art allowing an amplification of nuclei acid molecules.

[0018] The "amplification" of the individual sample templates refers to any kind of nucleic acid amplification method which results in the generation of multiple concatemers of the original template and the subsequent or simultaneous formation of individual continuous DNA strand particles.

[0019] "Continuous DNA strand particles" refers to a continuous DNA strand which intramolecularly forms secondary structures resulting in dense structures visible by imaging technologies such as microscopy. Continuous DNA strand particles may have average diameters of approx. 10 nm – 1 μ m, preferably approx. 100 nm - 800 nm, highly preferably approx. 300-400 nm. Continuous DNA strand particles include so-called DNA "nanoballs" or "rolonies", the latter if said particles result from rolling circle amplification (RCA).

[0020] The "labeling" of the continuous DNA strands can be realized by associating or incorporating any kind of marker detectable by conventional imaging techniques, e.g. a fluorescent marker.

[0021] The "quantification" as used herein can be realized by any kind of method allowing the counting of the individual labeled continuous DNA strands, such as a flow cytometry.

[0022] The object underlying the invention is herewith completely solved. The method according to the invention is instrument-independent and does not require a physically partitioning of the reaction mixture, e.g. by means of an emulsion-based process, or by means of a micro well chamber. Thereby the method according to the invention allows the elimination of an expensive and labor-intensive step used in many dPCR systems.

[0023] Just as for dPCR used so far which has many potential applications, in addition to clonal amplification, the method according to the invention can be applied for detection and quantification of low-level pathogens, rare genetic sequences, copy number variations, and relative gene expression in single cells, etc.

[0024] In an embodiment the method according to the invention is emulsion-free.

[0025] "Emulsion-free" refers to an amplification process not requiring a water oil emulsion technique to provide in the reaction mixture partitions in form of aqueous droplets dispersed in oil for individual amplification compartments. "Emulsion" generally refers to a reaction process, typically created by vigorously shaking or stirring a "water in oil" mix, to thereby applying sufficient input energy for generating a multitude of micron-sized aqueous compartments. Emulsions comprise aqueous compartments in a continuous oil phase (water-in-oil emulsion).

[0026] With this embodiment the disadvantages of droplet digital PCR (ddPCR) can be effectively avoided.

[0027] In an embodiment of the present invention said reaction mixture is an aqueous solution.

[0028] This measure creates a prerequisite for carrying out the method according to the invention *in vitro* (as e.g. as distinguished from carrying it out *in situ*). It allows the quantification of any kind of individual sample template. As used herein an "aqueous solution" is a solution in which the solvent is water.

[0029] In an embodiment of the present invention said individual sample templates is a circular single-stranded DNA template.

[0030] This measure has the advantage that the amplification of circular single-stranded DNA template can easily produce continuous DNA strand particles consisting of multiple concatemers of the original template, e.g. by applying rolling circle amplification (RCA).

[0031] According to the invention, the provision of a circular single-stranded DNA template can be realized by numerous ways. Preparation of a circular single-stranded DNA

template may include the circularization of dsDNA, ssDNA or RNA template, reverse transcriptase on circular RNA, single-stranded template ligation, double-stranded template ligation, the purification of nucleic acid circles from samples or the provision of synthetic nucleic acid circles.

[0032] In a variant, the template preparation comprises the purification of preexisting circular templates such as, but not limited to, natural biological nucleic acid circles, for example, virus DNA, plasmids, etc.

[0033] In a variant, preexisting circular RNA can be reverse transcribed by any reverse transcriptases to generate single-stranded DNA as a template for subsequent circularization (single-stranded ligation).

[0034] In a variant, the double-stranded DNA template can be denatured by heat or chemical process (like sodium hydroxide, but not limited to sodium hydroxide) to generate single-stranded DNA as a template for subsequent circularization (single-stranded ligation).

[0035] In another variant, a single-stranded DNA or RNA template may be provided with a 5' end phosphorylation for ligation. The phosphorylation treatment can be either by enzyme, like T4 polynucleotide kinase, or pre-phosphorylation through the last step of PCR amplification to add universal adaptors (amplifying with phosphorylated primer).

[0036] In a variant, single-strand template ligation can be carried out with either single-stranded DNA ligase (e.g. CircLigase ssDNA ligase from Epicentre) or double-stranded DNA ligase (e.g. T4 DNA ligase). When using double-stranded DNA ligase, a guide oligo is applied in the process of denaturation and ligation (Figure 2).

[0037] In another variant, double-strand template ligation can be applied with double-stranded DNA ligase (e.g. T4 DNA ligase) with either blunt ends or sticky ends. The double-stranded circles can be further treated with nicking enzymes to generate nicks across the circle templates, and then the nicked fragments can be removed by the enzymes with exonuclease activities. For example, but not limited to, the following enzymes are appropriate, namely T5 exonuclease, T7 exonuclease, DNA polymerases with proof-reading activities (3' to 5' exonuclease activity). Example polymerases can be, but are not limited to, Phi29 DNA polymerase, T4 DNA polymerase, T7 DNA polymerase, Phusion DNA polymerase, Q5 DNA polymerase.

[0038] In a variant, the circled single-stranded DNA fragments are selected/purified by exonuclease treatments (e.g. Exo I and/or Exo III) and column (QIAquick column, QIAGEN, Hilden, German) or beads-based purification processes.

[0039] In an embodiment of the present invention said amplification is carried out by means of isothermal amplification, preferably by rolling circle amplification (RCA).

[0040] Isothermal amplification enables rapid and specific amplification of DNA at constant temperature (e.g. 60-65 °C) avoiding the requirement of thermal cycling. Said measure has the advantage that an established method is used, in particular whenever the amplification of a circular single-stranded DNA template is envisaged with the intention to generate a continuous DNA strand consisting of multiple concatemers of the original template.

[0041] Rolling circle amplification ("RCA") is a type of isothermal amplification. The RCA products, also called "rolonies", are concatemers containing tens to thousands of tandem repeats that are complementary to the circular template. The power, simplicity, and versatility of the isothermal amplification technique have made it an attractive tool for biomedical research and nanobiotechnology. The long RCA products can reach approximately 1 μm size, each of which appearing as bright objects (about 100-fold brighter than the free probes in solution) upon fluorescent probe hybridization. These objects are individually counted either by flow cytometry or image cytometry. Rolonies may be sequenced using sequencing-by-synthesis (SBS) and/or sequencing-by-ligation (SBL) as described in International Patent Application Publication No. WO2011/044437.

[0042] The amplification process used in the invention resulting in the "rolonies" as referred to herein may be designated digital rolony or "dRolony". "dRolony", therefore, is a process for amplification, detection and quantification of nucleic acid molecules based on rolony structure without emulsion process.

[0043] RCA can be performed within a few minutes (for example, 10 minutes) or a couple of hours (for example, 4 hours). The typical sizes of rolonies are around 300-400 nm diameter which depends on amplification time, buffers and enzymes (Reads on Self-Assembling DNA Nanoarrays Human Genome Sequencing Using Unchained Base. Radoje Drmanac et al. (2010), Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. Science 327(5961), p. 78-81; Mingyan Xu (2013), Next-Generation Sequencing for Biomedical Applications. University of New Mexico UNM Digital

Repository: Electronic Theses and Dissertations. The size of the colonies are within the detection range of many instruments for rapid imaging, for example, but not limited to, microfluid detector, sequencer, flow cytometer, image cytometer or microplate fluorescence reader.

[0044] In another embodiment of the present invention said amplification is involving the use of a DNA polymerase with strong strand displacement activity.

[0045] This measure has the advantage that the provision of a single stranded DNA amplicon or, when using RCA, a colony is facilitated. Examples of DNA polymerases with strong strand displacement activity include, but are not limited to, phi 29 DNA polymerase, Bst DNA polymerase (large fragment), SensiPhi DNA polymerase, RNA polymerase for the case with RNA as a template.

[0046] In another embodiment of the present invention said amplification is involving one or more primers specifically hybridizing to the circular single-stranded DNA templates.

[0047] This measure allows the appropriate generation of said continuous DNA strands or colonies, respectively. In principle, when using RCA only a single primer is required. In an embodiment, more than one amplification primer can be used for amplification or RCA, respectively. If multiple primers bind to the targets at same direction (or same strand), linear RCA products are generated (rolling replication of short DNA circles. See Andrew Fire et al. (1995), Rolling replication of short DNA circles, Proc. Natl. Acad. Sci. 92(19), p. 4641-4645. If multiple primers bind to the targets at different direction (or different strands, for example, complementary strands), hyperbranched RCA products are generated (Mutation detection and single-molecule counting using isothermal rolling-circle amplification. See Paul Lizardi et al. (1998), Mutation detection and single-molecule counting using isothermal rolling-circle amplification, Nat. Genet. 19(3), p. 225-232.

[0048] In another embodiment of the invention said detectable marker is selected from the group consisting of: sequence-specific hybridization probe, non-specific hybridization probe, optical probe including fluorescent dyes, quantum dot, or magnetic probe including magnetic beads.

[0049] These measures allow an appropriate labeling of the continuous DNA strands with a detectable marker.

[0050] In another embodiment of the present invention said amplification and said labeling can occur simultaneously (real-time labeling) or consecutively (post labeling).

[0051] This measure provides preferred technical alternatives for an appropriate labeling.

[0052] In some embodiments, colony fluorescence labels can be non-specific by one of the following approaches, but not limited to the approaches. 1) Non-specific DNA binding dyes can be used during amplification or after amplification. For example, the following intercalating dyes can be applied, SYBR Green I, SYBR Green II, EvaGreen, etc. 2) Fluorescently labeled nucleotide can be used during RCA amplification to incorporate non-specific labeling. 3) Aminoallyl nucleotide, such as aminoallyl-dUTP, can be incorporated during RCA amplification. The resulting amine-containing DNA can be subsequently labeled with any amine-reactive fluorescent dye. 4) Modified fluorescent labeled nucleotide, for example, aha-dNTP (Thermo Fisher) can be incorporated during RCA amplification.

[0053] In some embodiments, colony fluorescence labels can be target-specific by one of the following approaches, but not limited to the approaches. 1) Dual labeled probes, such as TaqMan probe, MGB probe, LNA probe. 2) Single labeled probes, oligo fragment only labeled with fluorescent dye without quencher. If the detection probe is incorporated during post amplification process, denature at higher temperature (for example, more than 80°C) and then annealing at lower temperature (for example, lower than the T_m of the fluorescent probe) process is required.

[0054] In some embodiments, similar to fluorescent probe hybridization, nanoparticles including gold nanoparticles (AuNPs), magnetic beads, and quantum dots can be incorporated into colonies through complementary oligonucleotides to visualize and detect RCA products.

[0055] In some embodiments, multiplex colony detection can be applied for quantification. Target-specific probes with different fluorescent labels can be incorporated during amplification or after amplification.

[0056] In another embodiment of the present invention a secondary structure of said individual continuous DNA strand particles is stabilized intrinsically via incorporation of modified nucleotides during amplification, preferably of aminoallyl nucleotides. In another embodiment of the present invention a secondary structure of individual continuous DNA strand particles is stabilized extrinsically via additives in buffer, including hexamminecobalt (III) chloride.

[0057] These measures have the advantage that a shearing damage during handling and manipulation of the continuous DNA strand particles or colonies, respectively, is effectively reduced or even prevented. This allows sample transferring, passing through detection instrument, hydrodynamic focusing in a flow cytometer etc.

[0058] In order to increase the formation of secondary structures, the following further approaches can be applied: 1) incorporation of secondary structure during sample preparation (adaptor designed with enriched secondary structures); 2) amplification buffer and storage buffer of colonies can be optimized and adjusted to increase the secondary structure formation. The buffer can include, but not limited to, high salt (for example, PBS buffer), high magnesium, etc.

[0059] According to another embodiment of the invention said quantification by counting individual labeled continuous DNA strands involves a hydrodynamically focused stream of said individual continuous DNA strand particles and the consecutive detection of the latter.

[0060] By this measure an effective quantification by counting individual labeled continuous DNA strands is ensured.

[0061] In another embodiment of the invention said quantification by counting individual labeled continuous DNA strands involves a microfluidic microplate-based fluorescence reader, for example, the platform developed by Formulatrix (Bedford, MA).

[0062] This measures makes use of an particularly appropriate detection system and, therefore, ensures an effective quantification.

[0063] In another embodiment of the invention said quantification by counting individual labeled continuous DNA strands involves flow cytometry, preferably said flow cytometry involves a fluorescent system or electrical impedance.

[0064] Flow cytometry is a powerful tool because it allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. This makes the invention a rapid and quantitative method for analysis and purification of particles in solution.

[0065] A fluorescent system, for example, a flow cytometer instrument, typically consists of three core systems: fluidics, optics, and electronics. The fluidics system includes a flow cell, where the sample fluid is injected. The optics system consists of various filters, light detectors, and the light source, which is usually a laser line producing a single wavelength of light at a specific frequency. This is where the particles are passed through at least one laser beam. Lasers are available at different wavelengths and power levels (photon output/time). A detector in front of the light beam measures forward scatter light signals (FSC) and detectors to the side measure side scatter light signals (SSC). These light signals are converted by the electronics system to data that can be visualized and analyzed by software. Three critical parameters are measured by the instruments: FSC, SSC, and fluorescence emission signals. Both FSC and SSC measurements are influenced by multiple factors from samples and instrument settings.

[0066] In a fluorescent system, for example, a flow cytometer instrument, forward scattered light is most commonly used to detect the size of the object in the light path. Larger objects, producing more forward scattered light than smaller objects, will have a stronger forward scatter signal. Side scattered light passes from the illumination source into the flow channel, is refracted by cells in a direction that is outside of the original light path. Side-scattered light is usually used to make a determination regarding the granularity and complexity of the particle in the light path. Highly granular cells with a large amount of internal complexity, will produce more side-scattered light, and with a higher side-scatter signal.

[0067] A fluorescent system can have a wide selection of fluorophores; for example, FITC, PerCP, APC, PE, Cy5.5, Alexa Fluors, and more. Each type of fluorescent dye or label has its own characteristic excitation and emission spectrum which is important for designing flow cytometry experiments and optimization to detect and quantify colony objects.

[0068] A fluorescent system, for example, a flow cytometer instrument, can detect particles with the size typically from 0.1 μm to 40 μm . With recent development of the technologies, the type of small particles now being detected and evaluated by flow cytometry is increasing. Small particles can include platelets, which are typically 2-3 μm in diameter, bacteria, which can range from 0.3 μm -5 μm and cellular extravesicles, which can be further split into apoptotic bodies, microvesicles and exosomes, with the smallest being exosomes which are as little as 50 nm in diameter.

[0069] A fluorescent system, such as a flow cytometer instrument, can be applied with software for quantitative analysis of colonies populations detected by the system.

[0070] Impedance flow cytometry Impedance-based single cell analysis systems are commonly known as Coulter counters. They represent a well-established method for counting and sizing virtually any kind of cells and particles. The label-free technology has recently been enhanced by a "lab-on-a-chip" based approach and by applying high frequency alternating current (AC) in the radio frequency range (from 100 kHz to 30 MHz) instead of a static direct current (DC) or low frequency AC field. This technology allows a highly accurate cell analysis and provides additional information like membrane capacitance and viability. The relatively small size and robustness allow battery powered on-site use in the field.

[0071] In a further embodiment of the present invention said optical scanning is realized by imaging cytometry.

[0072] This measure provides an appropriate technical means to carry out step (c) of the method according to the invention.

[0073] Another subject-matter of the present invention is a kit for carrying out the method of any of claims 1-14, comprising

- reagents for generating individual continuous DNA strand particles each consisting of multiple concatemers of individual sample templates,
- reagents for labeling of the continuous DNA strands with a detectable marker, and

- a manual for carrying out the method of any of claims 1-14,

and, preferably, further comprising
- reagents for generating circular single-stranded DNA templates; and/or
- reagents for removing or reducing non-circular single-stranded DNA templates to minimize the background noises from non-specific amplification; and/or
- reagents for isothermal amplification based on the circular single-stranded templates, including DNA polymerases; and/or
- reagents for enhancing an isothermal amplification; and/or
- reagents for incorporation detection marker into amplified continuous DNA strands for detection and quantification; and/or
- reagents for stabilizing secondary structures of individual continuous DNA strand particles.

[0074] The features, characteristics, advantages and embodiments disclosed for the method according to the invention apply likewise to the kit according to the invention.

[0075] A kit is a combination of individual elements useful for carrying out the method of the invention, wherein the elements are optimized for use together in the methods. The kit may also contain additional reagents, chemicals, buffers, reaction vials etc. which may be useful for carrying out the method according to the invention. Such a kit unifies all essential elements required to work the method according to the invention, thus minimizing the risk of errors. Therefore, such kits also allow semi-skilled laboratory staff to perform the method according to the invention.

[0076] According to the invention, the above reagents include, but are not limited thereto, DNA polymerases with strong strand displacement activity such as phi 29 DNA polymerase, Bst DNA polymerase (large fragment), SensiPhi DNA polymerase, RNA polymerase; ssDNA and/or dsDNA ligases (e.g. CircLigase ssDNA ligase, T4 DNA ligase); reverse transcriptases; kinases such as T4 polynucleotide kinase; nicking enzymes;

exonucleases such as T5 exonuclease, T7 exonuclease; DNA polymerases with proof-reading activities (3' to 5' exonuclease activity) such as Phi29 DNA polymerase, T4 DNA polymerase, T7 DNA polymerase, Phusion DNA polymerase, Q5 DNA polymerase; buffers for afore-mentioned components.

[0077] It is to be understood that the before-mentioned features and those to be mentioned in the following cannot only be used in the combination indicated in the respective case, but also in other combinations or in an isolated manner without departing from the scope of the invention.

[0078] The invention is now described and explained in further detail by referring to the following non-limiting examples and drawings.

Figure 1: Rolony based digital detection: workflow comparison of ddPCR vs. the method according to the invention (dRolony)

Figure 2: Circularization based on guide oligo: hybridization schema of circle template and guide oligo.

Figure 3: Real-time detection probe incorporation into rolony. Example data from Bio-Rad C1000 Touch thermocycler.

Figure 4: Example of rolonies detected by a flow cytometer.

EMBODIMENTS

Overview

[0079] This disclosure describes, in one aspect, a method for digital amplification and detection for single molecule without physically partitioning the reaction mixture such as in droplet digital PCR (ddPCR). It is based on isothermal amplification, rolling circle amplification (RCA). Each DNA fragment is circled by a ligation process or purified from sample or synthetic process. Each circle is considered a single template within a sample, and is amplified by the RCA process. Target-specific fluorescent probe (more than one probe can be applied) or non-specific single-stranded DNA or double-stranded DNA fluorescent dye can be incorporated during amplification (real-time incorporation) or after amplification

(post hybridization). The amplified product, called rolonies, can be detected and quantified via fluorescence (e.g. flow cytometer, image cytometer).

[0080] Generally, the following steps may be carried out: (i) template preparation: circularization of dsDNA, ssDNA or RNA template or purification circles from samples or synthetic circles; (ii) signal amplification: RCA amplification of circled template with the incorporation of fluorescent dye or probe; (iii) signal detection via fluorescence (e.g. flow cytometer, image cytometer).

[0081] Comparing to the conventional dPCR such as ddPCR technology, the method addressed here has the advantages of an instrument-independent partition process, low costs and implementation simplicity.

[0082] In some embodiments of either aspect, the DNA fragment for digital detection is amplified by rolling circle amplification to generate rolonies. Just as to conventional dPCR which has many potential applications, in addition to clonal amplification, this rolony-based digital amplification and detection can be applied for detection and quantification of low-level pathogens, rare genetic sequences, copy number variations, and relative gene expression in single cells, etc.

[0083] The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

[0084] Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention.

Comparison of invention (dRolon) vs. ddPCR

[0085] The workflow difference comparison between the method according to the invention exemplified by dRolony vs. ddPCR is shown in Figure 1 and Table 1.

[0086] For the sample of molecules (either single-stranded or double-stranded), it requires an emulsion process to separate individual nanoparticles for ddPCR. However, for dRolony, each single-stranded circle molecule is a template for rolling circle amplification (RCA). This step is independent from an emulsion process. RCA is an isothermal amplification which only requires single primer. The amplified products, rolonies, can be incorporated with fluorescent probes, nucleotides or dyes, similar to dPCR, which can be detected and quantified by a fluorescent detection instrument.

Steps	ddPCR	dRolony
1. Sample preparation	DNA purification, fragmentation	DNA purification, fragmentation
2. Sample partition	Emulsion, require special instrument to generate nanoliter-sized droplets.	Circularization, do not require special instrument. Requirement: guide oligo, ligase, T4 Polynucleotide Kinase, buffers.
3. Amplification	PCR, two oligos with target-specific fluorescent probe, e.g. TaqMan probe.	Isothermal amplification by RCA, single primer with detection fluorescence probe, or dye (single-stranded DNA binding dye or double-stranded DNA binding dye) or fluorescent-labeled nucleotides, etc. Advantage: linear amplification with low error rate.
4. Detection	Fluorescence based detection system	Fluorescence based detection system. e.g. flow cytometer

Table 1: Comparison ddPCR vs. dRolony

dRolony detailed workflow

[0087] Certain embodiments pertain to methods of performing rolling circle amplification (RCA) on polynucleotides to produce concatemers. For example, linear or branched RCA amplifies circular single-stranded DNA by polymerase extension of a complementary primer. This process generates concatemerized copies of the circular DNA

template such that multiple copies of a DNA sequence are arranged end to end in a tandem repeat.

[0088] Generally, there are three steps for carrying out dRolony.

1) Sample preparation.

[0089] In some embodiments, the samples for dRolony can be any kinds of samples for ddPCR. It includes single-stranded or double-stranded DNA, and RNA.

[0090] In some embodiments, the samples can be fragmented by either mechanical or enzymatic process.

[0091] In some embodiments, the samples can be ligated or constructed with adaptors at both ends, similar to NGS library construction. With this kind of structure, universal RCA amplification primer and guide oligo can be designed for the sample library.

2) Circularization.

[0092] In some embodiments, the circled single-stranded templates are generated through one of the following approaches: synthetic oligos, sample purification, reverse transcriptase on circular RNA, single-stranded template ligation, double-stranded template ligation.

[0093] In some embodiments, the samples which can be purified to generate circle templates can include, but not limited to, the natural biological circles, for example, DNA virus, plasmids, etc.

[0094] In some embodiments, circular RNA can be reverse transcribed by any reverse transcriptases to generate single-stranded DNA as a template for circularization (single-stranded ligation).

[0095] In some embodiments, the double-stranded DNA template can be denatured by heat or chemical process (like sodium hydroxide, but not limited to sodium

hydroxide) to generate single-stranded DNA as a template for circularization (single-stranded ligation).

[0096] In some embodiments, the single-stranded DNA or RNA template may be with 5' end phosphorylation for ligation. The phosphorylation treatment can be either by enzyme, like T4 polynucleotide kinase, or pre-phosphorylation through the last step of PCR amplification to add universal adaptors (amplifying with phosphorylated primer).

[0097] In some embodiments, single-strand template ligation can be with either single-stranded DNA ligase (e.g. CircLigase ssDNA ligase from Epicentre) or double-stranded DNA ligase (e.g. T4 DNA ligase). When using double-stranded DNA ligase, guide oligo is applied in the process of denature and ligation (Figure 2).

[0098] In some embodiments, double-strand template ligation can be applied with double-stranded DNA ligase (e.g. T4 DNA ligase) with either blunt ends or sticky ends. The double-stranded circles can be further treated with nicking enzymes to generate nicks across the circle templates, and then the nicked fragments can be removed by the enzymes with exonuclease activities. For example, but not limited to, the following enzymes may be used, T5 exonuclease, T7 exonuclease, DNA polymerases with proof-reading activities (3' to 5' exonuclease activity). Example polymerases can be, but are not limited to, phi 29 DNA polymerase, T4 DNA polymerase, T7 DNA polymerase, Phusion DNA polymerase, Q5 DNA polymerase.

[0099] In some embodiments, the circled single-stranded DNA fragments are selected/purified by exonuclease treatments (e.g. Exo I and/or Exo III) and column (QIAquick column, QIAGEN, Hilden, German) or a beads-based purification process.

3) Amplification by RCA isothermal process.

[00100] The circled single-stranded DNA can be amplified through RCA process by the enzymes with strong strand displacement activities, for example, but not limited to, phi 29 DNA polymerase, Bst DNA polymerase (large fragment), SensiPhi DNA polymerase, RNA polymerase for the case with RNA as a template.

[00101] In some embodiments, more than one amplification primers can be used for rolling circle amplification. If multiple primers bind to the targets at the same direction (or

same strand), linear RCA products are generated (Rolling replication of short DNA circles. Andrew Fire et al. (1995). Proc. Natl. Acad. Sci, loc. cit.). If multiple primers bind to the targets at a different direction (or different strands, for example, complementary strands), hyperbranched RCA products are generated (Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Paul Lizardi et al. (1998), Nat. Genet., loc. cit.).

[00102] In some embodiments, colony fluorescence labels can be incorporated during amplification (real-time incorporation) or after amplification (post hybridization).

[00103] In some embodiments, colony fluorescence labels can be non-specific by one of the following approaches, but not limited to the approaches. 1) Non-specific DNA binding dyes can be used during amplification or after amplification. For example, the following intercalating dyes can be applied, SYBR Green I, SYBR Green II, EvaGreen, etc. 2) Fluorescently labeled nucleotide can be used during RCA amplification to incorporate non-specific labeling. 3) Aminoallyl nucleotide, such as aminoallyl-dUTP, can be incorporated during RCA amplification. The resulting amine-containing DNA can be subsequently labeled with any amine-reactive fluorescent dye. 4) modified fluorescent labeled nucleotide, for example, aha-dNTP (Thermo Fisher) can be incorporated during RCA amplification.

[00104] In some embodiments, colony fluorescence labels can be target-specific by one of the following approaches, but not limited to the approaches. 1) Dual labeled probes, such as TaqMan probe, MGB probe, LNA probe. 2) Single labeled probes, oligo fragment only labeled with fluorescent dye without quencher. If the detection probe is incorporated during post amplification process, denaturation at a higher temperature (for example, more than 80°C) and then the annealing at lower temperature (for example, lower than the T_m of the fluorescent probe) is required.

[00105] In some embodiments, similar to fluorescent probe hybridization, nanoparticles including gold nanoparticles (AuNPs), magnetic beads, and quantum dots can be incorporated into colonies through complementary oligonucleotides to visualize and detect RCA products.

[00106] In some embodiments, multiplex colony detection can be applied for quantification. Target-specific probes with different fluorescent labels can be incorporated during amplification or after amplification.

[00107] In some embodiments, RCA amplification can be performed within a few minutes (for example, 10 minutes) or a couple of hours (for example, 4 hours). The typical size of colonies are around 300-400 nm diameter which depends on amplification time, buffers and enzymes (Reads on Self-Assembling DNA Nanoarrays Human Genome Sequencing Using Unchained Base. Radoje Drmanac et al. Science 2009, loc. cit.; Next-Generation Sequencing for Biomedical Applications. Mingyan Xu, 2013, loc. cit.). The size of the colonies are within the detection range of many instruments for rapid imaging, for example, but not limited to, microfluid detector, sequencer, flow cytometer, image cytometer.

[00108] In some embodiments, in order to prevent shearing damage during colony manipulation (sample transferring, passing through detection instrument, hydrodynamic focusing in a flow cytometer, etc.), tighter nanoball structure is preferred in some cases, which can be enhanced by increasing secondary structures of colonies. In order to increase the secondary structures, the following approaches can be applied: 1) incorporate secondary structure during sample preparation (adaptor designed with enriched secondary structures); 2) amplification buffer and storage buffer of colonies can be optimized and adjusted to increase the secondary structure formation. The buffer can include, but not limited to, high salt (for example, PBS buffer), high magnesium, etc.

Detection by a fluorescent instrument

[00109] There are many instruments for fluorescent detections on particles, for example, flow cytometer, image cytometer. Originally developed in the late 1960s, flow cytometry is a powerful tool because it allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. This makes it a rapid and quantitative method for analysis and purification of particles in solution.

[00110] In some embodiments, a fluorescent system, for example, a flow cytometer instrument, typically consists of three core systems: fluidics, optics, and electronics. The fluidics system includes a flow cell, where the sample fluid is injected. The optics system consists of various filters, light detectors, and the light source, which is usually a laser line producing a single wavelength of light at a specific frequency. This is where the particles are passed through at least one laser beam. Lasers are available at different wavelengths and power levels (photon output/time). A detector in front of the light beam measures forward scatter light signals (FSC) and detectors to the side measure side scatter light signals (SSC). These light signals are converted by the electronics system to data that can be visualized and analyzed by software. Three critical parameters are measured by the instruments:

forward light scatter (FSC), side light scatter (SSC), and fluorescence emission signals. Both FSC and SSC measurements are influenced by multiple factors from samples and instrument settings.

[00111] In some embodiments, a fluorescent system, for example, a flow cytometer instrument, forward scattered light is most commonly used to detect the size of the object in the light path. Larger objects, producing more forward scattered light than smaller objects, will have a stronger forward scatter signal. Side scattered light passes from the illumination source into the flow channel, is refracted by cells in a direction that is outside of the original light path. Side-scattered light is usually used to make a determination regarding the granularity and complexity of the particle in the light path. Highly granular cells with a large amount of internal complexity will produce more side-scattered light, and with a higher side-scatter signal.

[00112] In some embodiments, a fluorescent system can have a wide selection of fluorophores; for example, FITC, PerCP, APC, PE, Cy5.5, Alexa Fluors, and more. Each type of fluorescent dye or label has its own characteristic excitation and emission spectrum which is important for designing flow cytometry experiments and optimization to detect and quantify colony objects.

[00113] In some embodiments, a fluorescent system, for example, a flow cytometer instrument, can detect particles with the size typically from 0.1 μm to 40 μm . With recent development of the technologies, the type of small particles now being detected and evaluated by flow cytometry is increasing. Small particles can include platelets, which are typically 2-3 μm in diameter, bacteria, which can range from 0.3 μm -5 μm and cellular extravesicles, which can be further split into apoptotic bodies, microvesicles and exosomes, with the smallest being exosomes which are as little as 50 nm in diameter.

[00114] In some embodiments, a fluorescent system, for example, a microplate reader, can be applied for quantitative detection on colonies.

[00115] In some embodiments, a fluorescent system can be applied with software for quantitative analysis of colonies populations detected by the system.

EXAMPLES

Example 1: dRolony protocol without fluorescent incorporation during amplification

An example of dRolony preparation can be performed with the following process.

1. Prepare Solution #1 with the following components: Mix input fragmented DNA (for example, 5-40ng, with prephosphorylation at 5' ends) with guide oligo (150 pmol) in 1X TE buffer with a volume as 50 μ L, annealing the guide oligo in a PCR tube at a thermocycler: 95°C for 3min, 4°C for 3 minutes.
2. Prepare solution #2: 2 μ L 120U/ μ L T4 DNA ligase (Enzymatics, Beverly, MA), 10 μ L T4 ligase buffer in a total volume of 50 μ L. For the DNA input without prephosphorylation, 2 μ L 10U/ μ L T4 Polynucleotide Kinase (Enzymatics, Beverly, MA) can be included.
3. Add Solution #2 to Solution #1, mix well, incubate at 25°C for 10 minutes.
4. Add 2 μ L 20U/ μ L Exo I and 2 μ L 100U/ μ L Exo III (Enzymatics, Beverly, MA), mix well, 37°C for 60 minutes. And then the sample is purified by QIAquick column (QIAGEN, Hilden, Germany), eluted in a volume of 50 μ L (circled template).
5. Prepare RCA amplification mix as following: 50 μ L circled template, 1 μ L 10U/ μ L phi 29 DNA polymerase in a total volume as 100 μ L with 0.4mM dNTP mix, 20nM amplification primer, 1X Phi29 buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM (NH₄)₂SO₄, 4 mM DTT, pH 7.5 @ 25°C). Mix well, set up reaction at 30°C for 30 minutes to 6 hours depending on needs. The reaction can be stopped by either heat inactivation (45°C for 5 minutes) or 5 μ L of 0.5M EDTA.

[00116] In some embodiments, with prephosphorylated library, the circularization efficiency can reach more than 90%.

Example 2: dRolony protocol with fluorescent incorporation during amplification

[00117] As an example of real-time incorporation of fluorescent detection probe, the dual labeled DNA probe (5' labeled with FAM, 3' labeled with black hole quencher) 500nM was added to step 5 amplification mix in Example 1. The reaction was set up in a real-time thermocycler (Bio-Rad C1000 Touch) at 30°C for 4hrs. An example data is shown in Figure 3

(data acquired at 2 minutes/cycle). The methods, disclosed here, can be applied for RCA amplification optimization and quality control.

Example 3: dRolony detection by a flow cytometer instrument

[00118] An example of detection of rolony by a flow cytometer is listed here.

[00119] The rolonies, generated by a protocol as Example 1, was diluted by PBS buffer to a concentration as 2.5ng/ μ L. In a volume of 100uL, detection probe (5' labeled Cy3 probe) was hybridized to the rolonies in a concentration as 60 nM at 75°C for 5 minutes.

[00120] The samples were then detected by Guava flow cytometer following the user's manual.

[00121] An example result is shown in Figure 4, which indicates the feasibility of using existing instrument, like flow cytometer, for rolonies based detection and quantification.

CLAIMS

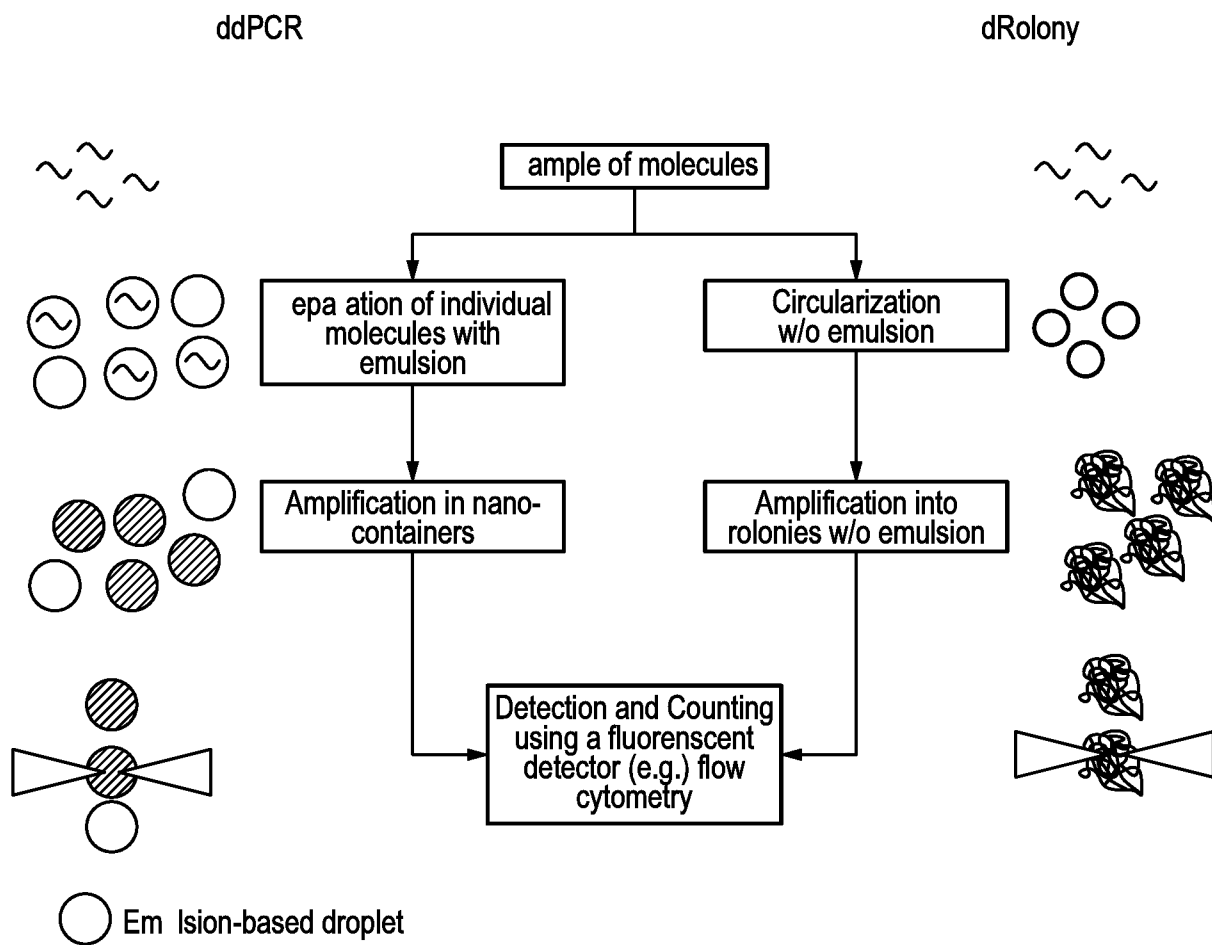
1. A method for digital quantification of individual sample molecules *in vitro* without physically partitioning the reaction mixture, comprising the following steps:
 - a. Amplification of individual sample templates in a reaction mixture to form individual continuous DNA strand particles each consisting of multiple concatemers of the original template;
 - b. Labeling of the continuous DNA strands with a detectable marker;
 - c. Quantification by counting individual labeled continuous DNA strands.
2. The method of claim 1, which is emulsion-free.
3. The method of claim 1, wherein said reaction mixture is an aqueous solution.
4. The method of any of claims 1-3, wherein said individual sample templates is a circular single-stranded DNA template.
5. The method of claim 4, wherein said amplification is carried out by means of isothermal amplification, preferably by rolling circle amplification (RCA).
6. The method of any of claims 1-5, wherein said amplification is involving the use of a DNA polymerase with strong strand displacement activity.
7. The method of any of claims 1-6, wherein said amplification is involving one or more primers specifically hybridizing to the circular single-stranded DNA templates.
8. The method of any of claims 1-7, wherein said detectable marker is selected from the group consisting of: sequence-specific hybridization probes, non-specific hybridization probes, optical probes including fluorescent dyes, quantum dots, or magnetic probes including magnetic beads.
9. The method of any of claims 1-8, wherein said amplification and said labeling can occur simultaneously (real-time labeling) or consecutively (post labeling).
10. The method of any of claims 1-9, wherein a secondary structure of said individual continuous DNA strand particles is stabilized intrinsically via incorporation of modified nucleotides during amplification, preferably of aminoallyl nucleotides, and/or wherein a

secondary structure of individual continuous DNA strand particles is stabilized extrinsically via additives in buffer, including hexamminecobalt (III) chloride.

11. The method of any of claims 1-10, wherein said quantification by counting individual labeled continuous DNA strands involves a hydrodynamically focused stream of said individual continuous DNA strand particles and the consecutive detection of the latter.
12. The method of claim 11, wherein said quantification by counting individual labeled continuous DNA strands involves flow cytometry, preferably said flow cytometry involves fluorescence or electrical impedance.
13. The method of any of claims 1-12, wherein said quantification by counting individual labeled continuous DNA strands involves an immobilization of said individual continuous DNA strand particles on a surface and a subsequent optical scanning.
14. The method of claim 13, wherein said optical scanning is realized by imaging cytometry.
15. A kit for carrying out the method of any of claims 1-14, comprising:
 - reagents for generating individual continuous DNA strand particles each consisting of multiple concatemers of individual sample templates,
 - reagents for labeling of the continuous DNA strands with a detectable marker, and
 - a manual for carrying out the method of any of claims 1-14,and, preferably, further comprising
 - reagents for generating circular single-stranded DNA templates; and/or
 - reagents for removing or reducing non-circular single-stranded DNA templates to minimize the background noises from non-specific amplification; and/or
 - reagents for isothermal amplification based on the circular single-stranded templates, including DNA polymerases; and/or
 - reagents for enhancing an isothermal amplification; and/or

- reagents for incorporation detection marker into amplified continuous DNA strands for detection and quantification; and/or
- reagents for stabilizing secondary structures of individual continuous DNA strand particles.

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**Fig. 1**

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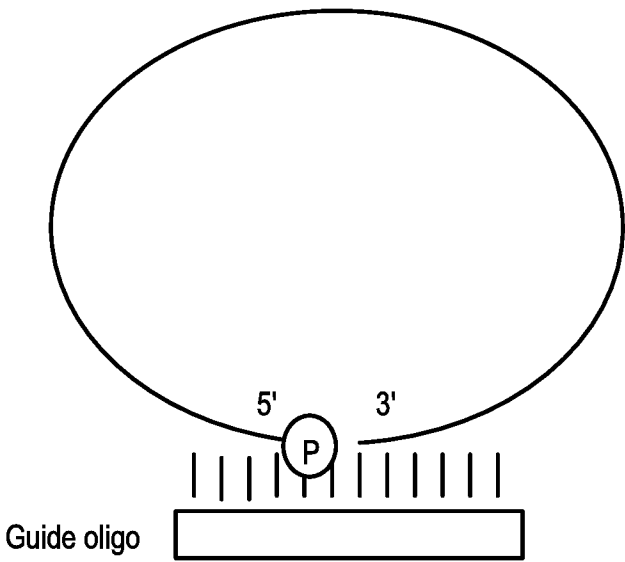


Fig. 2

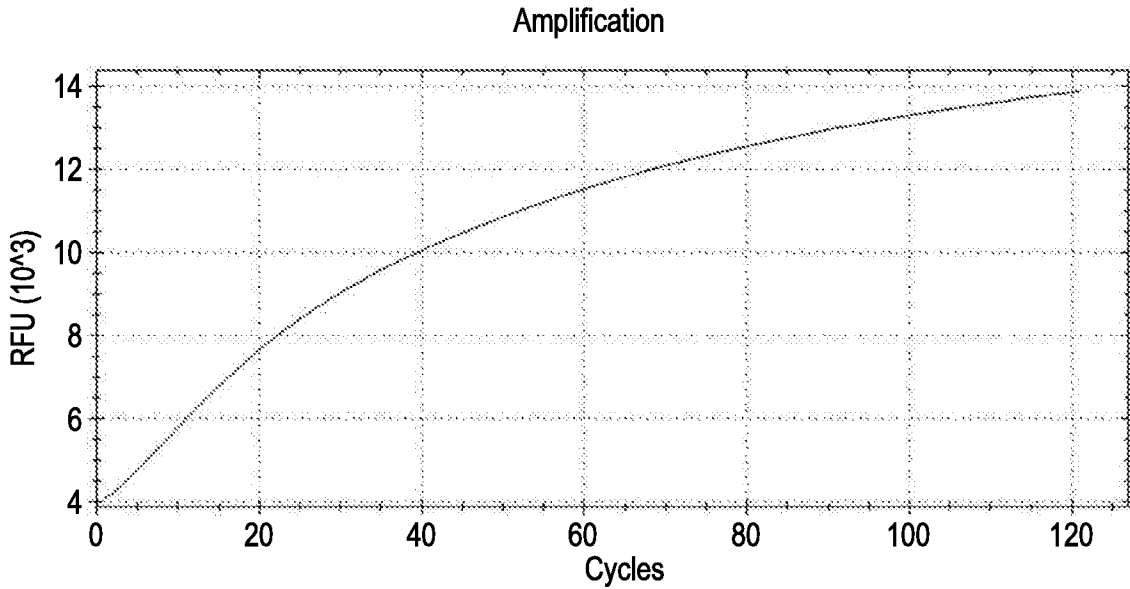
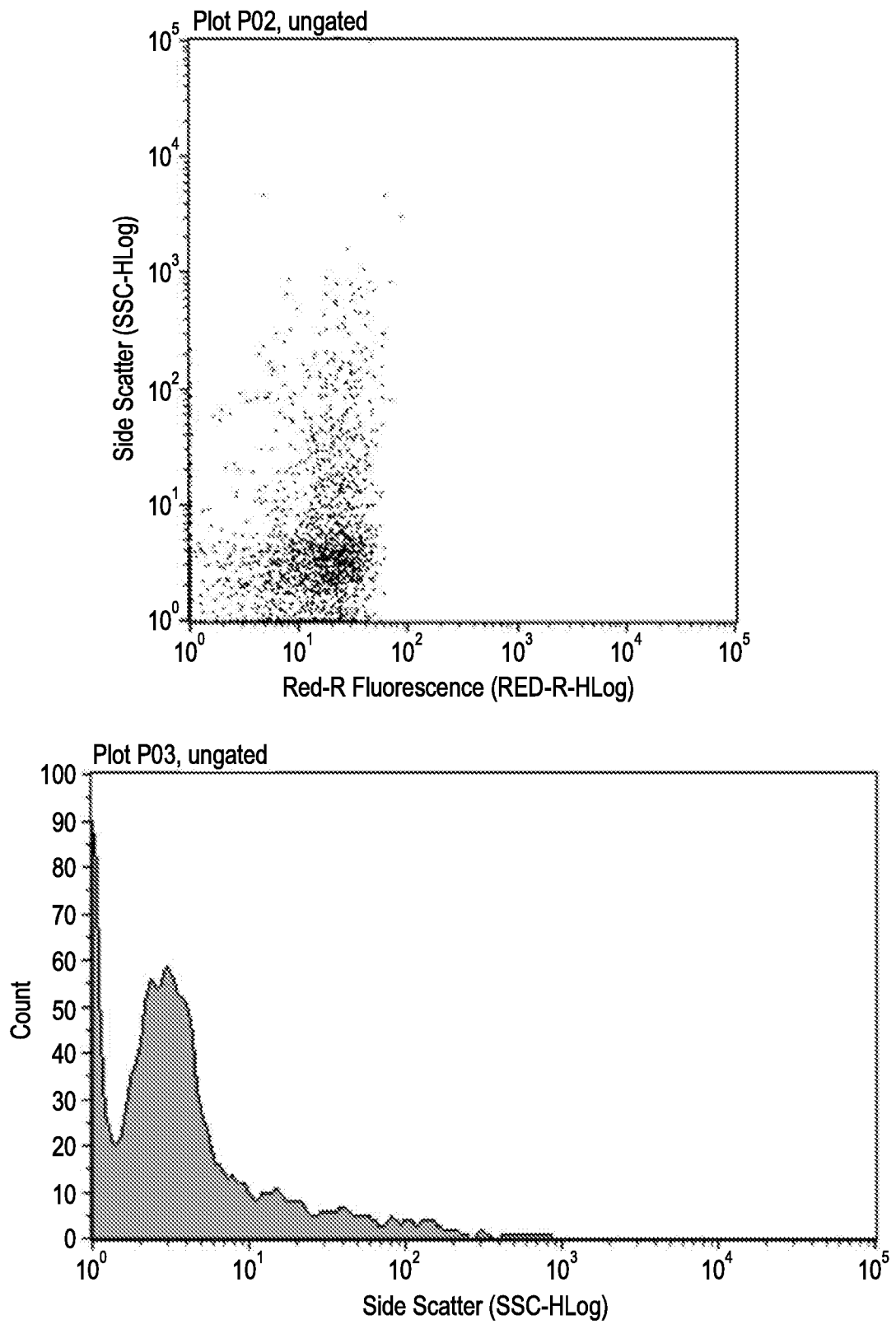


Fig. 3

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**Fig. 4**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/13955

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12Q 1/6844; C12Q 1/6851; C12Q 1/6876; C12Q 1/68 (2020.01)

CPC - C12Q 1/6844; C12Q 1/6851; C12Q 1/6876

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)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JARVIUS et al. Digital quantification using amplified single-molecule detection. Nat Methods, September 2006, Vol 3, No 9, Pages 725-727 [including online Supplementary Methods]. Especially pg 725 fig 1, pg 725 col 2 para 2, online Supplementary Methods PDF pg 4 para 3 continued to pg 5 para 1.	1-5
A	US 2016/0108458 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 21 April 2016 (21.04.2016). Especially abstract, claims 1 and 19.	1-5

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 March 2020

Date of mailing of the international search report

16 APR 2020

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/13955

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.