(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 November 2012 (08.11.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/151268 A1

(51) International Patent Classification:
G01N 33/543 (2006.01)

(21) International Application Number:
PCT/US2012/036105

(22) International Filing Date:
2 May 2012 (02.05.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/481,604 2 May 2011 (02.05.2011) US

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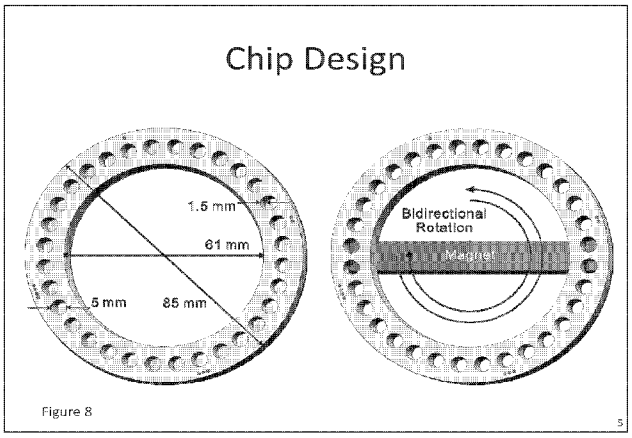
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title**: METHOD AND SYSTEM FOR HIGH THROUGHPUT OPTICAL AND LABEL FREE DETECTION OF ANALYTES



(57) **Abstract**: The invention provides a high throughput method and apparatus to simultaneously detect or determine the presence or amount of a polymeric analyte in multiple samples, which employ magnetic particles and subjects the sample and the magnetic particles to forms of energy that induce aggregate formation.

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METHOD AND SYSTEM FOR HIGH THROUGHPUT OPTICAL AND LABEL FREE DETECTION OF ANALYTES

Cross-Reference to Related Applications

This application claims the benefit of the filing date of U.S. application Serial No. 61/481,604, filed on May 2, 2011, the disclosure of which is incorporated by reference herein.

Background

Polymeric analytes can be detected using methods, such as chromatography, electrophoresis, binding assays, spectrophotometry, and the like. DNA detection, for instance, may require expensive, bulky optics for either absorbance-based techniques or intercalating-dye fluorescence based techniques. Although DNA concentration has routinely been detected spectrometrically by measuring absorbance ratio of a sample at 260/280 nm, the method suffers from poor sensitivity at low concentrations of DNA.

Other methods for DNA detection include DNA binding to a fluorescence dye and detecting the fluorescence using a fluorometer. Examples of such a dye are PicoGreen®, which is commercially available through Invitrogen (Carlsbad, CA) (see Ahn et al., Nucl. Acids Res., 24:2623 (1996); Vitzthum et al., Anal. Biochem., 276:59 (1999), and dyes disclosed in U.S. Patent Nos. 6,664,047; 5,582,977 and 5,321,130. Additional DNA quantification methods based on fluorescence have been developed and include oligonucleotide hybridization (Sanchez et al., J. Clin. Microbiol., 40:2381 (2002)) and real-time quantitative PCR (Heid et al., Genome Res., 6:986 (1996)). While highly sensitive, fluorometer-based methods are generally cumbersome, requiring reagent preparation and handling and a special fluorometer for exciting and measuring fluoro-emission.

Haque et al. (BMC Biotech., 3:20 (2003)) compared three popular DNA quantification methods with regard to accuracy: OD_{260}/OD_{280} (OD), PicoGreen® double stranded DNA (PG), and detection of fluorescent signal from a 5' exonuclease assay (quantitative genomic method (QG), based on the TaqMan® assay). Their exhaustive analysis, involving nearly 15,000 measurements, revealed that OD measurement was the most precise and least biased method for estimating DNA concentration. Among the benefits of that method are the relatively wide availability of absorbance spectrophotometers in contrast to fluorometers, that OD measurement does not consume sample or additional reagents, and that no time is required for incubation or reaction time, as is the case with a fluorophore. On the other hand, a large amount of sample is needed for OD measurement, and this method does not discriminate between single stranded and double stranded DNA (as PG does) (Singer et al., Anal. Biochem., 249:228 (1997)) or contaminating DNA (as the sequence specific QG method does). In addition, the presence of protein, RNA and salt can lead to an overestimate of DNA concentration from OD measurements.

Among the benefits of fluorometric methods are the use of very small sample volumes due to the high sensitivity of the methods and that fluorescence detection is easily implemented in microdevices. However, some reagents are not compatible with fluorescence based DNA quantification due to signal quenching.

Summary of the Invention

The invention provides a high throughput, optical and label-free detection technology based on aggregation of particles, e.g., magnetic particle (bead) aggregation, in the presence of polymeric molecules, such as DNA in biological samples, including those that have been subject to an amplification reaction, which are in a multi-well container (sample holder), and an energy source that induces particle aggregation in the wells, such as a bi-directional rotating magnetic field (RMF), thereby forming aggregates that can be detected and/or quantified. For example, magnetic beads useful in the apparatus and methods of the invention aggregate in a magnetic field through induced magnetic dipole and disaggregate due to loss of magnetic dipole after removal of the magnetic field. In one embodiment, bi-directional RMF and a circular arrangement of microwells on a chip allow for all the samples in the microwells to be exposed to the same RMF environment. The direction of RMF is reversed periodically, which drives the beads to migrate from one side of microwell to the other and distributes the beads evenly. The shape of the sample holder and/or position of the wells in the sample holder are selected to be employed with a magnet that provides for the same magnetic field in all wells, e.g., at the same time. That arrangement allows for simultaneous detection and quantitation of a polymeric analyte, e.g., DNA, or cells, in multiple samples, and significantly reduces the time required for analysis. For instance, a circular sample holder may have about 32 wells in a circular pattern, and aggregation of beads with samples having DNA may be observed in less than 5 minutes using bi-directional RMF. However, other magnetic fields may be employed. For example, a magnet may be moved back and forth over or under a multi-well container with wells in a linear conformation, including a single row of wells or multiple rows of wells, or an electromagnetic field can change direction periodically over or under a multi-well container with wells in a linear conformation.

As described hereinbelow, a plurality of individual mixtures of samples and particles is placed into wells of a multi-well container, e.g., one formed of glass, polymeric materials such as plastic, ceramic or other materials that do not substantially interact with the sample and/or alter the properties of the beads. In one embodiment, the magnetic beads are about 1 micron to about 10 microns in diameter, and may be modified, e.g., with silica or with molecules such as ssDNA, antibodies or apatamers that are useful to detect specific targets. In one embodiment, the wells are in a circular formation, near the outer edge of ring-like container, or may be in a linear formation, or any other formation that allows for exposure of a plurality of wells having samples and beads to be subjected to the same magnetic field. In one embodiment, the outer diameter of a circular container may be about 25 mm to about 100 mm, however, other outer diameters are envisioned. For containers with a ring configuration, in one embodiment, the outer diameter of the container may be about 25 mm to about 100 mm, and the inner diameter may be about 15 mm to about 90 mm, although other outer and inner diameters are envisioned. The outer diameter of a container useful in the apparatus and methods of the invention may be related to the size, shape and/or strength of the magnet, and/or the magnetic field. In one embodiment, the wells of the multi-well container may have a diameter of about 3 mm to about 7 mm and a depth of about 0.5 mm to about 5 mm, or any other dimensions that can accommodate a volume of about 10 µL to about 100 µL. Containers and wells of larger or smaller dimensions, e.g., those that can accommodate tens or hundreds of samples or smaller or larger volumes, may be employed. The container having the samples and the

particles is then subjected to a form of energy, e.g., bi-directional RMF, that induces aggregation of the particles and polymeric analyte in each well of the container, e.g., each sample is exposed to the same conditions and the aggregates are distributed evenly. In one embodiment, the RMF is reversed when substantially all of the beads in substantially all of the wells are on one side of the well, which is a function of the well diameter and the speed of the beads. The speed of the beads depends on the mass, size and magnetic property of the beads, the strength and direction of the magnetic field, and the viscosity of the sample, and in some embodiments its rotating speed. Then the presence or amount of the aggregates is detected, for instance, using image analysis. The method thus provides for simultaneous detection and quantitation of multiple samples, and so reduces the time required for analysis of those samples, e.g., in less than about 10 minutes. In addition, the sample holder may be configured to allow for sample preparation such as dilution using centrifugal force or any other applicable forces.

In one embodiment, under concentrated chaotropic salt conditions, e.g., salts such as guanidine hydrochloride, guanidine thiocyanate, ammonium perchlorate and the like, the aggregation of beads is specific for the presence of DNA and/or RNA (nucleic acid), such as amplified nucleic acid, in a sample and that formation is not inhibited by the presence of other cellular components, for example protein at orders of magnitude higher concentrations that greatly exceed that of the nucleic acid, e.g., DNA. In one embodiment, amplification of nucleic acid, e.g., amplification of phage or viruses in cells of interest that are to be detected, which occurs during phage or virus replication, e.g., during culture with host cells, or amplification of the nucleic acid of interest *in vitro*, such as polymerase chain reaction (PCR) amplification, ligase chain reaction (LCR) amplification or transcription mediated amplification (TMA), prior to induction of aggregate formation. Thus, the method and apparatus are useful to detect and quantify high molecular weight DNA (ss and dsDNA), e.g., genomic DNA, or RNA, or amplified nucleic acid, such as that in the presence of an abundance of protein, under chaotropic conditions.

As described herein, the use of nucleic acid amplification together with aggregate formation provides a different approach to organism detection. In one embodiment, this approach provides for the use of bacteriophage to detect specific organisms, e.g., phage D29 may be employed to detect *Mycobacterium smegmatis*. For example, the specificity of the assay can be increased by measuring the quantitative replication of host-specific bacteriophage following infection of bacteria by the phage, either DNA from the phage or the phage itself will induce aggregation. Aggregation induced by the intact phage improves sensitivity by roughly 100 fold. While aggregate based DNA detection in a chaotrope is generic, specificity and signal amplification in this context is provided by a phage or virus (which in one embodiment only infects one or a few types of bacteria or eukaryotic species) and a signal amplification of at least about 10^2 to about 10^{10}, e.g., about 10^4 to about 10^9, about 10^5 to about 10^8, or about 10^7 (e.g., 100 starting copies of phage may replicate to 10^9 copies in about 4 to about 5 hours). The number of amplification cycles for aggregate formation may be inversely related to the size of the phage genome, e.g., fewer amplification cycles may be needed for larger phage genomes. Moreover, whole phage (at sufficient concentrations) can induce a pinwheel effect at a 75-fold higher sensitivity than that observed for phage DNA alone.

Aggregate formation is not limited to nucleic acid; a positively-charged high molecular weight polysaccharide polymer, chitosan, that electrostatically binds to silica-coated beads under low ionic strength conditions, may also form aggregates when subjected to a rotating external magnetic field or other field that exerts a uniform field on all samples in a multi-well sample holder. Aggregate formation

may be detected visually, which requires minimal footprint or expensive optical equipment, and can be employed to quantify the amount of a polymeric analyte in a sample, such as a complex biological sample, e.g., one having protein, carbohydrates such as polysaccharides, nucleic acid, and/or lipid, or any combination thereof. Aggregate formation may be detected using microscopy, photography, scanners, magnetic sensing and the like.

Thus, the invention provides a method for detecting the presence or amount of a nucleic acid analyte in a plurality of biological samples, each in a well of a multi-well container. The method includes providing a multi-well contained where a plurality of the wells have a mixture having a biological sample and magnetic beads, e.g., from about 1 nm to about 300 micrometers (microns), about 5 nm to about 200 nm, or about 1 micrometer to about 10 micrometers, in diameter, which mixture is under conditions that allow for binding of an analyte in the sample to the beads. In one embodiment, the beads include a paramagnetic metal. The mixture is subjected to energy, e.g., a bi-directional rotating magnetic field or other magnetic field or magnet that subjects each sample to the same magnetic field, and the presence or amount of aggregates in each mixture is detected or determined. In one embodiment, aggregates are isolated from the mixture, thereby isolating the analyte. For example, the aggregates may be magnetically isolated. In one embodiment, after aggregate formation is detected or determined, in the absence of contact with a magnet or the rotating magnetic field (e.g., the field is turned off) or other applied energy, the aqueous solution in the mixture having the aggregates is removed and an elution buffer is added to form a second mixture having the aggregates, and the molecules bound to the beads eluted (isolated).

Thus, the invention also provides a method to isolate an analyte, e.g., from a complex sample. The method includes contacting the sample with magnetic beads in a solution, such as an aqueous solution, under conditions that allow for binding of the analyte to the beads so as to form a mixture. The mixture is subjecting to a rotating magnetic field or a magnet that results in aggregation of the beads having the bound analyte but not other molecules in the complex sample. For example, for a cellular sample where nucleic acid is the analyte for isolation, aggregation of the beads isolates the nucleic acid from other cellular components such as proteins, lipids, carbohydrates and the like. The cellular debris can be removed by removing the solution from the aggregate containing mixture and the nucleic acid can be eluted by adding a buffer, e.g., a Tris-EDTA containing buffer, to the aggregates, and the analyte containing buffer collected.

In one embodiment, the method for detecting the presence or amount of a polymeric analyte in a plurality of samples employs magnetic beads but not a bi-directional rotating magnetic field. In this embodiment, the samples are in a multi-well container where the wells are in linear alignment and a magnet moves back and forth parallel to the alignment of the wells or the direction of an electromagnetic field is changed periodically. In one embodiment, the sample is a complex biological sample. Aggregate formation is then detected or determined.

Thus, the invention provides a quantitative method, e.g., for nucleic acids under chaotropic conditions. Unlike methods that purify an analyte, such as DNA, before quantitation, methods described herein allow for quantitation without prior purification. Moreover, the quantitative behavior of particle, e.g., bead, aggregation can be used to directly determine the concentration of nucleated cells in whole blood. Based on the average mass of DNA contained within a WBC, the DNA quantitation values obtained from the aggregation results can be used to back-calculate the number of WBC in whole blood samples. In

one embodiment, concentrations as low as about 3 to about 10 pg/μL of nucleic acid in a sample may be detected. Smaller particles, e.g., beads, of about 5 microns (μm) may be useful in detecting and/or quantitating nucleic acid of about 1,000 to about 5,000 base pairs in length.

In one embodiment, the invention provides a method for detecting the presence or amount of a pathogen, e.g., a virus, fungus (e.g., yeast) or bacteria, in a plurality of samples. In one embodiment, samples, e.g., a physiological sample such as blood, serum, plasma, cerebrospinal fluid, tissue sample, nasal swab and the like, suspected of having a pathogen are subjected to nucleic acid amplification of pathogen-specific sequences, for instance, using the polymerase chain reaction, so as to yield pathogen-specific fragments of greater than about 1,000 bp in length, e.g., greater than about 3,000 bp up to 6,000 bp, in length. Only 6 to 7 amplification cycles, which cycles may be completed in about 10 minutes, may yield sufficient DNA for aggregate formation, using beads with a diameter, for instance, of about 8 microns. In one embodiment, at least about 1000 copies of the amplified viral genome, for instance, obtained after about 10 minutes of *in vitro* nucleic acid amplification, may be detected by aggregate formation

In one embodiment, the invention provides a method for detecting the presence or amount of a specific bacterium or eukaryotic cell. In one embodiment, samples suspected of having a bacterium, are contacted with phage that infect the bacterium. After phage replication, the phage containing supernatants are contacted with beads and subjected to conditions that result in aggregate formation. The method may detect as few as 10 bacterial cells in a sample.

The method may be applied to quantifying nucleic acids undergoing amplification, for instance, rolling circle amplification and whole genome amplification, where the products have higher molecular weights than products produced using some other nucleic amplification methods, such as the polymerase chain reaction. In one embodiment, the method is sensitive to about 20 human cells in 20 microliters of solution.

The quantification method may also be applied to non-nucleic acid polymeric analytes, such as the polysaccharide chitosan under non-chaotropic conditions. Under these conditions, the negatively charged silica bead surface is electrostatically attracted to the cationic chitosan (protonated amine) under low ionic strength conditions at physiological pH. The method may be altered to include fluorescently labeled magnetic beads or measurements of the magnetic susceptibility of the aggregates, to increase the sensitivity of the assay. .

The aggregation may be visually detected with the naked eye and/or quantified by image analysis. Moreover, the opaque nature of the aggregated particles makes the transition very easy to monitor optically, and simple image analysis techniques can be used to extract quantitative information. This may be accomplished with a camera and routine image processing software. The combination of high sensitivity and simplicity of the method provides in one embodiment a label-free approach to DNA or RNA detection and/or quantification, and thereby nucleic acid containing cell quantification. The observable effect for nucleic acid is also quite robust even in the presence of proteins and lipids at concentrations typically encountered in biological samples. The methods of the invention may have specific advantages for automated assays in microfluidic platforms.

For example, the stark differences in optical contrast of images in the absence and presence of DNA allows for the use simple digital image processing to define a quantitative relationship between the mass of DNA and the extent of particle (e.g., bead) aggregation. This relationship was determined via an

algorithm based on the gray value of the digital image. A threshold gray level is set such that dispersed beads and clusters are counted as "dark," whereas areas in the image cleared of beads are counted as "bright." The number of dark pixels in the image is then used as a measure of aggregation, with 100% dark area representing a sample without aggregation, whereas low dark area percentages correspond to nearly complete aggregation.

Brief Description of the Figures

Figure 1. HeLa cells were mixed with MagnaSil™ paramagnetic particles and imaging used to determine the normalized percent of dark area in the sample.

Figure 2. (A) Photograph of a blood sample analyzed by the pinwheel assay. The pinwheel results are given as an average (± SD) for an n=3 with image processing involving 5 photographs shot over 30 seconds. The pictures are analyzed using ImageJ v1.41. For each picture, a threshold value is set automatically by isodata algorithm, which defines pixels representing the particles, and then the number of these pixels is counted. (B) A graph of the percent of the dark area. Values are normalized by that of a negative control (no DNA) as a function of the amount of DNA or cell.

Figure 3. Blood samples analyzed by the pinwheel assay and by Coulter Counter cell count. The pinwheel results are given as an average (± SD) for an n = 3 with image processing involving 5 photographs shot over 30 seconds. (A) Bar graph of WBC per μL in three samples detected by the two methods. The results show that the pinwheel assay can be used to determine cell number. (B) The pinwheel effect can be utilized to define the concentration of DNA directly from blood samples. Using normalized percentage of 'dark' pixels with constant volume (3.5 nL) the comparison of three different human blood samples was accomplished. Different concentrations for each of the samples correlated with measurement of DNA via the conventional method (panel A). The inset shows the result of diluting each sample to equalize the number of nucleated cells (white blood cells-WBCs) per microliter in each sample. The results are displayed as the normalized percentage of 'dark' pixels with increasing amount of human blood (scaled by DNA amount).

Figure 4. (A) A negative control in a pinwheel assay. (B) A positive control in a pinwheel assay of purified phage DNA. (C) Correlation between the percent dark area and DNA in a pinwheel assay of purified phage DNA.

Figure 5. Quantitation of phage using a pinwheel assay. (A) Graph of percent dark area versus number of phage. (B) Graph of percent dark area versus phage concentration.

Figure 6. Detection of bacteria using phage. Graph of percent dark area versus percent of phage used to infect 100 bacterial cells.

Figure 7. Exemplary system to detect aggregation of magnetic beads in multiple samples subjected to a bi-directional RMF. Reversing switch controls direction of RMF.

Figure 8. Exemplary multi-well sample holder (container) showing one embodiment of dimensions of the container and wells. In this embodiment, the circular design and proper alignment (e.g., center of the holder overlaps with the center of the RMF) ensure identical RMF environment for all samples.

Figure 9. Images of bead migration in the absence of a polymeric analyte in a bi-directional RMF. For instance, for beads that travel from one side of the microwell to the center in 5 seconds, and migrate

from one side of the well to the other in 20 seconds, RMF is reversed every 20 seconds and measurements (dark area) taken every 5 seconds after the reversal.

Figure 10. Images of bead migration in the presence of DNA in a bi-directional RMF. The RMF is reversed every 20 seconds after mixing the DNA and beads, and measurements taken every 5 seconds after the reversal. Bi-directional RMF is as effective as unidirectional RMF in inducing aggregation.

Figure 11. Comparison of two samples with beads exposed to bi-directional RMF, one with DNA and the other without DNA. Aggregation occurred within two minutes of bi-directional RMF application.

Figure 12. Simultaneous detection of a polymeric analyte (DNA). A) Image of three DNA samples mixed with beads at t = 0 and at t = 5 minutes in a multi-well container with 27 samples. B) Image of the 27 samples.

Figure 13. Standard curve for DNA quantitation generated from the 27 samples in Figure 12. Down to 3 pg/uL can be detected for lambda DNA, which is comparable to the pinwheel protocol and conventional fluorescence spectroscopy.

Figure 14. Other embodiments of a magnetic field to induce aggregate formation in multiple samples simultaneously. A) A magnet moves back and forth between the two dashed lines, driving the beads moving in the well. B) An electromagnetic field changing the direction periodically.

Detailed Description of the Invention

Definitions

A "detectable moiety" is a label molecule attached to, or synthesized as part of, a solid substrate for use in the methods of the invention. These detectable moieties include but are not limited to radioisotopes, colorimetric, fluorometric or chemiluminescent molecules, enzymes, haptens, redox-active electron transfer moieties such as transition metal complexes, metal labels such as silver or gold particles, or even unique oligonucleotide sequences.

As used herein, the terms "label" refers to a marker that may be detected by photonic, electronic, opto-electronic, magnetic, gravimetric, acoustic, enzymatic, magnetic, paramagnetic, or other physical or chemical means. The term "labeled" refers to incorporation of such a marker, e.g., by incorporation of a radiolabeled molecule or attachment to a solid substrate that may be suspended in solution such as a bead.

A "biological sample" can be obtained from an organism, e.g., it can be a physiological fluid or tissue sample, such as one from a human patient, a laboratory mammal such as a mouse, rat, pig, monkey or other member of the primate family, by drawing a blood sample, sputum sample, spinal fluid sample, a urine sample, a rectal swab, a peri-rectal swab, a nasal swab, a throat swab, or a culture of such a sample, or from a plant or a culture of plant cells. Thus, biological samples include, but are not limited to, whole blood or components thereof, blood or components thereof, blood or components thereof, semen, cell lysates, saliva, tears, urine, fecal material, sweat, buccal, skin, cerebrospinal fluid, and hair. In one embodiment, the biological sample comprises cells.

"Analyte" or "target analyte" is a substance to be detected in a biological sample such as a physiological sample using the present invention. "Polymeric analyte" as used herein refers to macromolecules that are made up of repeating structural units that may or may not be identical. The polymeric analyte can include biopolymers or non-biopolymers. Biopolymers include, but are not limited

to, nucleic acids (such as DNA or RNA), proteins, polypeptides, polysaccharides (such as starch, glycogen, cellulose, or chitin), and lipids

"Capture moiety" is a specific binding member, capable of binding another molecule (a ligand), which moiety or its ligand may be directly or indirectly attached through covalent or noncovalent interactions to a substrate (bead). When the interaction of the two species produces a non-covalently bound complex, the binding which occurs may be the result of electrostatic interactions, hydrogen-bonding, or lipophilic interactions. The term "ligand" refers to any organic compound for which a receptor or other binding molecule naturally exists or can be prepared. Binding pairs useful as capture moieties and ligands include, but are not limited to, complementary nucleic acid sequences capable of forming a stable hybrid under suitable conditions, antibodies and the ligands therefore, enzymes and substrates therefore, receptors and agonists therefore, lectins and carbohydrates, avidin and biotin, streptavidin and biotin, and combinations thereof. In one embodiment, the affinity of a capture moiety and its ligand may be greater than about 10^{-5} M, such as greater than about 10^{-6} M, including greater than about 10^{-8} M and greater than about 10^{-9} M. In embodiment, oligonucleotides having biotin labels are bound to beads coupled to streptavidin.

The term "homology" refers to sequence similarity between two nucleic acid molecules. Homology may be determined by comparing a position in each sequence, which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences.

"Identity" means the degree of sequence relatedness between polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "homology" can be readily calculated by known methods. Suitable computer program methods to determine identity and homology between two sequences include, but are not limited to, the GCG program package (Devereux, et al., Nucleic Acids Research, 12:387 (1984)), BLASTN, and FASTA (Atschul et al., J. Molec. Biol., 215:403 (1990)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul et al., J. Mol. Biol., 215:403 (1990)).

As used herein, the term "amount" is intended to mean the level of a molecule. The term can be used to refer to an absolute amount of a molecule in a sample or relative to a control molecule. For example, when detecting specific sequences, a reference or control amount may be a normal reference level or a disease-state reference level. A normal reference level may be an amount of expression of a biomarker in a non-diseased subject or subjects. A disease-state reference level may be an amount of expression of a biomarker in a subject with a positive diagnosis for the disease or condition.

As used herein, the term "subject" means the subject is a mammal, such as a human, but can also be an animal, e.g., domestic animals (e.g., dogs, cats and the like), farm animals (e.g., cows, sheep, pigs, horses and the like) and laboratory.

A "paramagnetic metal" is a metal with unpaired electrons. Suitable paramagnetic metals include transition elements and lanthanide series inner transition elements. Additional suitable paramagnetic metals include, e.g., Yttrium (Y), Molybdenum (Mo), Technetium (Tc), Ruthenium (Ru), Rhodium (Rh), Tungsten (W), and Gold (Au). Additional specific suitable specific paramagnetic metals include, e.g., Y(III), Mo(VI), Tc(IV), Tc(VI), Tc(VII), Ru(III), Rh(III), W(VI), Au(I), and Au(III).

A lanthanide," "lanthanide series element" or "lanthanide series inner transition element" refers to Cerium (Ce), Praseodymium (Pr), Neodymium (Nd), Promethium (Pm), Samarium (Sm), Europium (Eu), Gadolinium (Gd), Terbium (Tb), Dysprosium (Dy), Holmium (Ho), Erbium (Er), Thulium (Tm), Ytterbium(Yb), or Lutetium (Lu). Specific suitable lanthanides include, e.g., Ce(III), Ce(IV), Pr(III), Nd(III), Pm(III), Sm(II), Sm(III), Eu(II), Eu(III), Gd(III), Tb(III), Dy(III), Ho(III), Er(III), Tm(III), Yb(II), Yb(III), and Lu(III).

Examples of transition metal oxides include, but are not limited to: CrO_2, $COFe_2O_4$, $CuFe_2O_4$, $Dy_3Fe_5O_{12}$, $DyFeO_3$, $ErFeO_3$, $Fe_5Gd_3O_{12}$, $Fe_5HO_3O_{12}$, $FeMnNiO_4$, Fe_2O_3, γ-Fe_3O_4 (magnetite), α-Fe_3O_4 (hematite), $FeLaO_3$, $MgFe_2O_4$, Fe_2MnO_4, MnO_2, $Nd_2O_7Ti_2$, $Al_02Fe_18NiO_4$, $Fe_2Ni_{0.5}O_4Zn_{0.5}$, $Fe_2Ni_{0\cdot4}Zn_{0.6}$, $Fe_2Ni_{0.8}Zn_{0.2}$, NiO, Fe_2NiO_4, $Fe_5O_{12}Sm_3$, $Ag_{0.5}Fe_{12}La_{0.5}O_{19}$, $Fe_5O_{12}Y_3$, and FeO_3Y. Oxides of two or more of the following metal ions can also be used: Al(+3), Ti(+4), V(+3), Mn(+2), Co(+2), Ni(+2), Mo(+5), Pd(+3), Ag(+1), Cd(+2), Gd(+3), Tb(+3), Dy(+3), Er(+3), Tm(+3) and Hg(+1).

As used herein, a "nucleic acid sequence," a "nucleic acid molecule," or "nucleic acids" refers to one or more oligonucleotides or polynucleotides as defined herein. As used herein, a "target nucleic acid molecule" or "target nucleic acid sequence" refers to an oligonucleotide or polynucleotide comprising a sequence that a user of a method of the invention desires to detect in a sample.

The term "polynucleotide" as referred to herein means a single-stranded or double-stranded nucleic acid polymer composed of multiple nucleotides. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single and double stranded forms of DNA.

The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and/or non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset comprising members that are generally single-stranded and have a length of 200 bases or fewer. In certain embodiments, oligonucleotides are 2 to 60 bases in length. In certain embodiments, oligonucleotides are 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 to 40 bases in length. In certain other embodiments, oligonucleotides are 25 or fewer bases in length. Oligonucleotides of the invention may be sense or antisense oligonucleotides with reference to a protein-coding sequence.

The term "naturally occurring nucleotides" includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" includes oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate, phosphoroamidate, and the like. See, e.g., LaPlanche et al., Nucl. Acids Res., 14:9081 (1986); Stec et al., J. Am. Chem. Soc., 106:6077 (1984); Stein et al., Nucl. Acids Res., 16:3209 (1988); Zon et al., Anti-Cancer Drug Design, 6:539 (1991); Zon et al., OLIGONUCLEOTIDES AND ANALOGUES: A PRACTICAL APPROACH, pp. 87-108 (F. Eckstein, Ed.), Oxford University Press, Oxford England (1991); U.S. Patent No. 5,151,510; Uhlmann and Peyman, Chemical Reviews, 90:543 (1990), the

disclosures of which are hereby incorporated by reference for any purpose. An oligonucleotide can include a detectable label to enable detection of the oligonucleotide or hybridization thereof.

The term "highly stringent conditions" refers to those conditions that are designed to permit hybridization of nucleic acid strands whose sequences are highly complementary, and to exclude hybridization of significantly mismatched sequences. Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of "highly stringent conditions" for solution (e.g., without bead aggregation) hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C. or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 50% formamide at 42°C. See Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual (2nd ed., Cold Spring Harbor Laboratory, 1989); Anderson et al., Nucleic Acid Hybridisation: A Practical Approach Ch. 4 (IRL Press Limited).

More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used--however, the rate of hybridization will be affected. Other agents may be included in the solution hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, $NaDodSO_4$, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or another non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4; however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH. See Anderson et al., Nucleic Acid Hybridisation: A Practical Approach Ch. 4 (IRL Press Limited).

Factors affecting the stability of duplexes include base composition, length, and degree of base pair mismatch. Hybridization conditions can be adjusted by one skilled in the art in order to accommodate these variables and allow nucleic acids of different sequence relatedness to form hybrids. For example, the melting temperature of a perfectly matched DNA duplex can be estimated by the following equation: $T_m(°C.)=81.5+16.6(\log[Na+])+0.41(\%\ G+C)-600/N-0.72(\%\ \text{formamide})$ where N is the length of the duplex formed, [Na+] is the molar concentration of the sodium ion in the hybridization or washing solution, % G+C is the percentage of (guanine+cytosine) bases in the hybrid. For imperfectly matched hybrids, the melting temperature is reduced by approximately 1°C for each 1% mismatch.

The term "moderately stringent conditions" refers to conditions under which a duplex with a greater degree of base pair mismatching than could occur under "highly stringent conditions" is able to form. Examples of typical "moderately stringent conditions" in solution are 0.015 M sodium chloride, 0.0015 M sodium citrate at 50-65°C. or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 20% formamide at 37-50°C. By way of example, "moderately stringent conditions" of 50 degree C in 0.015 M sodium ion will allow about a 21% mismatch.

It will be appreciated by those skilled in the art that there is no absolute distinction between "highly stringent conditions" and "moderately stringent conditions." For example, at 0.015 M sodium ion (no formamide), the melting temperature of perfectly matched long DNA is about 71°C. With a wash at 65°C. (at the same ionic strength), this would allow for approximately a 6% mismatch. To capture more

distantly related sequences, one skilled in the art can simply lower the temperature or raise the ionic strength.

A good estimate of the melting temperature in 1M NaCl* for oligonucleotide probes up to about 20 nt is given by: T_m=2°C. per A-T base pair+4°C. per G-C base pair *The sodium ion concentration in 6.times. salt sodium citrate (SSC) is 1M. See Suggs et al., Developmental Biology Using Purified Genes 683 (Brown and Fox, eds., 1981).

High stringency washing conditions for oligonucleotides may be at a temperature of 0-5°C below the Tm of the oligonucleotide, e.g., in 6 x SSC, 0.1% SDS.

Exemplary Methods

Efficient molecular analysis usually requires detecting the presence of an analyte in a very small sample at very low concentration. The use of an external magnetic field in microdevices to implement magnetic bead control has previously been disclosed, e.g., by U.S. Patent Nos. 7,452,726; 6,664,104; 6,632,655; and 6,344,326, PCT/US2010/002883 and PCT/US2009/036983; which are incorporated herein by reference. In one embodiment, the present invention uses magnetic beads in a magnetic field to provide a visual detection of the presence or quantity of a polymeric analyte, such as nucleic acids, lipids, polysaccharides, proteins, etc. The method arises from the observation that when a polymeric analyte binds to the magnetic beads, application of a magnetic field to the beads results in unique (aggregated) formations. Without the presence of the polymeric analyte, the movement and conformation of the magnetic beads induced by the magnetic field (non-aggregated) differs significantly from the aggregated formations. As such, the aggregated formation is specific to the presence of the binding between the polymeric analyte and the magnetic beads and the magnetic field, and therefore, can be used to detect the presence of the analyte. Aggregate formation is not specific for a rotating magnetic field, and may be induced by other means. Aggregate formation in a mixture with a polymeric analyte may be enhanced by applying other forms of energy, e.g., by vibrating the sample.

In one embodiment, the present invention relates to a method for detecting the presence of polymeric analyte in a plurality of samples by contacting the samples with magnetic beads, or another magnetic solid substrate that can be suspended in solution, and exposing the magnetic beads in each of the samples to an identical magnetic field. The presence of aggregates indicates the presence of the bound polymeric analyte. In one embodiment, the magnetic beads are coated or derivatized to specifically bind or to enhance the binding of the polymeric analyte to the magnetic beads. The environment can also be manipulated to enhance the binding of the polymeric analyte to the magnetic beads.

The present invention also relates to a system for detecting the presence of a polymeric analyte in a plurality samples. In one embodiment, the system contains a rotatable magnet, e.g., one mounted on a motor, so that, when activated, the motor rotates the magnet to create a bi-directional rotating magnetic field. The system also contains a detection chamber having a multi-well container with wells containing magnetic beads, located approximately at the center of the magnet, between its north and south poles. The motor is then activated to rotate the magnet around the detection chamber. The presence of aggregates in the wells indicates the presence of the polymeric analyte in the sample.

The method and apparatus of the invention can be added onto already existing assays or apparatuses, especially a micro-total analysis system (μ-TAS), to act as a polymeric analyte detector. For example, the presence of an antibody/antigen reaction may initiate the coupling of nucleic acids and the

presence/absence of the aggregates determines whether the antibody/antigen binding has occurred. This is analogous to an immuno-PCR method, where instead of using PCR and fluorescent probes for the detection of nucleic acids, the aggregates are employed.

The present invention is based on the observation that polymeric analytes, when bound to magnetic beads and in the presence a magnetic field, produce aggregates that allow for qualitative and quantitative analyses. "Pinwheel formation" as used herein refers to a rotating mass having a circular or disc-like cross-section. The mass is made of clumps or aggregates of magnetic beads tethered by a polymeric analyte. When viewed in a still photograph, the pinwheel formation looks like a disc shaped object made of an aggregate of magnetic beads. However, when viewed visually or by imaging, the disc shaped object actually spins around its center axis similar to that of a spinning pinwheel. Within a detection chamber, the pinwheel formations sometimes collide together to form larger pinwheels, and sometimes collide with the wall of the chamber to break up into smaller pinwheels.

In one embodiment, an apparatus for practicing the methods of the present invention includes a rotatable magnet, e.g., one mounted on a motor, and a detection chamber located approximately at the center of the magnet, between its north and south pole. In one embodiment, the apparatus contains a stir plate, having a rotatable magnet therein, and a detection chamber placed at the center of the stir plate. The stir plate has a top cover, on top of which the detection chamber sits. In one embodiment, underneath to top cover sits a magnet having a north pole and a south pole. The magnet may be a U-shaped magnet having its poles at either end of the U, however other magnet shapes may be used, e.g., I-shape or semicircular shape magnets. The magnet may be a motor that is capable of rotating the magnet around its center axis. The magnet may be located directly below the detection chamber, nevertheless other configurations may be used as long as the detection chamber is located approximately between the two poles of the magnet. The magnetic field may be positioned either parallel, orthogonal or at any angle to the detection chamber. The beads move in a defined form, and spin in a distinct direction correlating to the directional rotating of the magnetic field. A rotatable magnet or other devices that can produce a rotating magnetic field may be employed. Such devices may be an electromagnet or electronic circuitry that can produce a rotating magnetic field similar to that produced by the rotating magnet or electromagnetic induction.

The detection chamber maybe placed at approximately the center of the magnet (approximately the center of the magnetic field when the magnet is rotating). The detection chamber may be part of or a component of a microfluidic device or micro-total analysis system (μ-TAS). Generally, a microfluidic device or μ-TAS contains at least one micro-channel. There are many formats, materials, and size scales for constructing μ-TAS. Common μ-TAS devices are disclosed in U.S. Patent Nos. 6,692,700 to Handique et al.; 6,919,046 to O'Connor et al; 6,551,841 to Wilding et al.; 6,630,353 to Parce et al.; 6,620,625 to Wolk et al.; and 6,517,234 to Kopf-Sill et al.; the disclosures of which are incorporated herein by reference. Typically, a μ-TAS device is made up of two or more substrates that are bonded together. Microscale components for processing fluids are disposed on a surface of one or more of the substrates. These microscale components include, but are not limited to, reaction chambers, electrophoresis modules, microchannels, fluid reservoirs, detectors, valves, or mixers. When the substrates are bonded together, the microscale components are enclosed and sandwiched between the substrates. A detection chamber may include a microchannel. At both ends of the microchannel are inlet and outlet ports for

adding and removing samples from the microchannel. The detection chamber may be linked to other microscale components of a μ-TAS as part of an integrated system for analysis.

The wells in the multi-well container in the detection chamber may contain magnetic beads prior to the addition of the sample or the magnetic beads may be added to the wells along with the sample. The magnetic beads may contain a surface that is derivatized or coated with a substance that binds or enhances the binding of the polymeric analyte to the magnetic beads. Some coatings or derivatizations include, but are not limited to, amine-based charge switch, boronic acid, silanization, reverse phase, oligonucleotide, lectin, antibody-antigen, peptide-nucleic acid (PNA)-oligonucleotide, locked nucleic acid (LNA)-oligonucleotide, and avidin-biotin. For example, for the detection of nucleic acid, the magnetic beads can be silica coated to specifically bind nucleic acids when exposed to a high ionic strength, chaotropic buffer. A bead may also be coated with positively charged amines or oligomers for binding with nucleic acids.

To bind carbohydrates, the magnetic beads may contain a boronic acid- modified surface. Boronic acid bonds covalently and specifically to -cis dialcohols, a moiety common in certain carbohydrates including glucose.

To bind lipids, the magnetic beads may be modified with hydrophobic groups, such as benzyl groups, alkanes of various lengths (6-20), or vinyl groups. The lipids are bound to the beads by hydrophobic forces.

To bind proteins, the magnetic beads may contain a protein modified surface. For example, the surface of the beads may be coated with an antibody specific for the protein of interest. For general protein detection, the bead surface may be coated with avidin or biotin and the protein of interest may be derivatized with biotin or avidin. The avidin- biotin binding thus allows the protein to bind to the beads.

In addition to derivatization or coating of the magnetic beads, the physical environment where the polymeric analyte comes into contact with the magnetic beads may also be altered to allow the beads to specifically bind or to enhance the binding of the magnetic beads to the polymeric analyte. For example, a silica coated bead may be manipulated to specifically bind nucleic acid, carbohydrate, or protein depending on the conditions used: binding of DNA occurs in chaotropic salt solution, binding of positively charged carbohydrates occurs in low ionic strength solutions, and binding of proteins occurs under denaturing conditions (in the presence of urea, heat, and the like).

Depending on the concentration of polymeric analyte to be detected, the number of beads may be about 100 to about 10^8, such as about 10^4 to 10^7 for visual detection. Fluorescence detection may allow for a smaller number of beads, e.g., about 10. The higher the concentration of analyte in the sample, the higher the amount of magnetic beads that should be employed.

The magnetic field in the y-axis may have a strength of about 1 to 5,000 gauss, e.g., about 10 to 1000 gauss. Additionally, regardless of the shape of the magnet, the magnetic field component in the y-axis may obtain its maximum strength at the center of rotation and is at its minimum strength at both poles of the magnet. The field component may be maximized along the length of the magnet and may abruptly drop to its minimum at the poles. The field component does not significantly decrease off either side of the magnet. The magnetic field lines at the detection chamber may be parallel to the xy-plane in which the detection chamber lies.

To detect the polymeric analyte in a sample, in one embodiment, the samples and beads are added to the wells in the container and then the container is introduced to the detection chamber. With

the chamber locating at approximately the center of the magnet (between the two poles of the magnet), in one embodiment the magnet is rotated so that the chamber experiences a rotating magnetic field (the rotating magnetic field can also be effected using electronic circuitry rather than a magnet). The magnet may be rotated at about 10 to 10,000 rpm, such as at about 1000 to 3000 rpm or about 100 to about 300 rpm. The average size (diameter) of the aggregates may be proportional to the concentration of polymeric analyte, e.g., nucleic acids, in the sample. A calibration curve may be obtained for correlating the average size of the aggregates to the polymeric analyte concentration. Such a calibration curve may be generated, for example, by subjecting known concentrations of the polymeric analyte to the magnetic field and determining the average size of the aggregates for each concentration.

The presence of aggregates can be detected visually, or using optical or imaging instrumentation. One way to detect aggregates is to photograph or record a video of the detection chamber. This may be accomplished by the image or recording of one chamber at a time or multiple chambers. A computer program can then be used to detect the aggregates in the photograph or video. The program may initially upload and crop the image (photograph or frames of a video) so that only the detection chamber is shown. The cropped image may then converted to gray scale. An extended minima transformation is then performed with a threshold between about 40 to 70 to isolate the magnetic microparticles from the background pixels. Once holes within each object are filled in, each object may then be labeled, e.g., with a separate RGB color. A boundary is then created around each distinct object. For each boundary, a metric $m = 4\pi a/p^2$ is calculated, where a is the area of the object and p is the perimeter of the object. The metric m is a measure of the roundness of the object, for a perfect circle m = l. For each object, if m is greater than about 0.8, such as greater than about 0.95, that object is defined as a pinwheel. A centroid is then plotted over each object having m greater than about 0.8 (a pinwheel). If a photograph is used, the number of aggregates is then counted. If a video is used, the steps are repeated for each frame of the video and the average number of aggregates per frame is calculated. If the number of aggregates or average number of aggregates per frame is greater than a set value from 0.5 to 10 (depending upon the polymeric analyte and bead concentration), the program returns the result that polymeric analyte is present in the sample. See, for example, WO 2009/114709, the disclosure of which is incorporated by reference herein.

For software based automated detection, one possible system contains at least a camera and a computer for running the computer program. In this system, the camera takes pictures or video of the detection chamber and the images from the camera is analyzed by the computer. The computer may be electronically connected to the camera for automatically downloading and processing the images from the camera as discussed above. The automated detection is especially efficient when the detection chamber is part of a μ-TAS where the computer can also be use to control and sense other aspects of the μ-TAS, such as temperature, fluid flow, gating, reaction monitoring, etc.

Particles

Particles useful in the practice of the invention include metal (e.g., gold, silver, copper and platinum), semiconductor (e.g., CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (e.g., ferromagnetite) as colloidal materials, as well ZnS, ZnO, TiO2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs, and silica and polymer (e.g., latex) particles. The particles may have any shape, e.g., spheres (generally referred to as beads) or rods, or irregular shapes, and a population of particles may have particles that vary in shape or size, e.g., beads in a population of beads

may not have a uniform shape or diameter. The size of the particles may be from about 1 nm to about 300 micrometers (μm) (mean diameter for rods or spheres), such as from about 0.5 to about 250 μm, or from about 2 to about 10 μm. The particles may be coated or derivatized with agents, e.g., to enhance binding of a selected analyte. For example, particles may include a silica coating or be derivatized with streptavidin.

In various aspects, the methods provided include those utilizing particles which range in size from about 1 micrometers to about 250 micrometers in mean diameter, about 1 micrometers to about 240 micrometers in mean diameter, about 1 micrometers to about 230 micrometers in mean diameter, about 1 micrometers to about 220 micrometers in mean diameter, about 1 micrometers to about 210 micrometers in mean diameter, about 1 micrometers to about 200 micrometers in mean diameter, about 1 micrometers to about 190 micrometers in mean diameter, about 1 micrometers to about 180 micrometers in mean diameter, about 1 micrometers to about 170 micrometers in mean diameter, about 1 micrometers to about 160 micrometers in mean diameter, about 1 micrometers to about 150 micrometers in mean diameter, about 1 micrometers to about 140 micrometers in mean diameter, about 1 micrometers to about 130 micrometers in mean diameter, about 1 micrometers to about 120 micrometers in mean diameter, about 1 micrometers to about 110 micrometers in mean diameter, about 1 micrometers to about 100 micrometers in mean diameter, about 1 micrometers to about 90 micrometers in mean diameter, about 1 micrometers to about 80 micrometers in mean diameter, about 1 micrometers to about 70 micrometers in mean diameter, about 1 micrometers to about 60 micrometers in mean diameter, about 1 micrometers to about 50 micrometers in mean diameter, about 1 micrometers to about 40 micrometers in mean diameter, about 1 micrometers to about 30 micrometers in mean diameter, or about 1 micrometers to about 20 micrometers in mean diameter, about 1 micrometers to about 10 micrometers in mean diameter. In other aspects, the size of the particles is from about 5 micrometers to about 150 micrometers, from about 5 to about 50 micrometers, from about 10 to about 30 micrometers. The size of the particles is from about 5 micrometers to about 150 micrometers, from about 30 to about 100 micrometers, from about 40 to about 80 micrometers. In one embodiment, the magnetic particle may have an effective diameter of about 0.25 to 50 micrometers, including from about 0.5 to about 1.5 micrometers or from about 3 to about 15 micrometers. The size of the beads may be matched with the expected size of the polymeric analyte, e.g., nucleic acid, being detected. Smaller beads generally form aggregates with shorter polymer analytes and smaller beads may be more sensitive to shorter polymeric analytes. Bead size can be tuned to the specific cutoff in size needed for discrimination, including optical properties or amount surface area that can be derivatized.

In one embodiment, MagneSil particles (Promega Corp, Madison, WI) are employed. MagneSil particles are paramagnetic particles (iron-cored silicon dioxide beads) of about 8 micrometers in average diameter with the overall range of about 4 to about 12 microns in diameter. Those particles can be loaded into wells of a multi-well container and contacted with a sample, e.g., one having DNA, and then subjected to a magnetic field from an external magnet.

Oligonucleotides

Methods of making oligonucleotides of a predetermined sequence are well-known. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed. 1989) and F. Eckstein (ed.) Oligonucleotides and Analogues, 1st Ed. (Oxford University Press, New York, 1991). Solid-phase synthesis methods are contemplated for both oligoribonucleotides and oligodeoxyribonucleotides (the

well-known methods of synthesizing DNA are also useful for synthesizing RNA). Oligoribonucleotides and oligodeoxyribonucleotides can also be prepared enzymatically. Non-naturally occurring nucleobases can be incorporated into the oligonucleotide, as well. See, e.g., Katz, J. Am. Chem. Soc., 74:2238 (1951); Yamane, et al., J. Am. Chem. Soc., 83:2599 (1961); Kosturko, et al., Biochemistry, 13:3949 (1974); Thomas, J. Am. Chem. Soc., 76:6032 (1954); Zhang, et al., J. Am. Chem. Soc., 127:74-75 (2005); and Zimmermann, et al., J. Am. Chem. Soc., 124:13684-13685 (2002).

The term "oligonucleotide" as used herein includes modified forms as discussed herein as well as those otherwise known in the art which are used to regulate gene expression. Likewise, the term "nucleotides" as used herein is interchangeable with modified forms as discussed herein and otherwise known in the art. In certain instances, the art uses the term "nucleobase" which embraces naturally-occurring nucleotides as well as modifications of nucleotides that can be polymerized. Herein, the terms "nucleotides" and "nucleobases" are used interchangeably to embrace the same scope unless otherwise noted.

In various aspects, the methods may employ oligonucleotides which are DNA oligonucleotides, RNA oligonucleotides, or combinations of the two types. Modified forms of oligonucleotides are also contemplated which include those having at least one modified internucleotide linkage. In one embodiment, the oligonucleotide is all or in part a peptide nucleic acid (PNA) or includes LNA (see Koskin et al., Tetrahedron, 54:3607 (1998)). Other modified internucleoside linkages include at least one phosphorothioate linkage. Still other modified oligonucleotides include those comprising one or more universal bases. "Universal base" refers to molecules capable of substituting for binding to any one of A, C, G, T and U in nucleic acids by forming hydrogen bonds without significant structure destabilization. The oligonucleotide incorporated with the universal base analogues is able to function as a probe in hybridization, as a primer in PCR and DNA sequencing. Examples of universal bases include but are not limited to 5'-nitroindole-2'-deoxyriboside, 3-nitropyrrole, inosine, and hypoxanthine.

Modified Backbones. Specific examples of oligonucleotides include those containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are considered to be within the meaning of "oligonucleotide."

Modified oligonucleotide backbones containing a phosphorus atom include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Also contemplated are oligonucleotides having inverted polarity comprising a single 3' to 3' linkage at the 3'-most internucleotide linkage, i.e. a single inverted nucleoside residue which may be abasic (the nucleotide is missing or has a hydroxyl group in place thereof). Salts, mixed salts and free acid forms are also contemplated. Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717;

5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, the disclosures of which are incorporated by reference herein.

Modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages; siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts. See, for example, U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, the disclosures of which are incorporated herein by reference in their entireties.

Modified Sugar and Internucleoside Linkages. In still other embodiments, oligonucleotide mimetics wherein both one or more sugar and/or one or more internucleotide linkage of the nucleotide units are replaced with "non-naturally occurring" groups. In one aspect, this embodiment contemplates a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone. See, for example US Patent Nos. 5,539,082; 5,714,331; and 5,719,262, and Nielsen et al., *Science*, 1991, 254, 1497-1500, the disclosures of which are herein incorporated by reference.

In still other embodiments, oligonucleotides are provided with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and including —CH_2—NH—O—CH_2—, —CH_2—N(CH_3)—O—CH_2—, —CH_2—O—N(CH_3)—CH_2—, —CH_2—N(CH_3)—N(CH_3)—CH_2— and —O—N(CH_3)—CH_2—CH_2— described in US Patent Nos. 5,489,677, and 5,602,240. Also contemplated are oligonucleotides with morpholino backbone structures described in US Patent No. 5,034,506.

In various forms, the linkage between two successive monomers in the oligo consists of 2 to 4, desirably 3, groups/atoms selected from —CH_2—, —O—, —S—, —NR^H—, C=O, C=NR^H, >C=S, —Si(R")$_2$—, —SO—, —S(O)$_2$—, —P(O)$_2$—, —PO(BH_3) —, —P(O,S)—,—P(S)$_2$—, —PO(R")—, —PO(OCH_3) —, and —PO(NHR^H)—, where R^H is selected from hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl. Illustrative examples of such linkages are —CH_2—CH_2—CH_2—, —CH_2—CO—CH_2—, —CH_2—CHOH—CH_2—, —O—CH_2—O—, —O—CH_2—CH_2—, —O—CH_2—CH=(including R^5 when used as a linkage to a succeeding monomer), —CH_2—CH_2—O—, —NR^H—CH_2—CH_2—, —CH_2—CH_2—NR^H—, —CH_2—NR^H—CH_2— -, —O—CH_2—CH_2—NR^H—, —NR^H—CO—O—, —NR^H—CO—NR^H—, —NR^H—CS—NR^H—, —NR^H—C(=NR^H)—NR^H—, —NR^H—CO—CH_2—NR^H—O—CO—O—, —O—CO—CH_2—O—, —O—CH_2—CO—O—, —CH_2—CO—NR^H—, —O—CO—NR^H—, —NR^H—CO—CH_2—, —O—CH_2—CO—NR^H—, —O—CH_2—CH_2—NR^H—, —CH=N—O—, —CH_2—NR^H—O—, —CH_2—O—N=(including R^5 when used as a linkage to a succeeding monomer), —CH_2—O—NR^H—, —CO—NR^H—CH_2—, — CH_2—NR^H—O—, — CH_2—NR^H—CO—, —O—NR^H— CH_2—, —O—NR^H, —O— CH_2—S—, —S— CH_2—O—, — CH_2— CH_2—S—, —O— CH_2— CH_2—S—, —S— CH_2—CH=(including R^5 when used

as a linkage to a succeeding monomer), —S— CH_2— CH_2—, —S— CH_2— CH_2—- O—, —S— CH_2— CH_2—S—, — CH_2—S— CH_2—, — CH_2—SO— CH_2—, — CH_2—SO_2— CH_2—, —O—SO—O—, —O—$S(O)_2$—O—, —O—$S(O)_2$— CH_2—, —O—$S(O)_2$—NR^H—, —NR^H—$S(O)_2$— CH_2—; —O—$S(O)_2$— CH_2—, —O—$P(O)_2$—O—, —O—P(O,S)—O—, —O—$P(S)_2$—O—, —S—$P(O)_2$—O—, —S—P(O,S)—O—, —S—$P(S)_2$—O—, —O—$P(O)_2$—S—, —O—P(O,S)—S—, —O—$P(S)_2$—S—, —S—$P(O)_2$—S—, —S—P(O,S)—S—, —S—$P(S)_2$—S—, —O—PO(R")—O—, —O—$PO(OCH_3)$—O—, —O—PO(O CH_2CH_3)—O—, —O—PO(O CH_2CH_2S—R)—O—, —O—$PO(BH_3)$—O—, —O—$PO(NHR^N)$—O—, —O—$P(O)_2$—NR^H H—, —NR^H—$P(O)_2$—O—, —O—$P(O,NR^H)$—O—, — CH_2—$P(O)_2$—O—, —O—$P(O)_2$— CH_2—, and —O—$Si(R")_2$—O—; among which — CH_2—CO—NR^H—, — CH_2—NR^H—O—, —S— CH_2—O—, —O—$P(O)_2$—O—O—P(- O,S)—O—, —O—$P(S)_2$—O—, —NR^H $P(O)_2$—O—, —O—$P(O,NR^H)$—O—, —O—PO(R")—O—, —O—$PO(CH_3)$—O—, and —O—$PO(NHR^N)$—O—, where RH is selected form hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl, are contemplated. Further illustrative examples are given in Mesmaeker et. al., Current Opinion in Structural Biology, 5:343-355 (1995) and Susan M. Freier and Karl-Heinz Altmann, Nucleic Acids Research, 25:4429-4443 (1997).

Still other modified forms of oligonucleotides are described in detail in U.S. Patent Publication No. 20040219565, the disclosure of which is incorporated by reference herein in its entirety.

Modified oligonucleotides may also contain one or more substituted sugar moieties. In certain aspects, oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Other embodiments include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. In one aspect, a modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 78:486-504 (1995)) i.e., an alkoxyalkoxy group. Other modifications include 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O—CH_2—O—CH_2—$N(CH_3)_2$, also described in examples herein below.

Still other modifications include 2'-methoxy (2'-O—CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2—CH=CH_2), 2'-O-allyl (2'-O—CH_2—CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. In one aspect, a 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, for example, at the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. See, for example, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722;

5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, the disclosures of which are incorporated by reference in their entireties herein.

In one aspect, a modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is in certain aspects is a methylene $(—CH_2—)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Natural and Modified Bases. Oligonucleotides may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include other synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified bases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5 ,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5 ,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzox- azin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified bases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further bases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, *International Edition*, 1991, 30:613 (1991), and those disclosed by Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these bases are useful for increasing the binding affinity and include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. and are, in certain aspects combined with 2'-O-methoxyethyl sugar modifications. See, U.S. Pat. Nos. 3,687,808, U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,750,692 and 5,681,941, the disclosures of which are incorporated herein by reference.

A "modified base" or other similar term refers to a composition which can pair with a natural base (e.g., adenine, guanine, cytosine, uracil, and/or thymine) and/or can pair with a non-naturally occurring base. In certain aspects, the modified base provides a T_m differential of 15, 12, 10, 8, 6, 4, or 2°C or less. Exemplary modified bases are described in EP 1 072 679 and WO 97/12896.

An oligonucleotide, or modified form thereof, may be from about 20 to about 100 nucleotides in length. In one embodiment, the oligonucelotide is from 5 to 50 nucleotides in length or any integer in between. It is also contemplated wherein the oligonucleotide is about 20 to about 90 nucleotides in length, about 20 to about 80 nucleotides in length, about 20 to about 70 nucleotides in length, about 20 to about 60 nucleotides in length, about 20 to about 50 nucleotides in length about 20 to about 45 nucleotides in length, about 20 to about 40 nucleotides in length, about 20 to about 35 nucleotides in length, about 20 to about 30 nucleotides in length, about 20 to about 25 nucleotides in length, or about 15 to about 90 nucleotides in length, about 15 to about 80 nucleotides in length, about 15 to about 70 nucleotides in length, about 15 to about 60 nucleotides in length, about 15 to about 50 nucleotides in length about 15 to about 45 nucleotides in length, about 15 to about 40 nucleotides in length, about 15 to about 35 nucleotides in length, about 15 to about 30 nucleotides in length, about 15 to about 25 nucleotides in length, or about 15 to about 20 nucleotides in length, and all oligonucleotides intermediate in length of the sizes specifically disclosed to the extent that the oligonucleotide is able to achieve the desired result. Accordingly, oligonucleotides of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, and 100 nucleotides in length are contemplated.

"Hybridization," which is used interchangeably with the term "complex formation" herein, means an interaction between two or three strands of nucleic acids by hydrogen bonds in accordance with the rules of Watson-Crick DNA complementarity, Hoogstein binding, or other sequence-specific binding known in the art. Hybridization can be performed under different stringency conditions known in the art.

In various aspects, the methods include use of oligonucleotides which are 100% complementary to another sequence, i.e., a perfect match, while in other aspects, the individual oligonucleotides are at least (meaning greater than or equal to) about 95% complementary to all or part of another sequence , at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, at least about 50%, at least about 45%, at least about 40%, at least about 35%, at least about 30%, at least about 25%, at least about 20% complementary to that sequence, so long as the oligonucleotide is capable of hybridizing to the target sequence.

It is understood in the art that the sequence of the oligonucleotide used in the methods need not be 100% complementary to a target sequence to be specifically hybridizable. Moreover, an oligonucleotide may hybridize to a target sequence over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). Percent complementarity between any given oligonucleotide and a target sequence can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 215: 403-410 (1990); Zhang and Madden, Genome Res., 7:649-656 (1997)).

The stability of the hybrids is chosen to be compatible with the assay conditions. This may be accomplished by designing the nucleotide sequences in such a way that the T_m will be appropriate for standard conditions to be employed in the assay. The position at which the mismatch occurs may be chosen to minimize the instability of hybrids. This may be accomplished by increasing the length of perfect complementarity on either side of the mismatch, as the longest stretch of perfectly homologous base

sequence is ordinarily the primary determinant of hybrid stability. In one embodiment, the regions of complementarity may include G:C rich regions of homology. The length of the sequence may be a factor when selecting oligonucleotides for use with particles. In one embodiment, at least one of the oligonucleotides has 100 or fewer nucleotides, e.g., has 15 to 50, 20 to 40, 15 to 30, or any integer from 15 to 50, nucleotides. Oligonucleotides having extensive self-complementarity should be avoided. Less than 15 nucleotides may result in a oligonucleotide complex having a too low a melting temperature to be suitable in the disclosed methods. More than 100 nucleotides may result in a oligonucleotide complex having a too high melting temperature to be suitable in the disclosed methods. Thus, oligonucleotides are of about 15 to about 100 nucleotides, e.g., about 20 to about 70, about 22 to about 60, or about 25 to about 50 nucleotides in length.

The following examples are given to illustrate the present invention. It should be understood that the invention is not to be limited to the specific conditions or details described in those examples.

Example I

A RMF centered on a microfluidic chamber containing a minute mass of magnetic silica beads revealed the presence of a select polymeric analyte in the sample through bead aggregation and the formation of 'pinwheels'. When the sample is devoid of specific polymeric analytes, the beads remain in the 'dispersed' formation.

To characterize the pinwheel effect in the presence of DNA and protein, and provide evidence of a polymer size-dependence on pinwheel formation, the following experiments were conducted. Using commercially-available silica-coated, iron-cored magnetic beads (5 microns) added to a microfluidic chamber in 4 to 8 M guanidine hydrochloride, conditions for driving nucleic acids to bind the silica surface, the RMF circulates the beads freely in a manner that has them reasonably distributed. The dispersed formation is stable and reproducible upon addition of about 1 to about 10 mg/mL bovine serum albumin, representing a 1000-fold excess mass of protein. However, a distinct transition to the 'pinwheel' formation was observed upon addition of nanogram levels of human genomic DNA (hgDNA), even with protein present. This indicates that protein, even at excessively high concentrations, does not interfere with nucleic acid-induced pinwheel formation.

A dynamic range of hgDNA-induced pinwheel formation was observed over three orders of magnitude, from 10 ng/μL to 10 pg/μL. The mass of beads in the chamber was tuned to match the mass of hgDNA needed for pinwheel formation, e.g., 30 ng, 15 ng, 3 ng, 300 pg, and 30 pg of human genomic DNA with 4, 2, 1, 0.2, and 0.2 μL of beads, respectively, in a chaotropic, high salt solution.

To further support the premise that DNA is the only analyte causing pinwheel formation under chaotropic salt conditions, sheared and unsheared hgDNA were evaluated. While extracted hgDNA resulted in pinwheel formation, the same mass of sonicated DNA (40 ng) was similar to the negative control (dispersed). Interestingly, pinwheel formation was not exclusive to DNA or chaotropic conditions. Chitosan, a cationic polysaccharide (MW about 310 kDa), formed distinct pinwheels with the very same silica beads in a low-salt buffer (50 mM MES [2-(N-morpholino)ethanesulfonic acid] at pH 5). Here the binding is governed by electrostatic attraction, demonstrating that this detection method can be extrapolated with a different binding chemistry. This supports the position that this effect is a general phenomenon applicable to a wide variety of polymeric analytes.

The system described above provides a versatile, visual detection technique and related apparatus to detect and quantify polymeric molecules that bind to magnetic beads under certain conditions, e.g., conditions related to binding chemistries. Moreover, the technique may be conducted with only a minute mass of magnetic beads, e.g. as low as a few beads per assay, in a microfluidic chamber.

Example II

Exemplary Materials and Methods

Magnetic beads: MagneSil paramagnetic particle purchased from Promega Corporation, diameter = 8±4 μm.

PMMA array: 4×4 array made by laser engraver, diameter of each well = 0.2 in, capacity of each well = 20 μL

Camera: Canon EOS Rebel XS

Microscope: Leica S8 APO

Stir plate: Thermix Stirrer Model 120S purchased from Fisher Scientific, Inc.

Exemplary Procedure

1. Prepare GuHCl solution in 1× TE buffer with a concentration of 8 M. Concentrations of from about 100 mM to about 8 M may be employed. Other concentrations of guanidine hydrochloride, and other chaotropic salts, may be employed to drive nucleic acid to bind magnetic particles, such as magnetic particles having diameters disclosed herein. Moreover, different concentrations of salts may result in enhanced aggregation with certain diameters of magnetic beads, e.g., lower concentration of salts may result in enhanced aggregation of smaller diameter magnetic beads.
2. Prepare suspension of magnetic beads: take 30 μL of stock beads suspension, wash with water and GuHCl solution and resuspend in 1 mL GuHCl solution.
3. Prepare DNA sample:
    a. Pre-purified DNA: dilute using 8 M GuHCl solution to appropriate concentrations
    b. Cells or blood: mix cells or blood with copious 8 M GuHCl (e.g., volume ratio = 1:100) to ensure cells are lysed and all the DNA is released.
4. Use DNA with a known concentration and with the same size of unknown DNA as standard, and prepare standard DNA solutions by serial dilution.
5. Mix a certain number of beads (e.g., 2-15 μL of suspension, depending on desired detection limit, sensitivity, and dynamic range) and a certain volume of standard DNA solutions (typically 5 μL) in the wells of PMMA plate. Adjust the total volume to 20 μL and GuHCl concentration to 6 M using GuHCl and/or H_2O.
6. Repeat step 5 for unknown DNA samples. With the PMMA plate, up to 16 DNA-magnetic beads mixtures can be prepared and measured together.
7. Put the PMMA array on stir plate and turn on the stir plate to mix the beads and DNA until the mixture system reaches equilibrium (about 5 minutes).
8. Adjust the PMMA array position on the stir plate so that one of the wells is at the center of stir plate. Turn on the stir plate to disperse beads in the centered well and take pictures.

9. Repeat step 8 for all the other wells containing samples.
10. Collect 5 pictures for each well.
11. Analyze pictures using ImageJ (see image processing).
12. Normalize the dark area values acquired from ImageJ by the area of dispersed beads without DNA, and plot the area percentage versus concentration of DNA.

Exemplary Image Processing

Software: ImageJ v1.41 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009), with multithresholder plugin (http://rsbweb.nih.gov/ij/plugins/multi-thresholder.html, Nov 2nd, 2009).

Open 8-bit images, set threshold using triangle method in the multithresholder, click analyze->analyze particle to acquire the number of pixels below the threshold since beads are darker than background.

In triangle algorithm, the software sets the value of grey level that gives the maximum distance as shown below to be the threshold. (Zack et al., J. Histochem. Cytochem., 25:741 (1977)).

Results

Figure 1 shows the results of 5 and 10 µL of MagneSil paramagnetic particle suspension mixed with different amounts of HeLa cells. The graph is based on the assumption that there was 6.25 pg of DNA per cell.

Example III

Detection and Quantification of Nucleated Cells

To determine if cell number can be quantitated using the pinwheel assay, magnetic particles with DNA (concentrations may be at least 1 to 5 pg/uL, assuming 6.25 pg/cell) and without DNA from whole blood were subjected to RMF. As discussed above, detection of aggregates may be accomplished using a camera, a light source, a rotating magnetic field (RMF), a substrate for the sample such as PDMS-glass microwell chip, and magnetic particles, e.g., magnetic beads such as super paramagnetic silica-coated particles (about 5 µm in diameter).

Approximately 5 photographs are taken over 30 seconds after RMF is applied. For image analysis, a threshold value is set automatically by isodata algorithm. Pixels below threshold are considered dark, and the dark area of a sample without DNA is used to normalize DNA sample data. The dark area of a sample with DNA over the dark area of a sample without DNA times 100 is the dark area percent (%) (see Figure 2).

Figure 3 show a comparison of the use of aggregates versus a Coulter Counter to determine white blood cell counts in three samples. The results demonstrated that the pinwheel effect can be utilized to define the concentration of DNA directly from blood samples, and that different concentrations for each correlated with measurement of DNA via the conventional method. The results show that the pinwheel assay can be used to determine cell number.

Figure 3B shows the result of diluting each sample to equalize the number of nucleated cells per microliter in each sample. A pinwheel assay of the diluted samples yielded overlapping curves, certifying that a consistent pinwheel response was obtained and that the degree of bead aggregation tracked with the number of WBC in each sample.

The results for the pinwheel assay correlate quite well with those from the Coulter Counter. . WBC counts between 5,000 and 10,000 per μL of blood were generally within 25% error, while outside of that range, > 50% error was observed. This may be corrected by dilution of the blood sample. Thus, given an unknown blood sample, once could dilute it to a certain fold, and acquire a dark area value from the pinwheel assay. Based on a standard graph, the concentration of WBCs was plotted as a function of dark area and dilution factor, from which the concentration of WBC can be read directly after a pinwheel assay.

For example, one could dilute an unknown sample to a certain fold, obtain the dark area, and find its position. Thus, the method can be used to test if the concentration of WBCs in an unknown blood sample is within the normal range (4,000 to 11,000 per μL).

The following parameters were used for cell quantification.

```
SetDirectory["Desktop/Pinwheel X/2011_02_21_M13 samples"];
 func1[histodata_,threshold_]:=
  Module[{mean1,mean2,thr,greylevel,i},
   greylevel=Table[i,{i,256}];

If[Total[Take[histodata,threshold]]==0,mean1=0,mean1=Round[Total[Take[greylevel*histodata,threshold]]/
Total[Take[histodata,threshold]]]];
   If[Total[Take[histodata,threshold-
256]]==0,mean2=0,mean2=Round[Total[Take[greylevel*histodata,threshold-
256]]/Total[Take[histodata,threshold-256]]]];
   thr=Round[(mean1+mean2)/2]
   ];
 func2[filenames_]:=
  Module[{data1,data2,data3,threshold,darkarea,a},
   data1=ImageData[ImageResize[Import[filenames],Scaled[1/10]]];
   data2=data1 //. {a_,b_,c_}->a;
   data3=BinCounts[Flatten[data2],{0,1,1/256}];
   threshold=FixedPoint[func1[data3,#]&,128];
   darkarea=Total[Take[data3,threshold]]
   ];

 filenames=FileNames["*.JPG"];
filenumber=Total[Dimensions[filenames]];
results=Table[func2[filenames[[i]]],{i,filenumber}];

Export[DateString[{"Year","_","Month","_","Day","_","Hour","Minute","Second","_"}]<>"result.xls",{Transpos
e[Join[{filenames},{results}]]}];
```

Example IV

Method to Detect Cells Using Pathogen-Specific Virus or Phage

Phages specifically infect certain bacteria and reproduce many times over during the course of several hours. This reproduction can be quantified via a pinwheel assay of the DNA in the phage. Figure 4 shows a graph with results from a pinwheel assay using purified phage DNA.

Figure 5 shows the percent of dark area versus phage number (A) or concentration (B). M13 Phage (From Ph.D™ -7 Phage Display Peptide Library) were amplified through ER2738 *E. coli*, and phage were suspended in 1x TE (5 x 10^7 phage/μL). 2.5 to 3 μL beads + a 5 μL phage sample in TE + about 10 to 12.5 μL GuHCl (8M) were mixed. Figure 5B show the limit of detection for these phage under the tested conditions, which is around 10^5 phage per μL.

To detect bacteria in a sample using phage, a 10 mL sample with 100 *E. coli* bacteria was incubated with 1000 phage for 5 hours (Figure 6), and then the culture was centrifuged at 10,000 rpm for 10 minutes. The supernatant containing phage was transferred to a new tube and the phage were concentrated via standard polyethylene glycol precipitation (1/6 volume added). Phage samples were then analyzed by both titering and a pinwheel assay. 5 μL of the original sample, and of a 1:100 diluted, and a 1:1000 diluted sample, were mixed with 2.5 μL beads. From the data analysis, phage were able to produce a pinwheel at a concentration of 40,000 phage/μL, indicating the ability to positively detect a bacteria contaminated sample via this method. Although 100 *E. coli* in 10 mL did not result in pinwheel formation, the infection of the same number of cells with phage allowed for detection by a pinwheel assay, regardless of phage concentration. Phage diluted 1:1000 and 1:100 resulted in a 70% and 25% dark area, respectively, corresponding to about 10 and 100 x 10^4 phage per μL. Considering the dilution factor, it was calculated that 4 x 10^8 phage/μL were present in the nondiluted sample 5 hours after infection. The assay may detect as few as 1 to 10 cells in a sample.

Although exemplified with M13 phage and *E. coli*, other phage may be employed to detect *E. coli* or other bacteria. Phage useful in the invention include, but are not limited, to those in one of the following families: *Myoviridae*, *Siphoviridae, Podoviridae, Tectiviridae, Corticoviridae, Lipothrixviridae, Plasmaviridaa, Rudiviridae, Fuselloviridae, Inoviridae, Microviridae, Leviviridae,* or *Cystoviridae*.

Exemplary Myoviridae include Mycobacterium phage I3*, Enterobacteria* phage Mu*, Enterobacteria* phage P1, *Aeromonas* phage 43, Haemophilus phage HP1, *Halobacterium* phage phiH, *Pseudomonas* phage phiKZ, *Pseudomonas* phage EL, *Pseudomonas* phage Lin68, *Bacillus* phage SPO1, *Enterobacteria* phage T4, *Acinetobacter* phage 133, *Aeromonas* phage 44RR2.8*t*, *Aeromonas* phage 65, *Aeromonas* phage Aeh1, *Enterobacteria* phage SV14, *Enterobacteria* phage T2, *Pseudomonas* phage 42, *Vibrio* phage nt-1,*Bacillus* phage G, *Bacillus* phage PBS1, and *Microcystis aeruginosa* phage Ma-LMM01.

Exemplary Siphoviridae include *Lactococcus* phage c2*, Lactococcus* phage bIL6*, Mycobacterium* phage L5, *Mycobacteria* phage D29, *Enterobacteria* phage lambda*, Enterobacteria* phage HK022, *Enterobacteria* phage HK97, *Enterobacteria* phage N15, *Streptomyces* phage phiC31, *Methanobacterium* phage psiM1, *Bacillus* phage SPbeta, *Enterobacteria* phage T1, *Enterobacteria* phage T5 and *Vibrio* phage 149 (type IV).

Exemplary *Podoviridae* include *Salmonella* phage BPP-1*, Bordetella* phage BPP-1, *Burkholderia* phage BcepC6B, *Salmonella* phage epsilon15*, Escherichia* phage PhiV10, *Pseudomonas* phage LUZ24, Pseudomonas phage PaP3, *Enterobacteria* phage N4, *Enterobacteria* phage P22, *Salmonella* phage HK620, *Salmonella* phage ST64T, *Shigella* phage Sf6, *Enterobacteria* phage Phieco32*, Endosymbiont* phage APSE-1, *Lactococcus* phage KSY1, *Phormidium* phage Pf-WMP3, *Phormidium p*hage Pf-WMP4, *Pseudomonas* phage 119X, *Pseudomonas* phage F116, *Roseobacter* phage SIO1, and *Vibrio* phage VpV262.

Exemplary *Microviridae* include *Enterobacteria* phage φX174*, Spiroplasma* phage 4*, Bdellovibrio* phage MAC*1* and *Chlamydia* phage 1.

Specific phage tha may be useful include, but are not limited to, λ phage, T2 phage, T4 phage, T7 phage, T12 phage, R17 phage, MS2 phage, G4 phage, P1 phage, Enterobacteria phage P2, P4 phage, Phi X 174 phage, N4 phage, Φ6 phage, Φ29 phage, 186 phage or D29 phage.

Example V

For smaller genome viruses, e.g., those with genomes smaller than phage genomes, whole genome amplification (WGA) may be employed to replicate the entire genome or a substantial portion of the genome *in vitro*. In particular, for some viruses, PCR protocols have been employed for amplification of a sizable fraction of the genome, leading to about 6 Kbp amplified fragments. If fragments can be generated using PCR that are > about 3 Kbp, a pinwheel assay may be employed to detect those viruses (see Figure 5).

Example VI

High Throughput Method to Detect Polymeric Analytes

The aggregation observed in a RMF, as discussed above, allows for the qualitative detection of DNA and other (e.g., polysaccharides) polymeric analytes, the quantitative determination of DNA by coupling to simple image analysis,quantitative determination of polymeric analytes such as DNA in crude samples, cell counts in cells that contain DNA (e.g., WBCs, and cultured bacteria), coupling with PCR to amplify large sections of viral genomes for virus detection, and coupling with phage infection of bacteria to amplify the phage so that, upon quantitating the number of phage by the pinwheel effect, the original number of bacteria can be back-calculated. In uni-directional RMF, the sample is centered in RMF to evenly distribute beads in the microwell, so that only one sample can be analyzed at a time, which limits the throughput.

Figure 7 shows a schematic of an exemplary system to detect magnetic bead aggregation in multiple samples, and Figure 8 depicts an exemplary multi-well sample holder (container) showing one embodiment of dimensions of the container and wells. The method and apparatus allow for simualtaneous detection and quantitation of analytes in multiple samples an reduces the time for analysis. To detect aggregation in multiple samples at the same time, for beads in a sample holder such as shown in Figure 8 that travel from one side to the center of the well in 5 seconds and migrate from one side of a well to the other in 20 seconds in a RMF, RMF is reversed every 20 seconds and measurements are taken every 5 seconds after the reversal (see Figure 9).

3 µL silica coated paramagnetic beads (Magnesil beads) of about 8 microns in diameter, 12 µL 8 M GuHCl and 5 µL lambda DNA in 1xTE buffer (total volume of about 20 to about 25 µL) were added to wells of a 32 well ring shaped container formed of polymethylmethacylate (PMMA). The wells had a diameter of about 5 mm and a depth of about 2 mm to about 3 mm. The container was subjected to a RMF rotating at about 150 rpm and was reversed about every 20 seconds (see Figure 10).

Figure 11 is a comparison of two samples with beads exposed to bi-directional RMF, one with DNA and the other without DNA. Aggregation occurred within two minutes of bi-directional RMF application. Simultaneous detection of DNA in 27 samples is shown in Figure 12. A standard curve was generated from those samples (see Figure 13).

Figure 14 shows two other embodiments of a magnetic field useful to process multiple samples.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A method for detecting the presence or amount of a polymeric analyte in a plurality of samples, comprising:

a) providing a multi-well container having in each of a plurality of the wells, a sample suspected of having a polymeric analyte and magnetic beads under conditions that allow for binding of the analyte to the beads;

b) subjecting each well in the container to the same magnetic field at the same time so as to form aggregates in the wells; and

c) detecting the presence or amount of the aggregates in the wells, thereby detecting the presence or amount of the analyte in the samples.

2. The method of claim 1 wherein the container is formed of glass, synthetic or natural polymers, or ceramic.

3. The method of claim 1 or 2 wherein the polymeric analyte is nucleic acid, lipid, polysacharride, or protein.

4. The method of claim 3 wherein the nucleic acid is DNA or RNA.

5. The method of any one of claims 1 to 4 wherein the magnetic beads are coated or derivatized to bind or enhance binding to the polymeric analyte.

6. The method of any one of claims 1 to 5 wherein the magnetic beads are coated with silica, amine-based charge switch, boronic acid, silane, oligonucleotides, lectins, reverse phase, antibody, antigen, avidin, or biotin.

7. The method of any one of claims 1 to 6 wherein the polymeric analyte is nucleic acid, the magnetic beads are silica coated, and the conditions include the presence of a chaotropic agent.

8. The method of any one of claims 1 to 6 wherein the polymeric analyte is positively charged polysacharride, the magnetic beads are silica coated, and the conditions include low ionic strength.

9. The method of any one of claims 1 to 6 wherein the polymeric analyte is protein, the magnetic beads are silica coated, and the conditions include denaturing conditions for the protein.

10. The method of any one of claims 1 to 9 wherein one or more of the samples comprise nucleic acid and protein.

11. The method of any one of claims 1 to 9 wherein one or more of the samples comprise lysed cells.

12. The method of any one of claims 1 to 9 wherein one or more of the samples comprise amplified DNA.

13. The method of any one of claims 1 to 9 wherein one or more of the samples comprise a physiological fluid sample.

14. The method of any one of claims 1 to 9 wherein the analyte is genomic DNA.

15. The method of claim 14 wherein the genomic DNA is subjected to sonication, shearing or a nuclease.

16. The method of any one of claims 1 to 15 wherein the analyte is nucleic acid and the binding is not sequence specific.

17. The method of any one of claims 1 to 16 wherein the container has a ring like shape and the wells are in a circular formation.

18. The method of any one of claims 1 to 17 wherein the magnetic field is a bi-directional rotating magnetic field.

19. The method of any one of claims 1 to 16 wherein the magnetic field is an electromagnetic field.

20. The method of any one of claims 1 to 16 wherein the wells are in a linear formation.

21. An apparatus for detecting the presence of a polymeric analyte comprising

a. a device for generating a magnetic field;

b. a detection chamber containing a multi-well container; and

c. a system for simultaneously detecting aggregate formation in wells of the multi-well container.

22. The apparatus of claim 21 wherein the device comprises a motor connected to a magnet to rotate the magnet.

23. The apparatus of claim 21 where in the system for detecting aggregate formation contains a camera and a computer electronically connected to the camera for analyzing images from the camera.

24. The apparatus of claim 23 wherein the camera is a video camera.

25. The apparatus of claim 21 wherein the multi-well container has a circular conformation and the magnetic field is configured to generate an identical magnetic field for each well.

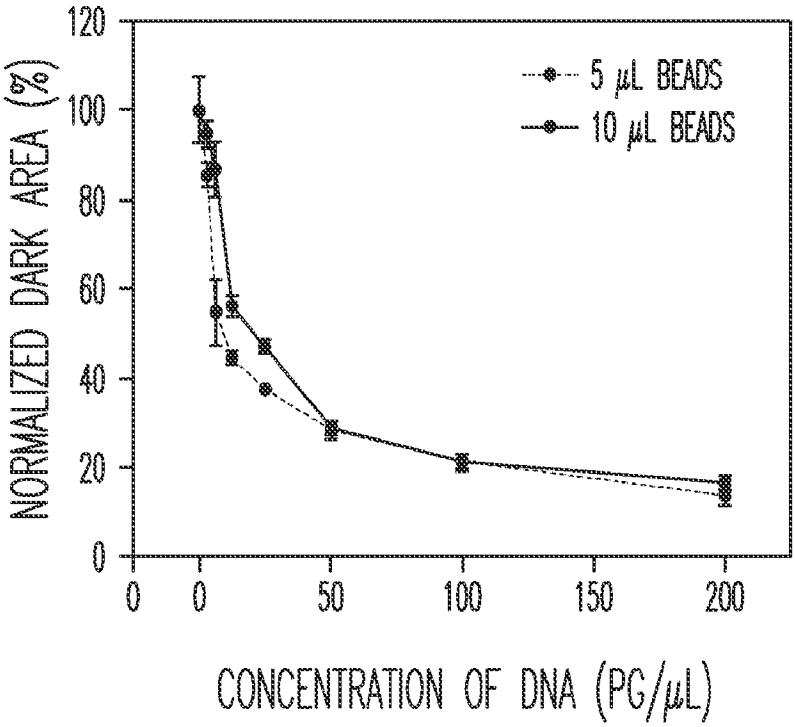

NORMALIZED DARK AREA (%)
120
100
80
60
40
20
0
0
0
50
100
150
200
CONCENTRATION OF DNA (PG/μL)
5 μL BEADS
10 μL BEADS

FIG. 1

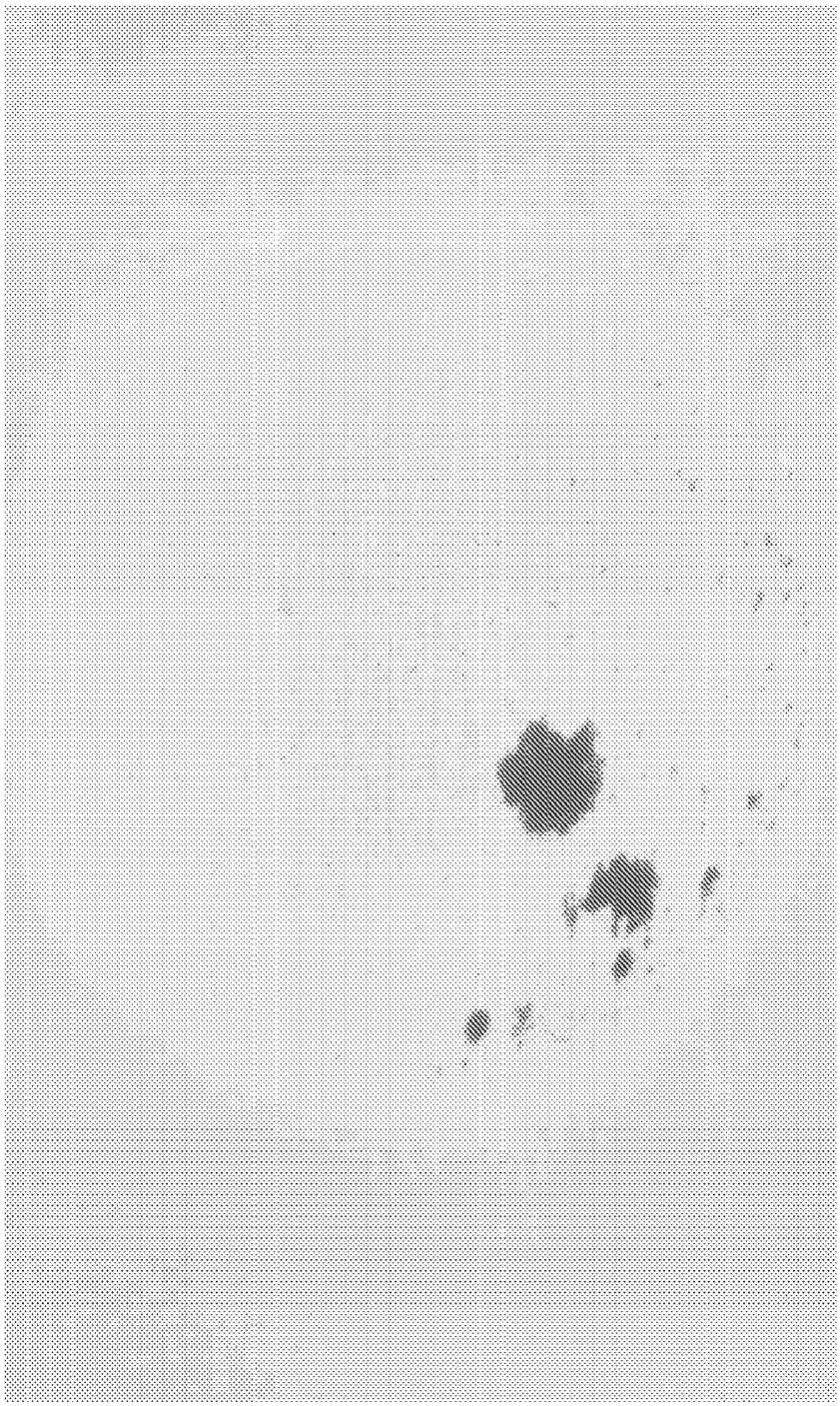


FIG. 2A

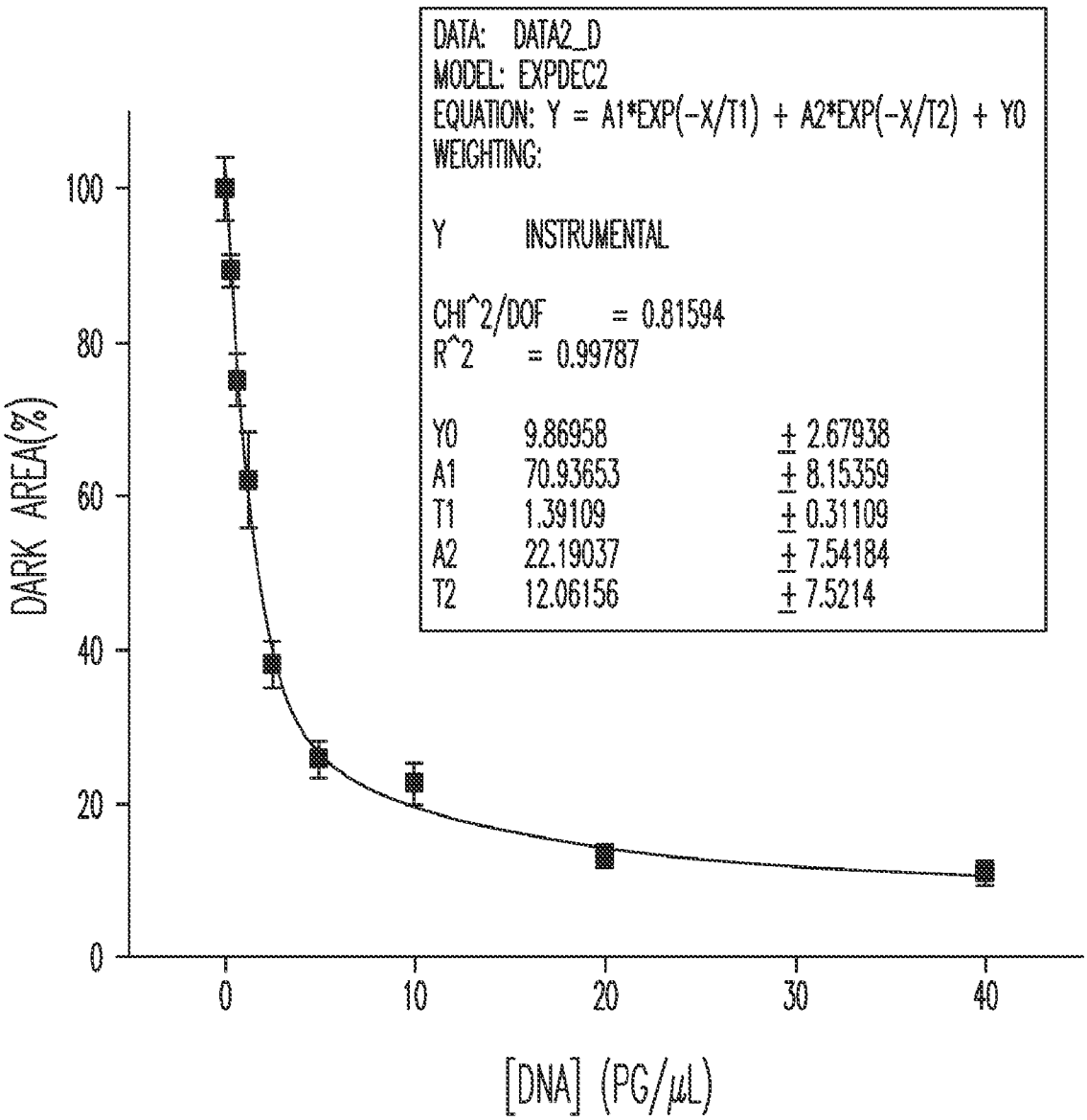

DATA: DATA2_D
MODEL: EXPDEC2
EQUATION: Y = A1*EXP(-X/T1) + A2*EXP(-X/T2) + Y0
WEIGHTING:
Y INSTRUMENTAL
CHI^2/DOF = 0.81594
R^2 = 0.99787
Y0 9.86958 ± 2.67938
A1 70.93653 ± 8.15359
T1 1.39109 ± 0.31109
A2 22.19037 ± 7.54184
T2 12.06156 ± 7.5214
DARK AREA(%)
100
80
60
40
20
0
0
10
20
30
40
[DNA] (PG/μL)

FIG. 2B

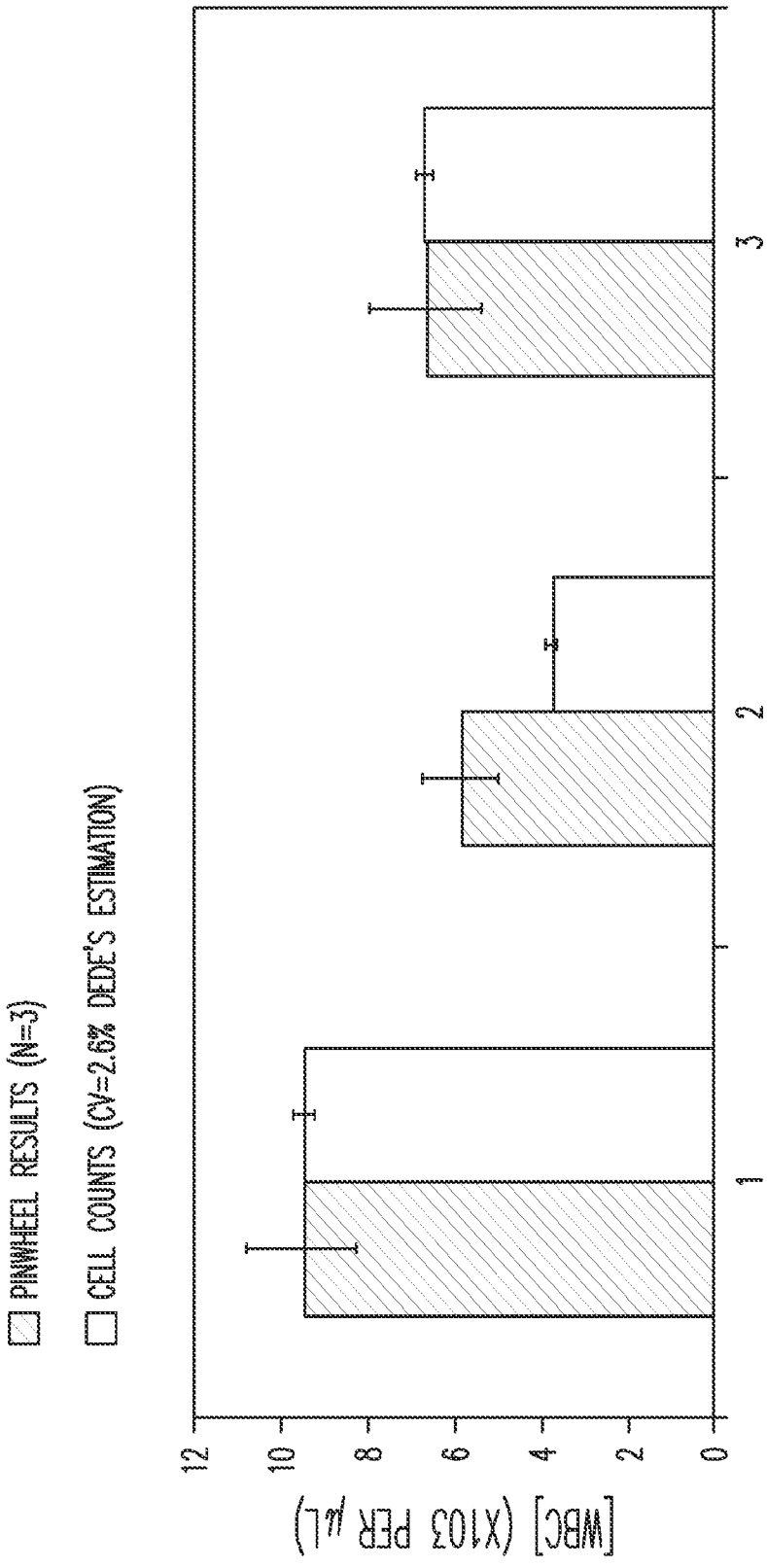

PINWHEEL RESULTS (N=3)
CELL COUNTS (CV=2.6% DEDE'S ESTIMATION)
[WBC] (X103 PER μL)
12
10
8
6
4
2
0
1
2
3
SAMPLE

FIG. 3A

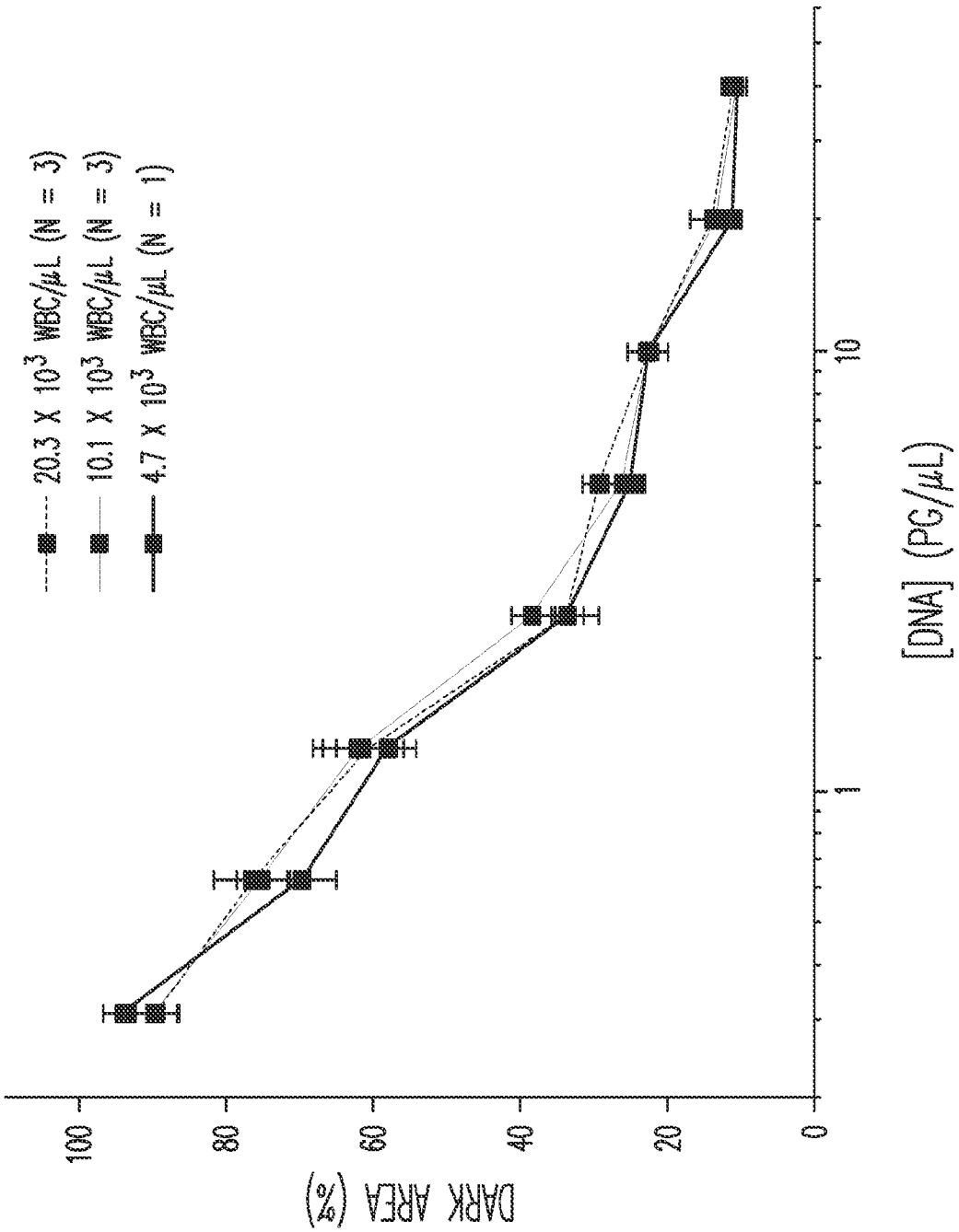

DARK AREA (%)
100
80
60
40
20
0
1
10
[DNA] (PG/μL)
20.3 X 10^3 WBC/μL (N = 3)
10.1 X 10^3 WBC/μL (N = 3)
4.7 X 10^3 WBC/μL (N = 1)

FIG. 3B

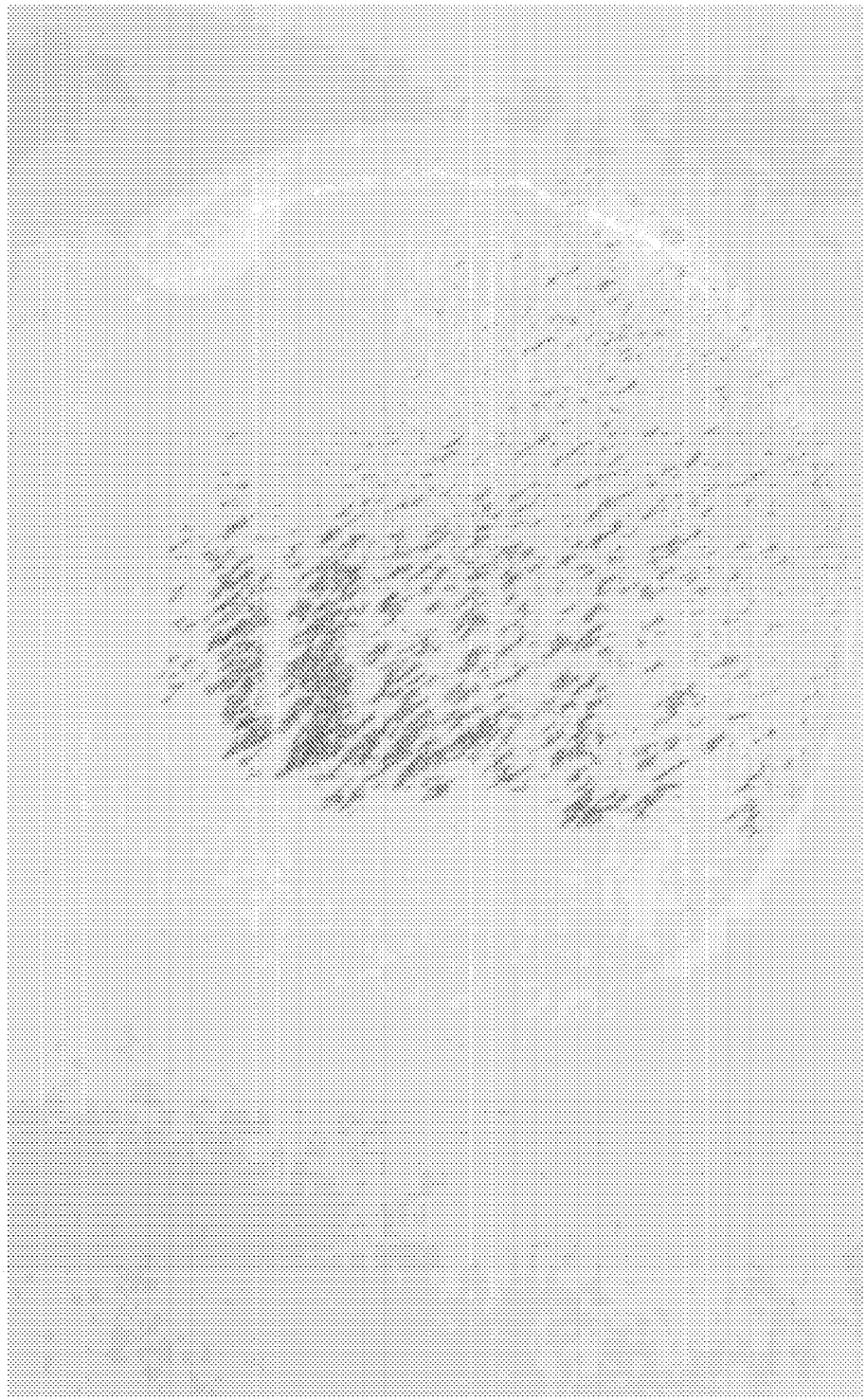


FIG. 4A

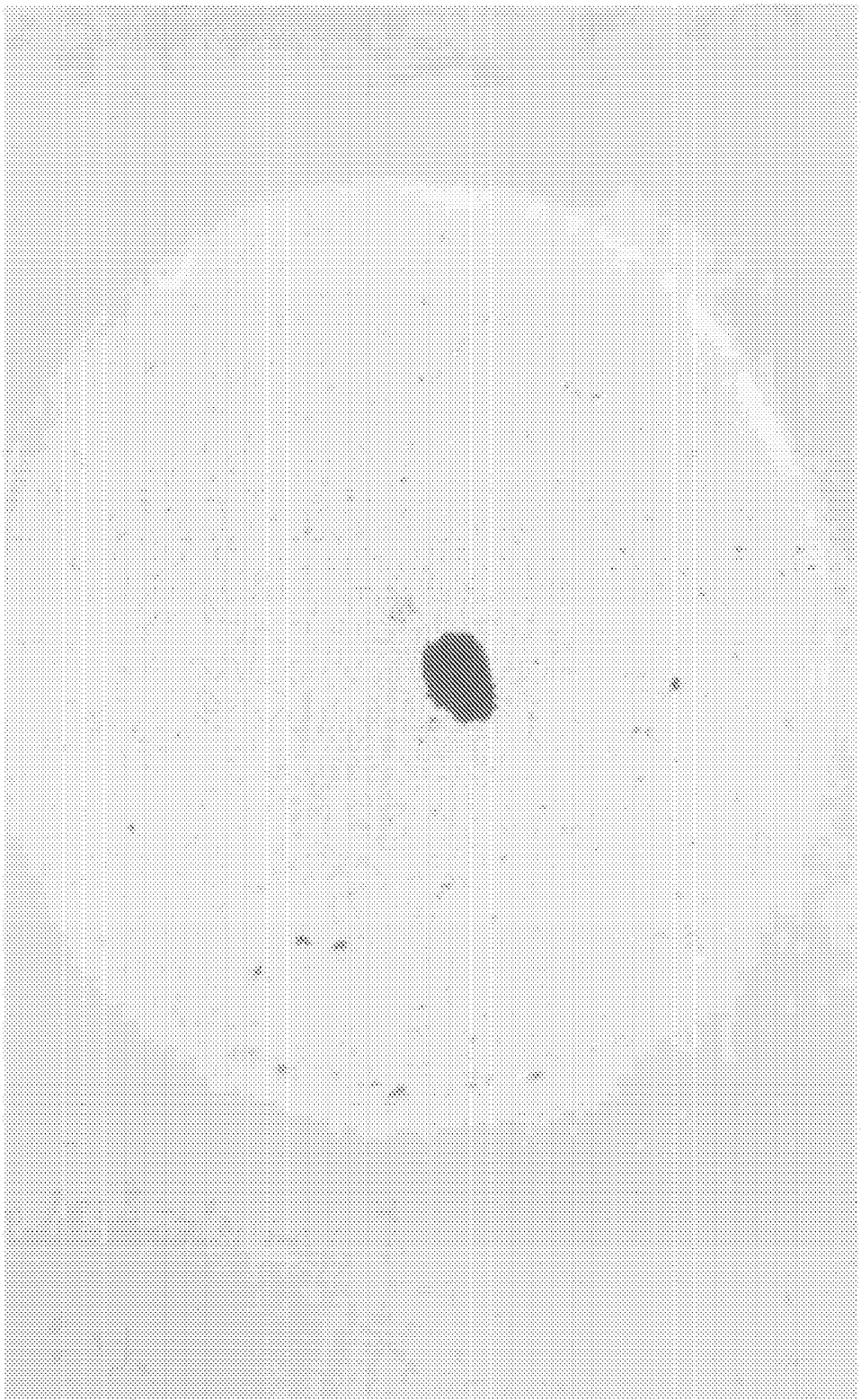


FIG. 4B

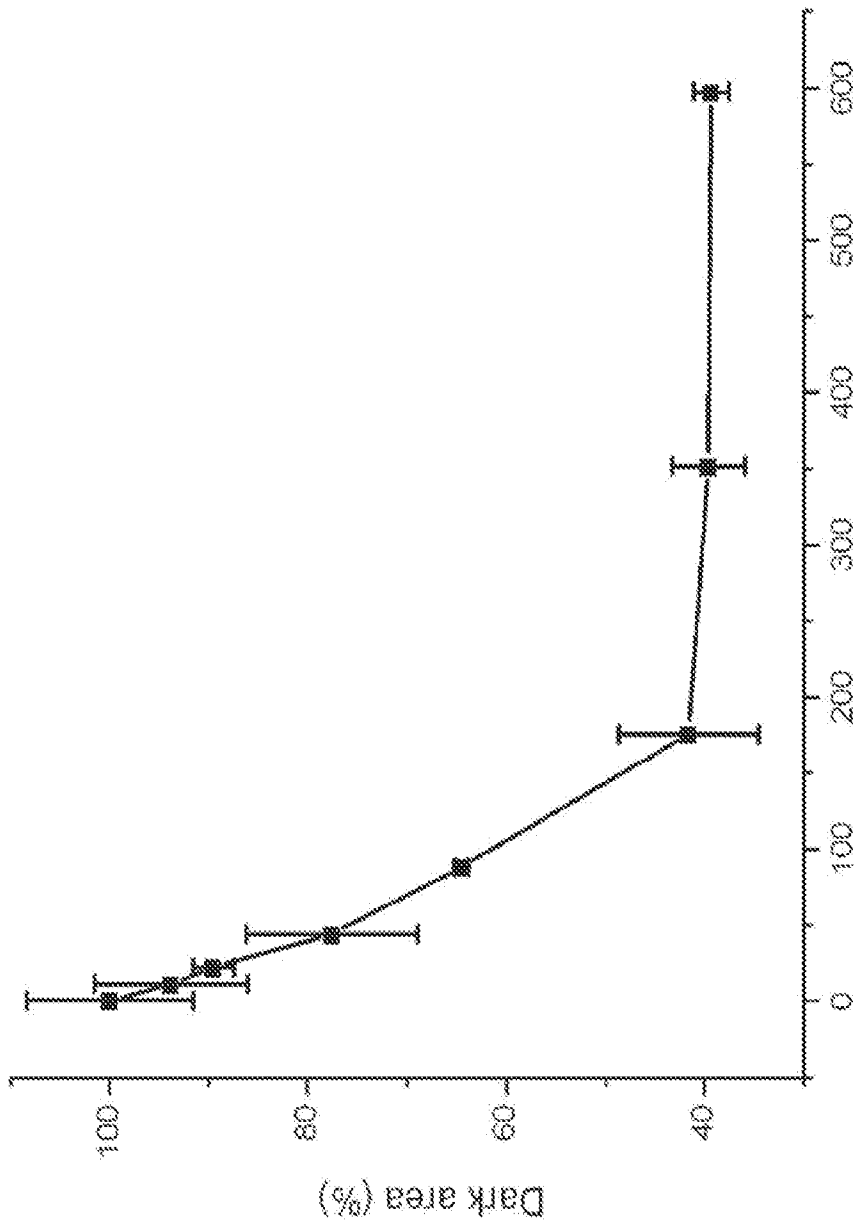
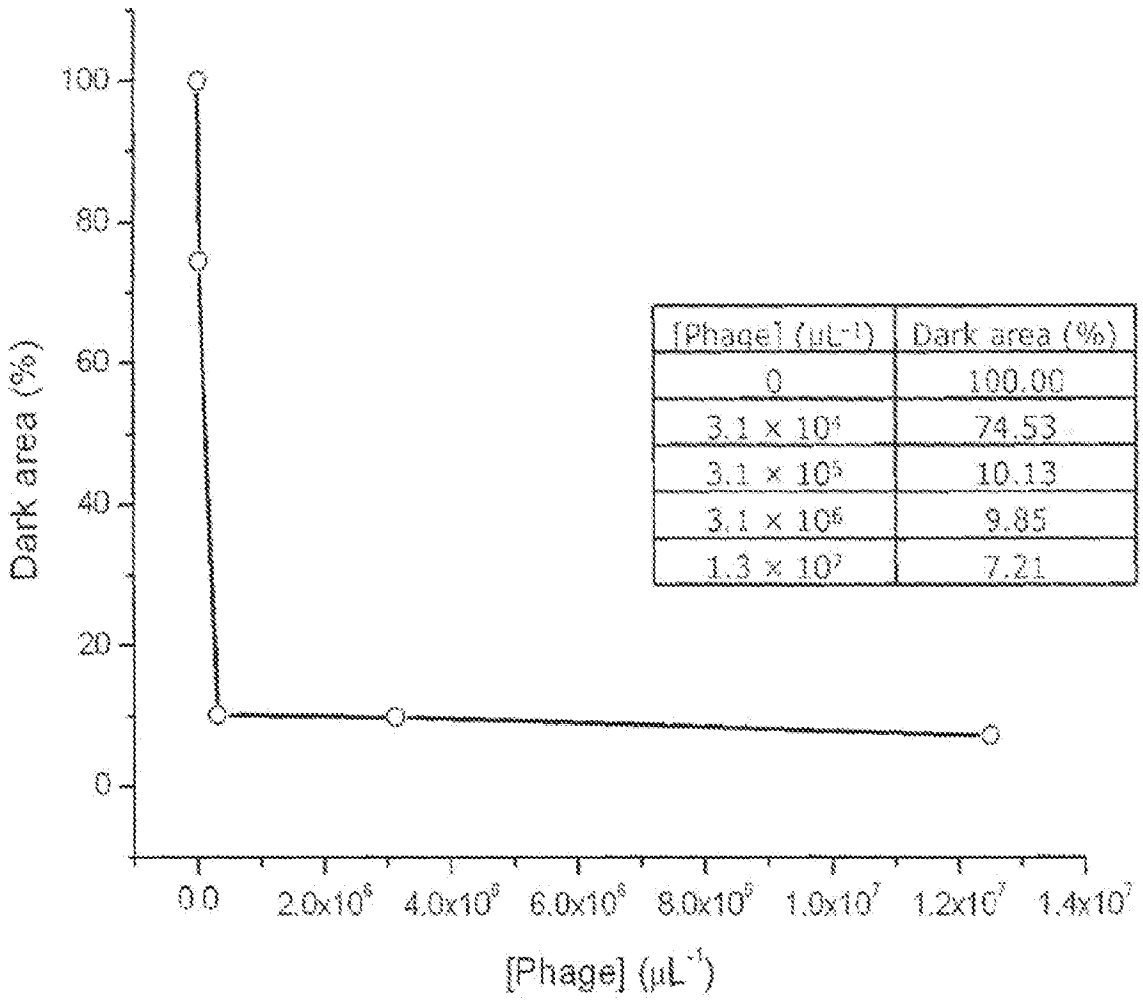


FIG. 4C



[Phage] (μL^{-1})	Dark area (%)
0	100.00
3.1×10^4	74.53
3.1×10^5	10.13
3.1×10^6	9.85
1.3×10^7	7.21

FIG. 5A

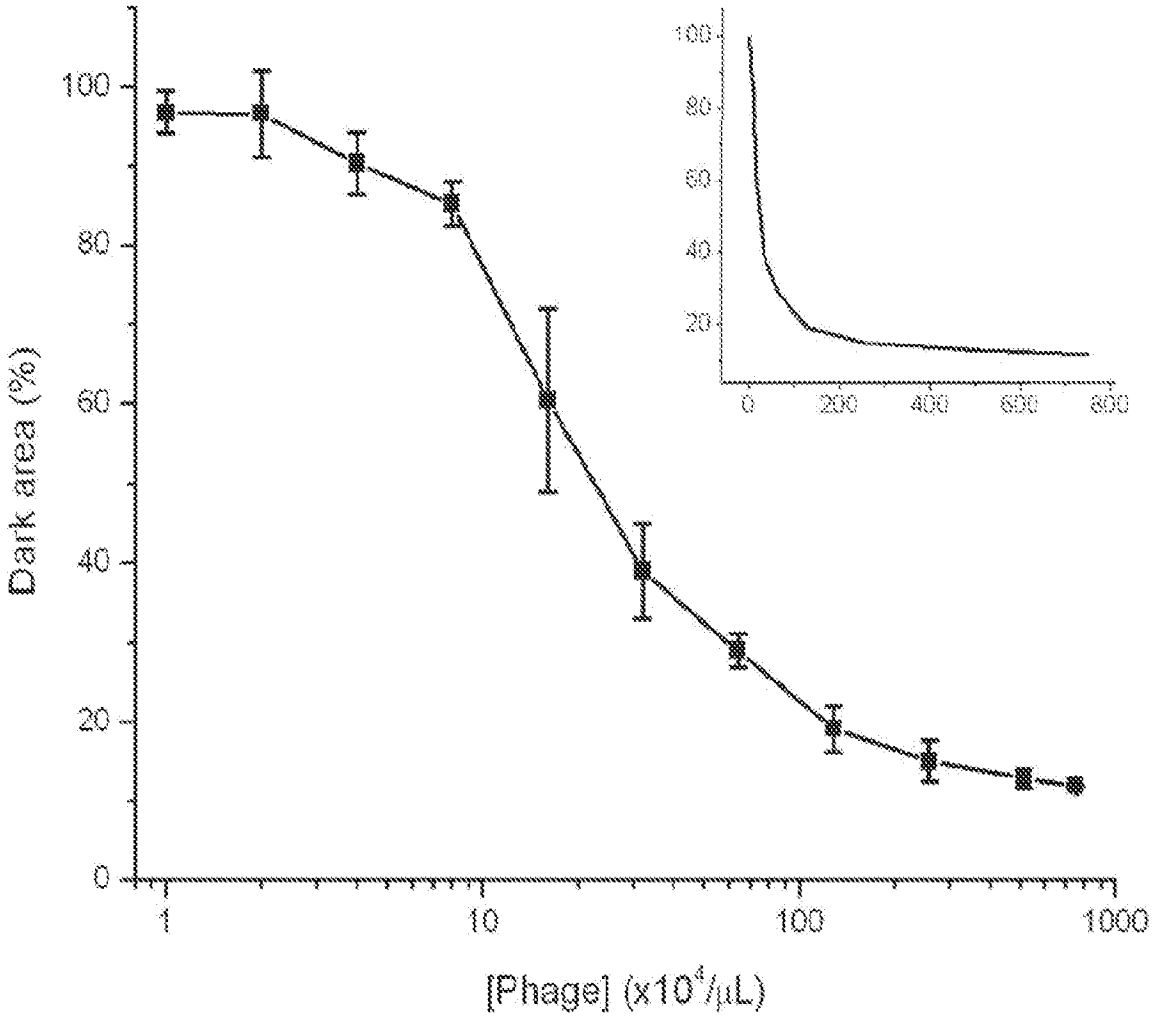

Dark area (%)
100
80
60
40
20
0
1
10
100
1000
[Phage] (x10⁴/μL)
100
80
60
40
20
0
200
400
600
800

FIG. 5B

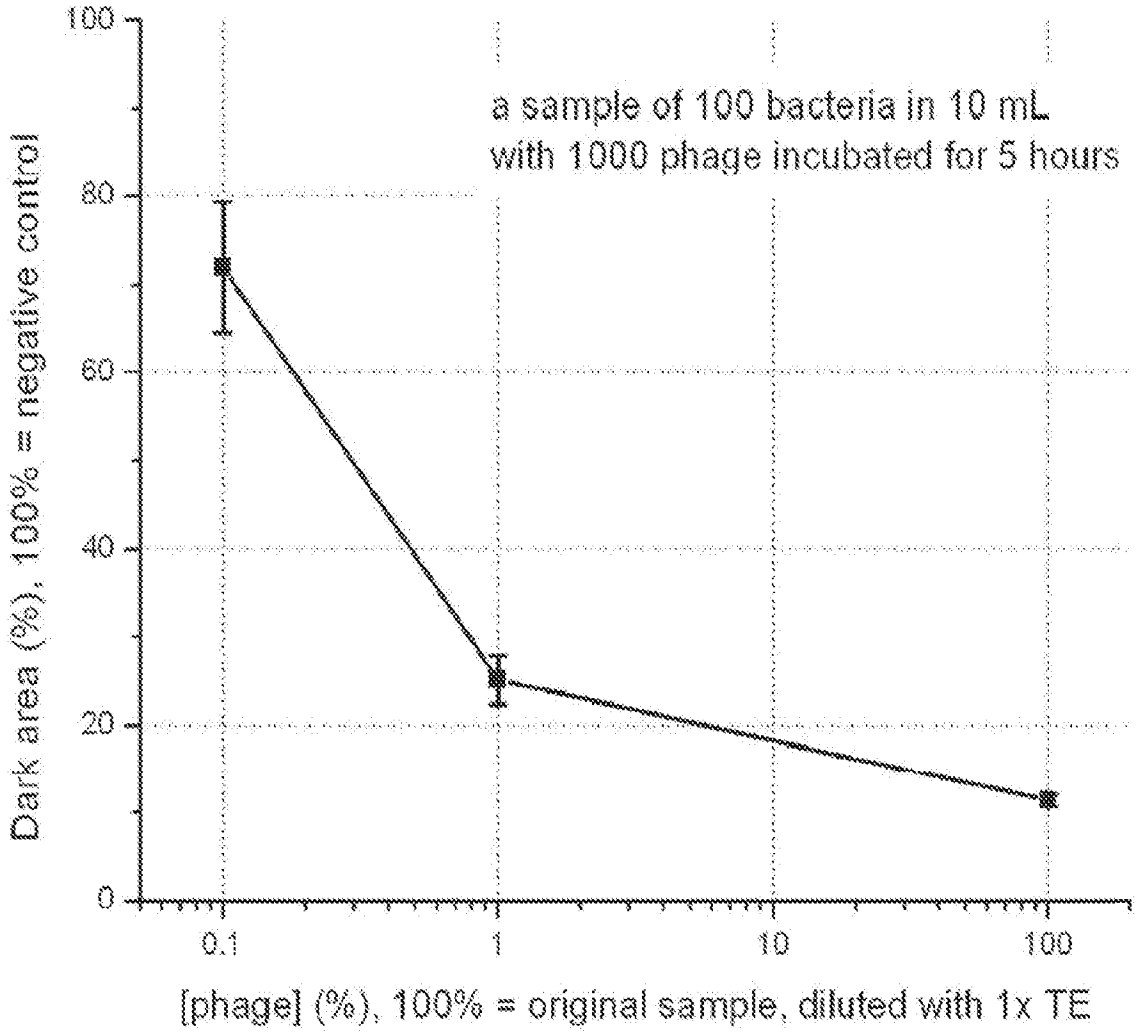

a sample of 100 bacteria in 10 mL
with 1000 phage incubated for 5 hours
Dark area (%), 100% = negative control
100
80
60
40
20
0
0.1
1
10
100
[phage] (%), 100% = original sample, diluted with 1x TE

FIG. 6

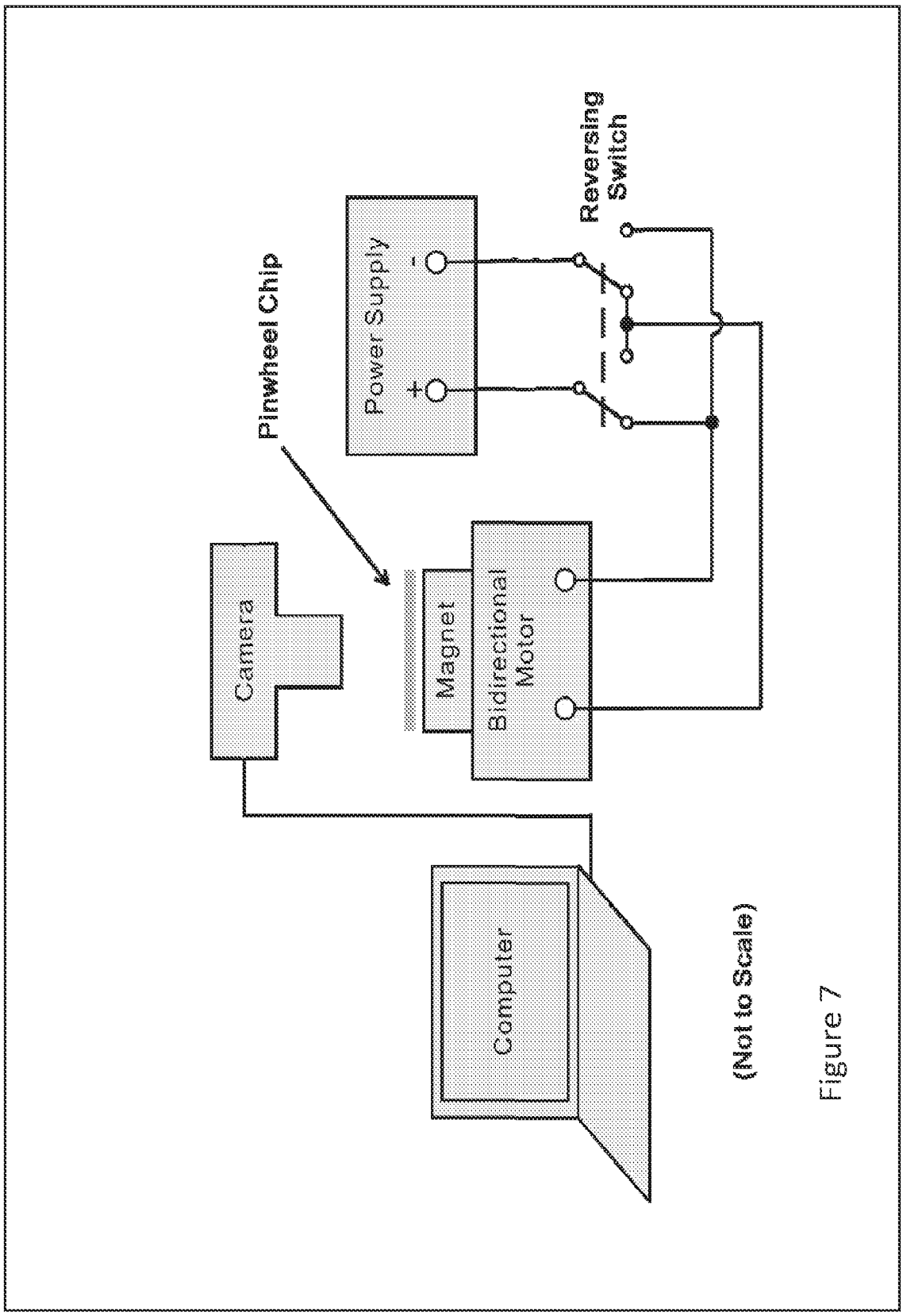

Reversing Switch
Power Supply
-
+
Pinwheel Chip
Camera
Magnet
Bidirectional Motor
Computer
(Not to Scale)

Figure 7

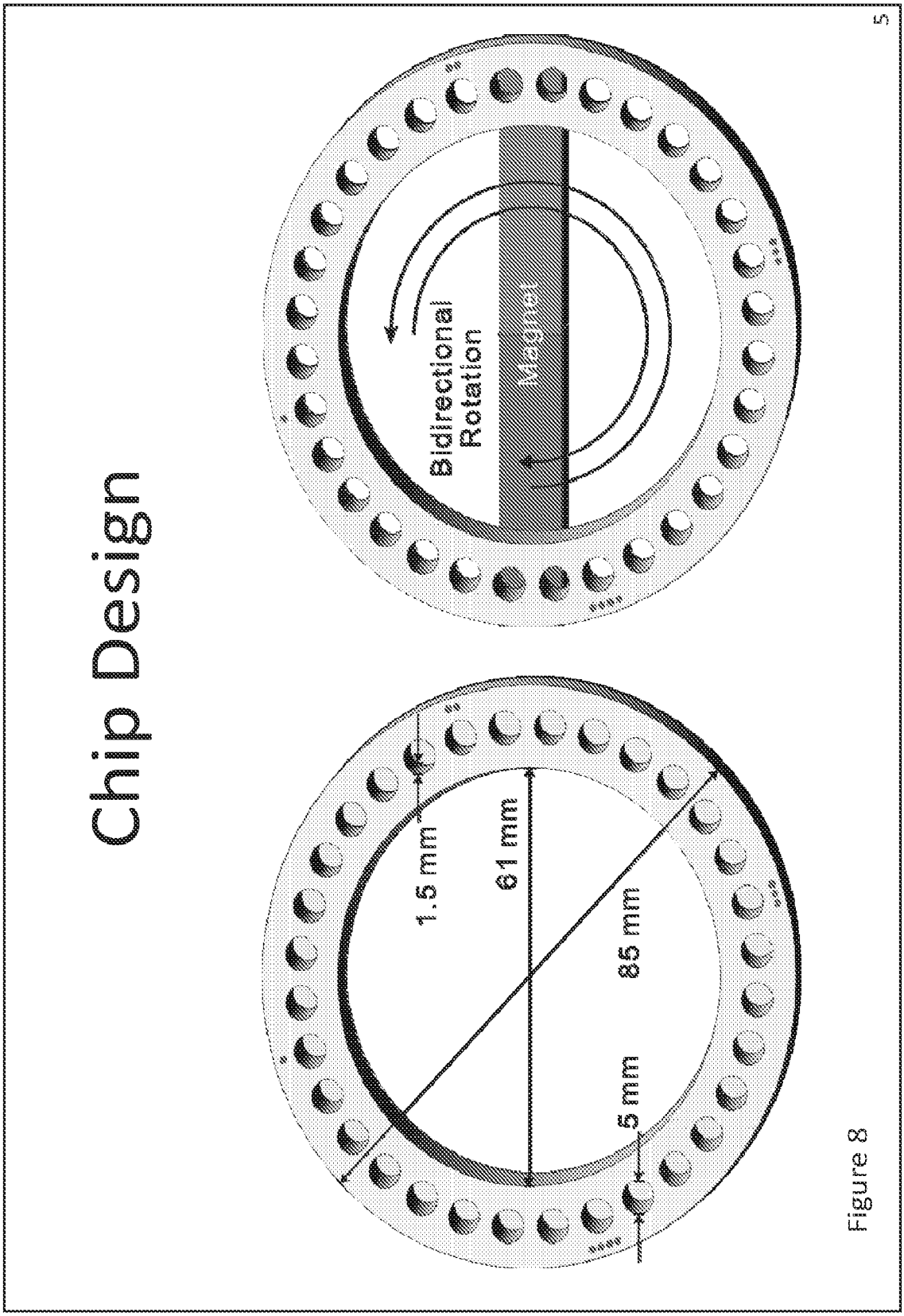

Chip Design
Bidirectional Rotation
Magnet
1.5 mm
61 mm
85 mm
5 mm

Figure 8

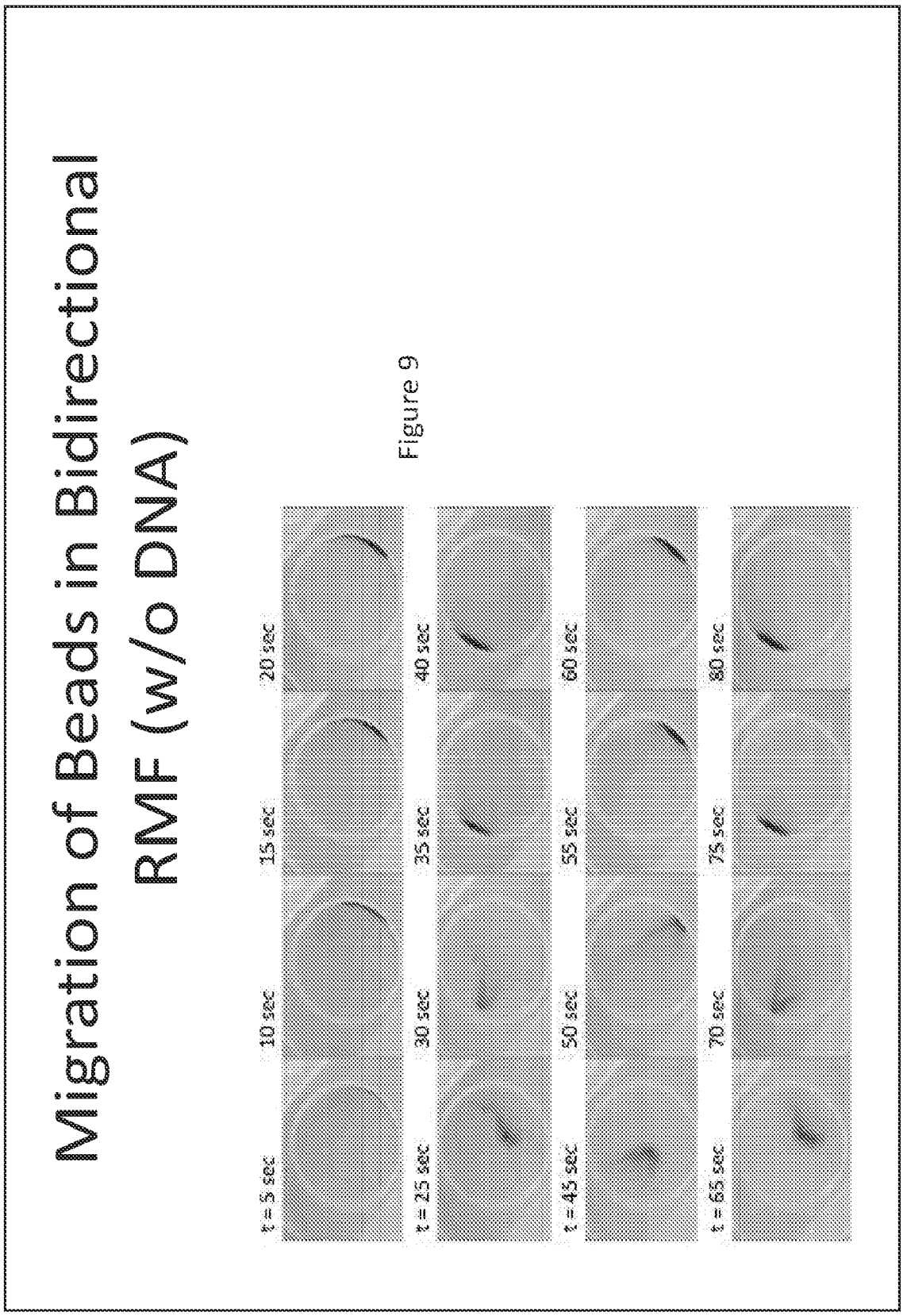

Migration of Beads in Bidirectional
RMF (w/o DNA)
t = 5 sec
10 sec
15 sec
20 sec
t = 25 sec
30 sec
35 sec
40 sec
t = 45 sec
50 sec
55 sec
60 sec
t = 65 sec
70 sec
75 sec
80 sec

Figure 9

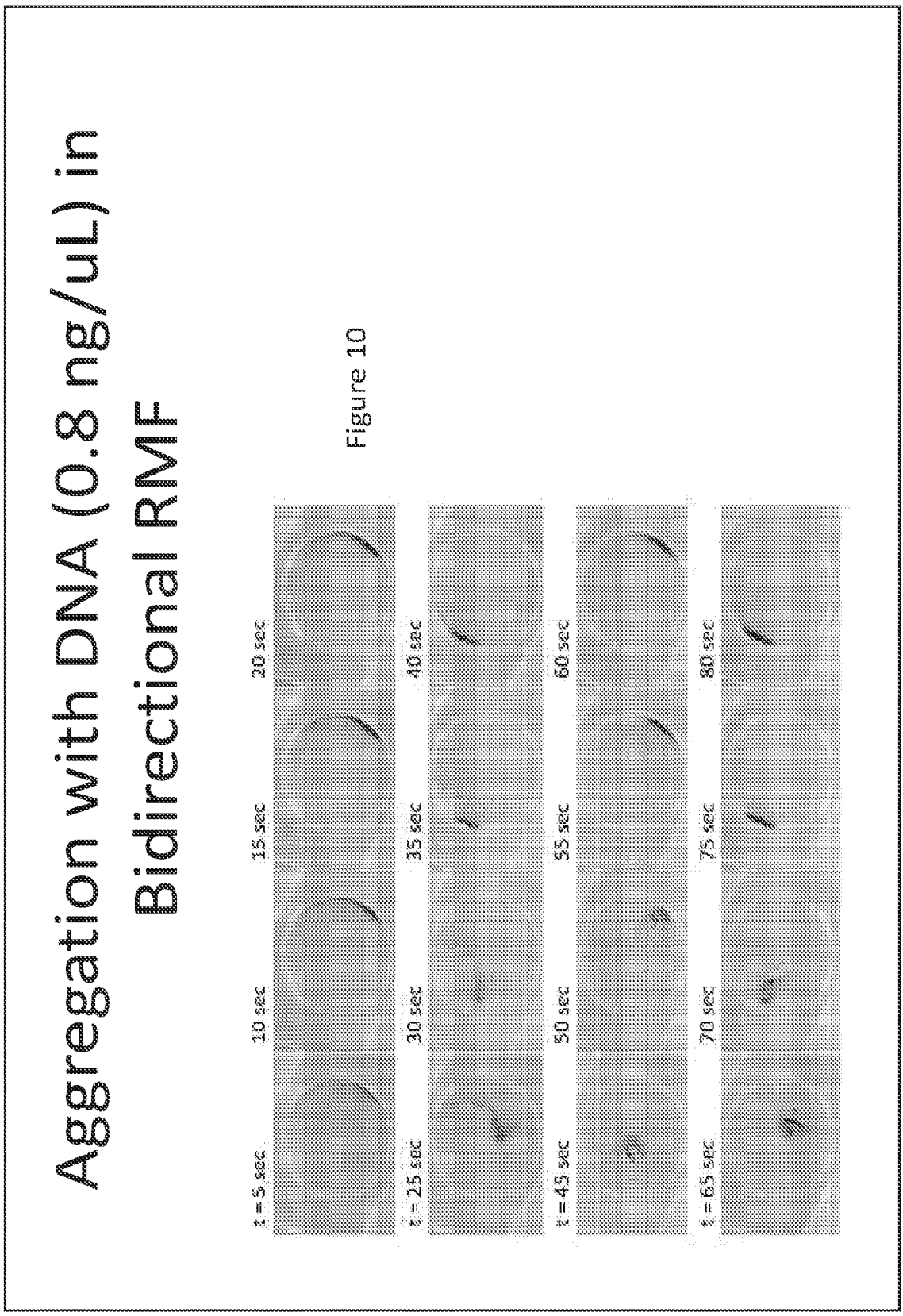

Aggregation with DNA (0.8 ng/uL) in Bidirectional RMF
t = 5 sec
10 sec
15 sec
20 sec
t = 25 sec
30 sec
35 sec
40 sec
t = 45 sec
50 sec
55 sec
60 sec
t = 65 sec
70 sec
75 sec
80 sec

Figure 10

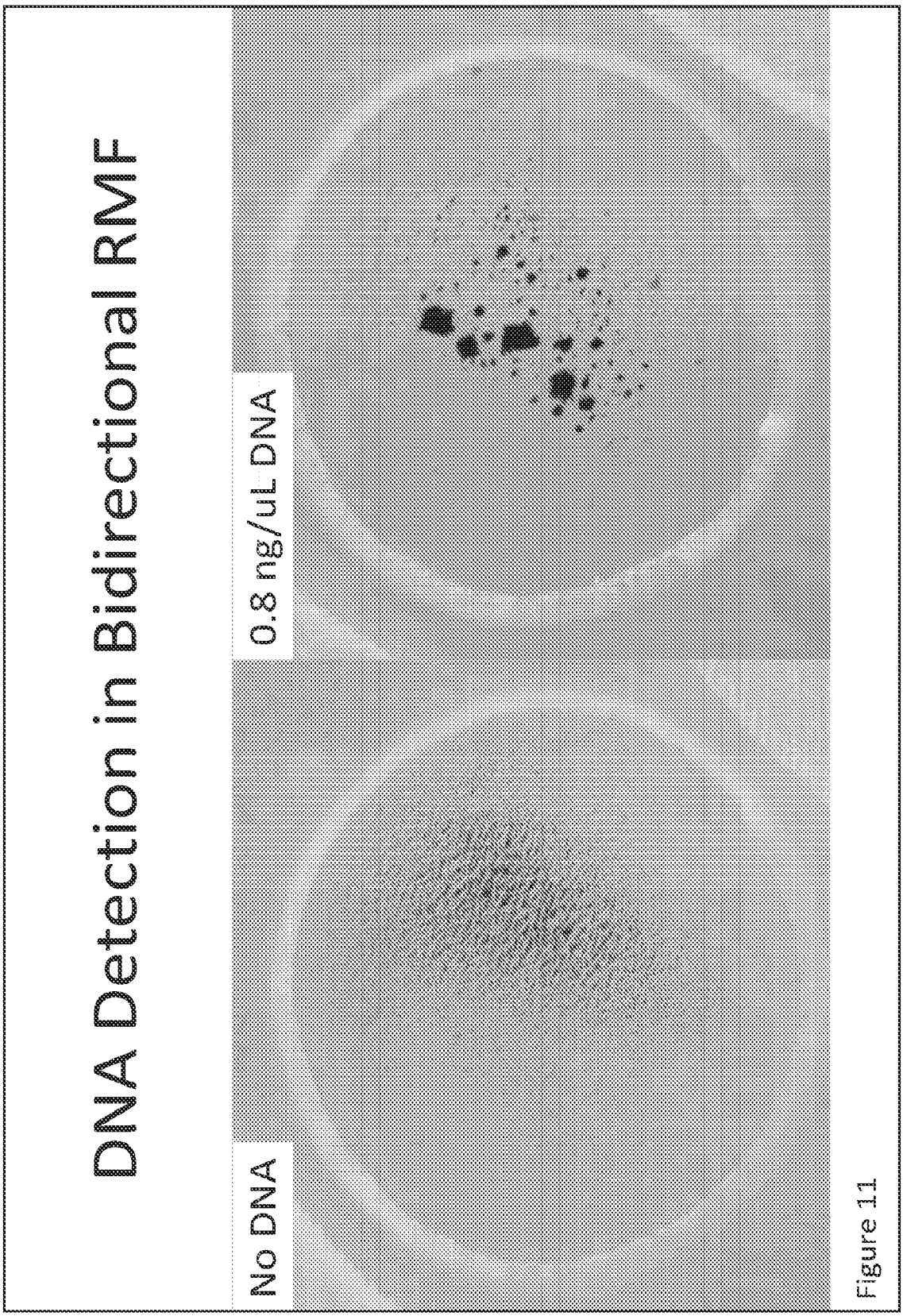


Figure 11

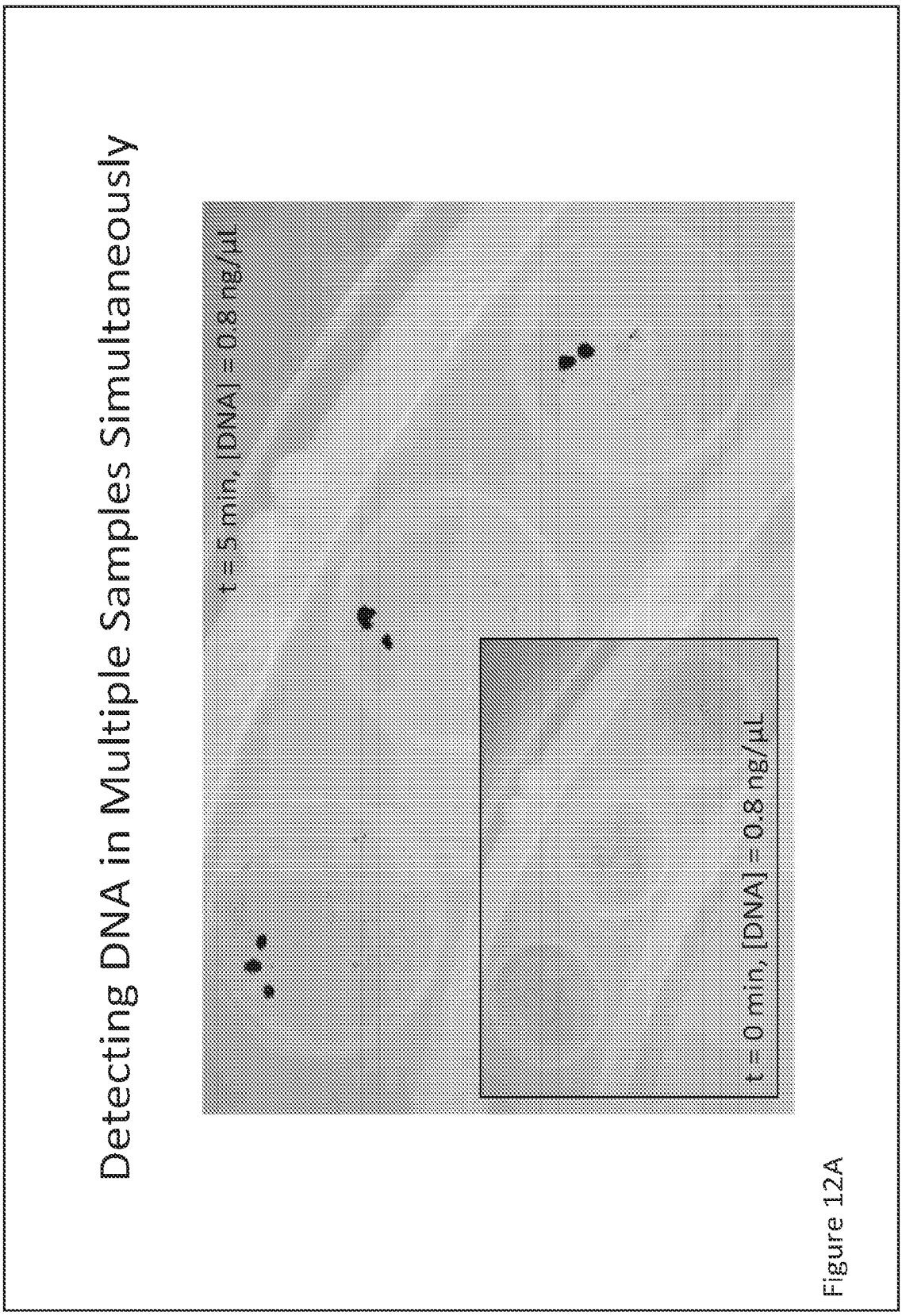

Detecting DNA in Multiple Samples Simultaneously
t = 5 min, [DNA] = 0.8 ng/μL
t = 0 min, [DNA] = 0.8 ng/μL

Figure 12A

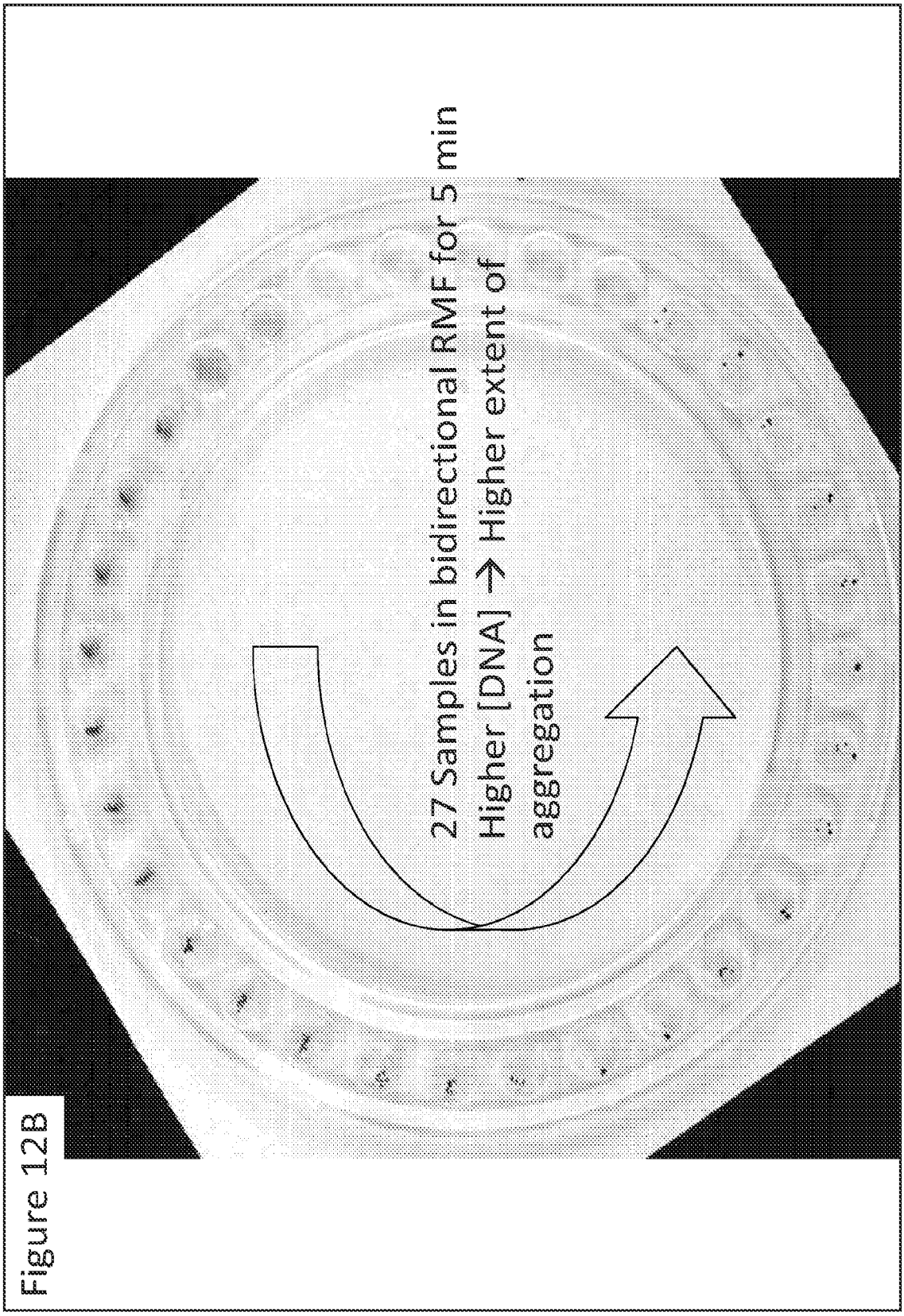

27 Samples in bidirectional RMF for 5 min
Higher [DNA] → Higher extent of aggregation

Figure 12B

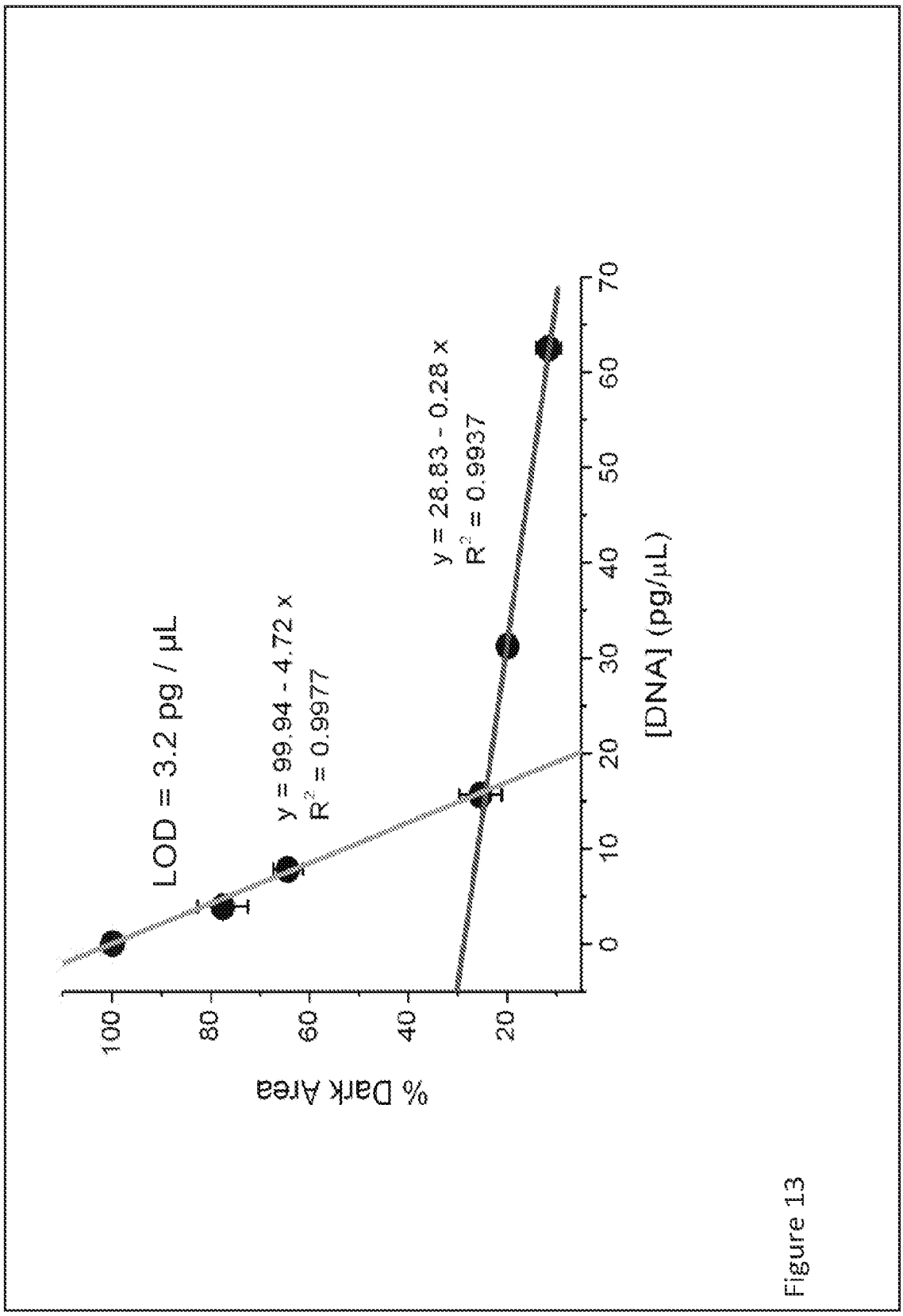

LOD = 3.2 pg / µL
y = 99.94 - 4.72 x
R2 = 0.9977
y = 28.83 - 0.28 x
R2 = 0.9937
% Dark Area
[DNA] (pg/µL)
100
80
60
40
20
0
10
20
30
40
50
60
70

Figure 13

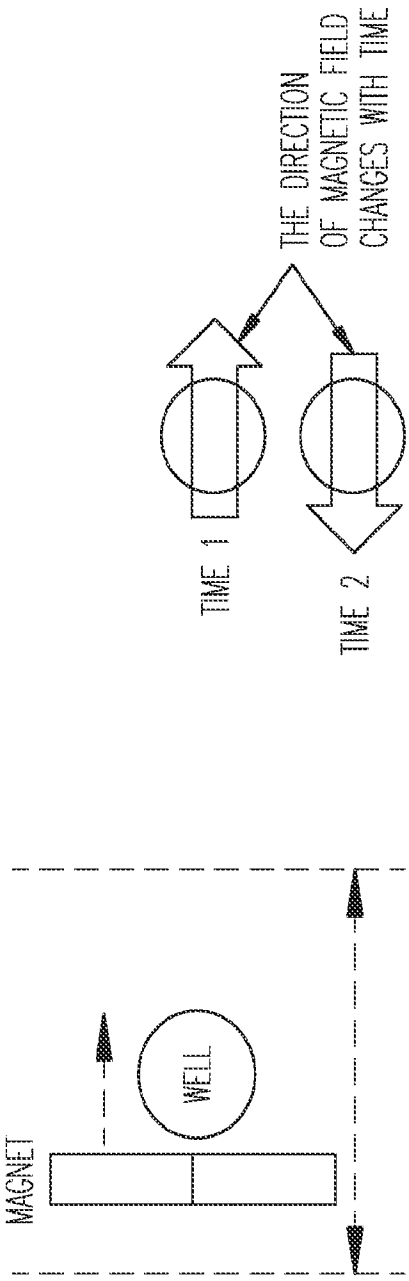

THE DIRECTION
OF MAGNETIC FIELD
CHANGES WITH TIME
TIME 1
TIME 2
WELL
MAGNET

Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/036105

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/543
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)
G01N

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, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2009/114709 A2 (UVA PATENT FOUNDATION [US]; LANDERS JAMES [US]; FINKLER DAVID [US]; BA) 17 September 2009 (2009-09-17) the whole document -----	21-24 1-20
Y	US 2006/252031 A1 (ABBOTT NICHOLAS [US] ET AL) 9 November 2006 (2006-11-09) fig 9 - 11, par. 0014, 0084, 0202, 0203, 0234-0236 -----	1-20
X,P	WO 2011/056215 A1 (LANDERS JAMES P [US]; FINKLER DAVID M [US]; BARKER NICOLAS SCOTT [US];) 12 May 2011 (2011-05-12) the whole document particularly p. 38, lines 2 - 6, claims, fig. 11/13 - 13/13 ----- -/--	1-20

[X] Further documents are listed in the continuation of Box C.

[X] 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

"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	Date of mailing of the international search report
8 August 2012	16/08/2012

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	Hoesel, Heidi

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/036105

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2012/027747 A2 (UNIV MICHIGAN [US]; MCNAUGHTON BRANDON H [US]; KINNUNEN PAIVO [US]; KO) 1 March 2012 (2012-03-01) par. 000115, 000132, 000135,000140 - 000149, 000180-000184, 000245 - 000253, claims 14 - 23	1-6,10, 19-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/036105

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009114709 A2	17-09-2009	AU 2009223122 A1	17-09-2009
		EP 2265942 A2	29-12-2010
		KR 20100135797 A	27-12-2010
		US 2011070660 A1	24-03-2011
		WO 2009114709 A2	17-09-2009
US 2006252031 A1	09-11-2006	NONE	
WO 2011056215 A1	12-05-2011	AU 2010315867 A1	21-06-2012
		WO 2011056215 A1	12-05-2011
WO 2012027747 A2	01-03-2012	US 2012164680 A1	28-06-2012
		WO 2012027747 A2	01-03-2012