of the concentration of analyte molecules in the fluid sample may be determined based on the information received when addressing the locations. In some cases, a measure of the concentration may be based at least in part on the number of locations determined to contain a capture object that is or was associated with at least one analyte molecule. In other cases and/or under differing conditions, a measure of the concentration may be based at least in part on an intensity level of at least one signal indicative of the presence of a plurality of analyte molecules and/or capture objects associated with an analyte molecule at one or more of the addressed locations.

In some embodiments, the number/fraction of locations containing a capture object but not containing an analyte molecule may also be determined and/or the number/fraction of locations not containing any capture object may also be determined. In such embodiments, a measure of the concentration of analyte molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with an analyte molecule to the total number of locations determined to contain a capture object not associated with an analyte molecule and/or a measure of the concentration of analyte molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with an analyte molecule to the number of locations determined to not contain any capture objects. In yet other embodiments, a measure of the concentration of analyte molecules in a fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object and an analyte molecule to the total number of locations addressed and/or analyzed.

In certain embodiments, at least some of the plurality of capture objects (e.g., at least some associated with at least one analyte molecule) are spatially separated into a plurality of locations, for example, a plurality of reaction vessels in an array format. The plurality of reaction vessels may be formed in, on and/or of any suitable material, and in some cases, the reaction vessels can be sealed or may be formed upon the mating of a substrate with a sealing component, as discussed in more detail below. In certain embodiments, especially where quantization of the capture objects associated with at least one analyte molecule is desired, the partitioning of the capture objects can be performed such that at least some (e.g., a statistically significant fraction) of the reaction vessels comprise at least one or, in certain cases, only one capture object associated with at least one analyte molecule and at least some (e.g., a statistically significant fraction) of the reaction vessels comprise an capture object not associated with any analyte

molecules. The capture objects associated with at least one analyte molecule may be quantified in certain embodiments, thereby allowing for the detection and/or quantification of analyte molecules in the fluid sample by techniques described in more detail herein.

An exemplary embodiment of an inventive assay method is illustrated in FIG. 1. A plurality of capture objects 2, are provided (step (A)). In this particular example, the plurality of capture objects comprises a plurality of beads. The beads are exposed to a fluid sample containing a plurality of analyte molecules 3 (e.g., beads 2 are incubated with analyte molecules 3). At least some of the analyte molecules are immobilized with respect to a bead. In this example, the analyte molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single analyte molecule and a statistically significant fraction of the beads do not associate with any analyte molecules. For example, as shown in step (B), analyte molecule 4 is immobilized with respect to bead 5, thereby forming complex 6, whereas some beads 7 are not associated with any analyte molecules. It should be understood, in some embodiments, more than one analyte molecule may associate with at least some of the beads, as described herein. At least some of the plurality of beads (e.g., those associated with a single analyte molecule or not associated with any analyte molecules) may then be spatially separated/segregated into a plurality of locations. As shown in step (C), the plurality of locations is illustrated as substrate 8 comprising a plurality of wells/reaction vessels 9. In this example, each reaction vessel comprises either zero or one beads. At least some of the reaction vessels may then be addressed (e.g., optically or via other detection means) to determine the number of locations containing an analyte molecule. For example, as shown in step (D), the plurality of reaction vessels are interrogated optically using light source 15, wherein each reaction vessel is exposed to electromagnetic radiation (represented by arrows 10) from light source 15. The light emitted (represented by arrows 11) from each reaction vessel is determined (and/or recorded) by detector 15 (in this example, housed in the same system as light source 15). The number of reaction vessels containing an analyte molecule (e.g., reaction vessels 12) is determined based on the light detected from the reaction vessels. In some cases, the number of reaction vessels containing a bead not associated with an analyte molecule (e.g., reaction vessel 13), the number of wells not containing a bead (e.g., reaction vessel 14) and/or the total number of wells addressed may also be determined. Such

determination(s) may then be used to determine a measure of the concentration of analyte molecules in the fluid sample.

A statistically significant fraction of capture objects that contain at least one analyte molecule (or no analyte molecules) will typically be able to be reproducibly detected and quantified using a particular system of detection and will typically be above the background noise (e.g., non-specific binding) that is determined when carrying out the assay with a sample that does not contain any analyte molecules, divided by the total number of objects (or locations) addressed. A "statistically significant fraction" as used herein for the present embodiments, may be estimated according to the Equation 1:

$$n \geq 3\sqrt{n} \qquad \text{(Eq. 1)}$$

wherein n is the number of determined events for a selected category of events. That is, a statistically significant fraction occurs when the number of events is greater than three times square root of the number of events. For example, to determine a statistically significant fraction of the capture objects not associated with any analyte molecules or particles, n is the number of capture objects detected that are not associated with any analyte molecules or particles. As another example, to determine a statistically significant fraction of the capture objects associated with at least one analyte molecule, n is the number of capture objects detected that are determined to be associated with an analyte molecule.

In some embodiments, the statistically significant fraction of capture objects (e.g., beads) associated with at least one analyte molecule (or a single analyte molecule in some cases where the ratio of mixing capture objects to analyte molecules would lead, statistically, to only zero or one analyte molecule associate with each capture object) to the total number of capture objects (e.g., beads) is less than about 1:2, less than about 1:3, less than about 1:4. is less than about 2:5, less than about 1:5, less than about 1:10, less than about 1:20, less than about 1:100, less than about 1:200, or less than about 1:500. Therefore, in such embodiments, the fraction of capture objects (e.g., beads) not associated with any analyte molecules to the total number of capture objects (e.g., beads) is at least about 1:100, about 1:50, about 1:20, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, about 5:1, about 10:1, about 20:1, about 50:1, about 100:1, or the like.

In some embodiments, the percentage of capture objects (e.g., beads) associated with at least one analyte molecule (or a single analyte molecule in some cases where the ratio of mixing capture objects to analyte molecules would lead, statistically, to only zero

or one analyte molecule associate with each capture objects) is less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.01%, or the like, the total number of capture objects. In some embodiments, the percentage of capture objects (e.g., beads) not associated with an analyte molecule to the total number of capture objects (e.g., beads) is at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or the like, the total number of capture objects.

In some embodiments, prior to spatially separating the plurality of capture objects, the capture objects may be exposed to a plurality of binding ligands which have an affinity for at least one type of analyte molecule (or particle). A "binding ligand," as used herein, is any molecule, particle, or the like which specifically binds to or otherwise specifically associates with an analyte molecule to aid in the detection of the analyte molecule. Binding ligands may be particularly useful in embodiments where at least some of the capture objects are associated with respect to more than one analyte molecule (e.g., two, three, four, five, or more, analyte molecules). In some cases, the binding ligand may be provided in a manner (e.g., at a concentration level) such that a statistically significant fraction of the capture objects comprising at least one analyte molecule associate with at least one binding ligand (or in some cases, a single binding ligand) and a statistically significant fraction of the capture objects (e.g., capture objects either associated with at least one analyte molecule or not associated with any analyte molecules) do not associate with any binding ligand.

A statistically significant fraction of the locations that contain a capture object (e.g., bead) associated with at least one analyte molecule and a single binding ligand is greater than or equal to the minimum number of locations that can be reproducibly determined to contain an capture object (e.g., bead) associated with a single binding ligand with a particular system of detection (i.e., substantially similar results are obtained for multiple essentially identical fluid samples comprising the capture objects associated with an analyte molecule and/or binding ligand) and that is above the background noise (e.g., non-specific binding) that is determined when carrying out the assay with a sample that does not contain any analyte molecules and/or binding ligands, divided by the total number of locations. The statistically significant fraction of locations that contain a capture object associated with at least one analyte molecule and a single binding ligand

can be determined according to Equation 1. The ratio of the number of capture objects to analyte molecules and/or binding ligands which may be provided such that substantially all of the capture objects are associated with zero or a single analyte molecule may be calculated using a Poisson distribution adjustment, as described herein.

In some embodiments, the statistically significant fraction of capture objects (e.g., beads) associated with at least one analyte molecule and at least one binding ligand to the total number of capture objects (e.g., beads) is less than about 1:2, less than about 1:3, less than about 1:4. is less than about 2:5, less than about 1:5, less than about 1:10, less than about 1:20, less than about 1:100, less than about 1:200, or less than about 1:500. In some cases, the statistically significant fraction of capture objects (e.g., beads) associated not associated with any binding ligand to the total number of capture objects at least about 1:100, about 1:50, about 1:20, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, about 5:1, about 10:1, about 20:1, about 50:1, about 100:1, or the like.

In some embodiments, the percentage of capture objects (e.g., beads) associated with at least one analyte molecule and at least one binding ligand to the total number of capture objects (e.g., beads) is less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.01%, or less. In some embodiments, the percentage of capture objects (e.g., beads) not associated with any binding ligand to the total number of capture objects is at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or greater.

A non-limiting example of an embodiment where a capture object is associated with more than one analyte molecule is illustrated in FIG. 2. A plurality of capture objects 20 are provided (step (A)). In this example, the plurality of capture objects comprises a plurality of beads. The plurality of beads is exposed to a fluid sample containing plurality of analyte molecules 21 (e.g., beads 20 are incubated with analyte molecules 21). At least some of the analyte molecules are immobilized with respect to a bead. For example, as shown in step (B), analyte molecule 22 is immobilized with respect to bead 24, thereby forming complex 26. Also illustrated is complex 30 comprising a bead immobilized with respect to three analyte molecules and complex 32 comprising a bead immobilized with respect to two analyte molecules. Additionally, in some cases, some of the beads may not associate with any analyte molecules (e.g., bead

28). The plurality of beads from step (B) is exposed to a plurality of binding ligands 31. As shown in step (C), a binding ligand associates with some of the analyte molecules immobilized with respect to a bead. For example, complex 40 comprises bead 34, analyte molecule 36, and binding ligand 38. The binding ligands are provided in a manner such that a statistically significant fraction of the beads comprising at least one analyte molecule become associated with at least one binding ligand (e.g., one, two, three, etc.) and a statistically significant fraction (i.e. as determined by Equation 1 above) of the beads comprising at least one analyte molecule do not become associated with any binding ligands. At least a portion of the plurality of beads from step (C) are then spatially separated into a plurality of locations. As shown in step (D), in this example, the locations comprise a plurality of reaction vessels 41 on a substrate 42. The plurality of reaction vessels may be exposed to the plurality of beads from step (C) such at each reaction vessel contains zero or one beads. The substrate may then be analyzed to determine the number of reaction vessels containing a binding ligand (e.g., reaction vessels 43), wherein in the number may be related to a measure of the concentration of analyte molecules in the fluid sample. In some cases, the number of reaction vessels containing a bead and not containing a binding ligand (e.g., reaction vessel 44), the number of reaction vessels not containing a bead (e.g., reaction vessel 45), and/or the total number of reaction vessels addressed/analyzed may also be determined. Such determination(s) may then be used to determine a measure of the concentration of analyte molecules in the fluid sample.

The foregoing exemplary methods may be performed using a number of different assay formats, different reaction conditions, and/or detection systems in different embodiments of the invention, several examples of which are described below. Additional components and/or method steps may be utilized as a substitute for and/or in combination with the exemplary methods and components described herein within the scope of the invention. It should be understood, while certain of the discussion herein focuses on a plurality of locations comprising a plurality of wells/reaction vessels in a substrate, this is by no means limiting and other materials may be used to segregate capture objects/molecules into a plurality of spatially distinct locations (e.g., regions in/on a hydrogel, points/regions on the surface of a planar substrate, etc.). As another example, while much of the discussion herein focuses on a plurality of capture objects comprising a plurality of beads, this is by no means limiting

and in other embodiments the capture objects may take other physical forms (e.g., nanotubes, disks, rings, microfluidic droplets, etc.).

Exemplary Assay Formats

The inventive assays may be carried our according to a very wide variety of basic protocols and formats. The particular format chosen can be based on the nature of the analyte molecules, the nature of the fluid sample containing the analyte molecules, and the availability and properties of binding partners of the analyte as well as other factors. Several exemplary basic formats were discussed previously in the context of the discussion of FIGS. 1-2. As would be apparent to those skilled in the art with the benefit of the teachings provided by the present disclosure, the invention may alternatively be performed according to protocols/formats not specifically described in the specific, exemplary embodiments illustrated in this detailed description, but which do not require undue burden or experimentation to practice.

As described above, an exemplary basic assay format/protocol comprises exposing a plurality of capture objects (e.g., beads) configured to capture an analyte molecule or particle to a sample containing or suspected of containing such analyte molecules (or particles). At least some of the analyte molecules may become immobilized with respect to a capture object. The plurality of capture objects may each include a binding surface having affinity for at least one type of analyte molecule. At least a portion of the capture objects may then be spatially segregated into a plurality of locations (e.g., reaction vessels/wells). Based at least in part on a determination of the number of locations comprising a capture object comprising at least one analyte molecule, a measure of the concentration of analyte molecules may be determined. Various other aspects of this basic assay format will now be discussed, including numerous considerations regarding the materials, concentrations, solutions, steps, and the like.

In certain embodiments, a plurality of capture objects is exposed to a sample containing or suspected of containing at least one type of analyte molecules, wherein the plurality of capture objects comprises a binding surface having an affinity for the at least one type of analyte molecule. In some cases, the binding surface may comprise a plurality of capture components. A "capture component", as used herein, is any molecule, other chemical/biological entity, or solid support modification disposed upon a solid support that can be used to specifically attach, bind or otherwise capture a target

molecule or particle (e.g., an analyte molecule), such that the target molecule/particle becomes immobilized with respect to the capture component and the support. The immobilization, as described herein, may be caused by the association of an analyte molecule with a capture component on the surface of the capture object. As used herein, "immobilized" means captured, attached, bound, or affixed so as to prevent dissociation or loss of the target molecule/particle, but does not require absolute immobility with respect to either the capture component or the object.

The number of analyte molecules which are immobilized with respect to a capture object may depend on the ratio of the total number of analyte molecules in the sample versus at least one of the total number, size, and/or surface density of capture components of capture objects provided. In some embodiments, the number of molecules or particles immobilized with respect to a single capture object may follow a standard Poisson distribution. In some cases, a statistically significant number of the capture objects associate with a single analyte molecule and a statistically significant number of capture objects do not associate with any analyte molecules. The total number of capture objects provided may be between about 10,000 and about 10,000,000, between about 50,000 and about 5,000,000, or between about 100,000 and about 1,000,000. In some cases, the total number of capture objects provided is at least about 10,000, at least about 50,000, at least about 100,000, at least about 1,000,000, at least about 5,000,000, or at least about 10,000,000. In some cases, the ratio of the number of analyte molecules in the fluid sample to capture objects provided is between about 10:1 and about 1:10,000,000, between about 8:1 and about 1:10,000,000, between about 10:1 and about 2:1, between about 2:1 and about 1:10, or less than about 1:10 (e.g., about 1:20, about 1:30, etc.). The ratio of analyte molecules in the fluid sample to capture objects provided may affect the assay steps and/or analysis carried out to determine a measure of the concentration of analyte molecules in the fluid sample, as described herein in the Quantification section.

In some cases, substantially all of the analyte molecules provided in the sample may become immobilized with respect to a capture object. That is, greater than about 90%, greater than about 95%, greater than about 97%, greater than about 98%, or greater than about 99% of the analyte molecules in the sample may become immobilized with respect to a capture object. In some cases, however, only a fraction of the analyte molecules in the sample may become immobilized with respect to a capture object. That is, in some cases, between about 1% and about 90%, between about 10% and about 90%,

between about 20% and about 80%, or between about 30% and about 70% of the analyte molecules provided in the sample are immobilized with respect to a capture object. In some embodiments, at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, or about 90%, or about 95% of the analyte molecules are immobilized with respect to a capture object.

In some formats of the assay, following immobilization, the plurality of capture objects (e.g., at least some of which are associated with at least one analyte molecule) may be exposed to a plurality of binding ligands. At least some of the analyte molecules immobilized with respect to a capture object may associate with a binding ligand. The number of binding ligands which associate with a capture object (e.g., via an analyte molecule) may depend on the ratio of the total number of analyte molecules immobilized with respect to a single capture object versus the total number of binding ligands exposed to the capture objects. For example, in embodiments where substantially all of the capture objects are associated with either zero or one analyte molecules, conditions may be selected such that substantially all of the analyte molecules associate with a single binding ligand, therefore each capture object associated with a single analyte molecule becomes associated with a single binding ligand (e.g., via the analyte molecule). Thus, the number of locations (e.g., reaction vessels) which contain a single analyte molecule may be determined by determining the number of locations (e.g., reaction vessels) which comprise a binding ligand. In such embodiments (e.g., where zero or at least one analyte molecules are associated with each capture object), the ratio of binding ligands provided (e.g., in a mixing step) to the total number of analyte molecules immobilized with respect to a capture object may be about 20:1, about 10:1, about 5:1, about 2:1, or about 1:1.

In some embodiments, however, a single capture object may be associated with zero, one, or more than one (e.g., two, three, four, etc.) analyte molecules. In such embodiments, the binding ligand may be provided at a concentration such that a statistically significant fraction of the capture objects comprising at least one analyte molecule associate with only a single binding ligand and a statistically significant fraction of the capture objects comprising at least one analyte molecule do not associate with any binding ligand. In other embodiments, however, the binding ligands may be provided at a concentration such that a statistically significant fraction of the capture objects comprising at least one analyte molecule associate with at least one binding ligand (e.g., one, two, three, etc.) and a statistically significant fraction of the

capture objects comprising at least one analyte molecule do not associate with any binding ligand. The concentration of analyte molecules in the fluid sample may then be determined, either with an analysis based at least in part of the number of locations containing a capture object associated with a binding ligand (e.g., by relating the concentration of analyte molecules in the fluid sample to the number of locations comprising a binding ligand), and/or an analysis based at least in part on an intensity reading of a signal indicative of the number of binding ligands at the addressed locations (e.g., in embodiments where at least some of the capture objects comprise more than one analyte molecule and/or more than one binding ligand, as described herein). In such embodiments (e.g., wherein more than one analyte molecule may be immobilized with respect to each capture object), the ratio of the number of binding ligands provided in solution to the number of analyte molecules immobilized with respect to a capture object may be about 1:50, about 1:40, about 1:30, about 1:20, about 1:10, about 1:5, about 1:3, about 1:2, about 1:1, or the like. In some cases, the ratio of the number of binding ligands provided in solution may be calculated based on the number of capture objects provided. In some cases, the ratio of binding ligands provided to the number of capture objects is about 1:50, about 1:40, about 1:30, about 1:20, about 1:10, about 1:5, about 1:3, about 1:2, about 1:1, or the like. In other cases, the ratio the number of capture objects to the number of binding ligands provided is about 1:50, about 1:40, about 1:30, about 1:20, about 1:10, about 1:5, about 1:3, about 1:2, or the like. In some embodiments, the quantification determination may comprise a Poisson distribution adjustment, as described herein.

In some embodiments, the concentration of binding ligand used in an assay may be selected as to minimize certain events which may occur when an excess of binding ligand is present, for example, non-specific binding of the binding ligand. In some cases, if the concentration of binding ligand is too high, an increase in background readings may occur due to non-specific interactions (e.g., with the capture objects, reaction vessels, etc.). In some cases, the concentration of binding ligand may be selected (or estimated, in the case of an unknown concentration of analyte molecule) such that a only a fraction of the analyte molecules immobilized with respect to a capture object associate with a binding ligand (e.g., about 0.1%, about 1%, about 2%, about 3%, about 4%, about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, or more). This may be especially useful in embodiments where the percentage of capture objects which associate with at least one analyte molecule is relatively high (e.g., greater than about

20%, greater than about 30%, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90%, or more). By providing the binding ligand at a lower concentration, in some cases, not every analyte molecule immobilized with respect to a capture object will associate with a binding ligand, which can be advantageous for quantification, for example when the presence of a binding ligand is required for detection, and especially when using a digital/binary read-out technique. For example, if the percentage of capture objects associated with an analyte molecule is about 50% or greater, a reduced number of binding ligands may be provided such that less than all of the immobilized analyte molecules associate with a binding ligand. In other cases, the percentage of binding ligands that associate with an analyte molecule may be reduced by decreasing the incubation time with the analyte molecule (e.g., limit the time of exposure such that only a fraction of the immobilized analyte molecules associate with an analyte molecule).

The total number of analyte molecules/binding ligands/capture objects/etc. in a solution may be determined using calculations with knowledge of the concentration of the analyte molecules/binding ligands/capture objects/etc. in solution. For example, the total number of binding ligands in a solution may be determined according to Equation 2:

$$\#of \quad binding \quad ligands = N_A \times [binding \quad ligand] \times volume \qquad \text{(Eq. 2)}$$

wherein N_A is Avogadro's number (6.022×10^{23} mol^{-1}), [binding ligand] is the concentration of the binding ligand in solution in moles per liter, and volume is the total volume of solution in liters employed. Similar calculations may be carried out for other components (e.g., analyte molecules (e.g., in a calibration sample), capture objects, etc.).

Following immobilization of a plurality of analyte molecules with respect to a plurality of capture objects and, in some cases, association of a binding ligand to at least some the immobilized analyte molecules, at least a portion of the capture objects may be spatially segregated into a plurality of locations. The percentage of capture objects which are spatially segregated into the plurality of locations may vary depending on numerous factors including, but not limited to, the ratio of the number of capture objects versus the total number of locations, the method of spatially segregating the capture objects, and/or the length of the time the capture objects are exposed to the locations. In some cases, at least about 0.5%, at least about 1%, at least about 2%, at least about 5%,

at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least above 90%, or more, of the capture objects are spatially segregated into the plurality of locations. In some cases, between about 0.1% and about 50%, between about 0.1% and about 30%, between about 0.5% and about 20%, between about 0.5% and about 10%, between about 0.5% and about 5%, between about 1% and about 10%, or about 0.5%, about 1%, about 2%, about 4%, about 5%, about 10%, about 20%, about 30%, about 50%, about 70%, or about 90% of the capture objects are spatially segregated into the plurality of locations. Following spatially segregating at least a portion of the capture objects into a plurality of locations, at least a portion of the locations may be addressed. The number of locations addressed may be about 0.5%, about 1%, about 2%, about 3%, about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more, of the total number of locations.

The portion of locations may be addressed to determine the number of locations containing an analyte molecule, or in some cases, a binding ligand. In some cases, the number of locations containing a capture object not associated with an analyte molecule (or a binding ligand), the number of locations containing and/or not containing a capture object, and/or the total number of locations analyzed/determined may also be determined. A measure of the concentration of analyte molecules in the fluid sample may be determined at least in part on the number of locations determined to contain an analyte molecule (or binding ligand). In some cases the measure of the concentration of analyte molecules in the fluid sample may be based at least in part on the ratio of the number of locations containing a capture object associated with an analyte molecule to the total number of locations addressed or the total number of locations addressed that contain a capture object. In other cases, a measure of the concentration of analyte molecules in the fluid sample may be based at least in part on the ratio of the number of locations containing a capture object associated with an analyte molecule to the number of locations containing a capture object not associated with an analyte molecule. Specific methods and calculations which may be used to determine the measure of the concentration of analyte molecules in the fluid sample are discussed in more detail below.

The ratios, percentages, and other parameters described herein with respect to the amount/quantity/ratio of a first component to a second component (for example, analyte molecules/capture objects, binding ligands/capture objects, binding ligands/analyte

molecules, capture objects/locations, precursor labeling agents/binding ligands, etc.) may be adjusted as desired to yield a desired ratio of analyte molecules/binding ligands captured per capture object, and/or may be controlled or determined using no more than routine experimentation, calculations (in some cases, including accounting for Poisson distributions), screening tests, etc., given the teaching and guidance provided by the present specification. For example, if the number of capture objects provided is known (e.g., as determined using a similar formula as given in Equation 1), the number of binding ligands that need to be provided may be determined based on the desired ratio of capture objects to binding ligands, and hence, the amount of moles of binding ligand that should be provided may be determined. As another example, in the case of an unknown concentration of analyte molecules, if a first assay method indicates that a significant number of capture objects comprise more than one analyte molecule (e.g., all or a significant number of locations are determined to contain an analyte molecule or there is less than a statistically significant number of beads determined to be free of analyte molecules), the fluid sample may be diluted and/or the number of capture objects may be increased such that the number of capture objects comprising at least one analyte molecule may be decreased.

Other aspects of the assay will now be discussed in detail. It should be understood, that none, a portion of, or all of the following steps may be performed at least once during the certain exemplary assay formats described herein. Non-limiting examples of additional steps not described which may be performed include, but are not limited to, washing and/or exposure to additional binding ligands, precursor labeling agents, and/or labeling agents, etc.

In some embodiments, the plurality of capture objects (e.g., at least some of which are associated with at least one analyte molecule) may be exposed to at least one additional reaction component prior to, concurrent with, and/or following spatially separating at least some of the plurality of capture objects into a plurality of locations. In some cases, the capture objects may be exposed to a plurality of binding ligands. In certain embodiments, a binding ligand may be adapted to be directly detected (e.g., the binding ligand comprises a detectable molecule or moiety) or may be adapted to be indirectly detected (e.g., including a component that can convert a precursor labeling agent into a labeling agent), as discussed more below. More than one type of binding may be employed in any given assay method, for example, a first type of binding ligand and a second type of binding ligand. In one example, the first type of binding ligand is

able to associate with a first type of analyte molecule and the second type of binding ligand is able to associate with the first binding ligand. In another example, both a first type of binding ligand and a second type of binding ligand may associate with the same or different epitopes of a single analyte molecule, as described below.

Certain binding ligands can comprise a component that is able to facilitate detection, either directly or indirectly. A component may be adapted to be directly detected in embodiments where the component comprises a measurable property (e.g., a fluorescence emission, a color, etc.). A component may facilitate indirect detection, for example, by converting a precursor labeling agent into a labeling agent (e.g., an agent that is detected in an assay). A "precursor labeling agent" is any molecule, particle, or the like, that can be converted to a labeling agent upon exposure to a suitable converting agent (e.g., an enzymatic component). A "labeling agent" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique.

In some embodiments, at least one binding ligand comprises an enzymatic component. In some embodiments, the analyte molecule may comprise an enzymatic component. The enzymatic component may convert a precursor labeling agent (e.g., an enzymatic substrate) into a labeling agent (e.g., a detectable product). A measure of the concentration of analyte molecules in the fluid sample can then be determined based at least in part by determining the number of locations containing a labeling agent (e.g., by relating the number of locations containing a labeling agent to the number of locations containing an analyte molecule). Non-limiting examples of enzymes or enzymatic components include horseradish peroxidase, beta-galactosidase, and alkaline phosphatase. Other non-limiting examples of systems or methods for detection include embodiments where nucleic acid precursors are replicated into multiple copies or converted to a nucleic acid that can be detected readily, such as the polymerase chain reaction (PCR), rolling circle amplification (RCA), ligation, Loop-Mediated Isothermal Amplification (LAMP), etc. Such systems and methods will be known to those of ordinary skill in the art, for example, as described in "DNA Amplification: Current Technologies and Applications," Vadim Demidov et al., 2004.

As an example of an assay method which comprises the use of a precursor labeling agent, as shown in FIG. 3, substrate 100 comprising a plurality of locations is provided, wherein the locations comprise reaction vessels. In reaction vessel 101 (e.g., location), analyte molecule 102 is immobilized with respect to bead 103 (e.g., capture

object). Binding ligand 104 is associated with analyte molecule 102. Binding ligand 104 comprises an enzymatic component (not shown). Precursor labeling agent 106 is converted to labeling agent 108 (upon exposure to the enzymatic component). Labeling agent 108 is detected using methods described herein. In contrast, reaction vessel 111 contains analyte molecule 112 immobilized with respect to bead 110. In this reaction vessel, analyte molecule 112 is not associated with a binding ligand comprising an enzymatic component. Therefore, precursor labeling agent 114 is not converted to a labeling agent in the reaction vessel. Thus this reaction vessel would give a different signal as compared to reaction vessel 101 where the precursor labeling agent was converted to a labeling agent. In some cases, there may also be reaction vessels which contain a bead not associated with an analyte molecule, for example, reaction vessel 121 contains bead 116. Additionally, some of the reaction vessels may not comprise any bead, for example, reaction vessel 123. Reaction vessels 121 and 123 may give different signals as compared to reaction vessel 101 as there would be no labeling agent present. However, reaction vessels 121 and 123 may contain precursor labeling agent 117. More than one precursor labeling agent may be present in any given reaction vessel.

In certain embodiments, solubilized, or suspended precursor labeling agents may be employed, wherein the precursor labeling agents are converted to labeling agents which are insoluble in the liquid and/or which become immobilized within/near the location (e.g., within the reaction vessel in which the labeling agent is formed). Such precursor labeling agents and labeling agents and their use is described in commonly owned U.S. Patent Application Serial No. 12/236,484, entitled "High Sensitivity Determination of the Concentration of Analyte molecules in a Fluid Sample," by Duffy, et al., filed September 23, 2008, incorporated herein by reference.

In some embodiments, during the assay, at least one washing step may be carried out. In one instance, a plurality of capture objects may be washed after exposing the capture objects to one or more solutions comprising analyte molecules, binding ligands, precursor labeling agents, or the like. For example, following immobilization of the analyte molecules with respect to a plurality of capture objects, the plurality of capture objects may be subjected to a washing step thereby removing any analyte molecules not specifically immobilized with respect to a capture object. In certain embodiments, the wash solution is selected so that it does not cause appreciable change to the configuration of the capture objects and/or analyte molecules and/or does not disrupt any specific binding interaction between at least two components of the assay (e.g., a capture

component and an analyte molecule). In other cases, the wash solution may be a solution that is selected to chemically interact with one or more assay components. As will be understood by those of ordinary skill in the art, a wash step may be performed at any appropriate time point during the inventive methods.

In some embodiments, assay methods may be carried out that do not comprise the use of a plurality of capture objects comprising a binding surface for at least one type of analyte molecule and/or a plurality of locations to which the capture objects may be spatially separated. For example, an assay according to the invention in certain embodiments may use any suitable method which is capable of isolating single analyte molecules and/or capture objects associated with one or more analyte molecules such that they can be individually addressed for detection. For example, an assay method may comprise providing a plurality of capture objects which are each associated with either a single analyte molecule or are free of any analyte molecules. At least a portion of the capture objects may be individually addressed to determine the number of the capture objects associated with an analyte molecule or particle. Based at least in part on the number of capture objects determined to be associated with an analyte molecule, a measure of the concentration of analyte molecules or particles in a fluid sample may be determined.

FIG. 4A illustrates a non-limiting embodiment where single analyte molecules are spatially segregating into a plurality of droplets. In FIG. 4A, plurality of analyte molecules 70 are provided, as shown in step (A). In this example, analyte molecules 70 are capable of being optically detected (e.g., the analyte molecules may be directly detected using optical interrogation). At least some of the plurality of analyte molecules 70 are contained within liquid droplets 72 (e.g., using microfluidic techniques) which comprise fluid 71, as shown in step (B). Additionally, some droplets may be present which do not contain any analyte molecules (e.g., droplets 74 comprising fluid 71). Plurality of droplets 75 are substantially surrounded by fluid 73 which is substantially immiscible with fluid 71. Plurality of droplets 75 can be optically interrogated by feeding droplets into column 74 such that each droplet passes by an optical detection system (e.g., comprising light source 76 and detector 78) single file, as shown in step (C). Each droplet may be determined to contain an analyte molecule when there is a change in the optical single (e.g., a change in optical signal due to the presence of an analyte molecule in the droplet).

As another example, as illustrated in FIG. 4B, plurality of analyte molecules 80 are provided, as shown in step (A). In this example, analyte molecules 70 are not capable of being optically detected, and must be indirectly detection (as described herein). Plurality of analyte molecules 80 are exposed to a plurality of binding ligands 82, such that at least one binding ligand associates with a significant portion of the analyte molecules, as shown in step (B), to form complex 83, as shown in step (B). In this example, each binding ligand 82 comprises enzymatic component 84. At least a portion of complexes 83 may be contained in droplets 85 (e.g., using microfluidic techniques), as shown in step (C), which comprise liquid 79. Additionally, some droplets may be present which do not contain any complexes (e.g., droplets 86 comprising fluid 79). Plurality of droplets 91 are substantially surrounded by fluid 87 which is substantially immiscible with fluid 79. Droplets 85 and 86 may additionally comprise precursor labeling agent 86, which is converted to labeling agent 88 upon exposure to enzymatic component 84, as indicated by arrow 87. Plurality of droplets 91 can be optically interrogated by feeding the plurality of droplets into column 89 such that each droplet passes by an optical detection system (e.g., comprising light source 90 and detector 92) single file, as shown in step (D). Each droplet may be determined to contain an analyte molecule when there is a change in the optical single (e.g., a change in optical signal due to the presence of a labeling agent in the droplet).

As yet another example, FIG. 4C illustrates and embodiment where single analyte molecules 282 are associated with respect to objects 280 via capture components 274. Additionally, in this example, the immobilized analyte molecules are associated with binding ligand 284. The droplets can be optically interrogated by feeding droplets into column 287 such that each droplet passed by the optical detection system (e.g., comprising light source 286 and detector 288) single file. Each droplet may be determined to contain a binding ligand when there is a change in the optical single (e.g., a change in optical signal due to the presence of a binding ligand in the droplet).

The following sections provide additional information regarding method steps, materials, and parameters that may be used to practice the assay methods described above.

Capture Objects and Spatial Locations for Capture Object Segregation

In some embodiments, the method and systems of the present invention utilize a plurality of capture objects that each includes a binding surface having affinity for at

least one type of analyte molecule. The plurality of capture objects may be configured to be able to be spatially segregated from each other, that is, the capture objects may be provided in a form such that the capture objects are capable of being spatially separated into a plurality of locations. For example, the plurality of capture objects may comprise a plurality of beads (which can be of any shape, e.g., sphere-like, disks, rings, cube-like, etc.), a dispersion or suspension of particulates (e.g., a plurality of particles in suspension in a fluid), nanotubes, or the like. In some embodiments, the plurality of capture objects is insoluble or substantially insoluble in the solvent(s) or solution(s) utilized in the assay. In some cases, the capture objects are solid or substantially solid (e.g., is essentially free of pores), however, in some cases, the plurality of capture objects may be porous or substantially porous, hollow, partially hollow, etc. The plurality of capture objects may be non-absorbent, substantially non-absorbent, substantially absorbent, or absorbent. In some cases, the capture objects may comprise a magnetic material, which as described herein, may facilitate certain aspect of the assay (e.g., washing step). In some cases, a capture object surface may also comprise a protective or passivating layer that can reduce or minimize non-specific binding events (e.g., analyte molecules, binding ligands, etc.).

In some embodiments, the capture objects each include a binding surface having affinity for at least one type of analyte molecule of interest. The portion of the capture object which comprises a binding surface may be selected or configured based upon the physical shape/characteristics and properties of the capture objects (e.g., size, shape), and the format of the assay. In some embodiments, substantially all of the outer surfaces of the capture objects form the binding surfaces. A binding surface having an affinity for at least one type of analyte molecule may be formed via the association of a plurality of capture components with a capture object. In some cases, an analyte molecule may associate with a capture component (e.g., become immobilized with respect to) via formation of at least one chemical bond and/or physical adsorption, or combination thereof. Non-limiting examples of types of chemical bonds include ionic bonds, covalent bonds (e.g., carbon-carbon, carbon-oxygen, oxygen-silicon, sulfur-sulfur, phosphorus-nitrogen, carbon-nitrogen, metal-oxygen, or other covalent bonds), hydrogen bonds (e.g., between hydroxyl, amine, carboxyl, thiol, and/or similar functional groups), dative bonds (e.g., complexation or chelation between metal ions and monodentate or multidentate ligands), Van der Waals interactions, or the like. Capture components which are useful or potentially useful for practicing certain aspects and embodiments of

the invention are discussed in more detail below. At least some of the analyte molecules, upon exposure to the plurality of capture objects comprising a plurality of capture components, become immobilized with respect to a capture component. In certain embodiments, substantially all of the plurality of analyte molecules in the fluid sample tested may become immobilized with respect to the capture components (and hence, the capture objects).

Without wishing to be bound by any theory, the use in certain embodiments of the invention of a capture step in which a plurality of capture objects having a large surface area for binding are exposed to a fluid sample containing the analyte molecules or particles such that the analyte molecules/particles become immobilized with respect to the capture objects may facilitate an increase in the speed and/or efficiency of the assay for detection and quantification of concentration of the analyte in the sample compared to assays where the analyte molecules themselves are segregated for detection without being exposed to and immobilized with respect to a capture object. This increase in binding speed and efficiency may be further enhanced if the solution in which the plurality of analyte molecules and capture objects are incubated for capture is agitated (e.g., stirred) to increase the collision frequency and mass transfer rate between the plurality of capture objects (e.g., plurality of beads) and the analyte molecules (e.g., as contrasted with a substrate comprising a stationary surface (e.g., a microtiter plate)).

The plurality of capture objects for analyte capture may be of any suitable size or shape. Non-limiting examples of suitable shapes include spheres, cubes, ellipsoids, tubes, sheets, and the like. In certain embodiments, the average diameter (if substantially spherical) or average maximum cross-sectional dimension (for other shapes) of a capture object may be greater than about 0.1 um (micrometer), greater than about 1 um, greater than about 10 um, greater than about 100 um, greater than about 1 mm, or the like. In other embodiments, the average diameter of a capture object or the maximum dimension of a capture object in one dimension may be between about 0.1 um and about 100 um, between about 1 um and about 100 um, between about 10 um and about 100 um, between about 0.1 um and about 1 mm, between about 1 um and about 10 mm, between about 0.1 um and about 10 um, or the like. The "average diameter" or "average maximum cross-sectional dimension" of a plurality of capture objects, as used herein, is the arithmetic average of the diameters/maximum cross-sectional dimensions of the capture objects. Those of ordinary skill in the art will be able to determine the average diameter/maximum cross-sectional dimension of a population of capture objects, for

example, using laser light scattering, microscopy, sieve analysis, or other known techniques. For example, in some cases, a Coulter counter may be used to determine the average diameter of a plurality of beads.

The capture objects for analyte capture may be fabricated from one or more suitable materials, for example, plastics or synthetic polymers (e.g., polyethylene, polypropylene, polystyrene, polyamide, polyurethane, phenolic polymers, or nitrocellulose etc.), naturally derived polymers (latex rubber, polysaccharides, polypeptides, etc), composite materials, ceramics, silica or silica-based materials, carbon, metals or metal compounds (e.g., comprising gold, silver, steel, aluminum, copper, etc.), inorganic glasses, silica, and a variety of other suitable materials. Non-limiting examples of potentially suitable configurations include beads (e.g., magnetic beads), tubes (e.g., nanotubes), plates, disks, dipsticks, or the like.

In some cases, the capture objects may be selected such that the capture object itself is detectable by the system. This may be useful in embodiments where the fraction or percentage of capture objects associated with an analyte molecule is to be determined (e.g., when the total number of capture objects interrogated and detected is used to determine the fraction of capture objects associated with an analyte molecule). For example, a capture object may be characterized as having an emission or absorption spectrum that can be exploited for detection so that capture objects may be interrogated to determine which spatial location contains a capture object. The properties of the emission spectrum (e.g., wavelength(s), intensity, etc.), may be selected such that the emission produced by the capture objects does not substantially alter and/or interfere with any other emission from components used in the assay (e.g., the emission of any labels used to determine the presence or absence of an analyte molecule). In some cases, dye molecules may be associated with a capture object using the binding ligands present on the surface of the capture object (e.g., a dye molecule may associate via a bond or interaction with a capture component). In some cases, between about 1 and about 50,000, or between about 1000 and about 50.000, or between about 1000 and about 20.000, or between about 10,000 and about 20,000, or between about 1,000 and about 10,000, or between about 10,000 and about 30,000, or between about 1 and about 10,000 dye molecules (e.g., fluorescent dye molecules) are associated with each capture object. See, for example, Example 21. In some cases, dye molecules might first be associated with the capture object, before the binding ligands are attached to the capture object. This approach reduces the chances that the affinity of the binding ligands are reduced by

attachment of dye molecules. In some cases, dye molecules are attached to an inert, "blocking" protein (e.g., bovine serum albumin) and the dye-labeled inert protein is used to block the beads after binding ligands are attached to the capture object. It should be understood, that in some cases, the capture objects may be detected and quantified using white light (e.g., as described herein, for example using bright field, dark field and/or phase contrast imaging).

In some embodiments, more than one type of capture object for analyte capture may be employed. In some cases, each type of capture object may include a surface with differing binding specificity. In these embodiments, more than one type of analyte molecule may be quantified and/or detected in a single, multiplexed assay method. For example, the plurality of capture objects for analyte capture may comprise a plurality of a first type of capture object comprising a binding surface having an affinity for a first type of analyte molecule and a plurality of a second type of capture objects comprising a binding surface having an affinity for a second type of analyte molecule. Upon exposure to a sample containing the first type of analyte molecule and the second type of analyte molecule, the first type of analyte molecule becomes immobilized with respect to the first type of capture object and the second type of analyte molecule becomes immobilized with respect to the second type of capture object. The first type of capture object and the second type of capture object may be encoded to be distinguishable from each other (e.g., to facilitate differentiation upon detection) by including a differing detectable property. For example, each type of capture object may have a differing fluorescence emission, a spectral reflectivity, shape, a spectral absorption, or an FTIR emission or absorption. In a particular embodiment, each type of capture object may comprise one or more dye compounds (e.g., fluorescent dyes) but at varying concentration levels, such that each type of capture object has a distinctive signal (e.g., based on the intensity of the fluorescent emission). Upon spatially segregating the capture objects after the capture step into a plurality of locations for detection, a location comprising a first type of capture object associated with a first type of analyte molecule may be distinguished from a location comprising a second type of capture object associated with a second type of analyte molecule via detection of the differing property. The number of locations comprising each type of capture object and/or the number of capture objects associated with an analyte molecule may be determined, enabling a determination of a measure of the concentration of both the first type of analyte molecule

and the second type of analyte molecules in the fluid sample based at least in part on these numbers.

For example, as illustrated in FIG. 5, step (A) a plurality of a first type of capture object 132 comprising a first type of capture component 134 and a plurality of a second type of capture object 136 comprising a second type of capture component 138 are provided. The plurality of capture objects is exposed to a fluid sample comprising a plurality of a first type of analyte molecule 140 and a second type of analyte molecule 142. As shown in step (B), at least some of the first type of analyte molecule 140 may associate with a capture object of the first type 132 via capture component 134 and at least some of the second type of analyte molecule 142 may associate with a capture object of the second type 136 via capture component 138. Some of the first type of capture objects and second type of capture objects may not associate with any first type of or second type of analyte molecules. At least some of the plurality of capture objects from step (B) may be spatially segregated into a plurality of locations (represented by reaction vessels 142 formed in substrate 146, as shown in step (C). Some of the reaction vessels may not comprise any capture objects. The plurality of reaction vessels may then be analyzed (e.g., for example, as illustrated in FIG. 1, step (D)) to determine the number of reaction vessels containing a first type of capture object associated with a first type of analyte molecule (e.g., reaction vessel 144) and the number of reaction vessels containing a second type of capture object associated with a second type of analyte molecule (e.g., reaction vessel 145). Additionally, the number of locations containing a first type of capture object or a second type of capture object not associated with any analyte molecules may also be determined. A measure of the concentration of the first type (or second type) of analyte molecule may be determined at least in part based on the number of the first type (or second type) of analyte molecule detected. Alternatively, a measure of the concentration of either first type (or second type) of analyte molecule in the fluid sample may be based on the ratio of the number of reaction vessels comprising the first type (or second type) of capture object associated with a first type (or second type) of analyte molecule to the number of reactions vessels comprising the first type (or second type) of capture objects not associated with any analyte molecules. Additional methods for determining the concentration of the first and/or second types of analyte molecules in the fluid sample may be carried out using methods similar to those described herein for samples comprising a single type of analyte molecule. Using optical detection, the first type of capture object may have a maximum wavelength of emission

at a first wavelength and the second type of capture object may maximum wavelength of emission at a second wavelength, and therefore, allow for the reaction vessels which contain a first type of capture object to be distinguished from the reaction vessels which contain a second type of capture object.

Alternatively, or in combination, with use of coded capture objects for multiplexing, as described above, in some multiplexed assays, similar capture object types may be employed that may, in certain cases, each include capture components specific for multiple types of analytes. In certain such assays, a first type of binding ligand, e.g., having affinity for a first analyte molecule type, and a second, third, etc. type of binding ligand having affinity for a second, third, etc., respectively type of analyte molecule type and configured to be detectably distinguishable from each other (e.g., through use of differing detectable markers, enzymatic components and/or labeling agents, etc.) may be used in conjunction with the assay, and the detection/quantification of the different types of binding ligands may be correlated to the presence/concentration of different types of analyte molecules in the fluid sample.

In a particular embodiment, the plurality of capture objects for analyte capture comprises a plurality of beads. The beads may each comprise a plurality of capture components via which a plurality of analyte molecules may be immobilized. The plurality of capture components may be present on the surface of the beads. In some embodiments, the beads may be magnetic beads. The magnetic property of the beads may help in separating the beads from a solution (e.g., comprising a plurality of unbound analyte molecules) and/or during washing step(s) (e.g., to remove excess fluid sample, labeling agents, etc.). Potentially suitable beads, including magnetic beads, are available from a number of commercial suppliers. As noted above, there are many other examples of potentially suitable capture objects for analyte capture including nanotubes (e.g., carbon nanotubes), microfluidic droplets (e.g., droplets of a first fluid substantially surrounded by a second fluid), etc.

Those of ordinary skill in the art will be aware of methods and techniques for exposing a plurality of capture objects to a fluid sample containing or suspected of containing an analyte molecule or particle for initial analyte capture. For example, the plurality of capture objects may be added (e.g., as a solid, as a solution) directly to a fluid sample. As another example, the fluid sample may be added to the plurality of capture objects (e.g., in solution, as a solid). In some instances, the solutions may be agitated (e.g., stirred, shaken, etc.).

Following immobilization of the analyte molecules with respect to a plurality of capture objects, the capture objects may be subjected to at least one wash step. The wash step may aid in the removal of any unbound molecules (e.g., analyte molecules, or other reaction components) from the solution. For example, referring to FIG. 1, following immobilization of analyte molecules 4 with beads 6, as shown in step (B), a wash step may be performed to remove any unbound analyte molecules not immobilized with respect to an analyte molecule. As another example, referring to FIG. 2, following association of binding ligands 31 with analyte molecules 36, as shown in step (C), a wash step may be performed to remove any unbound binding ligands. The wash step may be performed using any suitable technique known to those of ordinary skill in the art, for example, by incubation of the capture objects with a wash solution followed by centrifuging the solution comprising the capture objects and decanting off the liquid, or by using filtration techniques. In embodiments where the plurality of capture objects comprises a plurality of magnetic beads, the beads may be isolated from the bulk solution with aid of a magnet.

The plurality of capture objects subsequent to the capture step (e.g., at least some associated with at least one analyte molecule) may be exposed to one or more additional reagents, prior to spatially segregating the plurality of capture objects into a plurality of locations for detection. For example, as noted previously, the capture objects may be exposed a plurality of binding ligands, at least some of which may associate with an immobilized analyte molecule. The capture objects may be exposed to more than one type of binding ligand (e.g., a first type of binding ligand and a second, third, etc. type of a binding ligand), as noted above. The association of a binding ligand with an immobilized analyte molecule may aid in the detection of the analyte molecules, as described herein.

In some embodiments, in addition to a plurality of capture objects for analyte capture, a plurality of control objects may also be provided and/or employed. A control object(s) may be useful for a variety of purposes including, but not limited to, identification of the orientation of the plurality of locations (e.g., in the case where the plurality of locations is formed as an array of reaction sites, reaction vessels, etc.), to help determine the quality of the assay, and/or to help calibrate the detection system (e.g., optical interrogation system), as described below. It should be understood, that more than one type of control object may be present in any assay format (e.g., a first type of control object to determine quality of the assay and a second type of control object to

act as a location marker), or a single type of control object may have more than one of the above-described functions.

In some cases, the control objects used to identify the orientation of the plurality of locations (e.g., reaction vessels, sites, etc.) on an array (e.g., function as location marker(s) for an array). For example, a control object may be randomly or specifically distributed on an array, and may provide one or more reference locations for determining the orientation/position of the array. Such a feature may be useful when comparing multiple images of a portion of the array at different time intervals. That is, the positions of control objects in the array may be used to register the images. In some cases, the control objects may be use to provide reference locations in embodiments where a plurality of images of small overlapping regions are being combined to form a larger image.

The presence of control objects in an assay may provide information regarding the quality of the assay. For example, if a location is found to contain a control object comprising an enzymatic component but no labeling agent is present (e.g., the product of which would be present upon exposure of a control object comprising an enzymatic component to a precursor labeling agent), this gives an indication that some aspect of the assay may not be functioning properly. For example, the quality of the reagents may be compromised (e.g., concentration of precursor labeling agent is too low, decomposition of the precursor labeling agent, etc.), and/or perhaps not all of the locations were exposed to the precursor labeling agent.

In some embodiments, the control objects may be used to calibration the detection system. For example, the control objects may output an optical signal which may be used to calibration an optical detection system. In some embodiments, the control objects can be characterized and doped with a particular characteristic (e.g., fluorescence, color, absorbance, etc.) which can act as a quality control check for the detection system performance.

In some cases, the control objects may be used to standardize or normalize the system to account for variations of the performance and/or characteristics of different system components in different assays, over the course of time, etc. (e.g., detection system, arrays, reagents, etc.) between different potion of an array used in a test, and/or between two different arrays. For example, experimental set-up, parameters and/or variations may lead to changes the intensity of a signal (e.g., fluorescence signal) produced from a single array at different time points, or between at least two arrays at

simultaneous or different time points. In addition, in a single array, different portions of the array may produce different background signals. Such variations may lead to changes in calibration signals (e.g., determination of an average bead signal) between arrays, portions of and array or at multiple times, which can lead to inaccurate determinations in some cases. Non-limiting examples of parameters that may cause variation include labeling agent concentration, temperature, focus, intensity of detection light, depth and/or size of the locations in an array, etc. To account for the effects of some or all of such variations, in some embodiments, a plurality of control objects may be utilized. In certain instances, such control objects are essentially free of association with analyte molecules or particles. In certain embodiments, less than about 20%, about 10%, about 5%, about 1%, etc. of the control objects are associated with analyte molecules or particles. The control objects may be distinguishable from the capture objects (e.g., each may produce a distinguishable signal) and the system may be configured such that any analyte molecules associated with a control object are not accounted for in the concentration determination of the analyte molecules. The signals from the control objects may be used to normalize the interrogation values between different arrays, or in areas of a single array. For example, because the signals from the control objects should be approximately equal between arrays and/or about a single array, the control object signals may be normalized to an appropriate value and the signals of the non-control objects (e.g., the capture objects associated with an analyte molecule) may be adjusted accordingly.

The control object may be provided with the plurality of capture objects for analyte capture prior to exposure to a fluid sample containing analyte molecules, or may be added at another point in the assay (e.g., following exposure to the plurality of analyte molecules and/or binding ligands, and/or prior to spatially segregating the plurality of capture objects into a plurality of locations). The control objects may be distinguishable from the capture objects using techniques known to those of ordinary skill in the art. For example, in some embodiments, the control objects may comprise a unique property (e.g., are encoded) as compare to the capture objects comprising a binding surface for the analyte molecules. For example, the control object may have a different fluorescence emission, a spectral reflectivity, shape, a spectral absorption, or an FTIR emission or absorption, as compared to the capture objects. The percentage of control objects to total number of objects (e.g., capture objects and control objects) in the assay may be about

0.0001%, about 0.0005%, about 0.001%, about 0.005%, about 0.01%, about 0.05%, about 0.1%, about 0.5%, about 1%, about 5%, or the like.

In some embodiments, the control objects are configured as negative binding controls and may be of a similar shape and size as the capture objects used for immobilizing analyte molecules, however, the control objects may lack a binding surface for the analyte molecules (e.g., a plurality of capture components). For example, the control objects may comprise a plurality of beads, and the capture objects for immobilizing the analyte molecules may comprise the same or similar beads, additionally comprising at least one surface comprising a plurality of capture components.

In one embodiment, the control objects may comprise a positive control and include an enzymatic component. A precursor labeling agent may be converted to a labeling agent upon exposure to the enzymatic component. In some cases, the enzymatic component may be the same as the enzymatic component being used to detect the analyte molecules in a fluid sample (e.g., comprised in another component of the assay, for example, an enzymatic component comprised in a binding ligand, an analyte molecule, etc.). In such embodiments, the control object may be distinguishable from the capture objects such that the reaction vessels having a positive signal may be analyzed to determine whether the reaction vessel comprises a control object (e.g., having a first detectable signal) or a capture object (e.g., having a second detectable signal distinguishable from the first detectable signal). In other cases, the enzymatic component may be the different than an enzymatic component being used to detect the analyte molecules in a fluid sample (e.g., comprised in another component of the assay, for example, an enzymatic component comprised in a binding ligand, an analyte molecule, etc.). In this embodiment, the control object may or may not be distinguishable from the capture objects. Both a first type and a second type of precursor labeling agent may be provided to the reaction vessels, and the first type of precursor labeling agent may be converted to a first type of labeling agent upon exposure to the enzymatic component associated with the control beads and the second type of precursor labeling agent may be converted to a second type of labeling agent upon exposure to the other enzymatic component (e.g., comprised in the binding ligand/analyte molecule/etc.). The reaction vessels containing the first type of labeling agent correspond to the reaction vessels containing a control object and reaction vessels containing a second type of labeling agent correspond to the reaction vessels which contain a binding ligand/analyte

molecule/etc. The plurality of locations containing a control bead may be observed to analyze, for example, the effectiveness of the enzymatic conversion reaction

A variety of methods may be used to prepare the controls objects, for example, similar methods described herein for the capture objects (e.g., beads comprising a plurality of capture components). In some cases, some of the control objects may comprise an enzymatic component. The control objects may be prepared such that 1) the majority of control objects each comprise at least one enzymatic component (e.g., one, two, three, four, etc.) or 2) some of the control objects comprise a single enzymatic component and the remainder of the control objects do not comprise any enzymatic component. In the first case, during formation of the control objects, the ratio of enzymatic components provided in solution to objects may be greater than 1:1, greater than about 2:1, greater than about 5:1, greater than about 10:1, or the like. In such cases, it would be expected that after partitioning the control objects on a substrate, each location comprising a control object would give a positive signal on exposure to a precursor labeling agent. In the second case, during formation of the control objects, the ratio of enzymatic components provided in solution to objects may be less than about 1:5, less than about 1:10, less than about 1:20, less than about 1:50, or the like. In such cases, it would be expected that ratio of the number of locations comprising a control object and giving a positive signal to the number of locations comprising a control object not giving a positive signal would be approximately similar to the ratio of enzymatic components to objects during formation of the control objects and/or may follow a Poisson distribution.

As described above, following immobilization of a plurality of analyte molecules with respect to the plurality of capture objects in the analyte capture step, at least a portion of the capture objects may be spatially segregated into a plurality of locations, for example on a substrate. For example, each of capture objects of the portion of capture objects which are spatially segregated may be positioned in and/or associated with a location (e.g., a spot, region, well, etc. on the surface and/or in the body of a substrate) that spatially distinct from the locations in which each of the other capture objects are located, such that the capture objects and locations can be individually resolved by an analytical detection system employed to address the locations. As an example, each of a potion of the capture objects may be spatially segregated into an array of reaction vessels on a substrate, such that statistically only zero or one capture objects are located in at least some of the reaction vessels and in certain cases in essentially each reaction vessel.

Each location may be individually addressable relative to the other locations. Additionally, the locations may be arranged such that a plurality of locations may be addressed substantially simultaneously, as described herein, while still permitting the ability to resolve individual locations and capture objects.

It should be understood, that while much of the discussion herein focusing on locations containing a single capture object, this is by no means limiting, and in some embodiments, more than one capture object may be contained at a single location. In such embodiments, the ratio of capture objects to analyte molecules may be such that following spatial segregation of the plurality of capture objects into the plurality of locations, a statistically significant fraction of the locations contain no analyte molecules and a statistically significant fraction of locations contain at least one analyte molecule. That is, while a single location may contain a plurality of capture objects, in some cases, none of the capture objects are associated with any analyte molecules and only a single one of the capture objects in an addressed location is associated with at least one analyte molecule.

As noted above, in some embodiments, the plurality of locations comprises a plurality of reaction vessels/wells on a substrate. The reactions vessels, in certain embodiments, may be configured to receive and contain only a single capture object used for analyte capture. The plurality of capture objects can be partitioned across a plurality of such reaction vessels (e.g., configured as an array of reaction vessels on a substrate), in some cases, to facilitate determination of a measure of the concentration of analyte molecules in a fluid sample by means discussed in further detail below and in the examples.

In some embodiments of the present invention, the plurality of reaction vessels may be sealed e.g., after the introduction of the capture objects used for analyte capture, for example, through the mating of the second substrate and a sealing component. The sealing of the reaction vessels may be such that the contents of each reaction vessel cannot escape the reaction vessel during the remainder of the assay. In some cases, the reaction vessels may be sealed after the addition of the capture objects and, optionally, a precursor labeling agent to facilitate detection of the analyte molecules. For embodiments employing precursor labeling agents, by sealing the contents in some or each reaction vessel, a reaction to produce the detectable labeling agents can proceed within the sealed reaction vessels, thereby producing a detectable amount of labeling agents that is retained in the reaction vessel for detection purposes.

The plurality of locations comprising a plurality of reaction vessels may be formed using a variety of methods and/or materials. In some cases, the plurality of reaction vessels is formed as an array of depressions on a first surface. In other cases, however, the plurality of reaction vessels may be formed by mating a sealing component comprising a plurality of depressions with a substrate that may either have a featureless surface or include depressions aligned with those on the sealing component. Any of the device components, for example, the substrate or sealing component, may be fabricated from a compliant material, e.g., an elastomeric polymer material, to aid in sealing. The surfaces may be or made to be hydrophobic or contain hydrophobic regions to minimize leakage of aqueous samples from the microwells.

In some cases, the sealing component may be capable of contacting the exterior surface of an array of microwells (e.g., the cladding of a fiber optic bundle as described in more detail below) such that each reaction vessel becomes sealed or isolated such that the contents of each reaction vessel cannot escape the reaction vessel. According to one embodiment, the sealing component may be a silicone elastomer gasket that may be placed against an array of microwells with application of substantially uniform pressure across the entire substrate. In some cases, the reaction vessels may be sealed after the addition of the plurality of capture objects used for analyte capture and, optionally, any precursor labeling agent molecule that may be used to facilitate detection of the analyte molecule.

A non-limiting example of the formation of a plurality of reaction vessels containing assay solution on/in a substrate is depicted in FIG. 6. FIG. 6, panel (A) shows a surface comprising a plurality of microwells 139, which have been exposed to an assay solution 141 (e.g., a solution containing the capture objects used for analyte capture and/or control objects obtained after performance of the analyte capture step(s) and any washing step(s)), and a sealing component 143. Sealing component 143 in this example comprises a substantially planar bottom surface. Mating of substrate 139 with sealing component 143 forms a plurality of sealed reaction vessels 145. The areas between the reaction vessels 148 may be modified to aid in the formation of a tight seal between the reaction vessels.

A second embodiment is shown in FIG. 6, panel (B), in which sealing component 162 comprising a plurality of microwells 163 is mated with a substantially planar surface 158 which has been exposed to assay solution 162, thereby forming a plurality of reaction vessels 164.

In a third embodiment, as shown in FIG. 6, panel (C), substrate surface 166 comprising a plurality of microwells 167 is mated with sealing component 170 also comprising a plurality of microwells 171. In this embodiment, the microwells in the substrate and the microwells in the sealing components are substantially aligned so each reaction vessel 172 formed comprises a portion of the microwell from the sealing component and a portion of a microwell from the substrate. In FIG. 6, panel (D), the microwells are not aligned such that each reaction vessel comprises either a microwell from the sealing component 173 or a microwell from the substrate 175.

The sealing component may be essentially the same size as the substrate or may be different in size. In some cases, the sealing component is approximately the same size as the substrate and mates with substantially the entire surface of the substrate. In other cases, as depicted in FIG. 6, panel (E), the sealing component 176 is smaller than the substrate 174 and the sealing component only mates with a portion 178 of the substrate. In yet another embodiment, as depicted in FIG. 6, panel (F), the sealing component 182 is larger than the substrate 180, and only a portion 184 of the sealing component mates with the substrate 180.

In some embodiments, the reaction vessels may all have approximately the same volume. In other embodiments, the reaction vessels may have differing volumes. The volume of each individual reaction vessel may be selected to be appropriate to facilitate any particular assay protocol. For example, in one set of embodiments where it is desirable to limit the number of capture objects used for analyte capture contained in each vessel to a small number, the volume of the reaction vessels may range from attoliters or smaller to nanoliters or larger depending upon the nature of the capture objects, the detection technique and equipment employed, the number and density of the wells on the substrate and the expected concentration of capture objects in the fluid applied to the substrate containing the wells. In one embodiment, the size of the reaction vessel may be selected such only a single capture object used for analyte capture can be fully contained within the reaction vessel. In accordance with one embodiment of the present invention, the reaction vessels may have a volume between about 1 femtoliter and about 1 picoliter, between about 1 femtoliters and about 100 femtoliters, between about 10 attoliters and about 100 picoliters, between about 1 picoliter and about 100 picoliters, between about 1 femtoliter and about 1 picoliter, or between about 30 femtoliters and about 60 femtoliters. In some cases, the reaction vessels have a volume of less than about 1 picoliter, less than about 500 femtoliters, less than about 100

femtoliters, less than about 50 femtoliters, or less than about 1 femtoliter. In some cases, the reaction vessels have a volume of about 10 femtoliters, about 20 femtoliters, about 30 femtoliters, about 40 femtoliters, about 50 femtoliters, about 60 femtoliters, about 70 femtoliters, about 80 femtoliters, about 90 femtoliters, or about 100 femtoliters.

In embodiments where the plurality of capture objects used for analyte capture comprise a plurality of beads and the plurality of locations comprise a plurality of reaction vessels having a shape that is essentially that of a circular cylinder, the size of the reaction vessels may be based upon the size of the beads and may be designed so as to ensure that the number of wells containing more than a single bead is minimal. In some cases, the maximum permissible well diameter may be calculated according to Equation 3:

$$2 * BeadRadius + \sqrt{\left(3 * BeadRadius^2 - WellDepth^2 + 2 * WellDepth * BeadRadius\right)} \quad \text{(Eq. 3)}$$

and/or the maximum permissible well depth may be calculated according to Equation 4:

$$BeadRadius + \sqrt{\left(4 * BeadRadius * WellDiameter - WellDiameter^2\right)} \quad \text{(Eq. 4)}$$

The minimum permissible well depth and the minimum permissible well diameter to assure that a single bead can be contained in the well, in most embodiments, will not be less than the average diameter of the bead. Having a properly sized reaction vessel which allows for no more than a single bead to be present in a reaction vessel may provide better ability to resolve individual beads allowing for more accuracy with regard to determining a measure of the concentration of analyte molecules in a fluid sample by the means described in more detail below and in the Examples. For example, if the reaction vessels are too large, more than one bead may be able to fit in the reaction vessel, which may lead to an increase in the number of reaction vessels containing multiple analyte molecules, which may introduce inaccuracy in a concentration determination using an algorithm/statistical model based on single molecule detection (see below). In some cases, however, it may be desirable to have more than one bead fit in a reaction vessel. On the other hand, in the reaction vessel is too small, a bead may not be able to fit in the reaction vessel, thereby preventing proper sealing of the reaction vessel (e.g., in embodiments where the reaction vessel is sealed) and/or may lead to difficulties in addressing individual locations (e.g., in embodiments where a labeling agent is produced for detection, the labeling agent may disperse away from the reaction vessel it is produced in). In such cases, there may be false positives (e.g., a reaction vessel which does not contain an analyte molecule may be determined to contain an analyte molecule based on the labeling agent which has diffused away from the location

in which it was produced) which may lead to imprecise determination of a measure of the concentration of analyte molecules in a fluid sample.

In some embodiments, the average depth of the reaction vessels is between about 1.0 and about 1.7 times, between about 1.0 times and about 1.5 times, between about 1.0 times and about 1.3 times, or between about 1.1 times and about 1.4 times the average diameter of the beads. In some embodiments, the average diameter of the reactions vessels is between about 1.0 times and about 1.9 times, between about 1.2 times and about 1.7 times, between about 1.0 times and about 1.5 times, or between about 1.3 times and about 1.6 times the average diameter of the beads. In a particular embodiment, the average depth of the reaction vessels is between about 1.0 times and about 1.5 times the average diameter of the beads and the average diameter of the reactions vessels is between about 1.0 times and about 1.9 times the average diameter of the beads.

The total number of locations and/or density of the locations employed in an assay (e.g., the number/density of reaction vessels in an array) can depend on the composition and end use of the array. For example, the number of reaction vessels employed may depend on the number of capture objects employed, the suspected concentration range of the assay, the method of detection, the size of the capture objects, the type of detection entity (e.g., free labeling agent in solution, precipitating labeling agent, etc.). Arrays containing from about 2 to many billions of reaction vessels (or total number of reaction vessels) can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple types of analyte molecules to be assayed in parallel. The array may comprise between one thousand and one million reaction vessels per sample to be analyzed. In some cases, the array comprises greater than one million reaction vessels. In some embodiments, the array comprises between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 100,000 and about 500,000, between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000, or the like, reaction vessels. In some embodiments, the array comprises about 10,000, about 20,000, about 50,000, about 100,000, about 150,000, about 200,000, about 300,000, about 500,000, about 1,000,000, or more, reaction vessels.

The array of reaction vessels may be arranged on a substantially planar surface or in a non-planar three-dimensional arrangement. The reaction vessels may be arrayed in a regular pattern or may be randomly distributed. In a specific embodiment, the array is a regular pattern of sites on a substantially planar surface permitting the sites to be addressed in the X-Y coordinate plane.

In some embodiments, the reaction vessels are formed in a solid material. As will be appreciated by those in the art, the number of potentially suitable materials in which the reaction vessels can be formed is very large, and includes, but is not limited to, glass (including modified and/or functionalized glass), plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), Teflon®, polysaccharides, nylon or nitrocellulose, etc.), elastomers (such as poly(dimethyl siloxane) and poly urethanes), composite materials, ceramics, silica or silica-based materials (including silicon and modified silicon), carbon, metals, optical fiber bundles, or the like. In general, the substrate material may be selected to allow for optical detection without appreciable autofluorescence. In certain embodiments, the reaction vessels may be formed in a flexible material.

A reaction vessel in a surface (e.g., substrate or sealing component) may be formed using a variety of techniques known in the art, including, but not limited to, photolithography, stamping techniques, molding techniques, etching techniques, or the like. As will be appreciated by those of the ordinary skill in the art, the technique used can depend on the composition and shape of the supporting material and the size and number of reaction vessels.

In a particular embodiment, an array of reaction vessels is formed by creating microwells on one end of a fiber optic bundle and utilizing a planar compliant surface as a sealing component. In certain such embodiments, an array of reaction vessels in the end of a fiber optic bundle may be formed as follows. First, an array of microwells is etched into the end of a polished fiber optic bundle. Techniques and materials for forming and etching a fiber optic bundle are known to those of ordinary skill in the art. For example, the diameter of the optical fibers, the presence, size and composition of core and cladding regions of the fiber, and the depth and specificity of the etch may be varied by the etching technique chosen so that microwells of the desired volume may be formed. In certain embodiments, the etching process creates microwells by preferentially etching the core material of the individual glass fibers in the bundle such

that each well is approximately aligned with a single fiber and isolated from adjacent wells by the cladding material. Potential advantages of the fiber optic array format is that it can produce thousands to millions of reaction vessels without complicated microfabrication procedures and that it can provide the ability to observe and optically address many reaction vessels simultaneously.

Each microwell may be aligned with an optical fiber in the bundle so that the fiber optic bundle can carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers may provide the capability for simultaneous or non-simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber.

Alternatively, the equivalent structures of a plurality of reaction vessels may be fabricated using other methods and materials that do not utilize the ends of an optical fiber bundle as a substrate. For example, the array may be a spotted, printed or photolithographically fabricated substrate produced by techniques known in the art; see for example WO95/25116; WO95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637, 5,807,522, 5,445,934, 6,406,845, and 6,482,593. In some cases, the array may be produced using molding, embossing, and/or etching techniques as will be known to those of ordinary skill in the art.

In certain embodiments, the present invention provides a system equipped with a mechanical platform that applies a sealing component to a substrate. The platform may be positioned beneath a stage on the system. After the chosen reaction components have been added to an array of reaction vessels, the sealing component may be mated with the array. For example, the sealing component may be sandwiched between a flat surface (such as, for example, a microscope slide) and the array of reaction vessels using uniform pressure applied by the mechanical platform.

A non-limiting embodiment is illustrated in FIG. 7. A sealing component 300 is placed on top of mechanical platform 302. The assay solution 304 is placed on top of the sealing component 300. The mechanical platform is moved upwards towards the array 306 (e.g., fiber optic array) such that uniform pressure is applied. As shown in FIG. 8, the sealing component 300 forms a tight seal with the array 306. In other instances, varying pressure may be applied to the sealing component to form a tight seal between the sealing component and the array. The system may also comprise additional

components 312 that may be utilized to analyze the array (e.g., microscope, computer, etc.) as discussed more herein.

The plurality of capture objects used for analyte capture may be spatially separated into the plurality of reaction vessels using any of a wide variety of techniques known to those of ordinary skill in the art. In some cases, the plurality of reaction vessels may be exposed to a solution containing the plurality of capture objects. In some cases, force may be applied to the solution and/or capture objects, thereby aiding in the spatial separation of the capture objects from the fluid phase and/or the deposition of the capture objects in the vessels. For example, after application of an assay solution containing the capture objects to a substrate containing the reaction vessels, the substrate and solution may be centrifuged to assist in depositing the capture objects in the reaction vessels. In embodiments where the capture objects (e.g., beads) are magnetic, a magnet may be used to aid in containing the capture objects in the reaction vessels. In some cases, when the plurality of reaction vessels is formed on the end of a fiber optic bundle (or another planar surface), a material (e.g., tubing) may be placed around the edges of the surface of the array comprising the plurality of reaction vessel to form a container to hold the solution in place while the capture objects settle in the reaction vessels or are placed into the reaction vessels (e.g., while centrifuging). Following placement of the capture objects into at least some of the reaction vessels, the surrounding material may be removed and the surface of the array may be washed and/or swabbed to remove any excess solution/capture objects.

In some embodiments, the substrate does not include wells or reaction vessels forming the plurality of reaction vessels but uses/provides other means to spatially segregate the plurality of capture objects used for analyte capture. In some cases, a patterned substantially planar surface may be employed, wherein the patterned areas form a plurality of locations. In some cases, the patterned areas may comprise substantially hydrophilic surfaces which are substantially surrounded by substantially hydrophobic surfaces. A plurality of capture objects (e.g., beads) may be substantially surround by a substantially hydrophilic medium (e.g., comprising water), and the beads may be exposed to the pattern surface such that the beads associate in the patterned areas (e.g., the locations), thereby spatially segregating the plurality of beads. For example, in one such embodiment, a substrate may be or include a gel or other material able to provide a sufficient barrier to mass transport (e.g., convective and/or diffusional barrier) to prevent capture objects used for analyte capture and/or precursor labeling agent and/or

labeling agent from moving from one location on or in the material to another location so as to cause interference or cross-talk between spatial locations containing different capture objects during the time frame required to address the locations and complete the assay. For example, in one embodiment, a plurality of capture objects is spatially separated by dispersing the capture objects on and/or in a hydrogel material. In some cases, a precursor labeling agent may be already present in the hydrogel, thereby facilitating development of a local concentration of the labeling agent (e.g., upon exposure to a binding ligand or analyte molecule carrying an enzymatic component). As still yet another embodiment, the capture objects may be confined in one or more capillaries. In some cases, the plurality of capture objects may be absorbed or localized on a porous or fibrous substrate, for example, filter paper. In some embodiments, the capture objects may be spatially segregated on a uniform surface (e.g., a planar surface), and the capture objects may be detected using precursor labeling agents which are converted to substantially insoluble or precipitating labeling agents that remain localized at or near the location of where the corresponding capture object is localized. The use of such substantially insoluble or precipitating labeling agents is described herein.

Articles and Kits

In some embodiments of the present invention, an article or kit for determining a measure of the concentration of analyte molecules or particles in a fluid sample is provided. The article or kit may comprise a plurality of beads and a substrate comprising a plurality of reaction vessels. The reaction vessels may be configured to receive and contain the capture objects. The plurality of beads in certain embodiment have an average diameter between about 0.1 micrometer and about 100 micrometers and the size of the reaction vessels may be selected such that only either zero or one beads is able to be contained in single reaction vessels. In some cases, the average depth of the reaction vessels is between about 1.0 times and about 1.5 times the average diameter of the beads and the average diameter of the reactions vessels is between about 1.0 times and about 1.9 times the average diameter of the beads. In certain embodiments, the beads may have an average diameter between about between about 1 micrometer and about 10 micrometers, between about 1 micrometer and about 5 micrometers, or any range of sizes described herein.

The plurality of beads provided may have a variety of properties and parameters, as described herein. For example, the beads may be magnetic. The plurality of beads

may comprise a binding surface (e.g., a plurality of capture components) having an affinity for at least one type of analyte molecule or particle.

The plurality of reaction vessels may be formed in any suitable substrate, as described herein. In a particular embodiment, the plurality of reaction vessels is formed on the end of a fiber optic bundle. The fiber optic bundle may be prepared (e.g., etched) according to methods known to those of ordinary skill in the art and/or methods described herein. In other embodiments, the plurality of reactions vessels is formed in a plate or similar substantially planar material (e.g., using lithography or other known techniques). Exemplary suitable materials are described herein.

The average depth of the plurality of reaction vessels may be between about 1.0 and about 1.5 times, or between about 1.1 and about 1.3 times the average diameter of the beads, or any range described herein. The average diameter of the plurality of reaction vessels may be between about 1.0 times and about 1.9 times, or between about 1.3 times and about 1.8 times the average diameter of the beads, or any range described herein. The average depth and/or the average diameter of the plurality of reaction vessels may be chosen such that no more than one bead is able to be contained in a reaction vessels. Methods for calculating maximum depths and maximum diameters to facilitate single bead loading are described herein. The average volume of the plurality of reaction vessels may be between about 10 attoliters and about 100 picoliters, between about 1 femtoliter and about 1 picoliter, or any desired range. The substrate may comprise any number of reaction vessels, for example, between about 1,000 and about 1,000,000 reaction vessels, between about 10,000 and about 100,000 reaction vessels, or between about 100,000 and about 300,000 reaction vessels, or any other desired range.

The article or kit may comprise any number of additional components, some of which are described in detail herein. In some cases, the article or kit may further comprise a sealing component configured for sealing the plurality of reaction vessels. In certain embodiments, the plurality of reaction vessels may be formed upon the mating of at least a portion of a sealing component and at least a portion of the second substrate, as shown in FIGS. 7A-7F and as discussed in more detail herein. As another example, the kit may also provide solutions for carrying out an assay method as described herein. Non-limiting example of solutions include solutions containing one or more types of binding ligands and precursor labeling agents. In some cases, the article or kit may comprise at least one type of control bead.

In some embodiments, the kit may optionally include instructions for use of the plurality of beads and the plurality or reaction vessels (and any additional components provided). That is, the kit can include a description of use of the beads and reaction vessels, for example, for use with a system to determine a measure of the concentration of analyte molecules (or particles) in a fluid sample. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the invention. Instructions also can include any oral or electronic instructions provided in any manner such that a user of the kit will clearly recognize that the instructions are to be associated with the kit. Additionally, the kit may include other components depending on the specific application, as described herein. As used herein, "promoted" includes all methods of doing business including methods of education, hospital and other clinical instruction, scientific inquiry, drug discovery or development, academic research, pharmaceutical industry activity including pharmaceutical sales, and any advertising or other promotional activity including written, oral and electronic communication of any form, associated with the invention.

Capture Components

In some embodiments of the present invention, the surface of the capture objects may provide a binding surface having an affinity for at least one type of analyte molecule or particle. In some embodiments, the binding surface may comprise at least one type of capture component. Generally, the capture component allows the attachment of a molecule, particle, or complex to a solid support (that is, a surface of a capture object) for the purposes of immobilization, detection, quantification, and/or other analysis of the molecule, particle, or complex. A capture component is used in the present invention, in some cases, to immobilize an analyte molecule with respect to a capture object (e.g., a bead).

As will be appreciated by those in the art, the composition of the capture component will depend on the composition of the analyte molecule. Capture components for a wide variety of target molecules are known or can be readily found or developed using known techniques. For example, when the target molecule is a protein, the capture components may comprise proteins, particularly antibodies or fragments thereof (e.g., antigen-binding fragments (Fabs), Fab' fragments, pepsin fragments, $F(ab')_2$ fragments, full-length polyclonal or monoclonal antibodies, antibody-like fragments,

etc.), other proteins, such as receptor proteins, Protein A, Protein C, etc., or small molecules. In some cases, capture components for proteins comprise peptides. For example, when the target molecule is an enzyme, suitable capture components may include enzyme substrates and/or enzyme inhibitors. In some cases, when the target analyte is a phosphorylated species, the capture component may comprise a phosphate-binding agent. For example, the phosphate-binding agent may comprise metal-ion affinity media such as those describe in U.S. Patent No. 7,070,921 and U.S. Patent Application No. 20060121544. In addition, when the target molecule is a single-stranded nucleic acid, the capture component may be a complementary nucleic acid. Similarly, the target molecule may be a nucleic acid binding protein and the capture component may be a single-stranded or double-stranded nucleic acid; alternatively, the capture component may be a nucleic acid-binding protein when the target molecule is a single or double stranded nucleic acid. Alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867, 5,705,337, and related patents, nucleic acid "aptamers" may be developed for capturing virtually any target molecule. Also, for example, when the target molecule is a carbohydrate, potentially suitable capture components include, for example, antibodies, lectins, and selectins. As will be appreciated by those of ordinary skill in the art, any molecule that can specifically associate with a target molecule of interest may potentially be used as a capture component.

For certain embodiments, suitable target analyte molecule/capture component pairs can include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, nucleic acids/nucleic acids, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins and/or selectins, proteins/proteins, proteins/small molecules; small molecules/small molecules, etc. According to one embodiment, the capture components are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), and T-cell receptors and the target analyte molecules are one or more receptor target ligands.

In a particular embodiment, the capture component may be attached to the surface of a capture object via a linkage, which may comprise any moiety, functionalization, or modification of the binding surface and/or capture component that facilitates the attachment of the capture component to the surface. The linkage between the capture component and the surface may comprise one or more chemical or physical

(e.g., non-specific attachment via van der Waals forces, hydrogen bonding, electrostatic interactions, hydrophobic/hydrophilic interactions; etc.) bonds and/or chemical linkers providing such bond(s). In certain embodiments, the capture component comprises a capture extender component. In such embodiments, the capture component comprises a first portion that binds the analyte molecule and a second portion that can be used for attachment to the binding surface.

In certain embodiments, a capture object surface may also comprise a protective or passivating layer that can reduce or minimize non-specific attachment of non-capture components (e.g., analyte molecules, binding ligands) to the binding surface during the assay which may lead to false positive signals during detection or to loss of signal. Examples of materials that may be utilized in certain embodiments to form passivating layers include, but are not limited to: polymers, such as poly(ethylene glycol), that repel the non-specific binding of proteins; naturally occurring proteins with this property, such as serum albumin and casein; surfactants, e.g., zwitterionic surfactants, such as sulfobetaines; naturally occurring long-chain lipids; and nucleic acids, such as salmon sperm DNA.

The method of attachment of the capture component to a capture object surface depends of the type of linkage employed and may potentially be accomplished by a wide variety of suitable coupling chemistries/techniques known to those of ordinary skill in the art. The particular means of attachment selected will depend on the material characteristics of the capture object surface and the nature of the capture component. In certain embodiments, the capture components may be attached to the capture object surface through the use of reactive functional groups on each. According to one embodiment, the functional groups are chemical functionalities. That is, the binding surface may be derivatized such that a chemical functionality is presented at the binding surface which can react with a chemical functionality on the capture component resulting in attachment. Examples of functional groups for attachment that may be useful include, but are not limited to, amino groups, carboxy groups, epoxide groups, maleimide groups, oxo groups, and thiol groups. Functional groups can be attached, either directly or through the use of a linker, the combination of which is sometimes referred to herein as a "crosslinker." Crosslinkers are known in the art; for example, homo-or hetero-bifunctional crosslinkers as are well known (e.g., see 1994 Pierce Chemical Company catalog, technical section on crosslinkers, pages 155-200, or "Bioconjugate Techniques" by Greg T. Hermanson, Academic Press, 1996). Non-limiting example of crosslinkers

include alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), esters, amide, amine, epoxy groups and ethylene glycol and derivatives. A crosslinker may also comprise a sulfone group, forming a sulfonamide.

According to one embodiment, the functional group is a light-activated functional group. That is, the functional group can be activated by light to attach the capture component to the capture object surface. One example is PhotoLink™ technology available from SurModics, Inc. in Eden Prairie, MN.

In some cases, the capture object may comprise streptavidin-coated surfaces and the capture component may be biotinylated. Exposure of the capture component to the streptavidin-coated surfaces can cause association of the capture component with the surface by interaction between the biotin component and streptavidin.

In certain embodiments, attachment of the capture component to the binding surface may be effected without covalently modifying the binding surface of a capture object. For example, the attachment functionality can be added to the binding surface by using a linker that has both a functional group reactive with the capture component and a group that has binding affinity for the binding surface. In certain embodiments, a linker comprises a protein capable of binding or sticking to the binding surface; for example, in one such embodiment, the linker is serum albumin with free amine groups on its surface. A second linker (crosslinker) can then be added to attach the amine groups of the albumin to the capture component (e.g., to carboxy groups).

According to one embodiment in which a chemical crosslinker is used to attach the capture components to the capture object, the analyte molecule may be captured on the binding surface of a capture object using a capture component attached via chemical crosslinking in the following manner. First, the binding surface is derivatized with a functional group, such as, an amine group. Next, a crosslinker and the capture component are placed in contact with the binding surface such that one end of the crosslinker attaches to the amine group and the capture component attaches to the other end of the crosslinker. In this way, capture components comprising proteins, lectins, nucleic acids, small organic molecules, carbohydrates can be attached.

One embodiment utilizes proteinaceous capture components. As is known in the art, any number of techniques may be used to attach a proteinaceous capture component to a wide variety of solid surfaces. "Protein" or "proteinaceous" in this context includes proteins, polypeptides, peptides, including, for example, enzymes, and antibodies. A wide variety of techniques are known to add reactive moieties to proteins, for example,

the method outlined in U.S. Patent No. 5,620,850. The attachment of proteins to surfaces is known, for example, see Heller, Acc. Chem. Res. 23:128 (1990), and many other similar references.

In some embodiments, the capture component (or binding ligand) may comprise Fab' fragments. The use of Fab' fragments as opposed to whole antibodies may help reduce non-specific binding between the capture component and the binding ligand. In some cases, the Fc region of a capture component (or binding ligand) may be removed (e.g., proteolytically). In some cases, an enzyme may be used to remove the Fc region (e.g., pepsin, which may produce $F(ab')_2$ fragments and papain, which may produce Fab fragments). In some instances, the capture component may be attached to a binding surface using amines or may be modified with biotin (e.g., using NHS-biotin) to facilitate binding to an avidin or streptavidin coated capture object surface. $F(ab')_2$ fragments may be subjected to a chemical reduction treatment (e.g., by exposure to 2-mercaptoethylamine) to, in some cases, form two thiol-bearing Fab' fragments. These thiol-bearing fragments can then be attached via reaction with a Michael acceptor such as maleimide. For example, the Fab' fragments may then be treated with a reagent (e.g., maleimide-biotin) to attach at least one biotin moiety (i.e., biotinylated) to facilitate attachment to streptavidin-coated surfaces as described above.

Certain embodiments utilize nucleic acids as the capture component, for example for when the analyte molecule is a nucleic acid or a nucleic acid binding protein, or when the it is desired that the capture component serve as an aptamer for binding a protein, as is well known in the art.

According to one embodiment, each binding surface of a capture object comprises a plurality of capture components. The plurality of capture components, in some cases, may be distributed randomly on the binding surface like a "lawn." Alternatively, the capture components may be spatially segregated into distinct region(s) and distributed in any desired fashion.

Binding between the capture component and the analyte molecule, in certain embodiments, is specific, e.g., as when the capture component and the analyte molecule are complementary parts of a binding pair. In certain such embodiments, the capture component binds both specifically and directly to the analyte molecule. By "specifically bind" or "binding specificity," it is meant that the capture component binds the analyte molecule with specificity sufficient to differentiate between the analyte molecule and other components or contaminants of the test sample. For example, the capture

component, according to one embodiment, may be an antibody that binds specifically to some portion of an analyte molecule (e.g., an antigen). The antibody, according to one embodiment, can be any antibody capable of binding specifically to an analyte molecule of interest. For example, appropriate antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to as antibody mimetics), chimeric antibodies, humanized antibodies, antibody fusions (sometimes referred to as "antibody conjugates"), and fragments of each, respectively. As another example, the analyte molecule may be an antibody and the capture component may be an antigen.

According to one embodiment in which an analyte particle is a biological cell (e.g., mammalian, avian, reptilian, other vertebrate, insect, yeast, bacterial, cell, etc.), the capture component may be a ligand having specific affinity for a cell surface antigen (e.g., a cell surface receptor). In one embodiment, the capture component is an adhesion molecule receptor or portion thereof, which has binding specificity for a cell adhesion molecule expressed on the surface of a target cell type. In use, the adhesion molecule receptor binds with an adhesion molecule on the extracellular surface of the target cell, thereby immobilizing or capturing the cell. In one embodiment in which the analyte particle is a cell, the capture component is fibronectin, which has specificity for, for example, analyte particles comprising neural cells.

In some embodiments, as will be appreciated by those of ordinary skill in the art, it is possible to detect analyte molecules using capture components for which binding to analyte molecules is not highly specific. For example, such systems/methods may use different capture components such as, for example, a panel of different binding ligands, and detection of any particular analyte molecule is determined via a "signature" of binding to this panel of binding ligands, similar to the manner in which "electronic noses" work. This may find particular utility in the detection of certain small molecule analytes. In some embodiments, the binding affinity between analyte molecules and capture components should be sufficient to remain bound under the conditions of the assay, including wash steps to remove molecules or particles that are non-specifically bound. In some cases, for example in the detection of certain biomolecules, the binding constant of the analyte molecule to its complementary capture component may be between at least about 10^4 and about 10^6 M^{-1}, at least about 10^5 and about 10^9 M^{-1}, at least about 10^7 and about 10^9 M^{-1}, greater than about 10^9 M^{-1}, or the like. For example,

typical affinities for IgG antibodies for their antigens are in the range 10^5-10^{10} M^{-1}. The affinity of biotin for streptavidin is 10^{15} M^{-1}.

In certain embodiments, the capture component is chosen to be able to bind to a corresponding binding partner associated with or attached to the analyte molecule. For example, the capture component according to one embodiment is a chemical crosslinker as described above able to bind to proteins generally. According to one embodiment, every protein molecule in a fluid sample comprises an analyte molecule that attaches to such a chemical crosslinker. In another example, the capture component comprises streptavidin, which binds with high affinity to biotin, and thus captures any analyte molecules to which biotin has been attached. Alternatively, the capture component may be biotin, and streptavidin may be attached to or associated with the analyte molecules such that the analyte molecules can be captured by the biotin.

According to one embodiment, the binding surfaces of a capture object may be functionalized with capture components in the following manner. First, the surface of a capture object is prepared for attachment of the capture component(s) by being modified to form or directly bind to the capture components, or a linker may be added to the binding surface of the capture object such that the capture component(s) attaches to the binding surface of the capture object via the linker. In one embodiment, the binding surfaces of the capture object are derivatized with a chemical functionality as described above. Next, the capture component may be added, which binds to and is immobilized by the chemical functionality.

Exemplary Target Analytes

As will be appreciated by those in the art, a large number of analyte molecules and particles may be detected and, optionally, quantified using methods and systems of the present invention; basically, any analyte molecule that is able to be made to become immobilized with respect to a capture object (e.g., via a binding surface comprising a plurality of capture components) can be potentially investigated using the invention. Certain more specific targets of potential interest that may comprise an analyte molecule are mentioned below. The list below is exemplary and non-limiting.

In some embodiments, the analyte molecule may be an enzyme. Non-limiting examples of enzymes include, an oxidoreductase, transferase, kinase, hydrolase, lyase, isomerase, ligase, and the like. Additional examples of enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example,

AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When a system/method of the present invention is used to detect the presence of viral or bacterial agents, appropriate target enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases, or the like.

In other embodiments, the analyte molecule may comprise an enzymatic component. For example, the analyte particle can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte particle is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below. Non-limiting example of enzymatic components are horseradish peroxidase, beta-galactosidase, and alkaline phosphatase.

In yet other embodiments, the analyte molecule may be a biomolecule. Non-limiting examples of biomolecules include hormones, antibodies, cytokines, proteins, nucleic acids, lipids, carbohydrates, lipids cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, or combinations thereof. Non-limiting embodiments of proteins include peptides, polypeptides, protein fragments, protein complexes, fusion proteins, recombinant proteins, phosphoproteins, glycoproteins, lipoproteins, or the like. As will be appreciated by those in the art, there are a large number of possible proteinaceous analyte molecules that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analyte molecules include, but are not limited to, immunoglobulins, hormones, growth factors, cytokines (many of which serve as ligands for cellular receptors), cancer markers, etc. Non-limiting examples of biomolecules include PSA and TNF-alpha.

In certain embodiments, the analyte molecule may be a host-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with capture components comprising a multiplicity of specific antibodies and then the captured proteins may be further bound to a binding ligand comprising a secondary antibody with specificity to a post-translational modification. Alternatively, modified proteins may be captured with capture components comprising an antibody specific for a post-translational modification and then the captured proteins

may be further bound to binding ligands comprising antibodies specific to each modified protein.

In another embodiment, the analyte molecule is a nucleic acid. A nucleic acid may be captured with a complementary nucleic acid fragment (e.g., an oligonucleotide) and then optionally subsequently labeled with a binding ligand comprising a different complementary oligonucleotide.

Suitable analyte molecules and particles include, but are not limited to small molecules (including organic compounds and inorganic compounds), environmental pollutants (including pesticides, insecticides, toxins, etc.), therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.), biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc), whole cells (including prokaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells), viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.), spores, etc.

The fluid sample containing or suspected of containing an analyte molecule may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, fluid suspension of solid particles, supercritical fluid, and/or gas. In some cases, the analyte molecule may be separated or purified from its source prior to determination; however, in certain embodiments, an untreated sample containing the analyte molecule may be tested directly. The source of the analyte molecule may be synthetic (e.g., produced in a laboratory), the environment (e.g., air, soil, etc.), a mammal, an animal, a plant, or any combination thereof. In a particular example, the source of an analyte molecule is a human bodily substance (e.g., blood, serum, plasma, urine, saliva, tissue, organ, or the like). The volume of the fluid sample analyzed may potentially be any amount within a wide range of volumes, depending on a number of factors such as, for example, the number of capture objects used/available, the number of locations us/available, etc. In a few particular exemplary embodiments, the sample volume may be about 0.01 ul, about 0.1 uL, about 1 uL, about 5 uL, about 10 uL, about 100 uL, about 1 mL, about 5 mL, about 10 mL, or the like. In some cases, the volume of the fluid sample is between about 0.01 uL and about 10 mL, between about 0.01 uL and about 1 mL, between about 0.01 uL and about 100 uL, or between about 0.1 uL and about 10 uL.

In some cases, the fluid sample may be diluted prior to use in an assay. For example, in embodiments where the source of an analyte molecule is a human body fluid (e.g., blood, serum), the fluid may be diluted with an appropriate solvent (e.g., a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, or greater, prior to use. The sample may be added to a solution comprising the plurality of capture objects, or the plurality of capture objects may be added directly to or as a solution to the sample.

Binding Ligands and Precursor Labeling Agents/Labeling Agent

Binding ligands may be selected from any suitable molecule, particle, or the like, as discussed more below, able to associate with an analyte molecule and/or to associate with another binding ligand. Certain binding ligands can comprise an entity that is able to facilitate detection, either directly (e.g., via a detectable moiety) or indirectly. A component may facilitate indirect detection, for example, by converting a precursor labeling agent molecule into a labeling agent molecule (e.g., an agent that is detected in an assay). In some embodiments, the binding ligand may comprise an enzymatic component (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase, etc). A first type of binding ligand may or may not be used in conjunction with additional binding ligands (e.g., second type, etc.), as discussed herein.

In some embodiments, the plurality of capture objects, at least some of which comprise at least one analyte molecule, may be exposed to a plurality of binding ligands such that a binding ligand associates with at least some of the plurality of analyte molecules. In embodiments where a statistically significant fraction of the capture objects are associated with a single analyte molecule and a statistically significant fraction of the capture objects are not associated with any analyte molecules (e.g., where the number of analyte molecules is less than the total number of capture objects), a binding ligand may associate with substantially all of the analyte molecules immobilized with respect to a capture object. In some cases, greater than about 80%, greater than about 85%, greater than about 90%, greater than about 95%, greater than about 97%, greater than about 98%, greater than about 99%, or more, analyte molecules may become associated with a binding ligand.

In other embodiments where substantially all of the capture objects comprise at least one analyte molecule (e.g., in embodiments where the number of analyte molecules is about equal to or greater than the number of capture objects provided), the capture

objects may be exposed to the binding ligand such that a statistically significant fraction of the capture objects associate with at least one binding ligand (or in certain embodiments substantially only a single binding ligand) and a statistically significant fraction of the capture objects do not associate with any binding ligand. In some cases, the capture objects may be exposed to the binding ligands such that at least some of the capture objects associate with at least one binding ligand and a statistically significant fraction of the capture objects do not associate with any binding ligand. A screening test to determine an appropriate amount of binding ligand to use for a desired degree of binding ligand loading (e.g. to facilitate selection of an appropriate quantity of binding ligand to use for a particular situation) may be performed with calibration standards containing a known concentration of analyte molecule and varying quantities of binding ligand using a Poisson analysis. In certain embodiments, it is determined whether the analyte is essentially fully labeled or only partially labeled with binding ligand. The percentage active analyte molecules (i.e. those associated with binding ligand) detected can be converted to the percentage analyte molecules associated with zero, one, two etc. binding ligands using Poisson distribution adjustment as described elsewhere herein.

In some embodiments, more than one type of binding ligand may be used. In some embodiments, a first type of binding ligand and a second type of binding ligand may be provided. In some instances, at least two, at least three, at least four, at least five, at least eight, at least ten, or more, types of binding ligands may be provided. When a plurality of capture objects, some of which are associated with at least one analyte molecule, are exposed to a plurality of types of binding ligand, at least some of the plurality of immobilized analyte molecules may associate with at least one of each type of binding ligand. The binding ligands may be selected such that they interact with each other in a variety of different manners. In a first example, the first type of binding ligand may be able to associate with an analyte molecule and the second type of binding ligand may be able to associate with the first type of binding ligand. In such embodiments, the first type of binding ligand may comprise a first component which aids in association of the analyte molecule and a second component which aids in association of the second type of binding ligand with the first type of binding ligand. In a particular embodiment, the second component is biotin and the second type of binding ligand comprises an enzyme or an enzymatic component which associates with the biotin.

As another example, both the first type of binding ligand and the second type of binding ligand may associate directly with an analyte molecule. Without being bound by

theory or any particular mechanism, the association of both the first type and the second type of binding ligand may provide additional specificity and reliability in performing an assay, by identifying only locations which are determined to contain both the first type of binding ligand and/or the second type of binding ligand (e.g., either through direct or indirect detection) as containing an analyte molecule. Such assay methods may reduce the number of false positives caused by non-specific binding as locations that are found to only have a single type of binding ligand (e.g., only the first type of labeling agent or the second type of labeling agent) would be not be considered or counted as a location comprising an analyte molecule. The first type of binding ligand may comprise a first type of enzymatic component and the second type of binding ligand may comprise a second type of enzymatic component which differs from the first type of enzymatic component. A capture object comprising an analyte molecule, the first type of binding ligand, and the second type of binding ligand may be exposed to a first type of precursor labeling agent which is converted to a first type of labeling agent (e.g., comprising a first measurable property) upon exposure to the first type of enzymatic component and a second type of precursor labeling agent which is converted to a second type of labeling agent (e.g., comprising a measurable property which is distinguishable from the first measurable property) upon exposure to the second type of enzymatic component. Therefore, only locations which are determined to contain the first type of labeling agent and the second type of labeling agent are determined to contain an analyte molecule. As another example, the first type of binding ligand and the second type of binding ligand may each incorporate a component (e.g., such as a DNA label) and a third type of binding ligand may comprise two components complimentary to the components of the first type and second type of binding ligands (e.g., two types of complimentary DNA labels), wherein the third type of binding ligand also comprises an molecule or moiety for direct or indirect detection (e.g., the presence of the third type of binding ligand in a reaction vessel is required to determine the presence or absence of an analyte molecule in a location). When both the first type of binding ligands and the second types of binding ligands are present in substantially close proximity to each other (e.g., via association with an analyte molecule) association of the third type of binding ligand may occur, thus allowing detection of the analyte molecule. More information regarding the use of more than one type of binding ligand in a manner which may reduce certain negative affects associated with non-specific binding, are described in commonly owned U.S. Patent Application Serial No. 12/731,135, entitled "Ultra-Sensitive Detection of Molecules

using Dual Detection Methods" by Duffy et al., filed March 24, 2010, and International Patent Application No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules using Dual Detection Methods" by Duffy et al., filed March 1, 2011 (Attorney Docket No. Q0052.70012WO00), herein incorporated by reference.

Detection

The plurality of capture objects, some of which comprise at least one analyte molecule and/or at least one binding ligand can be detected and/or quantified, and the detection and/or quantification can be related to the presence and, optionally, the quantity and/or concentration of analyte molecules/particles in the sample being tested. In some embodiments, the plurality of capture objects may be detected and/or quantified by spatially segregating the plurality of capture objects into a plurality of locations. In some embodiments, the plurality of locations comprises a plurality of reaction vessels (e.g., in an array). In some cases, a detector may be configured to detect the capture objects in or at a plurality of locations (e.g., an array of reaction vessels). In some embodiments, the capture objects may be able to produce or be made to produce a detectable signal, for example, fluorescence emission, which may aid in the detection of the capture objects. In some cases, the capture objects may be detected using scattering techniques, as described herein.

In some embodiments, the number of capture objects spatially segregated may be substantially equal to the number of capture objects exposed to a fluid sample containing or suspected of containing analyte molecules. In some embodiments, however, the number of capture objects spatially segregated into a plurality of locations may be substantially less than the number of capture objects exposed to a fluid sample containing or suspected of containing analyte molecules. In some cases, about 1%, about 2%, about 3%, about 5%, about 10%, about 15%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, or more, of the capture objects exposed to a fluid sample are spatially segregated into a plurality of locations. In some instances, between about 1% and about 99%, between about 10% and about 90%, between about 20% and about 80%, between about 30% and about 70%, between about 50% and about 90%, between about 1% and about 50%, between about 5% and about 40%, or between about 10% and about 30% of the capture objects exposed to a fluid sample are spatially segregated into a plurality of locations.

The analyte molecules (or binding ligands) which are spatially segregated may be detected and/or quantified directly or indirectly. In the case of direct detection, the analyte molecules may comprise a molecule or moiety that may be directly interrogated and/or detected, for example, a fluorescent entity (e.g., a fluorescent moiety, fluorescent bead, fluorescent antibody, etc.), a metal nanoparticle or nanocluster (e.g., a gold nanocluster or nanoparticle, silver nanocluster or nanoparticle), a quantum dot (e.g., CdSe quantum dot, CdTe quantum dot, etc.), and radioactive isotopes. In embodiments where the assay comprises the use of one or more types of binding ligands, the binding ligands may comprise a molecule(s) or moiety(ies) that may be directly interrogated and/or detected. A location that comprises such an analyte molecule or binding ligand which comprises a moiety that may be directly interrogated and/or detected can be made to emit a signal upon interrogation of the location.

In some embodiments, non-enzymatic detection methods may be employed. Non-enzymatic detection methods will be known to those of ordinary skill in the art. Non-limiting examples include Raman scattering, electromagnetic radiation resonance methods (e.g., whispering gallery modes), spectroscopy (e.g., infrared, atomic spectroscopies), absorbance, piezoelectric transduction (e.g., quartz crystal microbalance (QCM)), circular dichroism, electron microscopies (e.g., scanning electron microscopy (SEM), x-ray photoelectron microscopy (XPS)), scanning probe microscopies (e.g., atomic force microscopy (AFM), scanning tunneling microscopy (STM)), light scattering; surface plasmon resonance (SPR), evanescent wave detection, optical interferometry and other methods based on measuring changes in refractive index, electrical transduction methods, such as conduction and capacitance; magnetic transduction effects (e.g., magnetoresistive effect), calorimetry (e.g., differential scanning calorimetry (DSC)), diffraction; nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), mass spectroscopy (e.g., matrix assisted laser desorption and ionization (MALDI)), fluorescence technologies (e.g., fluorescence resonance energy transfer (FRET), time-resolved fluorescence (TRF), fluorescence polarization (FP)), and luminescent oxygen channeling (LOCI).

In some embodiments, the plurality of analyte molecules (or binding ligands) are indirectly detected. The indirect approach can include, for example, exposing an analyte molecule, or a binding ligand associated with an analyte molecule, to a precursor labeling agent, wherein the precursor labeling agent is converted into a labeling agent upon exposure to the analyte molecule or the binding ligand associated with an analyte

molecule. The labeling agent may comprise a molecule or moiety that can be interrogated and/or detected. The presence or absence of an analyte molecule or binding ligand at a location may then be determined by determining the presence or absence of a labeling agent at/in the location. For example, the analyte molecule may comprise an enzymatic component and the precursor labeling agent molecule may be a chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agent molecule which is converted to a chromogenic, fluorogenic, or chemiluminescent product (each an example of a labeling agent) upon exposure to the converting agent. In this instance, the precursor labeling agent may be an enzymatic label, for example, a chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agent, that upon contact with the enzymatic component, is converted into a labeling agent, which is detectable. In some cases, the chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agent is provided in an amount sufficient to contact every location. In some embodiments, an electrochemiluminescent precursor labeling agent is converted to an electrochemiluminescent labeling agent. In some cases, the enzymatic component may comprise beta-galactosidase, horseradish peroxidase, or alkaline phosphatase.

As will be understood by those of ordinary skill in the art, a variety of appropriate chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agents may be selected for conversion by many different enzymes. Thus, any known chromogenic, fluorogenic, or chemiluminescent enzyme precursor labeling agent capable of producing a labeling agent in a reaction with a particular enzyme can potentially be used in the present invention as the precursor labeling agent in embodiments where the analyte molecule or a binding ligand associated with an analyte molecule comprises an enzymatic component. For example, many chromogenic, fluorogenic, or chemiluminescent precursor labeling agent suitable for use an enzymatic precursor labeling agent molecule are disclosed in *The Handbook -A Guide to Fluorescent Probes and Labeling Technologies,* Tenth Ed., Chapter 10.

In another embodiment, the analyte molecule may be a protein and the binding ligand may comprise a component which is capable of binding both to the analyte molecule and an enzymatic component. Exposure of the precursor labeling agent molecule to the enzymatic component bound to the binding ligand may convert the precursor labeling agent molecule to a chromogenic, fluorogenic, of chemiluminescent labeling agent molecule that may be detected.

Two non-limiting examples of indirect detection of an analyte molecule are illustrated in FIGS. 9A and 9B. In FIG. 9A, a location 150 (in this embodiment, represented by a reaction vessel) is provided which comprises capture object 152 (in this embodiment, represented by a bead). Analyte molecule 154 is immobilized with respect to capture object 152 via capture component 156. The reaction vessel is exposed to precursor labeling agent 158, which upon exposure to analyte molecule 154, is converted to labeling agent molecule 160, as indicated by arrow 159. As another example, in FIG. 9B, location 170 (in this embodiment, represented by a reaction vessel) is provided which comprises capture object 172 (in this embodiment, represented by a bead). Analyte molecule 174 is immobilized with respect to capture object 172 via capture component 176, and binding ligand 177 is associated with analyte molecule 174. The reaction vessel is exposed to precursor labeling agent 158, which upon exposure to binding ligand 177, is converted to a labeling agent molecule 180, as indicated by arrow 179.

In some embodiments, a plurality of locations may be addressed and/or a plurality of capture objects and/or species/molecules/particles of interest may be detected substantially simultaneously. "Substantially simultaneously" when used in this context, refers to addressing/detection of the locations/capture objects/species/molecules/particles of interest at approximately the same time such that the time periods during which at least two locations/capture objects/species/molecules/particles of interest are addressed/detected overlap, as opposed to being sequentially addressed/detected, where they would not. Simultaneous addressing/detection can be accomplished by using various techniques, including optical techniques (e.g., CCD detector). Spatially segregating capture objects/species/molecules/particles into a plurality of discrete, resolvable locations, according to some embodiments facilitates substantially simultaneous detection by allowing multiple locations to be addressed substantially simultaneously. For example, for embodiments where individual species/molecules/particles are associated with capture objects that are spatially segregated with respect to the other capture objects into a plurality of discrete, separately resolvable locations during detection, substantially simultaneously addressing the plurality of discrete, separately resolvable locations permits individual capture objects, and thus individual species/molecules/particles (e.g., analyte molecules) to be resolved. For example, in certain embodiments, individual molecules/particles of a plurality of molecules/particles are partitioned across a plurality of reaction vessels such that each

reaction vessel contains zero or only one species/molecule/particle. In some cases, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5% of all species/molecules/particles are spatially separated with respect to other species/molecules/particles during detection. A plurality of species/molecules/particles may be detected substantially simultaneously within a time period of less than about 1 second, less than about 500 milliseconds, less than about 100 milliseconds, less than about 50 milliseconds, less than about 10 milliseconds, less than about 1 millisecond, less than about 500 microseconds, less than about 100 microseconds, less than about 50 microseconds, less than about 10 microseconds, less than about 1 microsecond, less than about 0.5 microseconds, less than about 0.1 microseconds, or less than about 0.01 microseconds, less than about 0.001 microseconds, or less. In some embodiments, the plurality of species/molecules/particles may be detected substantially simultaneously within a time period of between about 100 microseconds and about 0.001 microseconds, between about 10 microseconds and about 0.01 microseconds, or less.

In some embodiments, techniques may be used to prevent or reduce dissociation of an analyte molecule from a capture component and/or capture object, and/or to prevent or reduce dissociation of a binding ligand from an analyte molecule and/or another binding ligand. As will be known to those of ordinary skill in the art, some reversible affinity interactions between selected analyte molecules, capture components, and/or binding ligands (e.g., between an antibody and an antigen) are governed by thermodynamics. Accordingly, at some point during certain assay methods, some dissociation may occur between an analyte molecule and a capture component and/or a binding ligand, and/or between a binding ligand and an analyte molecule and/or another binding ligand. This may result in a reduced number of analyte molecules (e.g., immunocomplexes) being detected than are actually present. The dissociation constant of a particular pair of components (e.g., antibody-antigen pair), washing and/or fluid exposure, time between exposure and interrogation, and/or other factors, may affect the degree to which a dissociation event alters determination of analyte molecules and/or particles. Accordingly, certain techniques may be used to reduce the effects of dissociation processes.

In a first embodiment, dissociation may be reduced or eliminated by removing fluids from the assay locations (e.g., wells) following spatial segregation of a plurality of analyte molecules (e.g., associated with a capture object via a capture component and/or

associated with at least one binding ligand) into a plurality of such locations. That is, all or substantially all of the fluid surrounding or substantially contained in or at the locations may be removed. For example. the fluid may be removed by air and/or vacuum drying. Removal of the fluid may reduce or eliminate dissociation. Immediately prior to interrogation of the locations, a fluid may be added to the locations thereby rehydrating the complexes to facilitate interrogation using a detector.

In a specific example, a plurality of analyte molecules is associated (e.g., via a capture object) with beads (one embodiment of capture objects). The beads, and thus the analyte molecules, are optionally associated with at least one binding ligand and/or exposed to a material (e.g., a buffer) to keep the analyte molecules and/or binding ligands hydrated (e.g., immunocomplexes may be exposed to a buffer containing a certain carbohydrate). Following loading of the beads into a plurality of wells (e.g., locations), excess solution (e.g., buffers) is removed by air and/or vacuum drying. In this embodiment, by eliminating the contact of the beads with bulk solution, the dissociation of the analyte molecules and/or binding ligands is essentially eliminated or substantially reduced as compared to a similar system where the fluid is not removed. In this case, the wells comprising the beads may be stored (e.g., for 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 12 hours, or more) with none or substantially none (e.g., less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.3%, less than about 0.1%, less than about 0.01%, or less) of the analyte molecules (and/or binding ligands) dissociating. Immediately prior to or close in time to the time when interrogations of the wells are performed (e.g., about 1 second, about 5 seconds, about 10 seconds, about 30 seconds, about 1 minute, about 2 minutes, or about 5 minutes prior), a solution may be provided to the wells, optionally followed by sealing (e.g., prior to interrogation). See, for example, Example 22.

In a second embodiment, dissociation may be reduced or eliminated by crosslinking an analyte molecule with a capture component, and/or crosslinking a binding ligand with an analyte molecule and/or a second binding ligand. For example, an analyte molecule comprising an antigen may be crosslinked with a binding ligand and/or capture component comprising an antibody. Crosslinking methods and techniques that may be employed are known to those of ordinary skill in the art. In some cases, following association of an analyte molecule with a capture component (e.g.,

associated with a capture object) and/or association of a binding ligand with the analyte molecule and/or a second binding ligand, the complex may be exposed to a crosslinking reagent (e.g., 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (i.e., EDC), glutaraldehyde, etc.), thereby promoting the desired crosslinking reaction. The crosslinking reagent may be selected according to the properties of the reagents used for assay, and various factors may be considered including the length of the crosslinking reagent and/or the necessary functional groups when selecting a crosslinking agent, as would be understood by those skilled in the art. Excess crosslinking reagent may optionally be removed and/or unreacted crosslinking groups may be quenched prior to interrogation and/or prior exposure to a labeling agent precursor or other reagent. The assay may be then carried out according to the methods as described herein. See, for example, Example 23.

A non-limiting example of a crosslinking reaction is shown in FIG. 10A. In this figure, bead 502 comprising capture component 504 (e.g., capture antibodies) is associated with analyte molecule 506. Analyte molecule 506 is also associated with binding ligand 508. Complex 507 is exposed to crosslinking reagent 510 and a reaction occurs as indicated by arrow 511. First crosslinking reagent 512 forms a crosslink between analyte molecule 506 and capture component 504 and crosslinking reagent 514 forms a crosslink between analyte molecule 506 and binding ligand 508.

As another example, the binding ligand may comprise a crosslinking component capable of forming a crosslink between two components (e.g., an analyte molecule and a binding ligand, or a capture component and an analyte molecule, or a first binding ligand and a second binding ligand), as shown in FIG. 10B. In this figure, bead 522 comprising capture component 524 (e.g., capture antibody) is associated with analyte molecule 526. Analyte molecule 526 is also associated with binding ligand 528 comprising crosslinking component 530. Upon exposure to an external stimulus (e.g., UV-light, chemical stimulus, etc.), as indicated by arrow 531, crosslink 532 forms between analyte molecule 526 and binding ligand 524. Non-limiting examples of crosslinking components useful for this purpose include N-succinimidyl-6-[4'-azido-2'-nitrophenylamino]) hexanoate (i.e., SANPAH) and succinimidyl 6-(4,4'-azipentanamido)hexanoate (i.e., LC-SDA).

During the step of the method where the locations into which the capture objects/analyte molecules have been segregated are addressed, any of a variety of parameters may be determined. In some embodiments, the number of locations which comprise a capture object and an analyte molecule (or binding ligand) is determined.

The number of locations which comprise a capture object but do not comprise an analyte molecule (or binding ligand) may also be determined. In some cases, the number of locations which are addressed which do not contain a capture object may also be determined. In still yet other cases, the total number of locations addressed may also be determined. A single interrogation or multiple interrogations of any subset or all of the locations ultimately addressed may be made at any given time to facilitate one or all of the above described determinations. For example, a first determination may be completed under a first range of wavelengths (e.g., white light) to determine the number of locations comprising a capture object, wherein the locations are not distinguished as to whether an analyte molecule (or binding ligand) is associated with the capture object, and a second determination of the same or some subset of the locations may be completed under a second range of wavelengths (e.g., fluorescence) to determine the number of locations which comprise a capture object associated with an analyte molecule (or binding ligand). Exemplary detection methods are described below.

Detection Methods

In some embodiments, in the systems/methods in which the species to be detected are partitioned across a plurality of locations, the locations can be interrogated using a variety of techniques, including techniques known to those of ordinary skill in the art.

In a specific embodiment of the present invention, the locations are optically interrogated. The locations exhibiting changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the detected species (e.g., labeling agent molecules, particles, etc.) and the operative wavelengths, optical filters designed for a particular wavelength may be employed for optical interrogation of the locations.

In embodiments where optical interrogation is used, the system may comprise more than one light source and/or a plurality of filters to adjust the wavelength and/or intensity of the light source. For example, in some cases, a first interrogation of the locations may be conducted using light of a first range of wavelengths (e.g., white light in embodiments where the capture objects are not fluorescent, or a wavelength range where the capture objects fluoresce), whereas a second interrogation is conducted using light of a second, differing range of wavelengths, such that the plurality of detectable molecules fluoresce. An exemplary system configuration is provided below (see FIG. 11).

In some embodiments, the optical signal from a plurality of locations is captured using a CCD camera Other non-limiting examples of camera imaging types that can be used to capture images include charge injection devices (CIDs), complimentary metal oxide semiconductors (CMOSs) devices, scientific CMOS (sCMOS) devices, and time delay integration (TDI) devices, as will be known to those of ordinary skill in the art. The camera may be obtained from a commercial source. CIDs are solid state, two dimensional multi pixel imaging devices similar to CCDs, but differ in how the image is captured and read. For examples of CIDs, see U.S. Patent No. 3,521,244 and U.S. Patent No. 4,016,550. CMOS devices are also two dimensional, solid state imaging devices but differ from standard CCD arrays in how the charge is collected and read out. The pixels are built into a semiconductor technology platform that manufactures CMOS transistors thus allowing a significant gain in signal from substantial readout electronics and significant correction electronics built onto the device. For example, see U.S. Patent No. 5,883,830. sCMOS devices comprise CMOS imaging technology with certain technological improvements that allows excellent sensitivity and dynamic range. TDI devices employs a CCD device that allows columns of pixels to be shifted into and adjacent column and allowed to continue gathering light. This type of device is typically used in such a manner that the shifting of the column of pixels is synchronous with the motion of the image being gathered such that a moving image can be integrated for a significant amount of time and is not blurred by the relative motion of the image on the camera. In some embodiments, a scanning mirror system coupled with a photodiode or photomultiplier tube (PMT) could be used to for imaging.

In one embodiment, the plurality of locations is formed directly as a plurality of reaction vessels in an end of a fiber optic bundle. According to one embodiment, the array of reaction vessels for the present invention can be used in conjunction with an optical detection system such as the system described in U.S. Publication No. 2003/0027126. For example, according to one embodiment, the array of reaction vessels of the present invention is formed in one end of a fiber optic assembly comprising a fiber optic bundle constructed of clad fibers so that light does not mix between fibers.

FIGS. 11A and 11B show non-limiting examples of a system of the present invention according to some embodiments. The system comprises a light source 452, excitation filter 454, dichromatic mirror 458, emission filter 460, objective 470, and array 472. Light 453 given off from light source 452 is passed through excitation filter 454. The light reflects off dichromatic mirror 458, passes through objective 470 and

shines on array 472. In some cases, stray light 464 may be reduced by a stray light reducing function 468, such as an iris or aperture. Light 471 emitted from the array passes through objective 470 and emission filter 460. Light 462 is observed. The system may comprise additional components (e.g., additional filters, mirrors, magnification devices, etc.) as needed for particular applications, as would be understood by those of ordinary skill in the art.

The system shown in FIG. 11A may additionally comprise components which aid in the determination of the number of reaction vessels which contain a capture object (e.g., using white light). Alternatively, the additional components may be used to determine the total number of locations and/or provide spatially information regarding the position of the locations (e.g., containing or not containing a capture object), which may help corroborate signals observed under different light regimes (e.g., fluorescence, white light) corresponding with the position of a location (e.g., a mask may be created).

In FIGS. 11A and 11B, excitation light is emitted from source 452 and collimated into a beam 453. The excitation filter 454 may be configured to transmit only the wavelength band that excites the fluorophore (e.g., 575 nm +/- 10 nm for resorufin). The excitation light is reflected downward by the dichroic filter 458 and excites the substrate 472 containing the sample through the objective lens 470. The image light is collected by the objective lens 470, collimated into a beam 471 and transmitted through the dichroic filter 458. Only the image light corresponding to the fluorescence wavelength band (e.g., 670 nm +/- 30 nm for resorufin) is transmitted through the emission filter 460. The remaining collimated beam 462 contains only the emitted fluorescence wavelengths which will subsequently be imaged through the camera system.

The same system may be used to determine the positioning of the locations containing sample (e.g., reaction vessels). The array comprising the reaction vessels containing capture objects may be illuminated with a "bright field" white light illumination. The array may be illuminated (e.g., using light source 475 shown in FIG. 11A) by directing a pseudo-collimated white light (e.g., white light LED) onto the array surface from an angle (e.g., θ_1 in FIG. 11A may be about 20 degrees, about 25 degrees, about 30 degrees, about 35 degrees, about 40 degrees, or greater) just outside the numerical aperture of the collection objective. Light that hits the surface of the array 472 (e.g., light 476) is reflected (and scattered) off the surface, collimated 471, and collected

by the objective lens (470). The collimated beam is subsequently imaged through the camera system.

The same system may also be used to determine which locations contain a capture object (e.g., bead). Any particular bead may or may not be associated with an analyte molecule and/or binding ligand. The array may be illuminated (e.g., using light source 473 as shown in FIG. 11A) with a "dark field" white light illumination. The array may be illuminated by aiming a pseudo-collimated white light (e.g., white light LED 473) onto the array surface from an angle (e.g., θ_2 in FIG. 11A is about 65 degrees, about 70 degrees, about 75 degrees, about 80 degrees, about 85 degrees) substantially outside the numerical aperture of the collection objective. Light that hits the surface of the array 472 (e.g., light 474) is reflected (and scattered) off the surface, collimated 471, and collected by the objective lens 470. The collimated beam is subsequently imaged by the camera system.

In some embodiments, an optical detection system may be employed, for example, as described in U.S. Publication No. 2003/0027126. In an exemplary system, light returning from an array of reaction vessels formed at the distal end of a fiber optic bundle is altered via use of a magnification changer to enable adjustment of the image size of the fiber's proximal or distal end. The magnified image is then shuttered and filtered by a shutter wheel. The image is then captured by charge coupled device (CCD) camera. A computer may be provided that includes and executes imaging processing software to process the information from the CCD camera and also optionally may be configured to control shutter and filter wheels. As depicted in U.S. Publication No. 20030027126, the proximal end of the bundle is received by a z-translation stage and x-y micropositioner.

For example, FIG. 12 shows a schematic block diagram of a system employing a fiber optic assembly 400 with an optical detection system. The fiber optic assembly 400 that comprises a fiber optic bundle or array 402 that is constructed from clad fibers so that light does not mix between fibers. An array of reaction vessels 401 is formed at/attached to the bundle's distal end 412, with the proximal end 414 being operatively connected with a z-translation stage 416 and x-y micropositioner 418. These two components act in concert to properly position the proximal end 414 of the bundle 402 for a microscope objective lens 420. Light collected by the objective lens 420 is passed to a reflected light fluorescence attachment with three pointer cube slider 422. The attachment 422 allows directs light from a 75 watt Xe lamp 424 through the objective

lens 420 to be coupled into the fiber bundle 402. The light from source 424 is condensed by condensing lens 426, then filtered and/or shuttered by filter and shutter wheel 428, and subsequently passes through a ND filter slide 430. Light returning from the distal end 412 of the bundle 402 passes through the attachment 422 to a magnification changer 432 which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer 432 is then shuttered and filtered by a second wheel 434. The light is collected by a charge coupled device (CCD) camera 436. A computer 438 executes imaging processing software to process the information from the CCD camera 436 and also optionally controls other components of the system, including but not limited to the first and second shutter and filter wheels 428, 434.

An array of reaction vessels used to practice some embodiments of the present invention may be integral with or attached to the distal end of the fiber optic bundle using a variety of compatible processes. In some cases, microwells are formed at the center of each individual fiber of the fiber optic bundle and the microwells may or may not be sealed. Each optical fiber of the fiber optic bundle may convey light from the single microwell formed at the center of the fiber's distal end. This feature enables the interrogation of the optical signature of individual reaction vessels to identify reactions/contents in each microwell. Consequently, by collecting the image of the end of the bundle with the CCD array, the optical signatures of the reaction vessels may be individually interrogated and/or imaged substantially simultaneously.

Quantification

According to some embodiments of the present invention, the methods, systems, and/or devices are used to determine the presence and/or a measure of the concentration of analyte molecules (or particles) in a fluid sample based at least in part on detecting and/or quantifying at least some of a plurality of capture objects used to capture the analyte molecules (and optionally at least one binding ligand). In certain embodiments where concentration is determined, a correlation and/or calibration relating the number (or fraction/percentage) of locations containing a capture object comprising at least one analyte molecule (and/or at least one binding ligand) to the quantity/concentration of analyte molecules in the fluid sample is employed. In some cases, the concentration of the analyte molecules in a fluid sample may be linearly proportional to the number/fraction of locations containing a capture object comprising at least one analyte molecule (and/or at least one binding ligand). In other cases, the measure of

concentration of the analyte molecules in a fluid sample may be related to the number/fraction of locations containing a capture object associated with at least one analyte molecule (and/or at least one binding ligand) by a non-linear relationship. In some embodiments, a measure of the concentration of analyte molecules in a fluid sample may be determined at least in part using a calibration curve developed using samples containing known concentrations of target analyte molecules. Methods to determine a measure of the concentration of analyte molecules in a fluid sample are discussed more below.

Certain embodiments of present invention are distinguished by the ability to detect and/or quantify low numbers/concentrations of capture objects comprising at least one analyte molecule (and/or at least one binding ligand) and may be well suited to determine a measure of the concentration of analyte molecules in a fluid sample containing very low concentrations of the analyte molecule. This capability may be facilitated, in certain embodiments, at least in part by spatially isolating individual capture objects, including at least some comprising at least one analyte molecule (and/or at least one binding ligand), for example, by partitioning a plurality of such capture objects across an array of locations (e.g., reaction vessels), and then detecting their presence in the reaction vessels. The presence of a capture object comprising at least one analyte molecule (and/or at least one binding ligand) in a reaction vessel, in some embodiments, can be determined and the number of such reaction vessels counted in a binary fashion. That is, in embodiments where a location (e.g., a reaction vessel) is found to contain at least one capture object associated with at least one analyte molecule (and/or binding ligand), the location is counted as one. In embodiments where a location (e.g., a reaction vessel) is found to contain a capture object, the location is counted as zero. For example, wells that are counted as "ones" may be determined by detecting the presence of a detectable molecule or particle in a reaction vessel that, as described above, indicates the presence of a capture object comprising at least one analyte molecule (and/or at least one binding ligand) in the well.

In embodiments where a fluid sample containing or suspected of containing is contacted with a plurality of capture objects such that any analyte molecules present in the sample are immobilized with respect to the plurality of capture objects such that a statistically significant fraction (e.g., as described above) of the capture objects associate with a single analyte molecule and a statistically significant fraction of the capture objects do not associate with any analyte molecules (e.g., as shown in FIG. 1, step (B)), a

determination of a measure of the concentration of analyte molecules in the fluid sample may be carried out as follows. First, at least a portion of the capture objects (at least some of which have a single analyte molecule immobilized) are spatially segregated into a plurality of locations (e.g., as shown in FIG. 1, step (C)). The number of locations that contain an analyte molecule immobilized with respect to a capture object is determined, either directly (e.g., by detection of the analyte molecule itself (e.g., see FIG. 1, step (D)) or indirectly (e.g., by detection of a binding ligand associated with the analyte molecule, by detection of a labeling agent (e.g., formed via conversion of a precursor labeling agent upon exposure to an analyte molecule see FIG. 4A), etc.). In some embodiments, a measure of the concentration of analyte molecules in a fluid sample is determined at least in part on the determination of the number of the plurality of locations that contain an analyte molecule (e.g., reaction vessels 12 in FIG. 1, step (D)). In certain such embodiments, a measure of the concentration of analyte molecules in the fluid sample is determined at least in part by comparison of this measured parameter to a calibration standard and/or by using a Poisson and/or Gaussian distribution analysis of the number of locations that would be expected to contain an analyte molecule.

In some embodiments the number of locations which comprise a capture object not associated with an analyte molecule may also be determined (e.g., reaction vessel 13 in FIG. 1, step (D)). In such cases, a measure of the concentration of analyte molecules in a fluid sample may be determined based at least in part on the ratio of locations comprising an analyte molecule immobilized with respect to a capture object, to the number of locations comprising a capture object not associated with an analyte molecule. In some cases, the number of locations which do not comprise a capture object may also be determined (e.g., reaction vessel 14 in FIG. 1, step (D)). In such cases, a measure of the concentration of analyte molecules in a fluid sample may be determined based at least in part on the ratio of locations comprising an analyte molecule immobilized with respect to a capture object to the number of locations not comprising a capture object and/or the number of locations not comprising an analyte molecule – whether or not such location contains a capture object (in either case above or elsewhere, the denominator for the ratio/fraction may or may not include the positive ("on" or "one") locations added to the nil ("off" or "zero") locations depending upon preference). In yet other cases, the total number of locations addressed/analyzed may be determined (e.g., reaction vessels 12, 13, and 14 in FIG. 1, step (D)) and a measure of the concentration of analyte molecules in a fluid sample may be based on the ratio of the locations comprising an

analyte molecule immobilized with respect to a capture object to the total number of locations addressed/analyzed.

It should be understood, that in some assay methods, a measure of the concentration of analyte molecules in a fluid sample may be carried out using more than one type of analysis (e.g., a first analysis based on the number of locations comprising an analyte molecule immobilized with respect to a capture object, and a second analysis based on the ratio of the ratio/fraction of locations comprising an analyte molecule immobilized with respect to a capture component, to the total number of locations comprising a capture object, etc.). In such embodiments, the second analysis may be used as a quality control measure (e.g., to confirm that the first analysis provided a reasonable result) and/or the two analysis results may be averaged.

In some embodiments, the determination of a measure of the concentration of analyte molecules in a fluid sample being tested may be carried out using a similar analysis as described above, but by determining the number of reaction vessels which comprise a binding ligand as opposed to the number of reaction vessels which comprise an analyte molecule immobilized with respect to a capture object. As described herein, in some cases, following immobilization of a plurality of analyte molecules to a plurality of capture objects, the plurality of capture objects may be exposed to at least one type of binding ligand such that at least some of the immobilized analyte molecules associate with at least one binding ligand (e.g., see FIG. 2, step (B). This assay method may be especially useful in embodiments where more than one analyte molecule is expected to become associated with each capture object, but binary quantification may still be desired. In some cases, the binding ligand may be provided at a concentration such that at least some of the capture objects containing at least one analyte molecule do not associate with any binding ligands (e.g., see FIG. 2, step (C). In such embodiments, the number of locations containing a capture object associated with a binding ligand (e.g., via an analyte molecule) can replace the number of locations containing a capture object associated with an analyte molecule in the analysis and methods described above.

A measure of the concentration of analyte molecules or particles in a fluid sample may be determined using a variety of calibration techniques, and the particular technique resulting in the most accuracy and reliability can depend on the relative number/concentration of analyte molecules in the sample to the number/concentration of capture objects exposed to the sample (and or, for embodiments using binding ligands, the relative number/concentration of binding ligands to the number/concentration of

capture objects exposed to each other during/after capture of the analyte molecules by the capture objects). Non-limiting examples of concentration determination methods that may be useful in particular analyte concentration regimes include the above described binary read-out methods, and/or methods in which the relative positive signal intensity measured for the locations ("intensity read-out methods") is employed. Either or both of the above methods – or alternative methods – may further employ a comparison of the measured parameter with a calibration curve.

It is currently believed that the most accurate method of determination may depend at least in part on the concentration of analyte molecules contained in the fluid sample. For example, in embodiments in which the concentration of the analyte molecules in the sample being tested results in a statistically significant fraction of locations to which that capture objects are partitioned comprising a single analyte molecule or binding ligand and a statistically significant fraction of locations not comprising any analyte molecules or binding ligands (e.g., at or approaching a regime where essentially no locations comprise more than one analyte molecule or binding ligand), a binary read-out method may be particularly useful, and in some cases, may be used in conjunction with a calibration curve. In other embodiments, where a larger number of locations comprise more than one analyte molecule and/or more than one binding ligand, a determination based at least in part on an intensity read-out may provide a more accurate measure of the concentration of analyte molecules in a fluid sample. Such a determination may also be used in conjunction with a calibration curve.

In certain embodiments, the fraction of locations (e.g., the statistically significant fraction) which comprises at least one capture object associated with an analyte molecule and/or binding ligand is less than about 50%, less than about 40%, less than about 25%, less than about 10%, less than about 5%, less than about 1%, less than about 0.5%, or less than about 0.1% of the total number of locations containing a capture object. In such embodiments, a measure of the concentration of analyte molecules in the fluid sample may be determined using a binary read-out method. In some cases, the percentage of locations which do not contain a capture object associated with an analyte molecule and/or binding ligand is at least about 20%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, or at least about 95%, at least about 99%, at least about 99.5%, at least about 99.9%, or greater, of the total number of locations.

While the discussion below focuses primarily on the use of a binary read-out system (e.g., based on counting the number of "on" and "off" locations) for ultra low level detection capability, this is by no means limiting, and the inventive methods and assays may also in certain embodiments employ instead of or in addition to a binary quantification protocol , one based in measurement of intensity (i.e. an intensity read-out method) (e.g., to extend dynamic range). As noted, in some cases, the detection systems and quantification methods may be configured so that the system can use either or both of a binary read-out determination and an intensity read-out determination, depending on the assay format and/or the concentration of analyte molecules in the fluid sample. For example, the method and/or system may be able to determine a base parameter from a first measurement and decide to use either a binary read-out determination or an intensity read-out determination depending on the result of the first determination, as described in more detail below and as is described in commonly owned U.S. Patent Application Serial No. 12/731,136, entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles" by Rissin et al., filed March 24, 2010, and International Patent Application No. (not yet determined), entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles" by Rissin et al., filed March 1, 2011 (Attorney Docket No. Q0052.70013WO00), each herein incorporated by reference.

According to one embodiment, the quantification method of the present invention can be performed as follows. A fluid sample containing or suspected of containing an analyte molecule of interest is contacted with a plurality of capture objects and, optionally, one or more binding ligands and the capture objects are partitioned across an array of locations, such as reaction vessels/wells (as described previously). In some embodiments, where a binary read-out method is desired to be used for determination, in the step of contacting the fluid sample with the capture objects, the relative amounts/concentrations of fluid sample and capture object containing solution are selected (e.g., based on a known or estimated/suspected approximate concentration range of analyte molecules in the sample) so that the ratio of analyte molecules in the fluid sample to total number of capture objects provided to the solution will be less than about 1:5, less than about 1:10, less than about 1:12, less than about 1:15, less than about 1:20, less than about 1:50, less than about 1:100, or less. With such ratios, at least some of the capture objects statistically will be expected associate with a single analyte molecule and the majority of the remainder of the capture objects will not associate with

any analyte molecules. The number of capture objects associating with multiple analyte molecules under such conditions may be low enough to be neglected, such that capture object determined to comprise an analyte molecule can be assumed to comprise a single analyte molecule. Under such conditions, an analysis system configured to perform a binary read out quantification may be used to determine the number of locations which comprise a capture object associated with an analyte molecule by any detection method as described herein. The number of locations which comprise a capture object associated with an analyte molecule is then counted (e.g., FIG. 1, step (D), the total number of reaction vessels comprising an analyte molecules is two, e.g., reaction vessels 12) and, in some cases, the fraction of the total number of locations containing a capture object which contain a capture object associated with an analyte molecule is calculated (e.g., in FIG. 1, total number of reaction vessels comprising a capture object is three, reactions vessels 12 and 13; thus, fraction of the total number of locations comprising a capture object associated with an analyte molecule is 2:3). Utilization of a zero (no analyte molecule detected) or one (an analyte molecule detected) response, in conjunction with using an array with a large number of locations can permit a determination of bulk concentrations of analyte molecules in the sample by counting the actual number of molecules contained in the volume of sample partitioned across and contained in the locations. In some cases, the analyte molecule may be detected indirectly (e.g., the read-out is accomplished by counting the number of locations containing at least one labeling agent molecule, wherein the labeling agent has been converted from a precursor labeling agent upon exposure to an analyte molecule). In instances where a large number of locations (e.g., at least about 10,000 locations) are substantially simultaneously interrogated, the ratio of locations comprising an analyte molecule associated with a capture object to total number of locations determined (e.g., in some cases, the locations which contain a capture object associate with or not associated with any analyte molecules) may be at least about 1:100, at least about 1:1000, at least about 1:10,000 or less. Utilizing an array with a large number of locations (e.g., at least about 10,000, at least about 50,000, at least about 100,000, at least about 500,000, etc.) may provide a statistically significant signal even at this low ratio.

In some assays, a Poisson distribution adjustment may be applied to numbers and/or ratios determined by a binary read-out method to facilitate and/or improve accuracy of determining a concentration of analyte molecules in a fluid sample. For example, in embodiments where the ratio of analyte molecules in the fluid sample to the

total number of capture objects contacted with the fluid sample is greater than about 1:10, greater than about 1:5, greater than about 1:4, greater than about 1:3, or greater than about 1:2, or between about 1:10 and about 1:2, between about 1:5 and about 1:2, the number of analyte molecules immobilized per capture may be zero or one, with a greater proportion containing more than one than for the regime described in the paragraph above. In some such cases, performance and accuracy of the concentration determinations may be improved over use of an assumption that all positive locations contain only a single analyte molecule (as described in the paragraph above) by employing a Poisson distribution adjustment to predict the number of locations expected to contain 0, 1, 2, 3, 4, etc., analyte molecules per capture object.

A Poisson distribution describes the likelihood of a number of events occurring if the average number of events is known. If the expected number of occurrences is μ, then the probability ($P_\mu(\nu)$) that there are exactly ν occurrences (ν being a non-negative integer, $\nu = 0, 1, 2, ...$) may be determined by Equation 5:

$$P_\mu(\nu) = e^{-\mu}\left(\frac{\mu^\nu}{\nu!}\right) \qquad \text{(Eq. 5)}$$

In some embodiment of the present invention, μ is equal to the fraction of the number of locations determined to contain an analyte molecule associated with an analyte to the total number of capture objects detected (e.g., either associated with or not associated with any analyte molecules), and ν is the number of capture objects associated with a certain number of analyte molecules (e.g., the number of capture objects associated with either 0, 1, 2, 3, etc. analyte molecule). By determining μ from interrogating the array of locations during an assay, the concentration of analyte molecules in the sample can be determined using a Poisson distribution adjustment. For example, in an assay using the binary mode of measurements where capture objects associated with 1, 2, 3, 4, etc. analyte molecules are not distinguished from each other (e.g., where $\nu = 1, 2, 3, 4$ are not differentiated from each other) and the wells (e.g., locations, reaction vessels) are simply characterized as "on" wells, then occurrences of $\nu = 0$ can by determined definitively as the number of "off" wells. ($P_\mu(0)$) may be calculated according to Equation 6:

$$P_\mu(0) = e^{-\mu}\left(\frac{\mu^0}{0!}\right) = e^{-\mu} \qquad \text{(Eq. 6)}$$

and the number of expected occurrences, μ, may be determined based on a rearrangement of Equation 5, as given in Equation 7:

$$\mu = -\ln[P_\mu(0)] \quad \text{(Eq. 7).}$$

The number of occurrences of capture objects associated with no analyte molecules (or binding ligands), $P_\mu(0)$, is equal to 1 minus the total number of capture objects with all other occurrences (e.g., capture objects associated at least one analyte molecule or binding ligand) then μ is given by Equation 8:

$$\mu = \frac{Number\ of\ Analyte\ molecules}{Number\ of\ Capture\ objects} = -\ln(1 - fraction\ of\ \text{"on"}\ wells) \quad \text{(Eq. 8).}$$

Rearranging Equation 8, the total number of analyte molecules in the fluid sample contained in the locations interrogated containing a capture object can be determined using Equation (9):

$$Number\ of\ Analyte\ molecules = -\ln(1 - fraction\ of\ \text{"on"}\ wells) \times Number\ of\ Capture\ objects \quad \text{(Eq. 9).}$$

Therefore, the total number of molecules can be determined from the fraction of "on" wells for a given number of wells containing capture objects, and a measure of the concentration of analyte molecules in the fluid sample may be based at least in part on this number (as well as, e.g., any dilutions of the sample during the assay, the number and volume of the wells containing capture objects interrogated, etc.). The number of capture objects with 1, 2, 3, 4 etc. associated analyte molecules can also be determined by calculating $P_\mu(1)$, $P_\mu(2)$, $P_\mu(3)$ etc. from the μ determined and Equation 5.

As a non-limiting example of use of a Poisson distribution adjustment, in an assay where 26% of 50,000 capture objects interrogated were "on" (i.e., contained one or more analyte molecules and/or binding ligands) then the total number of analyte molecules present is calculated as $-\ln(1-0.26)\times 50{,}000 = 15{,}056$ molecules. Of these 15,056 molecules, using $\mu = -\ln(1-0.26) = 0.3011$ in the Eq. 5 for $v = 1$, 11,141 capture objects are calculated to have 1 analyte molecule, 1,677 capture objects 2 analyte molecules, 168 capture objects 3 analyte molecules, 13 capture objects 4 analyte molecules, and 1 capture object 5 analyte molecules. A similar analysis may be applied to embodiments where a statistically significant fraction of the spatially separated capture objects are associated with at least one binding ligand and a statistically significant fraction of spatially separated capture objects are not associated with any binding ligands.

In some embodiments, wherein the ratio of locations comprising a capture object associated with at least one analyte molecule and/or a binding ligand to locations containing a capture object free of any analyte molecule/binding ligand is high (e.g., greater than about 1:2, greater than about 1:1, greater than about 2:1, greater than about

4:1, greater than about 8:1, or greater), the determination of the concentration of analyte molecules in the fluid sample may be based at least in part on an intensity read-out determination. In such an embodiment, the total intensity of the array (e.g., total fluorescence) may be determined and a measure of the concentration of analyte molecules in the fluid sample is based at least in part on this determination.

In some embodiments, a measure of the concentration of analyte molecules or particles in the fluid sample may be determined at least in part by comparison of a measured parameter to a calibration standard. In some cases, a calibration curve may be used, similar to as described herein, wherein the total intensity is determined for a plurality of samples comprising the analyte molecule at a known concentration using a substantially similar assay format. For example, the number and/or fraction of locations that comprise a capture object associated with an analyte molecule (e.g., based on a binary read-out), or alternatively, the total intensity of the array, may be compared to a calibration curve to determine a measure of the concentration of the analyte molecule in the fluid sample. The calibration curve may be produced by completing the assay with a plurality of standardized samples of known concentration under similar conditions used to analyze test samples with unknown concentrations. A calibration curve may relate the fraction of the capture objects determined to be associated with an analyte molecule and/or binding ligand with a known concentration of the analyte molecule. The assay may then be completed on a sample containing the analyte molecule in an unknown concentration, and number/fraction of capture objects determined to be associated with an analyte molecule and/or binding ligand may be compared to the calibration curve, (or a mathematical equation fitting same) to determine a measure of the concentration of the analyte molecule in the fluid sample.

In one exemplary embodiment for performing a calibration, four standardized fluid samples comprising an analyte molecule in varying concentration (w, x, y, and z) are used. An assay (e.g., immobilizing the analyte molecules with respect to a plurality of capture objects, optionally exposing the capture objects to at least one type of binding ligand, partitioning at least a portion of the capture objects into a plurality of discrete, separately addressable locations, detecting at least a portion of the capture objects, etc.) is carried out for each calibration sample, and the number/fraction of capture objects comprising an analyte molecule and/or binding ligand (b, c, d, and e) is determined. A plot/equation/look-up table, etc. is produced relating the values b, c, d, and e to concentrations w, x, y, and z, respectively, as depicted in FIG. 13. The assay may be

then be carried out under substantially identical conditions on a fluid sample containing an analyte molecule of unknown concentration, wherein the resulting value of number/fraction of capture objects comprising an analyte molecule and/or binding ligand determined to detection of the capture objects is f. This value (f) may be plotted on the graph and a measure of the unknown concentration of the target analyte in the fluid sample (t) may be determined. In some cases, the calibration curve may have a limit of detection, wherein the limit of detection is the lowest concentration of analyte molecules in a fluid sample that may be accurately determined. In some cases, the r^2 value of the calibration curve may be greater than about 0.5, greater than about 0.75, greater than about 0.8, greater than about 0.9, greater than about 0.95, greater than about 0.97, greater than about 0.98, greater than about 0.99, greater than about 0.9999, or about 1. Values b, c, d, and e may be based on the absolute number of measured locations/capture objects associated with an analyte molecule (or binding ligand), or a ratio of the number of locations containing a capture object associated with an analyte molecule (or binding ligand) to the number of locations containing a capture object not associated with any analyte molecules or a ratio of the number of locations containing a capture object associated with an analyte molecule (or binding ligand) to the number of locations containing a capture object or a ratio of the number of locations containing a capture object associated with an analyte molecule (or binding ligand) to the total number of locations addressed, etc. Any number of calibration standards may be used to develop the calibration curve (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, calibration standards).

In some embodiments, the concentration of analyte molecules in the fluid sample may be determined through use of a calibration curve using an assay system employing a computer. The computer may execute software that may use the data collected to produce the calibration curve and/or to determine a measure of the concentration of analyte molecules in a test fluid sample from such calibration curve. For example, a fluorescence image of an array comprising the plurality of capture objects partitioned across the array may be collected and analyzed using image analysis software (e.g., IP Lab, BD Biosciences). The analysis software may automatically calculate the number of locations that have fluorescence intensity over the background intensity (e.g., a number that correlates to the number of locations which comprise an analyte molecule). The number of locations which comprise fluorescence intensity over the background intensity may be divided by the total number of locations addressed, for example, to determine the fraction of locations which comprise an analyte molecule. The active location fraction

may be compared to a calibration curve to determine a measure of the concentration of analyte molecules in the fluid sample.

In certain embodiments, it may be possible to increase both the dynamic range and the sensitivity of the assay by expanding the number of locations into which the capture objects are partitioned and/or by adjusting the ratio of capture objects (e.g. beads) to analyte molecules in the initial capture step. In certain cases, decreasing or increasing the analyte-to bead ratio may result in more dynamic range. In some cases, as the volume of a sample increases, detecting small numbers of analyte molecule with accuracy, may, in some cases, become more challenging for example, due to limitations of equipment, time constraints, etc. For example, to achieve the same efficiencies in larger volume sample (e.g., 1 mL, 10 mL) as achieved with a smaller volume sample (e.g., 100 μL), more beads (e.g., 10 and 100 times more beads, respectively) may be necessary, and thus, the beads may need to be spatially segregated into larger number of locations, wherein the larger number of locations may require an increased imaging area.

For the capture step, the choice of bead concentration may depend on several competing factors. For example, it can be advantageous if sufficient beads are present to capture most of the target analyte from thermodynamic and kinetic perspectives. As an exemplary illustration, thermodynamically, 200,000 beads in 100 μL that each have about 80,000 capture components (e.g. antibodies) bound to correlates to an antibody concentration of about 0.3 nM, and the antibody-protein equilibrium at that concentration may give rise to a relatively high capture efficiency of target analyte molecules in certain cases (e.g. >70%). Kinetically, for 200,000 beads dispersed in 100 μL, the average distance between beads can be estimated to be about 80 μm. Proteins the size of TNF-α and PSA (17.3 and 30 kDa, respectively), as exemplary analyte molecules, for example, will typically tend to diffuse 80 μm in less than 1 min, such that, over a 2 hour incubation, capture of such analyte molecules will tend not to be limited kinetically. In addition, it can also be advantageous to provide sufficient beads loaded onto the arrays to limit Poisson noise to a desired or acceptable amount. Considering as an example a situation where 200,000 beads in a in 10 μL volume are loaded onto an array, typically about 20,000–30,000 beads may become trapped in femtoliter sized wells of the array. For a typical background signal (e.g. due to non specific binding, etc.) of 1% active beads, this loading would be expected to result in a background signal of 200–300 active beads detected, corresponding to a coefficient of variation (CV) from Poisson noise of 6–7%, which may be acceptable in typical embodiments. However, bead concentrations

above certain concentrations may be undesirable in certain cases in that they may lead to: a) increases in non-specific binding that may reduce signal-to-background; and/or b) undesirably low ratios of analyte-to-bead such that the fraction of active beads is too low, resulting in high CVs from Poisson noise. In certain embodiments, considering a balance of factors such as those discussed above, providing about 200,000 to 1,000,000 beads per 100 µL of test sample may be desirable or, in certain cases optimal, for performing certain assays of the invention.

For embodiments of the inventive assay employing one or more binding ligand(s) to label the captured analyte molecules, it may be advantageous to, in certain instances, adjust the concentrations used to yield desirable or optimal performance. For example, considering an embodiment involving an analyte molecule that is a protein (captured protein) and employing a first binding ligand comprising a detection antibody and a second binding ligand comprising an enzyme conjugate (e.g. SβG), the concentrations of detection antibody and enzyme conjugate (SβG) used to label the captured protein may in some cases be limited or minimized to yield an acceptable background signal (e.g. 1% or less) and Poisson noise. The choice of the concentrations of detection antibody and enzyme conjugate (SβG) used to label the captured protein can be factors in improving the performance of or optimizing certain of the inventive assay methods. In certain cases, it may be desirable for only a fraction of the capture proteins to be labeled so as to avoid saturating signals produced by the assay. For example, for a particular assay where background levels observed are equivalent to ~1–2 fM of target protein, such that the ratio of analyte to bead may be about 0.3–0.6, the number of active beads may be in the range of about 25–40% if every protein was labeled with an enzyme, which may be higher than desirable in some cases. To produce background signals that may be closer to a lower end of the dynamic range for a digital detection assay— considering e.g. that in certain cases 1% active beads may provide a reasonable noise floor for background in digital detection assays of the invention—appropriate labeling of the captured protein can potentially be achieved by kinetic control of the labeling steps, either by limiting or minimizing the concentrations of both labeling reagents or by using shorter incubation times. For example, in an embodiment where label concentrations are minimized, use of a standard ELISA incubation time may provide acceptable results; e.g. using a total assay time of ~6 h. This length of time may be acceptable for testing that tolerates a daily turnaround time for samples. For shorter turnaround times of, for example, <1 hour

(e.g., for point-of-care applications), the assay could be performed with shorter incubations with higher concentrations of labels.

In some embodiments, accuracy of a particular method of determining concentration with the inventive assays may be compromised, i.e. both above and below the thresholds of the dynamic range for the particular method. For example, as the concentration of the capture objects associated with an analyte molecule goes down, eventually, when below the lower limit of the dynamic range, the number of capture objects associated an analyte molecule may be too low to observe a sufficient number of occupied locations to obtain a reliable and reproducible measurement. In such a situation, the number of locations could be decreased in order to make sure that at least some (e.g., a statistically significant number) of them are occupied by a capture object associated with an analyte molecule, and/or the sample tested could be concentrated and/or the number of capture objects incubated with the sample could be decreased, etc. to increase the number/fraction of positive capture objects detected. On the other hand, a binary read-out system/method may be above its upper threshold of accuracy and/or utility when, for example, loading approaches saturation with "on" capture objects such that substantially 100% of the locations contain at least one capture object associated with an analyte molecule. At this limit, discrimination between two samples with concentrations falling in this range may not be feasible using a binary read-out system/method. In such a situation, to provide a more accurate result, a greater number of locations could be used, the concentration of the sample could be reduced, for example, through serial dilutions, the number of capture objects incubated with the sample could be increased, etc. to decrease the number/fraction of positive capture objects detected, and/or an intensity read-out system/method could be employed.

In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is less than about 5000 fM, less than about 3000 fM, less than about 2000 fM, less than about 1000 fM, less than about 500 fM, less than about 300 fM, less than about 200 fM, less than about 100 fM, less than about 50 fM, less than about 25 fM, less than about 10 fM, less than about 5 fM, less than about 2 fM, less than about 1 fM, less than about 500 aM (attomolar), less than about 100 aM, less than about 10 aM, less than about 5 aM, less than about 1 aM, less than about 0.1 aM, less than about 500 zM (zeptomolar), less than about 100 zM, less than about 10 zM, less than about 5 zM, less than about 1 zM, less than about 0.1 zM, or less. In some cases, the limit of detection (e.g., the lowest concentration of an

analyte molecule which may be determined in solution substantially accurately) is about 100 fM, about 50 fM, about 25 fM, about 10 fM, about 5 fM, about 2 fM, about 1 fM, about 500 aM (attomolar), about 100 aM, about 50 aM, about 10 aM, about 5 aM, about 1 aM, about 0.1 aM, about 500 zM (zeptomolar), about 100 zM, about 50 zM, about 10 zM, about 5 zM, about 1 zM, about 0.1 zM, or less. In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is between about 5000 fM and about 0.1 fM, between about 3000 fM and about 0.1 fM, between about 1000 fM and about 0.1 fM, between about 1000 fM and about 0.1 zM, between about 100 fM and about 1 zM, between about 100 aM and about 0.1 zM. The concentration of analyte molecules or particles in a fluid sample may be considered to be substantially accurately determined if the measured concentration of the analyte molecules or particles in the fluid sample is within about 10% of the actual (e.g., true) concentration of the analyte molecules or particles in the fluid sample. In certain embodiments, the measured concentration of the analyte molecules or particles in the fluid sample may be within about 5%, within about 4%, within about 3%, within about 2%, within about 1%, within about 0.5%, within about 0.4%, within about 0.3%, within about 0.2% or within about 0.1%, of the actual concentration of the analyte molecules or particles in the fluid sample. In some cases, the measure of the concentration determined differs from the true (e.g., actual) concentration by no greater than about 20%, no greater than about 15%, no greater than 10%, no greater than 5%, no greater than 4%, no greater than 3%, no greater than 2%, no greater than 1%, or no greater than 0.5%. The accuracy of the assay method may be determined, in some embodiments, by determining the concentration of analyte molecules in a fluid sample of a known concentration using the selected assay method.

In some embodiments, more than one of the above described types of analysis and quantification methods may be employed with the same system and in a single assay. For example, in embodiments where the analyte molecules are present at lower concentration ranges, single analyte molecules can be detected, and the data may be analyzed using a digital analysis method (binary quantification). In some cases using binary quantification, as previously described, the data may be processed using a Poisson distribution adjustment. At higher concentration ranges (e.g., where it may become challenging or inaccurate to perform binary quantification), the data may be analyzed using an analog analysis method, based, for example on measured relative signal intensities (intensity read-out determination). In certain embodiments, the results of the

two analysis methods (digital and analog) may both be utilized by a single assay system/protocol of the invention by linking the two methods using a single calibration curve. For example, in some embodiments, at low concentration levels (e.g., in the digital/binary concentration range), a measure of the concentration of analyte molecules in a fluid sample may be determined at least in part by counting beads as either "on" (e.g., by determining if the reaction vessel contains a bead associated with an analyte molecule) or "off" (e.g., by determining if the reaction vessel contains a bead not associated with any analyte molecule). At low ratios of analyte molecules to beads (e.g., less than about 1:5, less than about 1:10, less than about 1:20, etc.), substantially all of the beads are associated with either zero or a single analyte molecule. In this range, the percentage of active beads (e.g., "on" reaction vessels) increases linearly with increasing analyte concentration, and a digital analysis method may be most suitable.

As the analyte concentration increases, however, more of the beads will associate with more than one analyte molecule. Therefore, as the analyte concentration increases (but still in the digital range), the percentage of active beads in a population generally will not be linearly related to the bulk analyte concentration as some of the beads may associate with more than one analyte molecule. In these concentration ranges, the data may be advantageously analyzed using a digital analysis method with the above described Poisson distribution adjustment. The above described non-linear effect can be accounted for using Poisson distribution adjustment across substantially the concentration range in which there remains a statistically significant fraction of beads not associated with any analyte molecules or particles in the sample. For example, ranges of percentage of active beads (i.e. "on" beads divided by total beads multiplied by 100%) for which a digital analysis method may be able to accurately determine a measure of the concentration, include up to about 20% active beads, up to about 30% active beads, up to about 35% active beads, up to about 40% active beads, up to about 45% active beads, up to about 50% active beads, up to about 60% active beads, up to about 70% active beads, up to about 80% active beads, or more. In many cases when operating in the ranges above, using a Poisson distribution adjustment will improve accuracy.

Above a certain active bead percentage (i.e., where there is no longer a statistically significant fraction of beads present in the population that are not associated with any of analyte molecules or particles, or, potentially advantageously for situations where there may be a statistically significant fraction of beads present in the population that are not associated with any of analyte molecules or particles but that result in active

bead percentages above a certain level – e.g., greater than or substantially greater than about 40%) (or active location percentage, in embodiments where beads are not employed)) improvements in accuracy and/or reliability in the determination of analyte molecule concentration may potentially be realized by employing an intensity measurement based analog determination and analysis rather than or supplementary to a digital/binary counting/Poisson distribution adjustment as previously described. At higher active bead percentages, the probability of an active bead (e.g., positive reaction vessel) being surrounded by other active beads (e.g., positive reaction vessels) is higher and may in certain assay set ups create certain practical challenges to exclusively using the digital/binary determination method. For example, in certain embodiments, leakage of a detectable component into a reaction vessel from an adjacent reaction vessel may occur to some extent. Use of an analog, intensity level based technique in such situations can potentially yield more favorable performance. In an intensity measurement based analog determination and analysis, the association of multiple analyte molecules at relatively high concentrations with single beads is quantified. The intensity of at least one signal from the plurality of reaction vessels which contain at least one analyte molecule may be determined. In some cases, the intensity is determined as the total overall intensity for the reaction vessels containing at least one analyte molecule (e.g., the intensity of the reaction vessels in determined as a whole). In other cases, the intensity of each reaction vessel with a signal may be determined and averaged, giving rise to an average bead signal (ABS).

According to certain embodiments, an inventive assay system may include a link between the results/parameters of the two analysis methods/systems (i.e. digital and analog), for example, with the aid of a calibration curve, so that the system is able to operate in multiple modes of quantification depending on the signal relating to the number/fraction of "on" beads detected. Such systems can have substantially expanded dynamic ranges in certain cases. Further description of such systems which can combine and use more than one quantification method for a single assay is provided in commonly owned U.S. Patent Application Serial No. 12/731,136, entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles" by Rissin et al., filed March 24, 2010, and International Patent Application No. (not yet determined), entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles" by Rissin et al., filed March 1, 2011 (Attorney Docket No. Q0052.70013WO00), each herein incorporated by reference.

The following examples are included to demonstrate various features of the invention. Those of ordinary skill in the art should, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments which are disclosed while still obtaining a like or similar result without departing from the scope of the invention as defined by the appended claims. Accordingly, the following examples are intended only to illustrate certain features of the present invention, but do not necessarily exemplify the full scope of the invention.

Example 1

This following example describes materials used in Examples 2-19. Optical fiber bundles were purchased from Schott North America (Southbridge, MA). Non-reinforced gloss silicone sheeting was obtained from Specialty Manufacturing (Saginaw, MI). Hydrochloric acid, anhydrous ethanol, and molecular biology grade Tween-20 were purchased from Sigma-Aldrich (Saint Louis, MO). 2.8-um (micrometer)-diameter tosyl-activated magnetic beads were purchased from Invitrogen (Carlsbad, CA). 2.7-um-diameter carboxy-terminated magnetic beads were purchased from Varian, Inc. (Lake Forest, CA). Monoclonal anti-human TNF-α capture antibody, polyclonal anti-human TNF-α detection antibody, and recombinant human TNF-α were purchased from R&D systems (Minneapolis, MN). Monoclonal anti-PSA capture antibody and monoclonal detection antibody were purchased from BiosPacific (Emeryville, CA); the detection antibody was biotinylated using standard methods. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), and SuperBlock® T-20 Blocking Buffer were purchased from Thermo Scientific (Rockford, IL). DNA was purchased from Integrated DNA Technologies (Coralville, IA) and/or purified DNA was ordered from Integrated DNA Technologies (Coralville, IA). Streptavidin-β-galactosidase (SβG) was purchased from Invitrogen or conjugated in house using standard protocols. Resorufin-β-D-galactopyranoside (RGP) was purchased from Invitrogen (Carlsbad, CA). The fiber polisher and polishing consumables were purchased from Allied High Tech Products (Rancho Dominguez, CA).

Example 2

The following describes a non-limiting example of the preparation of 2.8-um-diameter magnetic beads functionalized with protein capture antibody. 600 uL (microliter) of 2.8-um-diameter tosyl-activated magnetic bead stock (1.2×10^9 beads)

was washed three times in 0.1 M sodium borate coating buffer pH 9.5. 1000 ug (microgram) of capture antibody was dissolved in 600 uL of sodium borate coating buffer. 300 uL of 3M ammonium sulfate was added to the antibody solution. The 600 uL of bead solution was pelleted using a magnetic separator and the supernatant was removed. The antibody solution was added to the beads and the solution was allowed to mix at 37°C for 24 hours. After incubation, the supernatant was removed and 1000 uL of PBS buffer containing 0.5% bovine serum and 0.05% Tween-20 was added to the beads. The beads were blocked overnight (~8 hours) at 37°C. The functionalized and blocked beads were washed three times with 1 ml PBS buffer containing 0.1% bovine serum and 0.05% Tween-20. Finally, 1 mL of PBS containing 0.1% bovine serum, 0.05% Tween-20, and 0.02% sodium azide was added to the functionalized and blocked beads. 50 uL aliquots were stored at 4°C for later use.

Example 3

The following describes a non-limiting example of the preparation of 2.7-um-diameter magnetic beads functionalized with protein capture antibody. 500 uL of 2.7-um-diameter carboxy-terminated magnetic beads stock (1.15×10^9 beads) was washed twice in 0.01 M sodium hydroxide, followed by three washes in deionized water. Following the final wash, the bead solution was pelleted and the wash solution was removed. 500 uL of a freshly prepared 50 mg/mL solution of NHS in 25 mM MES, pH 6.0, was added to the bead pellet and mixed. Immediately, a 500 uL of a freshly prepared 50 mg/mL solution of EDC in 25 mM MES, pH 6.0, was added to the bead solution and mixed. The solution was then allowed to mix for 30 min at room temperature. After activation, the beads were washed twice with 25 mM MES at pH 5.0. Meanwhile, 1000 uL of 25 mM MES at pH 5.0 was used to dissolve 1000 ug of capture antibody. The antibody solution was then added to the activated beads and the coupling reaction was allowed to proceed for 3 hours at room temperature. After incubation, the supernatant was removed using the magnetic separator, and 1000 uL of 100 mM Tris-HCl (pH 7.4) was added and allowed to mix at room temperature for one hour to block any remaining reactive sites. Finally, the functionalized beads were stored in 1 mL of SuperBlock blocking buffer, and 0.02% sodium azide was added to the functionalized and blocked beads. 50 uL aliquots were stored at 4°C for later use.

Example 4

The following describes a non-limiting example of the preparation of 2.7-um-diameter magnetic beads functionalized with DNA. 120 μL of 2.7-um-diameter carboxy-terminated magnetic beads was washed three times with 0.01 M NaOH, followed by deionized water for another three times. 500 uL of freshly prepared 50 mg/mL NHS in cold 25 mM MES (pH 6) was added to the pellet of beads after the final wash, and the beads were re-suspended by vortexing briefly. 500 uL of freshly prepared 50 mg/mL EDC solution in cold 25 mM MES (pH 6) was immediately added to this bead suspension and mixed for 30 min. After activation, the beads were washed three times with cold 25 mM MES (pH 5). DNA capture probe with amine modification at 5' end (5'-NH2/C12-GTT GTC AAG ATG CTA CCG TTC AGA G-3' (SEQ ID NO. 1)) was dissolved in nuclease-free water to make a 2.6 mM stock solution. 60 μL of the DNA stock was added to 600 uL of the coupling buffer that contains 0.1 M sodium phosphate and 0.5 M NaCl, pH 8. The resulting DNA solution was added to the washed beads and mixed for 3 hours at room temperature. The bead suspension was vortexed every 30 min during the reaction. After incubation, the DNA supernatant was removed and 1 mL of 100 mM Tris-HCL (pH 7.4) was added to the pellet and mixed for 1 hour to inactivate the remaining binding sites on the beads. Finally, the beads were washed in Tris-EDTA (TE) buffer and 0.05% Tween-20 for three times, and stored in TE buffer containing 0.05% Tween-20 and 0.02% sodium azide at 4 °C.

Example 5

The following describes a non-limiting example of the capture of proteins on magnetic beads and formation of enzyme-labeled immunocomplex. Test solutions containing the protein of interest were incubated with suspensions of magnetic beads functionalized with capture antibody (e.g., see Example 2) for 1 h at 37 °C. The beads were then separated and washed three times in PBS. The beads were resuspended and incubated with solutions containing detection antibodies for 30 min at 37 °C. The beads were then separated and washed three times in PBS. The beads were incubated with solutions containing SβG (e.g., target analyte) for 30 min at 37 °C, separated, and washed six times in PBS and 0.1% Tween-20. The beads were then resuspended in 10 uL of PBS in order to load into the wells of the fiber bundle arrays.

Example 6

The following describes a non-limiting example of the capture of DNA on magnetic beads and formation of enzyme-labeled complex (FIG. 14). Beads functionalized with DNA capture probe (e.g., see Example 4) that is specific to the complementary target DNA of interest were incubated with solutions containing the target DNA (5'-TT GAC GGC GAA GAC CTG GAT GTA TTG CTC C TCT GAA CGG TAG CAT CTT GAC AAC-3' (SEQ ID NO. 2)) (e.g., target analyte) for 2 hrs. After incubation, the DNA target solution was removed and the beads were washed three times in 0.2× SSC buffer containing 0.1% Tween-20. The beads were then resuspended and mixed with 10 nM biotinylated signal probe (5'-TAC ATC CAG GTC TTC GCC GTC AA/Biotin/-3' (SEQ ID NO. 3)) (e.g., first type of binding ligand) that is also specific to the target DNA for 1 hr. The beads were then washed three times in 0.2× SSC buffer containing 0.1% Tween-20 after removing the signal probe. A solution 10 pM containing SβG (e.g., second type of binding ligand comprising an enzymatic component) was then added to the bead pellet, resuspended, and mixed for 1 hr. The beads were then separated and washed six times in 5× PBS buffer containing 0.1% Tween-20. The beads were then resuspended in 10 μL of PBS and loaded onto a femtoliter well array.

Example 7

The following example describes the capture of biotin-labeled DNA on magnetic beads and formation of enzyme-labeled complex, according to a non-limiting embodiment (see FIG. 15). Beads functionalized with DNA capture probe that is specific to DNA of interest were incubated with 1 uM target DNA-biotin (5'-biotin-C TCT GAA CGG TAG CAT CTT GAC AAC-3' (SEQ ID NO. 4)) overnight (16 hrs) in TE buffer containing 0.5M NaCl and 0.01% Tween-20. After incubation, the DNA target solution was removed and the beads were washed three times in PBS buffer containing 0.1% Tween-20. The bead stock was distributed into a microtiter plate giving 400,000 beads per well in 100 uL. The buffer was aspirated from the microtiter plate wells, the beads were resuspended and incubated with various concentrations of SβG in Superblock containing 0.05% Tween-20 for 5 hr. In some cases, the beads were resuspended every 30 min during the incubation. The beads were then separated and washed six times with 5x PBS buffer containing 0.1% Tween-20. Finally, the beads were resuspended in 10 uL of PBS containing 0.1% Tween-20. In some embodiments,

the beads were then separated and washed six times with 5× PBS buffer containing 0.1% Tween-20. For detection of enzyme, the beads were either: a) resuspended in 20 μL of PBS containing 0.1% Tween-20, and 10 μL aliquots were loaded onto two femtoliter well arrays for detection, or; b) resuspended in 100 μL of 100 μM RGP in PBS, incubated for 1 h at room temperature, and read on a fluorescence plate reader (Infinite M200, Tecan).

Example 8

The following describes a non-limiting example of the preparation of microwells arrays. Optical fiber bundles approximately 5-cm long were sequentially polished on a polishing machine using 30-, 9-, and 1-micron-sized diamond lapping films. The polished fiber bundles were chemically etched in a 0.025 M HCl solution for 130 seconds, and then immediately submerged into water to quench the reaction. To remove impurities from etching, the etched fibers were sonicated for 5 s and washed in water for 5 min. The fibers were then dried under vacuum and exposed to air plasma for 5 min to clean and activate the glass surface. The arrays were silanized for 30 minutes in a 2% solution of silane to make the surfaces hydrophobic.

Example 9

The following describes a non-limiting example of the loading of beads into microwells. To apply the solution of beads to the etched wells in a fiber bundle, clear PVC tubing (1/16" I.D. 1/8" O.D.) and clear heat shrink (3/16" ID) were cut into approximately 1 cm long. A piece of PVC tubing was first put onto the etched and functionalized end of a fiber bundle to create a reservoir to hold the bead solution, followed by the application of heat shrink around the interface between the PVC tubing and fiber bundle to provide a tight seal. 10 uL of the concentrated bead solution was pipetted into the reservoir created by the PVC tubing. The fiber bundle was then centrifuged at 3000 rpm (~1333 g) for 10 minutes to force the beads into the etched wells. The PVC tubing/heat shrink assembly was removed after centrifugation. The distal end of the fiber bundle was dipped in PBS solution to wash off excess bead solution, followed by swabbing the surface with deionized water.

Example 10

The following describes a non-limiting example of the detection of beads and enzyme-labeled beads in microwell arrays. A custom-built imaging system containing a mercury light source, filter cubes, objectives, and a CCD camera was used for acquiring fluorescence images. Fiber bundle arrays were mounted on the microscope stage using a custom fixture. A droplet of β-galactosidase substrate (100 μM RPG) was placed on the silicone gasket material, and put into contact with the distal end of the fiber array. The precision mechanical platform moved the silicone sheet into contact with the distal end of the etched optical fiber array, creating an array of isolated femtoliter reaction vessels. Fluorescence images were acquired at 577 nm with an exposure time 1011 ms. Five frames (at 30 seconds per frame) were taken for each fiber bundle array. The fluorescent images were analyzed using image analysis software to determine the presence or absence of enzymatic activity within each well of the microwell array. The data was analyzed using a developed image processing software using MathWorks MATLAB and MathWorks Image Processing toolbox. The software aligned acquired image frames, identified reaction vessel positions, located reaction vessels with beads and measured the change in reaction vessel intensity over a predefined time period. Reaction vessels containing beads with sufficient intensity growth over all data frames were counted and the final number of active reaction vessels wes reported as a percentage of all identified reaction vessels.

As well as fluorescence, the arrays were imaged with white light to identify those wells that contain beads. After acquiring the fluorescence images, the distal (sealed) end of the fiber bundle arrays were illuminated with white light and imaged on the CCD camera. Due to scattering of light by the beads, those wells that contained a bead appeared brighter in the image than wells without beads. Beaded wells were identified using this method by software.

Example 11

The following describes a non-limiting example of the loading of beads into an array of microwells (FIG. 16). Arrays of 50,000 microwells were prepared as described above. 2.8 um beads were prepared as described above. 10-uL solutions containing different numbers of beads (from 80,000 to 2 million beads) were prepared as described above. Beads were loaded into the arrays of microwells as described above. The array loaded with a solution comprising 2 million beads was imaged using scanning electron

microscopy (SEM). SEM showed that >99% of the 50,000 wells contained a bead, and each of these well only contained a single bead. The arrays loaded with 80,000 to 200,000 beads were imaged using white-light microscopy and image analysis was used to identify wells that contained a bead. The number of beads per array was determined over three arrays and plotted as a function of number of beads in solution (FIG. 16B). From FIG. 16B, in this embodiment, the number of beads loaded is a fraction of those provided in solution and not every well contains a bead at these assay-relevant bead loading concentrations. In some cases, the presence of a bead in a well (using white light images) may be correlated to those wells that contain enzymatic activity. In such cases, the read-out may be ratiometric (%active beads) and normalized for variation in bead loading.

Example 12

The following describes a non-limiting example of bead filling as a function of well depth (FIG. 17). In some embodiments, a single bead containing single analyte molecules can be delivered into a microwell so that they can be spatially isolated and sealed. To achieve this situation, the well depth and width may be carefully controlled to parameters optimized for a given bead diameter. FIGS. 17A-17C show SEM images of beads loaded as described above into arrays of microwells where the well depth was controlled by etching for different times. On average, the wells etch at a rate of approximately 1.5 to 1.7 μm per minute. Therefore, wells of 3.25 um depth are produced in about 115 to 130 s. For a well depth of 2.5 um (FIG. 17A), very few beads are retained in the microwells as they are too shallow and detection of single analytes may be poor. At 3-um depth (FIG. 17B), SEM images show good filling of single beads into single wells, and a low occurrence of two beads in one well; this array may seal well and allow large numbers of single beads to be interrogated for the presence of a single analyte. For 3.5-um deep wells (FIG. 17C), many of the wells contain two beads as well as those that contain one. The presence of a second bead above the plane of the array may deteriorate the sealing of the array as described above and may denigrate the quality of single bead isolation. These experiments indicated that an optimal well depth for 2.8-um diameter beads, in this embodiment, is between about 3 and about 3.25 um. While this range is optimal, it is also possible to perform the inventive measurements using well depths of 3.6 um, i.e., the upper limit as indicated by Eq.(4).

Example 13

The following example describes the comparison of a non-limiting method of the present invention versus a conventional plate reader for detecting enzyme (see FIGS. 18A, 18B, and 19). Biotin-DNA beads were prepared as described above. These beads were then incubated with a low concentration of SβG such that beads statistically contained either zero or one enzymes. These beads were loaded into microwells, sealed, and imaged as described above; FIG. 18A and 18B show representative images. In some cases, an increase in sensitivity to enzyme label that comes from isolating single beads is observed as compared to traditional bulk measurements. Beads coated with DNA were prepared, incubated with biotinylated DNA, and then incubated with various concentrations of SβG (from 350 aM to 320 fM) as described above. Enzymes on these beads were then measured in two ways. First, the beads were loaded into microwell arrays, sealed and imaged as described above. The fraction of active wells was determined as described above and is plotted as a function of the concentration of SβG in FIGS. 19A, the lower range expanded in FIG. 19B. Second, the beads were incubated with 100 uL of RPG in a microtiter plate for one hour and read on a fluorescence plate reader. The fluorescence signal as a function of the concentration of SβG is plotted in FIG. 19C. The lower limit of detection (LOD) of the inventive method (defined as the concentration at which the signal rises above three standard deviations over the background) in this experiment was 384 zM. The LOD of the bulk measurement on the plate reader was 14.5 fM. The single molecule array approach of the present invention, therefore, provided an increase of 37,760-fold in sensitivity to enzyme label over the plate reader. It should be noted that at the concentrations tested statistically only zero or single analytes should be detected on beads; for example, the ratio of enzymes to beads at 350 aM was 21,070/400,000 = 0.053.

Example 14

The following example illustrates the precision of detection in a method as described herein, in a non-limiting embodiment. Detection of single molecules may allow for high precision. In theory, the lowest variance in the measurement is the Poisson noise associated with counting small numbers of events. In this non-limiting example, the %Poisson Noise is given by $\sqrt{N}/N$, where N is the number of active (enzyme-associated) beads. FIG. 20 shows a plot of the %Poisson Noise against the experimental variance over three measurements (%CV) from the experimental data in

FIG. 19B. As can be seen, the imprecision of the measurement (%CV) tracks closely with the Poisson Noise, suggesting that the Poisson noise may limit the precision of the methods in some cases.

Example 15

The following non-limiting example describes the detection of PSA in serum (FIG. 21). 2.8-um-diameter beads coated in anti-PSA antibody were prepared as described above. These beads were incubated with 25% bovine serum or 25% bovine serum spiked with 50 fM PSA. The beads were then labeled with anti-PSA detection antibody and three different concentrations of SβG (1, 10, or 100 pM). The beads were then loaded into microwell arrays, sealed and imaged as described above. Image analysis was used to determine the fraction of beads that contained an enzyme. These data show that the invention can be used to detect low concentrations of proteins in serum by performing ELISAs on single beads and detecting single enzyme labels. Because of the high efficiency of the capture of analyte using beads in this invention, the concentration of enzyme label used can be varied to only label a fraction of the analytes captured on the beads in order to optimize the signal-to-background and dynamic range of the measurement. In this data set, 1 pM of enzyme label gave an optimal signal-to-background ratio.

Example 16

The following non-limiting example describes the detection of TNF-α (FIG. 22). 2.8-um-diameter beads coated in anti-TNF-α antibody were prepared as described above. These beads were incubated with 25% bovine serum or 25% bovine serum spiked with 100 fM TNF-α. The beads were then labeled with anti-TNF-α detection antibody (e.g., first binding ligand) and two different concentrations of SβG (1 or 100 pM) (e.g., second binding ligand comprising an enzymatic component). The beads were then loaded into microwell arrays, sealed and imaged as described above. Image analysis was used to determine the fraction of beads that contained an enzyme. These data show that the invention can be used to detect low concentrations of TNF-α in serum by performing ELISAs on single beads and detecting single enzyme labels. As in the previous example, the amount of enzyme label can be varied to ensure that the measurement detects only single enzyme labels on the beads and optimize the signal-to-background ratio. In this

particular example, the backgrounds are very low so the signal-to-background ratio is optimal at an enzyme label concentration of 1 pM and 100,000 beads.

Example 17

The following non-limiting example describes the detection of DNA in buffer (FIG. 23). 2.7-um-diameter beads functionalized with a capture sequence of DNA were prepared as described as above. These beads were then incubated with various concentrations of target DNA and then labeled with a biotinylated signal probe DNA sequence as described above. The beads were then labeled by incubating with various concentrations of SβG (1, 10, or 100 pM). The beads were then loaded into microwell arrays, sealed and imaged as described above. Image analysis was used to determine the fraction of beads contained an enzyme. These data show that the invention can be used to detect low concentrations of DNA by forming sandwich-like complexes on single beads and detecting single enzyme labels. As in the case of protein detection, the amount of enzyme label can be varied to ensure that there are statistically one or zero enzymes per bead even in the case where there are more than one target DNA molecules captured. This allows the dynamic range and signal-to-background of the single molecule measurement to be optimized. In this case, 10 pM of SβG gave the optimal signal-to-background.

Comparative Example 18

The following non-limiting example describes a method using detection comprising chemiluminscence from alkaline phosphatase (FIG. 24). Different concentrations of 10 uL of alkaline phosphatase were mixed with 90 uL of a solution containing the most sensitive chemiluminescent substrate available (APS-5; Lumigen Inc.) in a microtiter plate and incubated for 5 mins. The microtiter plate was then read in chemiluminescence mode of a plate reader. FIG. 24 shows a plot of chemiluminescence as a function of concentration of alkaline phosphatase. The lowest concentration of enzyme that could be detected above background was 100 aM and the calculated limit of detection was 50 aM, close to the reported value of 30 aM. Single molecule detection of β-galactosidase on beads in this invention (LOD = 220 zM) is, therefore, more than 100 times more sensitive than chemiluminscent detection of alkaline phosphatase, the most sensitive enzyme label system that is commercially available.

Example 19

The clinical use of protein biomarkers for the differentiation of healthy and disease states, and for monitoring disease progression, requires the measurement of low concentrations of proteins in complex samples. Certain current immunoassays can measure proteins at concentrations above 10^{-12} M, whereas the concentration of the majority of proteins important in cancer, neurological disorders, and the early stages of infection are thought to circulate in the range from 10^{-16} to 10^{-12} M. For example: a 1 mm^3 tumor composed of a million cells that each secrete 5000 proteins into 5 L of circulating blood translates to ~2×10^{-15} M (or 2 femtomolar, fM); early HIV infection with sera containing 2–3000 virions equates to concentrations of p24 antigen ranging from 60×10^{-18} M (60 attomolar, aM) to 15×10^{-15} M (15 femtomolar). Attempts to develop protein-based detection methods capable of detecting these concentrations have focused on the replication of nucleic acid labels on proteins, or on measuring the bulk, ensemble properties of labeled protein molecules. Sensitive methods for detecting proteins have, however, lagged behind those for nucleic acids, such as the polymerase chain reaction (PCR), limiting the number of proteins in the proteome that have been detected in blood. The isolation and detection of single protein molecules provides the most direct method for measuring extremely low concentrations of proteins, although the sensitive and precise detection of single protein molecules has proven challenging. The following describes a non-limiting exemplary method for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for detecting proteins, namely, the enzyme-linked immunosorbent assay (ELISA). The method can detect proteins in serum at attomolar concentrations and may enable the measurement of a single molecule in blood.

The method makes use of arrays of femtoliter-sized reaction chambers (FIG. 25) that can isolate and detect single enzyme molecules. In the first step, a sandwich antibody complex is formed on microscopic beads, and the bound complexes are labeled with an enzyme reporter molecule, as in a conventional bead-based ELISA. When assaying samples containing extremely low concentrations of protein, the ratio of protein molecules (and the resulting enzyme label complex) to beads is small (typically less than 1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution, leading to single immunocomplexes on individual beads. For example, if 50 aM of a protein in 0.1 mL (3000 molecules) was captured on 200,000 beads, then 1.5% of the beads would have one protein molecule and 98.5% would have

zero protein molecules (FIG. 25B). It is typically not possible to detect these low numbers of proteins using conventional detection technology (e.g., a plate reader), because the fluorophores generated by each enzyme diffuse into a large assay volume (typically 0.1–1 mL), and it takes hundreds of thousands of enzyme labels to generate a fluorescence signal above background (FIG. 15A). The method of this Example enables the detection of very low concentrations of enzyme labels by confining the fluorophores generated by individual enzymes to extremely small volumes (~50 fL), leading to a high local concentration of fluorescent product molecules. To achieve this localization in an immunoassay, in the second step of the method the immunoassay beads are loaded into an array of femtoliter-sized wells (FIG. 25B). The loaded array is then sealed against a rubber gasket in the presence of a droplet of fluorogenic enzyme substrate, isolating each bead in a femtoliter reaction chamber. Beads possessing a single enzyme-labeled immunocomplex generate a locally high concentration of fluorescent product in the 50-fL reaction chambers. By using standard fluorescence imaging on a microscope, it is possible to detect single enzyme molecules, and to image tens to hundreds of thousands of immunocomplexes substantially simultaneously. By isolating the enzymes associated with each immunocomplex, each complex can give rise to a high measurable signal which can result in substantially improved sensitivity over bulk measurements. The protein concentration in the test sample, in some cases, is determined by simply counting the number of wells containing both a bead and fluorescent product relative to the total number of wells containing beads (FIG. 25D). The concentration is then determined digitally rather than by using the total analog signal.

FIG. 25A shows the capturing and labeling single protein molecules on beads using standard ELISA reagents. FIG. 25B shows the loading of beads into femtoliter microwell arrays for isolation and detection of single molecules. FIG. 25C shows an SEM image of a small section of a femtoliter well array after bead loading. 2.7-μm-diameter beads were loaded into an array of wells with diameters of 4.5 μm and depths of 3.25 μm. FIG. 25D shows a fluorescence image of a small section of the femtoliter well array after signals from single molecules are generated. While the majority of femtoliter chambers contain a bead from the assay, only a fraction of those beads possess catalytic enzyme activity, indicative of a single, bound protein. The concentration of protein in bulk solution may be correlated to the percentage or number of beads that have bound a protein molecule. The exemplary assay was capable of providing linearity over ~4.5 logs for 50,000 beads.

FIG. 15 shows the digitization of enzyme-linked complexes can increase sensitivity substantially compared to bulk, ensemble measurements. FIG. 15 shows a log-log plot of signal output (% active beads for assay comprising the use of capture objects; Relative Fluorescence Units (r.f.u.) for plate reader) as a function of the concentration of SβG. SβG concentrations for the ensemble readout ranged from 3 fM to 300 fM, with a detection limit of 15×10^{-15} M (15 fM; line (i)). For the exemplary assay according to the current invention, SβG concentrations ranged from 350 zM to 7 fM, demonstrating a linear response over 4.5 logs, with a detection limit of 220×10^{-21} M (220 zM; line (ii)). Error bars are based on the standard deviation over three replicates for both technologies. LODs were determined from the signal at three standard deviations above background. Table 1 provides information regarding the imprecision of this exemplary assay according to the current invention relating to Poisson noise of counting single events. The intrinsic variation (Poisson noise) of counting single active beads is given by $\sqrt{n}$. Comparing the Poisson noise associated coefficient of variation (%CV) with the %CV for this exemplary assay according to the current invention over three measurements shows that the imprecision of the assay is determined only by counting error. This observation suggests that this assays according to the current invention, in some cases, may have imprecision <20% as long as at least 25 active beads are detected, equating to an enzyme concentration of 4.5 aM.

Table 1.

[SbG] (aM)	Average # single complexes	Average % active	Measurement %CV	Poisson %CV
0	1	0.0016%	87%	122%
0.35	3	0.0086%	75%	55%
0.7	5	0.0099%	63%	46%
3.5	22	0.0413%	10%	21%
7	38	0.0713%	15%	16%
35	237	0.4461%	1%	7%
70	385	0.8183%	5%	5%
350	1787	3.3802%	2%	2%
700	4036	7.5865%	5%	2%
3500	15634	30.6479%	3%	1%
7000	24836	44.5296%	1%	1%

To quantify the potential sensitivity that may be achieved by singulating enzyme-labeled molecules compared to conventional ensemble measurements, a model sandwich assay was developed to capture enzyme molecules on beads; the population of beads were either singulated and read using methods of the current invention, or read as an

ensemble population on a conventional plate reader. Beads were functionalized with DNA capture molecules, and subsequently saturated with biotinylated complementary DNA target molecules in a one-step hybridization (see Methods section below). These beads were used to capture various concentrations of an enzyme conjugate, streptavidin-β-galactosidase (SβG), commonly used as a label in ELISA. While the exemplary assay according to the current invention and conventional assay incubations were performed under the substantially similar conditions, in these example the assays diverged at the bead readout step. For the comparative conventional assay, beads were read out in 100 uL on a fluorescence plate reader after 1 h incubation with 100 μM resorufin-β-D-galactopyranoside (RGP), a fluorogenic substrate for β-galactosidase. The detection limit of the capture assay on the microtiter plate reader was 15 fM of SβG (FIG. 15). For single molecule detection according to the present invention, the beads were loaded into the femtoliter arrays and, after sealing a solution of RGP into the wells of the array, signal generated from single enzymes accumulated in the reaction chambers for 2.5 min, with fluorescent images acquired every 30 s. A white light image of the array was acquired at the end of the experiment. These images were analyzed to identify wells that contained beads (from the white light image) and determine which of those beads had an associated enzyme molecule bound (from time-lapsed fluorescent images). FIG. 15 shows a log-log plot of the percentage of beads that contained an enzyme as a function of bulk SβG concentration. The limit of detection (LOD) for the assay according to the invention was 220 zeptomolar (13 molecules in 100 μL, or 22 yoctomoles), corresponding to an improvement in sensitivity over the plate reader of a factor of 68,000, showing that singulation can result in a dramatic increase in sensitivity over conventional ensemble measurements. The concentration of SβG detected using the assay of the present invention was a factor of 140 lower than chemiluminescence detection of alkaline phosphatase (30 aM), the current most sensitive ELISA system. The high thermodynamic and kinetic efficiency that may be achieved for the present process (Table 2) can enable the detection of very low numbers of enzyme labels and indicates that the measurement of a single labeled molecule from blood is a possibility.

Table 2. Calculations of the capture efficiency of enzyme label from 0.35 aM to 7000 aM for the assay performed according to the invention (FIG. 15).

	Column A	Column B	Column C	Column D	Column E	Column F
[SβG] (aM)	Average % active beads observed (Figure 2)	Enzyme/bead ratio from Poisson distribution	Total # of enzymes on 400,000 beads	Background corrected # of enzymes on beads	Calculated # of enzymes in 100-µL sample	Capture Efficiency
0	0.0016%	0.000016	7	---	---	---
0.35	0.0086%	0.000086	34	28	21	132%
0.7	0.0099%	0.000099	40	33	42	79%
3.5	0.0413%	0.000413	165	159	211	75%
7	0.0713%	0.000713	285	279	421	66%
35	0.4461%	0.004471	1789	1782	2107	85%
70	0.8183%	0.008217	3287	3280	4214	78%
350	3.3802%	0.034387	13755	13748	21070	65%
700	7.5865%	0.078897	31559	31552	42140	75%
3500	30.6479%	0.365974	146390	146383	210700	69%
7000	44.5296%	0.589320	235728	235722	421400	56%

In Table 2, for the experiments, on average 50,000 beads (~12.5%) were interrogated out of the 400,000 incubated with the solutions of enzyme. The low fraction of beads detected was limited by the number of reaction vessels used in this example (50,000 wells). By accounting for bead loss, the number of active beads observed experimentally (Column A) can be used to estimate the total number of active beads out of the 400,000 used (Column B). After background subtraction (Column C) and applying a Poisson distribution adjustment based on the distribution of 0, 1, 2, 3, 4, etc., enzyme molecules per bead, the total number of molecules captured on beads can be determined (Column D). The ratio of this number to the total number of enzymes in 100 uL at the start of the experiment (Column E; volume × concentration × Avogadro's number) yields a capture efficiency (Column F). The overall efficiency of capture and detection of enzyme using the present assay is high (65-85%) and in some embodiments, may be minimally limited in this experiment by the slow diffusion of the large SβG conjugate (MW ~ 515 kDa).

A sandwich-based assay was developed for two clinically-relevant protein biomarkers, namely prostate specific antigen (PSA) and tumor necrosis factor-α (TNF-α). These assays show that the high enzyme label sensitivity of the assay of this Example translates to highly sensitive (<1 fM) assays suitable for detecting proteins in blood. An assay for DNA was also developed to show that the assay of this Example can be used to directly detect single nucleic acid molecules without requiring replication of the target. All assays were performed as outlined in FIG. 25A and FIG. 25B, apart from the DNA assay where a capture sequence and a biotinylated signal sequence were used in place of capture and detection antibodies, respectively. FIGS. 14A-14C show data from

the assays for A) PSA, B) TNF-α, and C) DNA. The human forms of the proteins were spiked into 25% bovine serum to be representative of clinical test samples; a four-fold dilution factor was used which may reduce matrix effects in immunoassays. DNA was detected in buffer to be representative of purified nucleic acid preparation techniques. Using digital detection to detect PSA in 25% serum, an LOD of 46 aM (1.5 fg/mL) was attained, equating to 184 aM in whole serum. A leading commercial PSA assay (ADVIA Centaur, Siemens) reports an LOD of 3 pM (0.1 ng/mL) in human serum, and ultra-sensitive assays have been reported with LODs in the range of 10–30 fM. The single molecule assay of the present Example was, therefore, more sensitive than the commercial assay by a factor of 15,000, and more sensitive than other ultra-sensitive methods by a factor of at least 50. The detection limit in the TNF-α determination was 148 aM (2.5 fg/mL), corresponding to 590 aM in whole serum. The highest sensitivity commercially-available ELISA for TNF-α has an LOD of 21 fM (0.34 pg/mL) in serum (Table 1). The assay of the present Example, therefore, imparted an improvement over the most sensitive TNF-α assay of a factor of 35. The LOD of the digital DNA sandwich assay was 135 aM, corresponding to about 8000 copies. The ability of certain assays of the present invention to potentially measure much lower concentrations of proteins compared to conventional techniques arises from the extremely low background signals that may be achieved by digitizing the detection of proteins.

FIGS. 14A-14C shows the attomolar detection of proteins in serum and DNA in buffer using digital detection. Plots of % active beads against analyte concentration for: (FIG. 14A) human PSA spiked into 25% serum, (FIG. 14B) human TNF-α spiked into 25% serum, and (FIG. 14C) DNA in buffer. The bottom row of plots shows the low-end of the concentration range. Assays were performed by sequentially incubating specific capture beads with target solution, biotinylated detector, and SβG conjugate. After completion of the assay, beads were loaded into femtoliter well arrays and interrogated for the presence of single immunocomplexes.

Background in the inventive digital detection immunoassays may arise, at least in part from non-specific binding (NSB) of detection antibody and enzyme conjugate to the capture bead surface (Table 3). Because the inventive assays can provide improved label sensitivity over conventional assays (FIG. 15), significantly less detection antibody (~1 nM) and enzyme conjugate (1–50 pM) can be used to detect binding events compared to conventional assays (labeling reagent concentrations ~10 nM). The decreased label concentration may result in substantially reduced NSB to the capture

surface, resulting in much lower background signals and lower LODs. For example, in the TNF-α and PSA determinations as described above, the NSB levels were equivalent to the signal produced by 1.8 fM and 1.2 fM of target protein, respectively. The highest sensitivity commercial TNF-α assay has an NSB level equivalent to 85 fM of TNF-α (FIG. 26), a factor of 50 higher. The ability to reduce backgrounds in certain assays of the present invention by lowering the concentration of labeling reagents can translate to immunoassays with improved sensitivity over conventional assays.

Table 3. NSB dropout data.

NSB Dropout Experiment (SiMoA PSA assay)					
	Average	SD	CV	% NSB	
No dAb; No PSA	0.110%	0.162%	147%	from SbG	20%
No SbG; No PSA	0.000%	0.000%	---	from dAb	80%
NSB	0.541%	0.194%	36%	Total NSB	100%

In Table 3, a dropout experiment isolating the sources of NSB in the PSA digital assay. By comparing 'no detection antibody NSB' (No dAb) and 'no SβG NSB' (No SβG) to total NSB (NSB), the contribution of detection antibody and SβG to the NSB of the assay was determined.

To demonstrate the unique diagnostic measurements that could be afforded by detecting single molecules of a protein in human clinical samples, PSA was measured in serum samples from patients who had undergone radical prostatectomy (RP). PSA is a serum biomarker for prostate cancer used as both a screening tool and to monitor for the biochemical recurrence of patients who have undergone radical prostatectomy. After radical prostatectomy, the vast majority of PSA is eliminated, and levels fall below the detection limit of standard commercial assays (3 pM or 0.1 ng/mL). Regular monitoring of these patients for return of PSA can detect recurrence of prostate cancer, but several years may pass post-surgery for biochemical recurrence to be detected by current immunoanalyzers. The ability to accurately quantify PSA levels in post-prostatectomy patients at very low concentrations (<3 fM or 100 fg/mL) may provide early indication of recurrence should PSA levels increase. FIG. 27 shows PSA levels measured using the assay of this Example in the serum of thirty patients (age 60–89) who had undergone radical prostatectomy and whose blood was collected at least 6 weeks post-surgery. The PSA levels in the sera of all 30 patients were below the detection limit of commercial assays. Here, whole serum samples were diluted 1:4 in buffer and measured using the

PSA digital assay of this Example (FIG. 14A). PSA was successfully detected in all 30 patients using the present assay. Nine of the thirty samples fell below the LOD of the previously highest sensitivity PSA assay. These data demonstrate a potential clinical utility of certain inventive assays for measuring proteins in serum at concentrations well below the capability of current technology. Table 4 summarizes the patient results.

FIG. 27 shows digital detection of PSA in serum samples of patients who had undergone radical prostatectomy. The concentrations of PSA were determined using the assay of the present Example in serum samples from RP patients (circles (iv)), healthy control samples (circles (ii)), and Bio-Rad PSA control samples (circles (i)). RP patient samples were obtained from SeraCare Life Sciences (Milford, MA) and all had undetectable PSA levels as measured by a leading clinical diagnostic assay (ADVIA Centaur); line (iv) represents the detection limit of the ADVIA Centaur PSA assay (100 pg/mL or 3 pM). All 30 patient samples were above the detection limit of the inventive PSA digital assay, shown by the line (v) (0.00584 pg/mL or 184 aM), with the lowest patient PSA concentrations measured at 0.014 pg/mL (420 aM) using the present assay. Patient samples with the lowest PSA levels were detectable, but approached the LOD of the assay resulting in a high dose %CV. The present assay was validated for specificity to PSA using control standards (Bio-Rad) and serum from healthy individuals (ProMedDx) that had been assayed using the ADVIA Centaur PSA assay (See Table 5).

Table 4. Summary of Patient Results

Patient ID	[PSA] (pg/mL)	Dose %CV	Patient ID	[PSA] (pg/mL)	Dose %CV	Patient ID	[PSA] (pg/mL)	Dose %CV
S600	9.39	6%	**S590**	0.22	22%	**S580**	0.30	4%
S599	0.75	10%	**S589**	0.85	17%	**S579**	1.22	15%
S598	2.71	12%	**S588**	2.33	3%	**S578**	0.090	91%
S597	1.79	12%	**S587**	1.06	13%	**S577**	1.92	6%
S596	2.46	17%	**S586**	1.29	22%	**S576**	0.014	286%
S595	0.32	21%	**S585**	0.49	84%	**S575**	0.79	63%
S594	1.63	15%	**S584**	0.056	136%	**S574**	1.62	20%
S593	1.15	12%	**S583**	1.33	26%	**S573**	0.22	32%
S592	3.46	9%	**S582**	4.76	9%	**S572**	1.04	20%
S591	0.21	25%	**S581**	1.57	31%	**S571**	0.24	21%

Table 5.

	Centaur (ng/mL)	Assay Method of the present invention (ng/mL)
Bio-Rad Control 1	0.838	1.06 ± 0.21
Bio-Rad Control 2	2.47	2.66 ± 0.36
Normals		
ProMedDx S376	2.1	1.60
ProMedDx S378	2.3	1.70
ProMedDx S381	2.9	2.14
ProMedDx S388	4.1	3.95
ProMedDx S395	0.93	0.63
ProMedDx S396	0.9	0.77
ProMedDx S397	1.2	0.66

Table 5 shows the specificity of the present assay was confirmed using PSA samples from Bio-Rad (controls) and ProMedDx (serum from healthy individuals) that had previously been tested on a commercial immunoanalyzer (ADVIA Centaur, Siemens). The PSA concentrations of the healthy serum samples determined using the exemplary method were (24±12)% lower than those originally determined on the ADVIA Centaur. There are two possible explanations for this systematic bias between the two technologies. First, the ADVIA Centaur values were obtained on fresh serum, whereas values for the present assay were obtained after the sera had been frozen for extended periods of time and had experienced a freeze-thaw cycle. Second, there may be differences between the PSA calibrators used to generate the calibration curves that would result in differences in PSA concentrations determined. Complex PSA from the World Health Organization (WHO) was used as calibrators; the ADVIA Centaur calibration PSA is unknown.

By isolating and detecting single immunocomplexes formed in ELISA, certain assays of the invention can impart sensitivity, precision, and dynamic range improvements over standard readout methods and other known ultra-sensitive approaches. While certain of the inventive assays can provide sensitivity below the detectable limit of standard and ultra-sensitive readout formats in serum, potentially two more logs of sensitivity may be available based on the enzyme label LOD (FIG. 15). The ability to isolate and interrogate single molecules on individual beads according to certain embodiments of the invention may facilitate distinguishing true antibody-antigen binding events from non-specifically bound complexes. Identifying and differentiating these two populations may permit the detection of a single biomarker molecule in a human serum sample.

Capture of proteins on magnetic beads and formation of enzyme-labeled immunocomplex (FIG. 14 and 27). Beads functionalized with an antibody to the target protein were prepared according to the manufacturer's instructions. Test solutions containing the protein of interest were incubated with suspensions of 200,000 magnetic beads for 2 h at room temperature. The beads were then separated and washed three times in PBS and 0.1% Tween-20. The beads were resuspended and incubated with solutions containing detection antibody (typically, 1–3 nM) for 45 min at room temperature. The beads were then separated and washed three times in PBS and 0.1% Tween-20. The beads were incubated with solutions containing SβG (1–50 pM) for 30 min at room temperature, separated, and washed six times in PBS and 0.1% Tween-20. The beads were then resuspended in 10 μL of PBS and loaded onto a femtoliter well array.

Example 20

FIG. 28 shows a histogram of the average fluorescence intensity of reaction vessels in an experiment of the present invention. A representative set of images from the experiment were analyzed to determine populations of reaction vessels that: (i) contained no beads; (ii) contained a bead (from white light scatter) but no enzyme (no increase in fluorescence intensity); and, (iii) contained a bead (from white light scatter) and an enzyme (increasing fluorescence intensity over four consecutive images, and an overall increase in fluorescence of at least 20%). Specifically, line (i) represents data from empty reaction vessels, line (ii) represents reaction vessels with a bead but no enzyme ("off" beads), and line (iii) represents reaction vessels with both a bead and enzymatic activity ("on" beads).

Example 21

The following example describes determination of assay locations comprising a capture object using capture objects (e.g., beads) which are fluorescent.

In some cases, it may be advantageous to determine the presence or absence of beads in a location (e.g., well in this example) using methods other than dark field detection methods. In some cases, dark field detection methods may not be ideal because 1) the scattering light source may send a non-uniform field of light to the beaded array surface and thus, the intensity distribution of scattered light produced by wells comprising beads may be broad and beaded wells that are in the extreme high or extreme

low intensity region of the distribution may not be accurately determined to contain a bead.; 2) small differences in scatter from beaded and empty wells may result in detection algorithms based on local, well-to-well differences in scattering lacking desired accuracy at high bead occupancy because the well-to-well differences are to small to be consistently detected; and 3) the use of dark field scatter to detect beads may eliminate using different bead types on the same array for multiplexing purposes

Microspheres of many different colors and fluorescent emission wavelengths are commercially available and some were employed in an assay as described herein. Emission wavelengths were chosen so there was no spectral overlap with readout wavelengths for detecting resorufin dye produced by single enzyme molecule catalysis (570/590 excitation/emission) which were used in this example as the detected entity,. In experiments investigating commercially available fluorescent beads (e.g., FITC labeled and Qdot 525 labeled beads), the amount of fluorescent dye loaded into the beads, in some cases, was so high that the bead fluorescence bled into the resorufin channel (570/590). It is important in some embodiments to have low background fluorescence over the wavelengths of detection of the analyte molecules because the amount of fluorescence product in the wells (e.g., from the analyte molecules) may be relatively small.

Accordingly, in this example, beads were loaded (e.g., as described below) with varying quantities of dyes so that the beads could be easily counted by the imaging system, while keeping the amount of dye low enough so it did not interfere with the current background level of the system. In this example, each bead surface comprised about 100,000 antibody molecules (e.g., capture components), and only a very small fraction of these antibodies are then used to associate an analyte molecule or particle. For example, if about 10 analyte molecules associate with a bead on average, only 10 capture antibody molecules (0.01 % of capture components) have bound a target molecule which are then detected by the system. Due to the large fraction of unused antibodies available on the bead surface, the dye labeling approach in this example employed covalent attachment of dye molecules directly to the antibodies on the bead surface.

Alexa Fluor® 488 sulfodichlorophenol ester (AF-488 SDP ester) is an amine reactive dye molecule purchased from Invitrogen that was used in this example. Antibody coated beads were incubated with various molar ratios of the AF-488 SDP ester. Antibody:AF-488 SDP ester molar ratios from 1:27 to 1:800 were tested, all of

which gave bright signal in the Alexa 488 channel. FIG. 29A shows the antibody beads modified at a 1:27 ratio. The beads were seen in the Alexa 488 channel, while the background resorufin channel fluorescence of the AF-488 modified beads appears to be comparable to the standard unmodified antibody beads. Specifically, FIG. 29A shows an image of a portion of the standard array using the resorufin channel; FIG. 29B an image of a portion of the Alexa 488 modified 1:27 molar ratio beads using the resorufin channel; and FIG. 29C an image of a portion of the Alexa 488 modified 1:27 molar ratio beads using the Alexa 488 channel.

Table 6 shows the relative intensities of both the Alexa 488 modified beads and the standard unmodified beads in both fluorescent channels. The standard beads give a similar background fluorescence in both channels, and the Alexa 488 beads give a bright signal in the Alexa channel (~400 counts) while maintaining a resorufin background signal that is comparable to the standard beads.

Table. 6

Upright Imager Results			
standard beads			
	average	stdev	%cv
Alexa intensity beaded	98.37	8.13	8%
Alexa intensity empty	52.14	8.22	16%
Net signal	46.23		
	average	stdev	%cv
Resorufin intensity beaded	134.16	16.28	12%
Resorufin intensity empty	97.09	3.86	4%
Net signal	37.08		
Alexa488 labeled beads			
	average	stdev	%cv
Alexa intensity beaded	469.85	89.04	19%
Alexa intensity empty	64.53	1.44	2%
Net signal	405.32		
Resorufin intensity beaded	114.93	5.45	5%
Resorufin intensity empty	85.06	3.79	4%
Net signal	29.86		

By comparing the intensity of the modified beads to an Alexa-488 calibration curve, each bead was calculated to have approximately 5,000 Alexa-488 dye molecules

bound. With such a low number of dye molecules on the bead surface, experiments were performed to assess if the Alexa-488 labeling procedure negatively impacted the antibody beads in a protein detection assay. The antibody beads that were labeled with Alexa-488 dye specifically bind Tau protein, for a protein relevant to Alzheimer's disease. Control beads and Alexa-488 beads with Tau specific capture antibody were compared in an assay similar to those described in Examples 3, 5, 8, 9, and 10 above but with anti-Tau antibodies The results shown in FIG. 29D suggest that the Alexa-488 conjugation has little or no negative impact on bead performance in the assay. Specifically, FIG. 29D shows results comparing signal from control beads and Alexa-488 labeled beads for the Tau assay.

While the initial assay results show that the Alexa-488 conjugation has little or no negative impact on the performance of the antibody beads used in the assay, it was also determined whether the Alexa-488 labeling resulted in a bead counting advantage over unlabeled assay beads. The results of this determination are summarized in Table 7. The results were determined using two modes of analysis of a single strip using Alexa-488 conjugated beads in the Tau assay. This strip was analyzed using the standard dark field method, and also the Alexa-488 detection method. Results comparing the dark field method to the fluorescence detection method for counting beads reveal an improvement in the Alexa-488 detection method over the dark field method. Across the arrays, anywhere from 1300 to 6500 beads were not counted using dark field imaging.

Table 7.

Strip 242, beads counted using dark field imaging					
Conc.	T. Pos. PreMorph	Beads	% Active		
0.00 pg/mL	586	25806	2.271		
0.00 pg/mL	532	28584	1.861		
0.00 pg/mL	685	31899	2.147		
6.25 pg/mL	14969	28625	52.293		
6.25 pg/mL	15988	26330	60.722		
6.25 pg/mL	17456	30850	56.583		
25.00 pg/mL	32482	32741	99.209		
25.00 pg/mL	28028	28155	99.549		
Strip 242, beads counted using Alexa channel					
Conc.	T. Pos. PreMorph	Beads	% Active	bead increase	% increase
0.00 pg/mL	735	31890	2.305	6084	23.58%
0.00 pg/mL	553	29954	1.846	1370	4.79%
0.00 pg/mL	756	35299	2.142	3400	10.66%
6.25 pg/mL	17277	33045	52.283	4420	15.44%
6.25 pg/mL	19735	32528	60.671	6198	23.54%
6.25 pg/mL	21086	37385	56.402	6535	21.18%
25.00 pg/mL	36222	36497	99.247	3756	11.47%
25.00 pg/mL	33144	33245	99.696	5090	18.08%

Example 22

In the certain embodiments of assay methods of the present invention, there may be a variation in the elapsed time between addition of detector antibody and interrogation of certain arrays or regions of arrays versus interrogation of other arrays or regions. In certain cases, dissociation that may occur over such time intervals may result in inaccuracies in later made versus earlier made determinations of analyte molecule concentrations. This example investigated the effect of measures to reduce dissociation on the time variant accuracy of assay determinations. In this example, the assay comprised the following steps:

1. Immunocomplexes of enzyme-labeled PSA molecules (capture anti-PSA, PSA in serum, biotinylated detection anti-PSA, and SβG) were formed on magnetic beads and washed using methods similar to those described in Examples 3 and 5. Magnetic beads presenting immunocomplexes were exchanged into buffer containing a certain carbohydrate (e.g., sucrose, tehalose, etc.), or any other molecular species that can help maintain hydration state of proteins.
2. After loading beads into the microwells of fiber bundle arrays using methods similar to those described in Example 9, excess of buffer was removed by air/vacuum drying.
3. The fiber bundle with beads loaded were stored for various periods of time.
4. Right before signal generating/array read out, the dried fiber bundles were dipped in PBS buffer, wiped with a swab, dipped in PBS buffer again, and then dipped in a solution containing 100 μM RGP. The arrays were then sealed and read out using methods similar to those described above in Example 10.

Dissociation of the biotinylated detection antibody from the captured analyte molecule, in particular, can cause a continual signal drop over time. In certain cases, dissociation can account for more than a 50% signal drop over the course of interrogation of an array or series of arrays during conduct of an assay (e.g., see FIG. 30A). FIG. 30A shows data from the similar experiments carried out over four different days. In these experiments, immunocomplexes of enzyme-labeled PSA were prepared simultaneously on beads in 64 wells of a 96-well plate that were arranged in 8-well columns. The beads from each column were then tested on a strip of 8 arrays using single molecule detection (Example 10) at increasing times (shown on the x-axis). Each data point is the average concentration of PSA determined from a column of 8 wells by comparison to a calibration curve that was obtained before the first column was tested. .

This signal drop over time (which is manifested as a drop in the concentration of PSA that is determined) may be more pronounced in assays using proteins as reagents/targets than in assays involving nucleic acids (e.g. DNA). The decrease of signal fits to a dissociation model in certain cases.

By carrying out drying of the wells in the present Example, the dissociation effects was eliminated and/or reduced by up to 90% (e.g., see FIG. 30B).

Those of ordinary skill in the art will be aware that while traditional immunoassays (e.g., ELISA) can suffer from signal loss due to dissociation of detection entities, in general, they do not require special treatment for the dissociation of immunocomplex because the plates are read out very quickly. For example, within a minute or so, a microplate reader can generate signal for a full 96-well plate. The difference in dissociation between well 1 and well 96 within this time frame is negligible. However, for assays like the methods described herein wherein interrogation of the assay sites may in certain cases take a longer time period, dissociation may affect the precision and/or accuracy of the methods. Accordingly, the process of drying the assay sites can prevent loss of an analyte molecule and/or binding ligand and reduce effects of dissociation.

In some embodiments, additional components may be provided which maintain the native configuration and/or biological activity of analyte molecules (e.g., proteins) in the microwells and which can protect the immunocomplexes and/or labeling agent(s) (e.g. enzyme(s)) during the drying/rehydration process. For example, routine screening tests may be used to identify agents that can keep an antibody/antigen/enzyme complex intact and/or maintain enzyme activity after drying/rehydration. Without such agents, drying may denature and/or deactivate enzymes or other components of the assay. For example, screening of known nonionic kosmotropes revealed that carbohydrates help keep 90-100% of the immunocomplex signal after drying/rehydration treatment. Carbohydrates and other molecules may be used to maintain antibody active during dehydration in protein array assay and/or may be used to keep protein activity during dehydration/lyophilization.

Example 23

The following example describes the crosslinking of a binding ligand to an analyte molecule to aid in the prevention of disassociation of the binding ligand from the analyte molecule. In this assay, enzyme labeled PSA immunocomplexes were formed on

beads using methods similar to those described in Example 3, 5, and 22, using concentrations of PSA of 0, 0.1, and 1 pg/mL spiked into serum. One set of beads were reacted with 10 mg/mL of EDC for 10 minutes to cross-link the detection antibody to other proteins associated with the beads, e.g., PSA, capture antibody and blocking proteins. Control beads were not reacted with EDC. Control bead were then read using methods similar to those described above in Example 10 at t = 0 and then 2 hours later. The cross-linked beads were also read using methods similar to as described in Example 10 at about 2 hours after crosslinking The result showed that after 2 hours the average number of enzymes per bead on the control beads was reduced because of dissociation of the detection antibody. The crosslinked beads, however, maintained the high AEB even after 2 hours. These data suggest that crosslinking the detection antibody to proteins irreversibly associated with the beads substantially eliminated the dissociation effects. In addition, crosslinking boosted the signal, also as shown in FIG. 31 (EDAC-treated; (ii)) relative to the 0 hour wait (i) presumably because of the extra dissociation time the control beads experienced before being read.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or

methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively.

What is claimed:

Claims

1. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at least one type of analyte molecules or particles;

immobilizing analyte molecules or particles with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one analyte molecule or particle and a statistically significant fraction of the capture objects do not associate with any analyte molecule or particle;

spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of separate locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing an analyte molecule or particle; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations determined to contain an analyte molecule or particle.

2. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at least one type of analyte molecules or particles to form capture objects comprising at least one immobilized analyte molecule or particle;

mixing the capture objects prepared in the exposing step to a plurality of binding ligands such that at least some of the capture objects associate with a single binding ligand and a statistically significant fraction of the capture objects do not associate with any binding ligand;

spatially segregating at least a portion of the capture objects subjected to the mixing step into a plurality of locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of locations containing a binding ligand; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations determined to contain a binding ligand.

3. The method of claim 1 or 2, wherein the percentage of capture objects which associate with at least one analyte molecule and/or binding ligand is less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%, or less than about 5%, or less than about 1%, or less than about 0.5%, or less than about 0.1% or the total number of capture objects.

4. The method of claim 1 or 2, wherein the percentage of capture objects which do not associated with any analyte molecules and/or binding ligands is at least about 20%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 99%, or at least about 99.5%, or at least about 99.9%, of the total number of capture objects.

5. The method of claim 1 or 2, wherein in the addressing step, the number of said locations containing a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle not containing an analyte molecule or particle or a binding ligand is determined.

6. The method of claim 5, wherein the measure of the concentration of analyte molecule or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle containing an analyte molecule or particle or a binding ligand, to the total number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle.

7. The method of claim 5, wherein the measure of the concentration of analyte molecule or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle containing an analyte molecule or particle or a binding ligand, to the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle but not to contain any capture objects that include a binding surface having affinity for at least one type of analyte molecule or particle containing an analyte molecule or particle or a binding ligand.

8. The method of claim 1 or 2, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle containing an analyte molecule or particle or binding ligand, to the number of locations addressed in the addressing step that do not contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle.

9. The method of claim 1 or 2, wherein the plurality of capture objects that include a binding surface having affinity for at least one type of analyte molecule or particle comprises a plurality of beads.

10. The method of claim 1 or 2, wherein the plurality of locations comprises a plurality of reaction vessels.

11. The method of claim 10, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

12. The method of claim 1, wherein at least a portion of the analyte molecules or particles are associated with at least one binding ligand.

13. The method of claim 1, wherein the analyte molecules or particles comprise an enzymatic component.

14. The method of claim 2 or 12, wherein the binding ligand comprises an enzymatic component.

15 The method of claim 1 or 2, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15} M, or less than about 40×10^{-15} M, or less than about 30×10^{-15} M, or less than about 20×10^{-15} M, or less than about 10 $\times 10^{-15}$ M, or less than about 5×10^{-15} M, or less than about 1×10^{-15} M.

16. The method of claim 1 or 2, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard.

17. The method of claim 1, wherein during the immobilizing step, at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95% of the analyte molecules or particles are immobilized with respect to a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle.

18. The method of claim 2, wherein during the mixing step, at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the immobilized analyte molecules or particles associate with a binding ligand.

19. The method of claim 1 or 2, wherein during the spatially segregating step, at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the capture objects subjected to the immobilizing steps are spatially separated into the plurality of locations.

20. The method of claim 10, wherein the number of reaction vessels addressed in the addressing step is at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the total number of reaction vessels.

21. The method of claim 1 or 2, wherein the portion of the capture objects that include a binding surface having affinity for at least one type of analyte molecule or particle are spatially separated by exposing the plurality of locations to a solution comprising the plurality of capture objects.

22. The method of claim 2 or claim 12, wherein following the spatially segregating step, the locations are exposed to precursor labeling agent.

23. The method of claim 22, wherein the precursor labeling agent is converted to a labeling agent upon exposure to a binding ligand.

24. The method of claim 23, wherein the number of locations containing a capture object containing an analyte molecule or particle or a labeling agent is determined by determining the number of locations comprising a labeling agent.

25. The method of claim 23, wherein the labeling agent is a chromogenic, fluorescent, or chemiluminescent.

26. The method of claim 1 or 2, wherein the analyte molecules or particles are proteins.

27. The method of claim 1 or 2, wherein the analyte molecules or particles are nucleic acids.

28. The method of claim 10, wherein the plurality of reaction vessels are formed upon the mating of at least a portion of a sealing component and at least a portion of a substrate.

29. The method of claim 10, wherein the plurality of reaction vessels are formed in a planar substrate.

30. The method of claim 10, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters.

31. The method of claim 10, wherein the average volume of the plurality of reaction vessels is between about 1 femtoliter and about 1 picoliter.

32. The method of claim 9, wherein the average diameter of the plurality of beads is between about 0.1 micrometer and about 100 micrometers.

33. The method of claim 9, wherein the average diameter of the plurality of beads is between about 1 micrometer and about 10 micrometers.

34. The method of claim 1 or 2, further comprising performing at least one wash step.

35. The method of claim 10, further comprising sealing the plurality of reaction vessels.

36. The method of claim 1 or 2, wherein the plurality of locations is addressed using optical techniques.

37. The method of claim 1 or 2, wherein the binding surface comprises a plurality of capture components.

38. The method of claim 1, wherein the percentage of locations addressed in the addressing step determined to contain an analyte molecule or particle is less than about 10%, or less than about 5%, or less than about 1%, or less than about 0.1% of the total number of locations addressed.

39. The method of claim 2, wherein the percentage of locations addressed in the addressing step determined to contain a binding ligand is less than about 10%, or less

than about 5%, or less than about 1%, or less than about 0.1% of the total number of locations addressed.

40. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

providing a substrate comprising a plurality of locations, at least a portion of which locations contain a bead, wherein with respect to the total number of beads present on the substrate, the ratio of beads comprising at least one analyte molecule or particle to beads comprising no analyte molecules or particles is between about 8:1 and about 1:10,000,000;

addressing at least a portion of the plurality of locations, wherein during the addressing step at least two of the plurality of locations is addressed at least partially concurrently;

detecting at each addressed location the presence or absence of a bead and whether, if present, the bead comprises any analyte molecules or particles; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample at least in part by determining the number of locations addressed containing a bead comprising at least one analyte molecule or particle.

41. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

providing a substrate comprising a plurality of locations, at least a portion of which contain a bead, wherein with respect to the total number of beads present on the substrate, the ratio of beads comprising at least one analyte molecule or particle associated with a binding ligand to beads comprising no analyte molecules or particles associated with a binding ligand is between about 8:1 and about 1:10,000,000;

addressing at least a portion of the plurality of locations, wherein during the addressing step at least two of the plurality of locations is addressed at least partially concurrently;

detecting at each addressed location the presence or absence of a bead and whether, if present, the bead comprises any analyte molecules or particles associated with a binding ligand; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample at least in part by determining the number of locations addressed

containing a bead comprising at least one analyte molecule or particle associated with a binding ligand.

42. The method of claim 40 or 41, wherein the ratio is between about 2:1 and about 1:1,000,000.

43. The method of claim 40 or 41, wherein the ratio is between about 1:10 and about 1:100,000.

44. The method of claim 40 or 41, wherein the ratio is between about 2:1 and about 1:10.

45. The method of claim 40 or 41, wherein in the addressing step, the number of said locations containing a bead but not containing any beads comprising an analyte molecule or particle or a binding ligand is determined.

46. The method of claim 40 or 41, wherein in the addressing step, the number of said locations containing a bead is determined.

47. The method of claim 46, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a bead comprising an analyte molecule or particle or binding ligand, to the number of locations addressed in the addressing step determined to contain a bead.

48. The method of claim 45, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a bead comprising an analyte molecule or particle or binding ligand, to the number of locations addressed in the addressing step determined to contain a bead but not to contain any beads comprising an analyte molecule or particle or a binding ligand.

49. The method of claim 40 or 41, wherein the measure of the concentration of analyte molecule or particles in the fluid sample is based at least in part on the ratio of

the number of locations addressed in the addressing step determined to contain a bead comprising an analyte molecule or particle or binding ligand, to the total number of locations addressed.

50. The method of claim 40 or 41, wherein the plurality of locations comprise a plurality of reaction vessels.

51. The method of claim 50, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

52. The method of claim 3 or 4, wherein the percent of locations comprising at least one bead is greater than about 50%, or greater than about 60%, or greater than about 70%, or greater than about 80%, or greater than about 90%, or greater than about 95%.

53. The method of claim 40 or 41, wherein the analyte molecules or particles are proteins or nucleic acids.

54. The method of claim 40, wherein at least a portion of the analyte molecules or particles are associated with at least one binding ligand.

55. The method of claim 41 or 54, wherein the binding ligand comprises an enzymatic component.

56 The method of claim 40 or 41, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15} M.

57. The method of claim 40 or 41, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard.

58. The method of claim 40 or 41, wherein each of the capture objects comprising at least one analyte molecule comprise one, two, three, four, or five analyte molecules.

59. The method of claim 41 or 54, wherein following the providing step, the locations are exposed to precursor labeling agent.

60. The method of claim 59, wherein the precursor binding ligand is converted to a labeling agent upon exposure to a binding ligand.

61. The method of claim 60, wherein the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle containing an analyte molecule or particle or a labeling agent is determined by determining the number of locations addressed comprising a labeling agent.

62. The method of claim 59, wherein the labeling agent is a chromogenic, fluorescent, or chemiluminescent.

63. The method of claim 50, wherein the plurality of reaction vessels is formed upon the mating of at least a portion of a sealing component and at least a portion of a second substrate.

64. The method of claim 50, wherein the volume of each of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters.

65. The method of claim 40 or 41, wherein the average diameter of the beads is between about 0.1 micrometer and about 100 micrometers.

66. The method of claim 40 or 41, wherein the average diameter of the beads is between about 1 micrometer and about 10 micrometers.

67. The method of claim 40 or 41, further comprising performing at least one wash step.

68. The method of claim 40 or 41, wherein the plurality of locations is addressed using optical detection.

69. The method of claim 40 or 41, wherein the bead comprises a plurality of capture components.

70. The method of claim 50, wherein the number of reaction vessels addressed is at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% of the total number of reaction vessels.

71. An article or kit, comprising:

a plurality of beads having an average diameter between about 0.1 micrometer and about 100 micrometers; and

a substrate comprising a plurality of reaction vessels, wherein the average depth of the reaction vessels is between about 1.0 times and about 1.5 times the average diameter of the beads and the average diameter of the reactions vessels is between about 1.0 times and about 1.9 times the average diameter of the beads.

72. The article or kit of claim 71, wherein the average diameter of the plurality of beads is between about 1 micrometer and about 10 micrometers.

73. The article or kit of claim 71, wherein the average diameter of the plurality beads is between about 1 micrometer and about 5 micrometers.

74. The article or kit claim 71, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

75. The article or kit claim 71, wherein the article further comprises a sealing component.

76. The article or kit claim 71, wherein the average depth of the plurality of reaction vessels is between about 1.1 and about 1.3 times the average diameter of the beads.

77. The article or kit claim 71, wherein the average diameter of the plurality of reaction vessels is between about 1.3 and about 1.8 times the average diameter of the beads.

78. The article or kit of claim 71, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters.

79. The article or kit of claim 71, wherein the average volume of the plurality of reaction vessels is between about 1 femtoliter and about 1 picoliter.

80. The article or kit of claim 71, wherein the plurality of beads comprise binding surfaces having an affinity for at least one type of analyte molecule or particle.

81. The article or kit of claim 80, wherein the binding surfaces comprise a plurality of capture components.

82. The article or kit of claim 71, wherein the substrate comprises between about 1,000 and about 1,000,000 reaction vessels.

83. The article or kit of claim 71, wherein the substrate comprises between about 10,000 and about 100,000 reaction vessels.

84. The article or kit of claim 71, further comprising a plurality of control beads.

85. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at least one type of analyte molecules or particles, wherein at least some of the capture objects become associated with at least one analyte molecule or particle;

mixing the plurality of capture objects prepared in the exposing step to a plurality of binding ligands comprising an enzymatic component such that a statistically significant fraction of the capture objects associated with at least one analyte molecule or particle associate with a single binding ligand;

spatially segregating at least a portion of the capture objects subjected to the mixing step into a plurality of separate locations; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part by addressing at least a portion of the plurality of locations subjected to the spatially segregating step to determine the presence of the enzymatic component or a product of a reaction involving the enzymatic component.

86. The method of claim 85, wherein the analyte molecule or particle is a protein.

87. The method of claim 85, wherein the binding surface comprises a plurality of capture components.

88. The method of claim 85, wherein the reaction involving the enzymatic component comprises exposing the enzymatic component to a precursor labeling agent.

89. The method of claim 88, wherein the precursor labeling agent is converted to a labeling agent upon exposure to the enzymatic component.

90. The method of claim 89, wherein the labeling agent is chromogenic, fluorescent, or chemiluminescent.

91. The method of claim 85, wherein the enzymatic component comprises horseradish peroxidase, beta-galactosidase, or alkaline phosphatase.

92. The method of claim 85, wherein the plurality of locations comprise a plurality of reaction vessels.

93. The method of claim 92, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

94. The method of claim 85, further comprising determining the number of locations addressed in the determining step determined to contain an enzymatic component or a product of a reaction involving the enzymatic component.

95. The method of claim 94, further comprising determining the number of locations addressed in the determining step determined to contain a capture object that includes a

binding surface having affinity for at least one type of analyte molecule or particle but determined not to contain an enzymatic component or a product of a reaction involving the enzymatic component.

96. The method of claim 94, further comprising determining the number of locations addressed in the determining step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle.

97. The method of claim 94, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the number of locations addressed in the determining step determined to contain an enzymatic component or a product of a reaction involving the enzymatic component.

98. The method of claim 96, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the determining step determined to contain an enzymatic component or a product of a reaction involving the enzymatic component, to the number of locations addressed in the determining step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle.

99. The method of claim 95, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is based at least in part on the ratio of the number of locations addressed in the determining step determined to contain an enzymatic component or a product of a reaction involving the enzymatic component, to the number of locations addressed in the determining step determined to contain a capture object that includes a binding surface having affinity for at least one type of analyte molecule or particle but determined not to contain an enzymatic component or a product of a reaction involving the enzymatic component.

100. The method of claim 94, wherein the measure of the concentration of analyte molecules or particle in a fluid sample is based at least in part on the ratio of the number of locations addressed in the determining step determined to contain an enzymatic

component or a product of a reaction involving the enzymatic component to the total number of locations addressed.

101. The method of claim 85, wherein the locations are addressed optically.

102. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

immobilizing a plurality of analyte molecules or particles with respect to a plurality of beads;

spatially segregating at least a portion of the plurality of beads into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a bead;

further determining the number of said locations containing a bead and an analyte molecule or particle; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a bead and an analyte molecule and particle, to the number of locations containing a bead.

103. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

immobilizing a plurality of analyte molecules or particles with respect to a plurality of beads;

spatially segregating at least a portion of the plurality of beads into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a bead;

further determining the number of said locations containing a bead and an analyte molecule or particle; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a bead and an analyte molecule and particle, to the number of locations containing a bead but not containing any analyte molecules or particles.

104. The method of claim 102 or 103, wherein the number of said locations containing a bead is determined using optical techniques.

105. The method of claim 104, wherein the number of said locations containing a bead is determined using white light.

106. The method of claim 104, wherein the number of said locations containing a bead and an analyte molecule or particle is determined using fluorescence.

107. The method of claim 104, wherein the optical techniques comprises the use of a CCD detector.

108. The method of claim 104, wherein the optical techniques comprises the use of a CID, CMOS, sCMOS, or TDI device.

109. The method of claim 102 or 103, wherein the plurality of locations comprises a plurality of reaction vessels.

110. The method of claim 109, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

111. The method of claim 102 or 103, wherein at least a portion of the analyte molecules or particles are associated with at least one binding ligand.

112. The method of claim 102 or 103, wherein the analyte molecules or particles comprise an enzymatic component.

113. The method of claim 112, wherein the binding ligand comprises an enzymatic component.

114 The method of claim 102 or 103, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15} M, or less than about 40×10^{-15} M, or less than about 30×10^{-15} M, or less than about 20×10^{-15} M, or less than about 10×10^{-15} M, or less than about, or less than about 1×10^{-15} M.

115. The method of claim 102 or 103, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard.

116. The method of claim 102 or 103, wherein during the spatially segregating step, at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the capture objects subjected to the immobilizing steps are spatially separated into the plurality of locations.

117. The method of claim 102 or 103, wherein the number of locations addressed in the addressing step is at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the total number of location.

118. The method of claim 102 or 103, wherein the plurality of beads each include a binding surface having affinity for at least one type of analyte molecule or particle.

119. The method of claim 102 or 103, wherein following the spatially segregating step, the locations are exposed to precursor labeling agent.

120. The method of claim 119, wherein the precursor binding ligand is converted to a labeling agent upon exposure to a binding ligand.

121. The method of claim 120, wherein the number of locations containing a capture object containing an analyte molecule or particle or a labeling agent is determined by determining the number of locations comprising a labeling agent.

122. The method of claim 120 wherein the labeling agent is a chromogenic, fluorescent, or chemiluminescent.

123. The method of claim 102 or 103, wherein the analyte molecules or particles are proteins.

124. The method of claim 102 or 103, wherein the analyte molecules or particles are nucleic acids.

125. The method of claim 109, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters.

126. The method of claim 109, wherein the average volume of the plurality of reaction vessels is between about 1 femtoliter and about 1 picoliter.

127. The method of claim 102 or 103, wherein the average diameter of the plurality of beads is between about 0.1 micrometer and about 100 micrometers.

128. The method of claim 102 or 103, wherein the average diameter of the plurality of beads is between about 1 micrometer and about 10 micrometers.

129. The method of claim 102 or 103, further comprising performing at least one wash step.

130. The method of claim 109, further comprising sealing the plurality of reaction vessels.

131. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

providing a plurality of capture objects that each are associated with either a single analyte molecule or particle or are free of any analyte molecules or particles;

individually addressing at least a portion of the capture objects and determining the number of said capture objects associated with an analyte molecule or particle; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of capture objects subjected to the addressing step determined to be associated with an analyte molecule or particle.

132. The method of claim 131, wherein the capture objects each include a binding surface having affinity for at least one type of analyte molecule or particle and the forming step comprises exposing the plurality of capture objects to a solution containing or suspected of containing the at least one type of analyte molecules or particles.

133. The method of claim 132, comprising a step of immobilizing analyte molecules or particles with respect to the plurality of capture objects such that a statistically significant fraction of the capture objects associate with a single analyte molecule or particle and a statistically significant fraction of the capture objects are free of any analyte molecule or particle.

134. The method of claim 133, comprising a step of individually isolating at least a portion of the capture objects subjected to the immobilizing step from the remainder of said portion of the capture objects.

135. The method of claim 134, wherein the capture objects comprise or are each contained within a liquid droplet that is immiscible within a fluid in which the droplet is suspended during at least the addressing step.

136. The method of claim 131, wherein at least a portion of the analyte molecules or particles are associated with at least one binding ligand.

137. The method of claim 131, wherein the analyte molecules or particles comprise an enzymatic component.

138. The method of claim 136, wherein the binding ligand comprises an enzymatic component.

139. The method of claim 131, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15} M, or less than about 40×10^{-15} M, or less than about 30×10^{-15} M, or less than about 20×10^{-15} M, or less than about 10×10^{-15} M, or less than about 5×10^{-15} M, or less than about 1×10^{-15} M.

140. The method of claim 131wherein the measure of the concentration of analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standards.

141. The method of claim 131, wherein the plurality of locations is addressed using optical techniques.

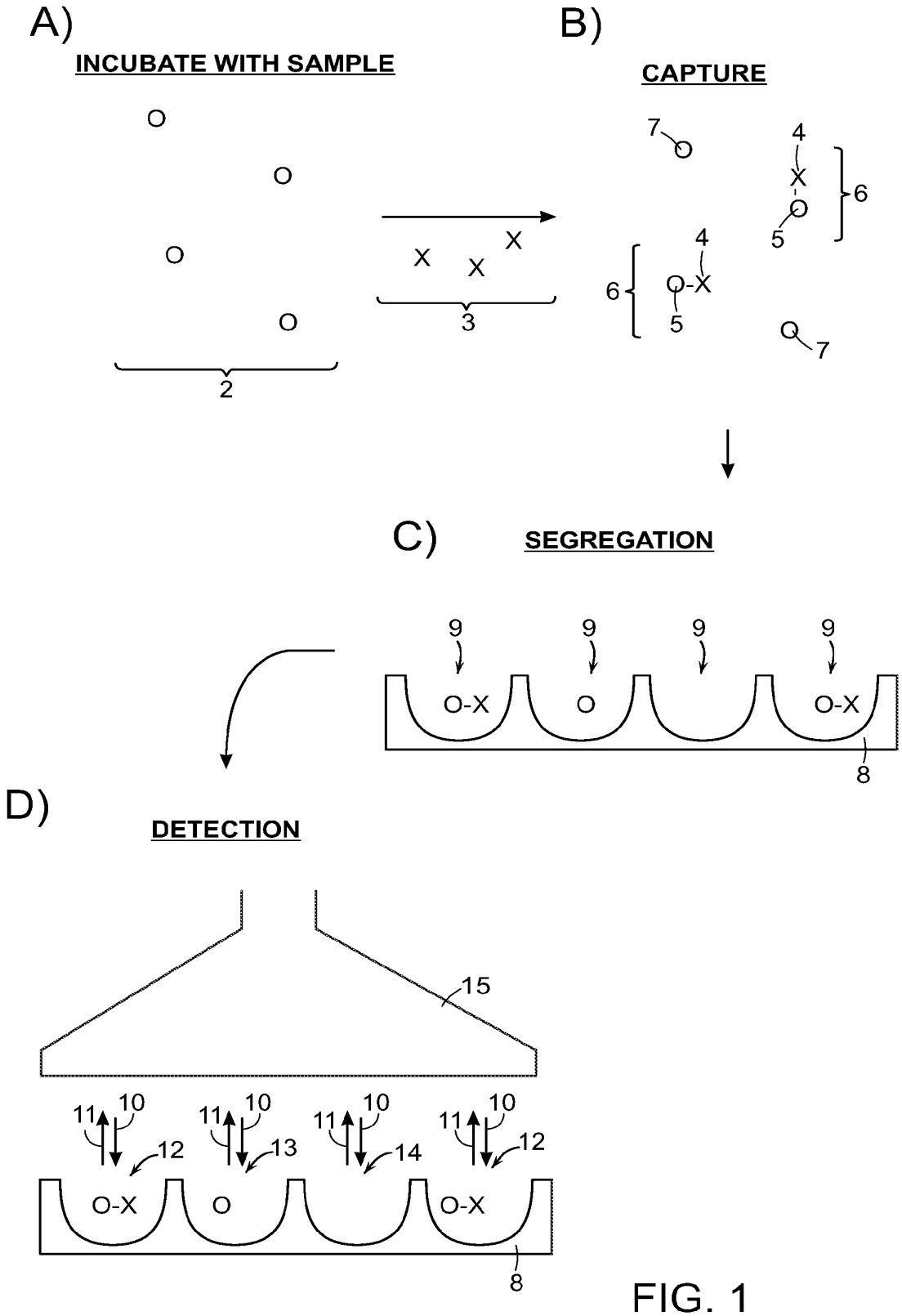


FIG. 1

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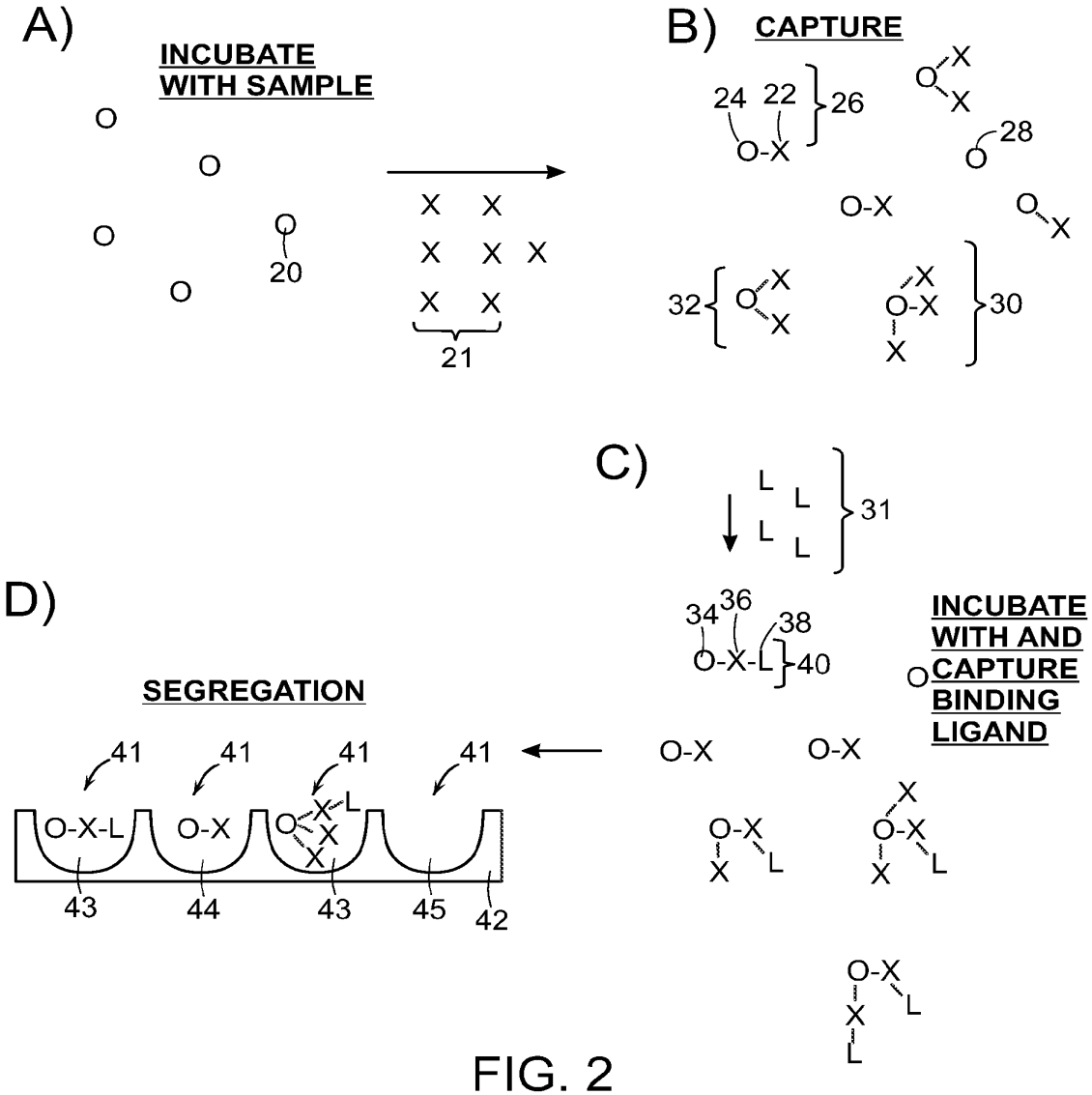


FIG. 2

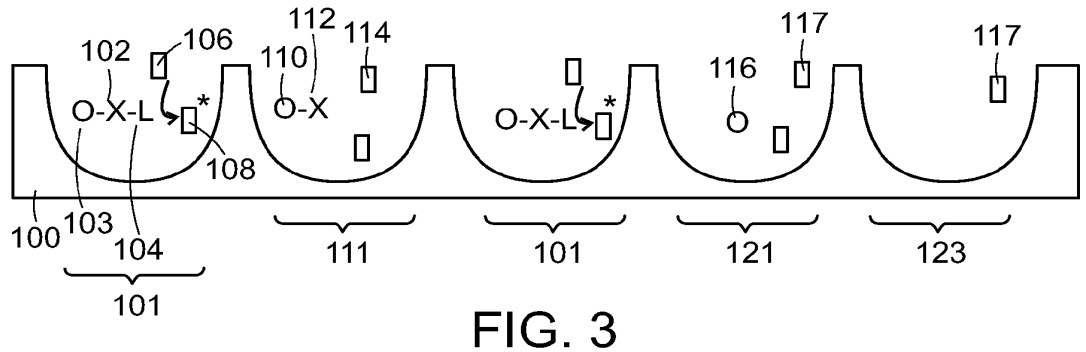


FIG. 3

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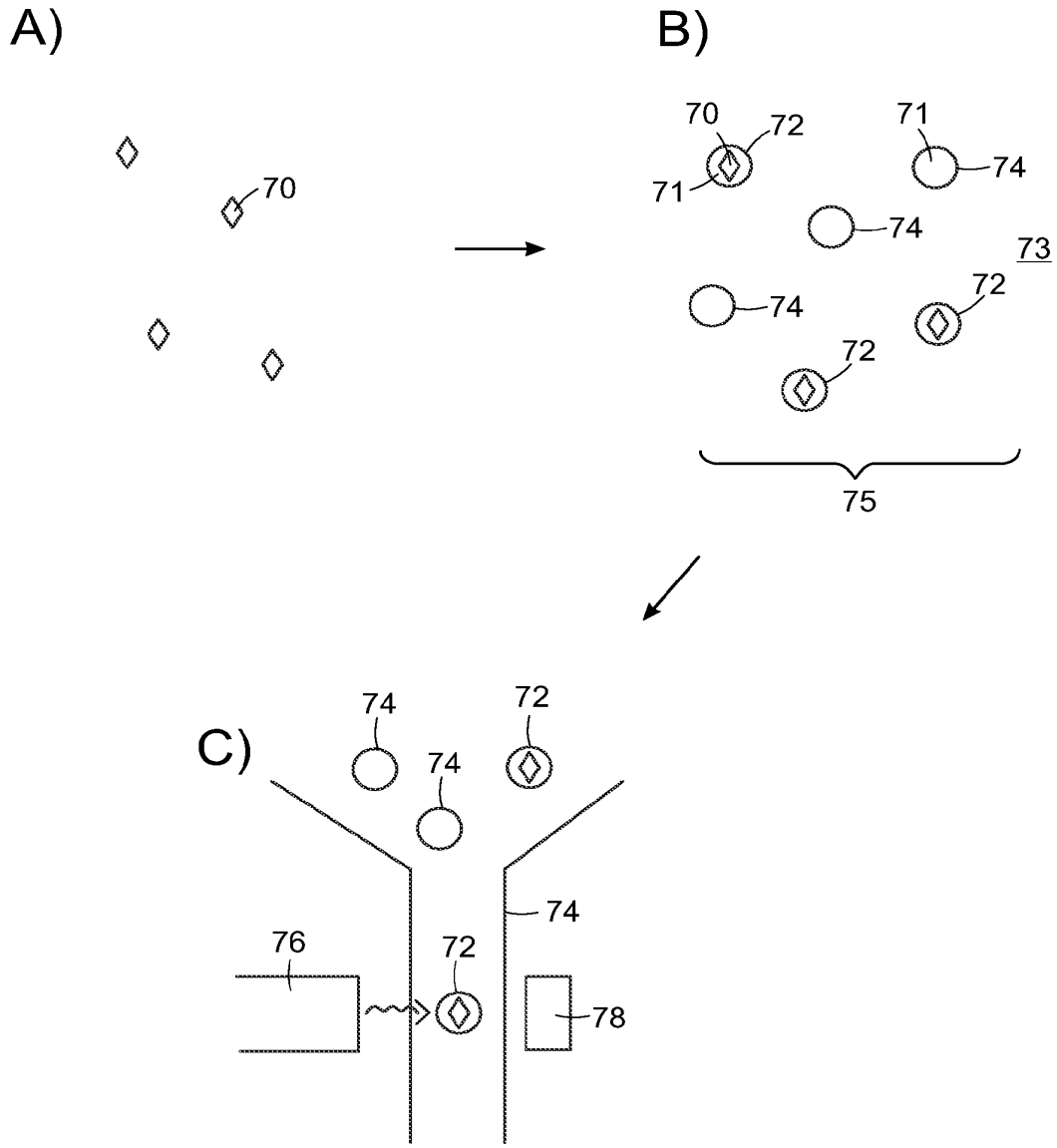


FIG. 4A

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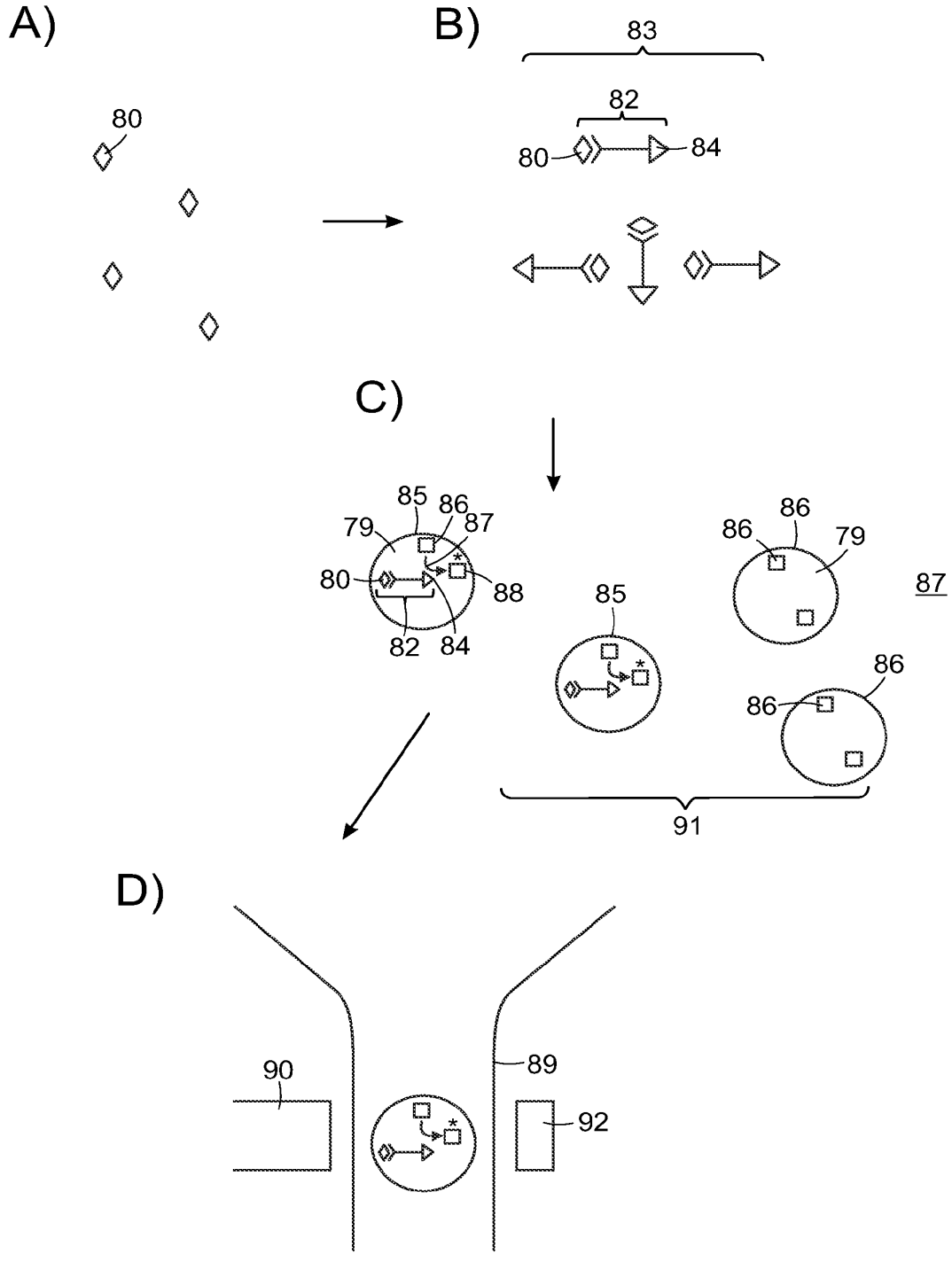


FIG. 4B

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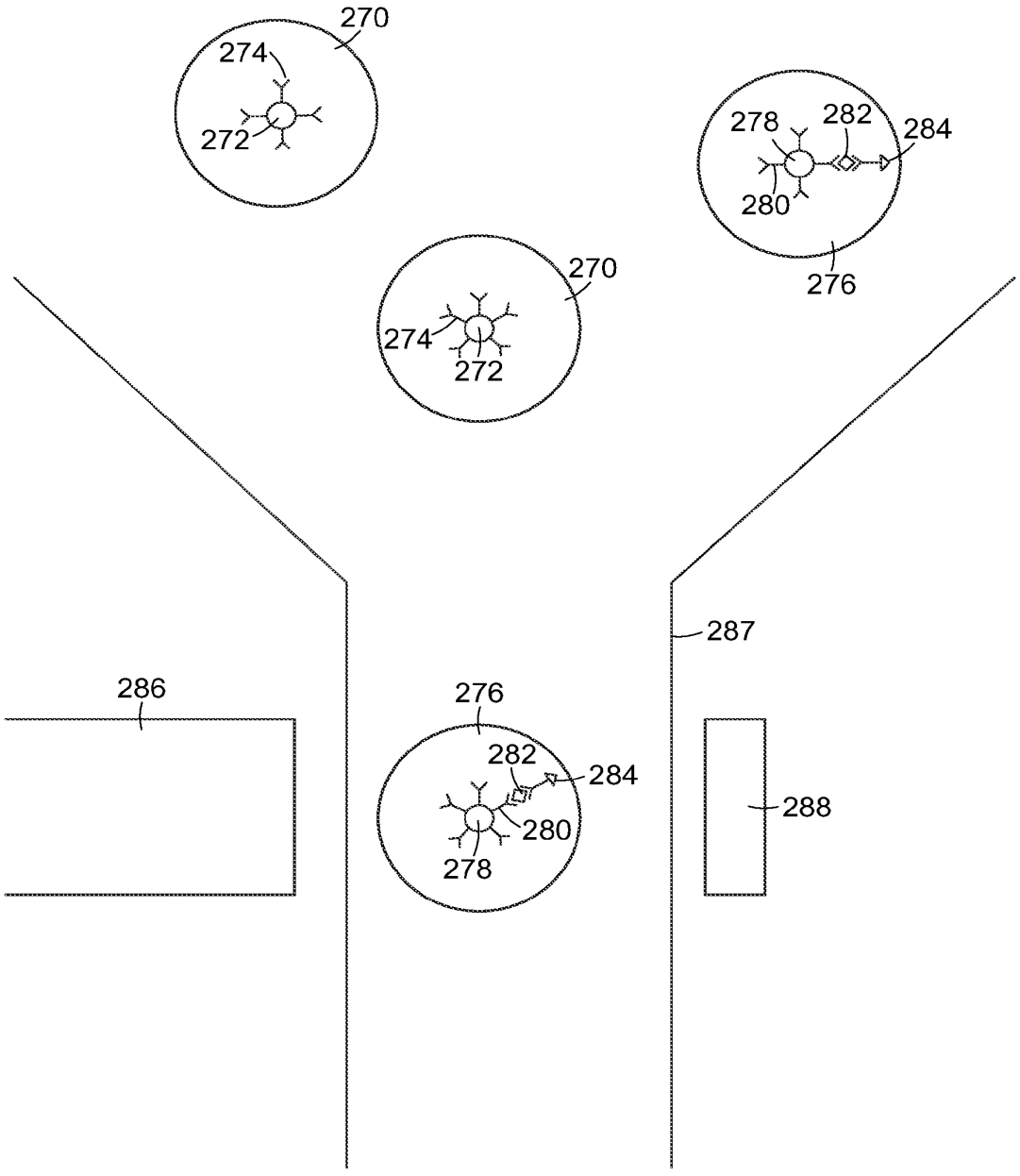


FIG. 4C

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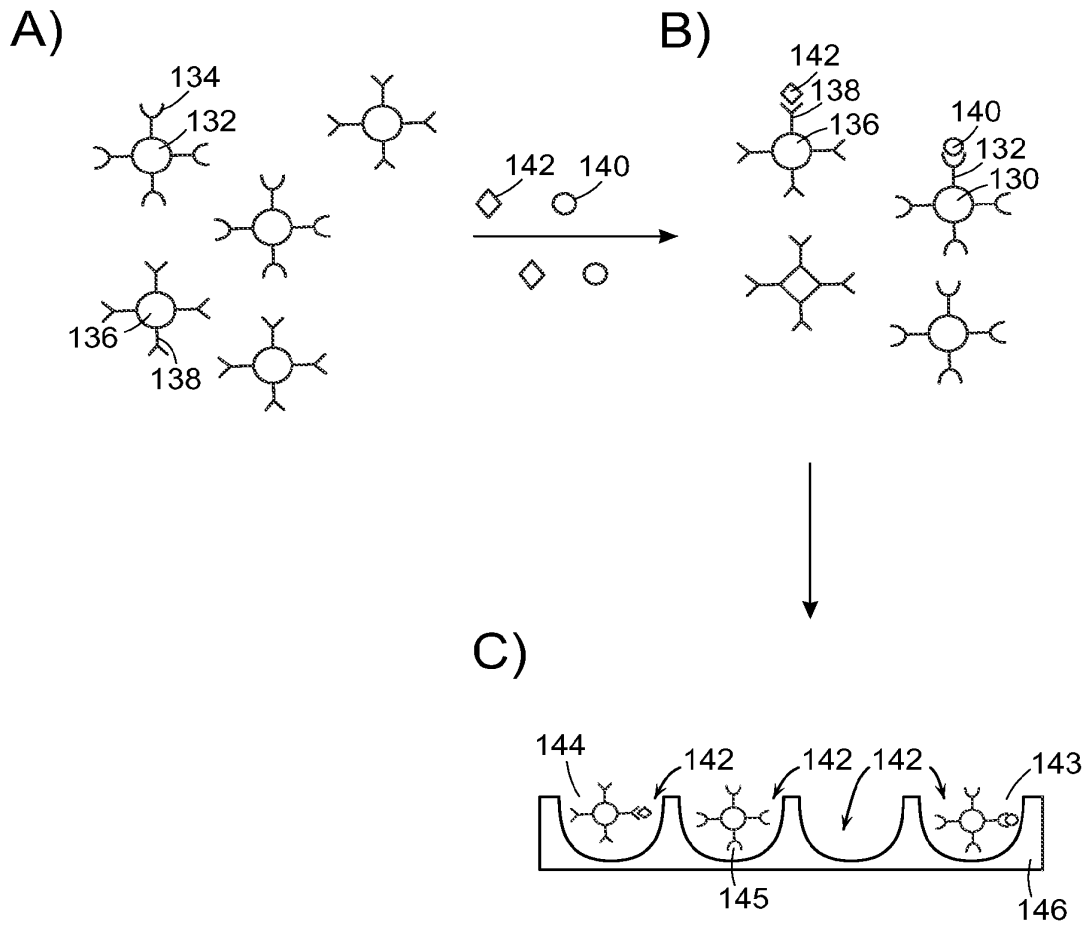

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FIG. 5

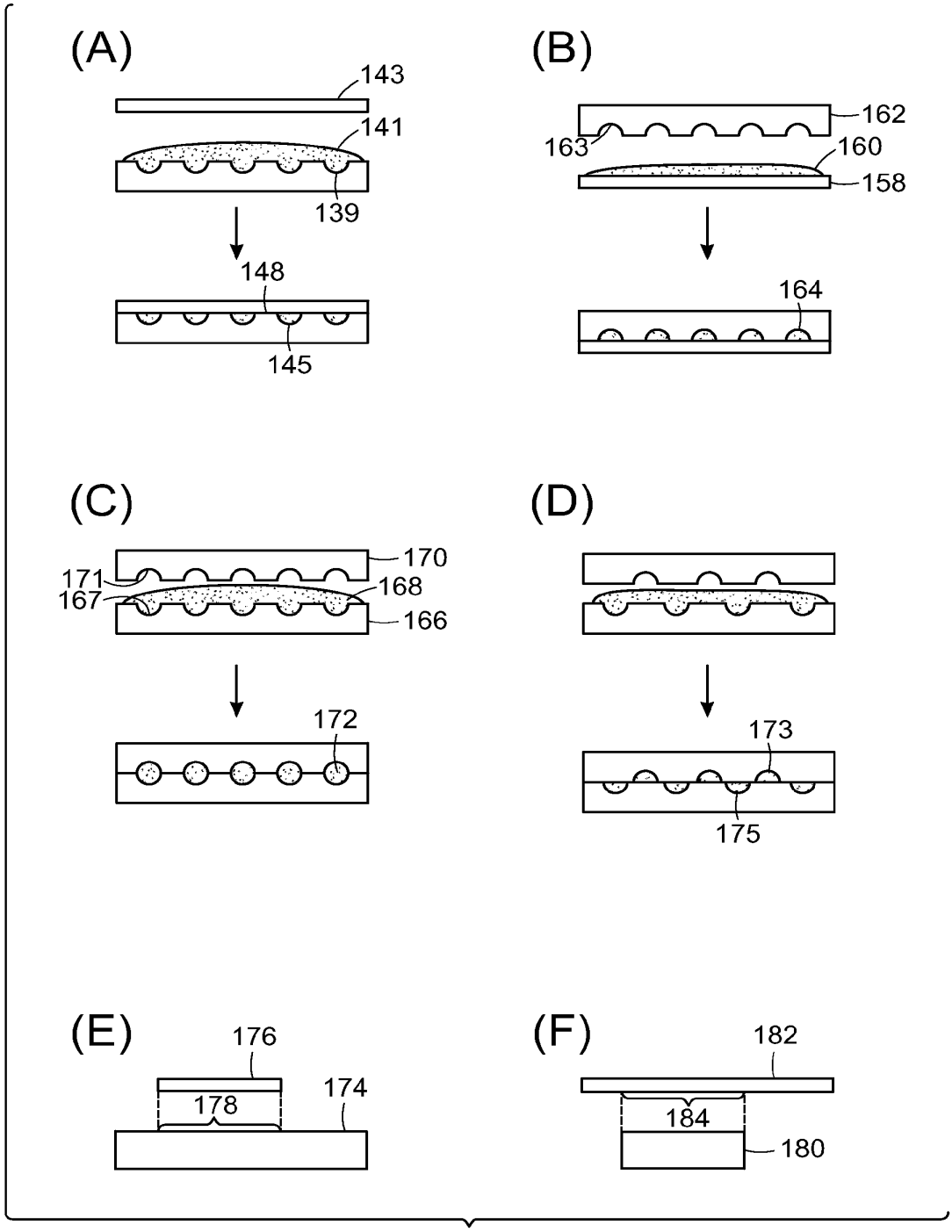


FIG. 6

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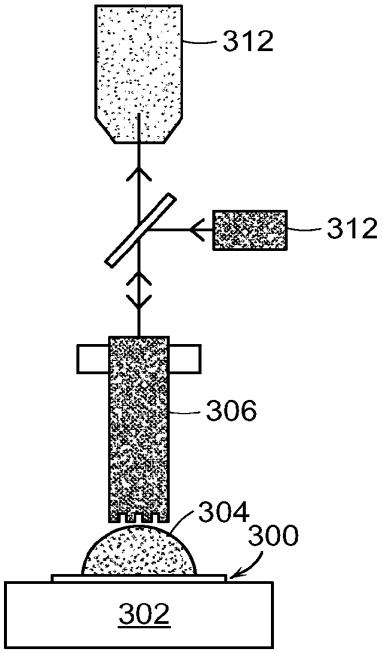


FIG. 7

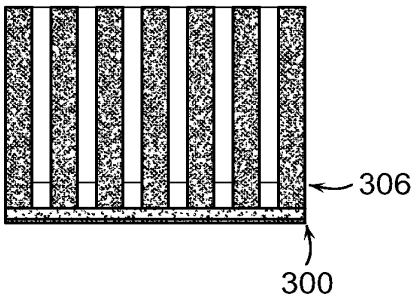


FIG. 8

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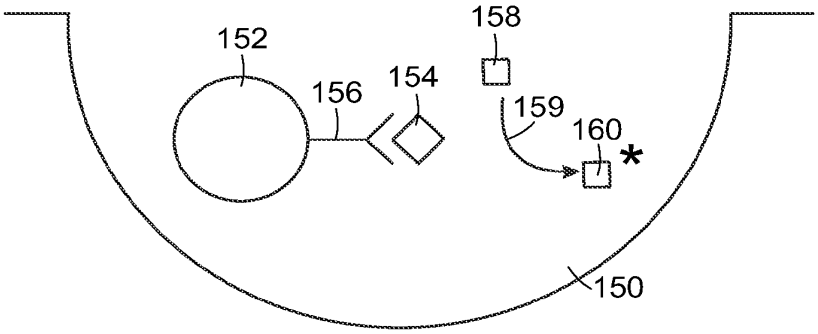


FIG. 9A

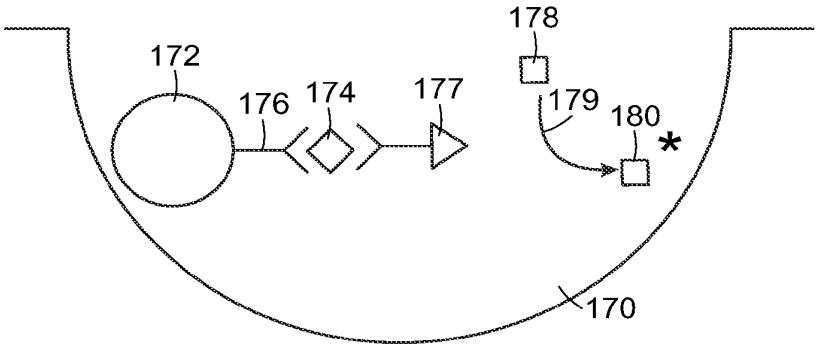


FIG. 9B

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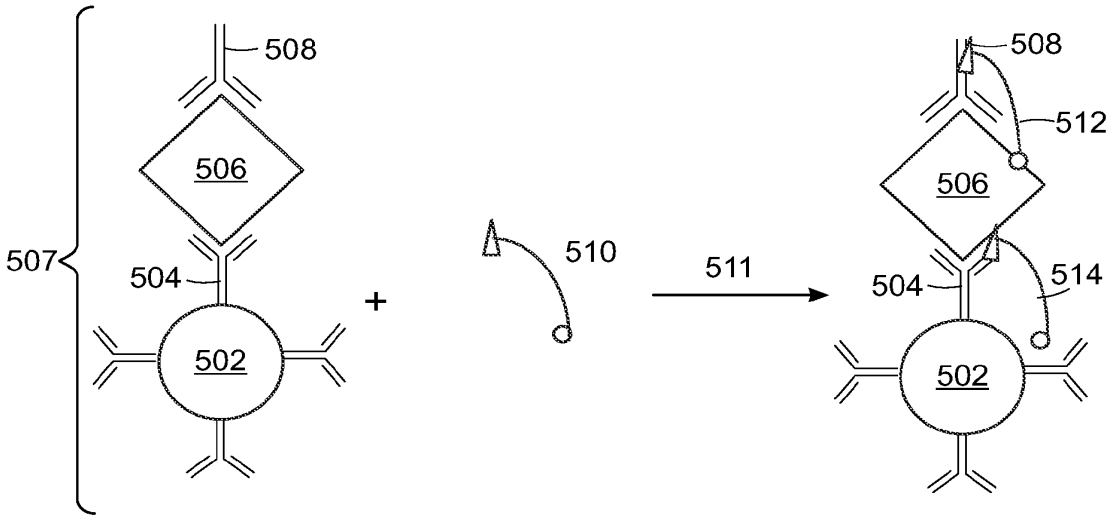


FIG. 10A

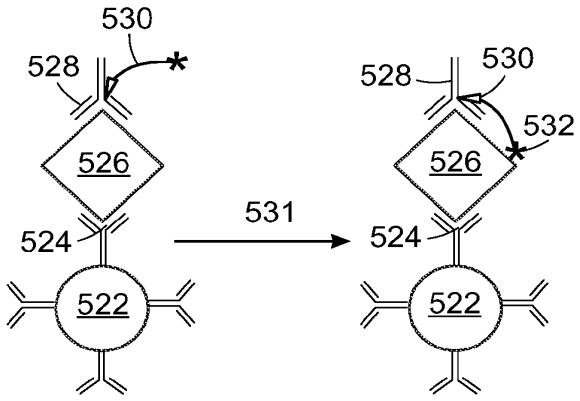


FIG. 10B

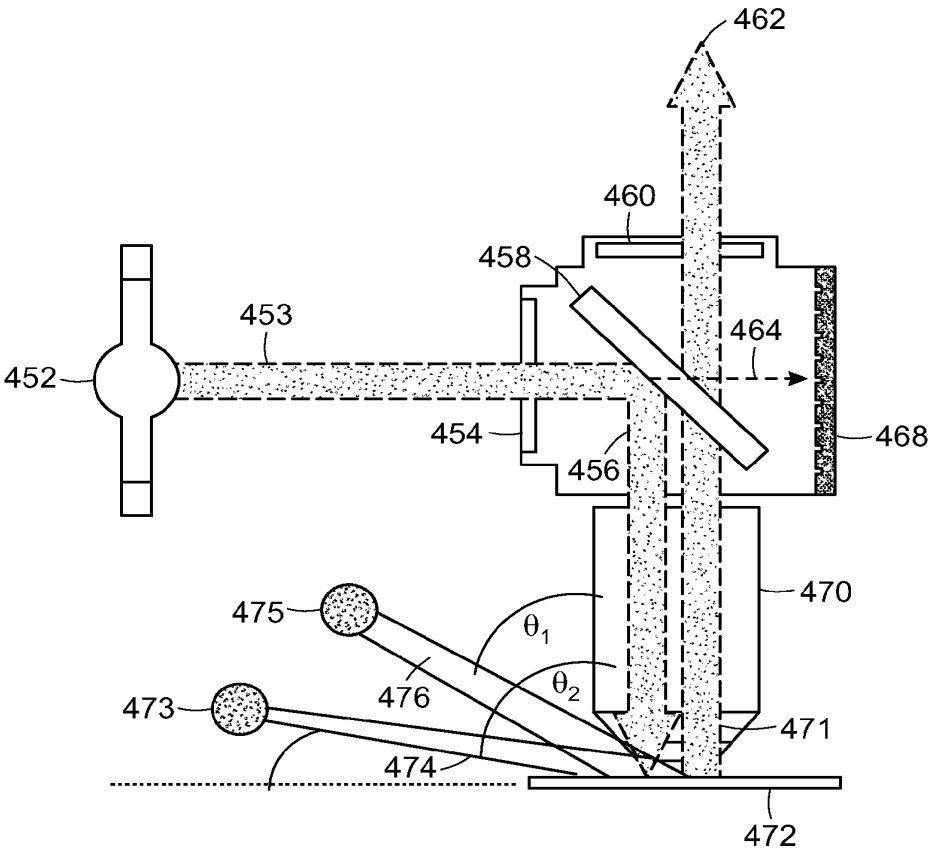


FIG. 11A

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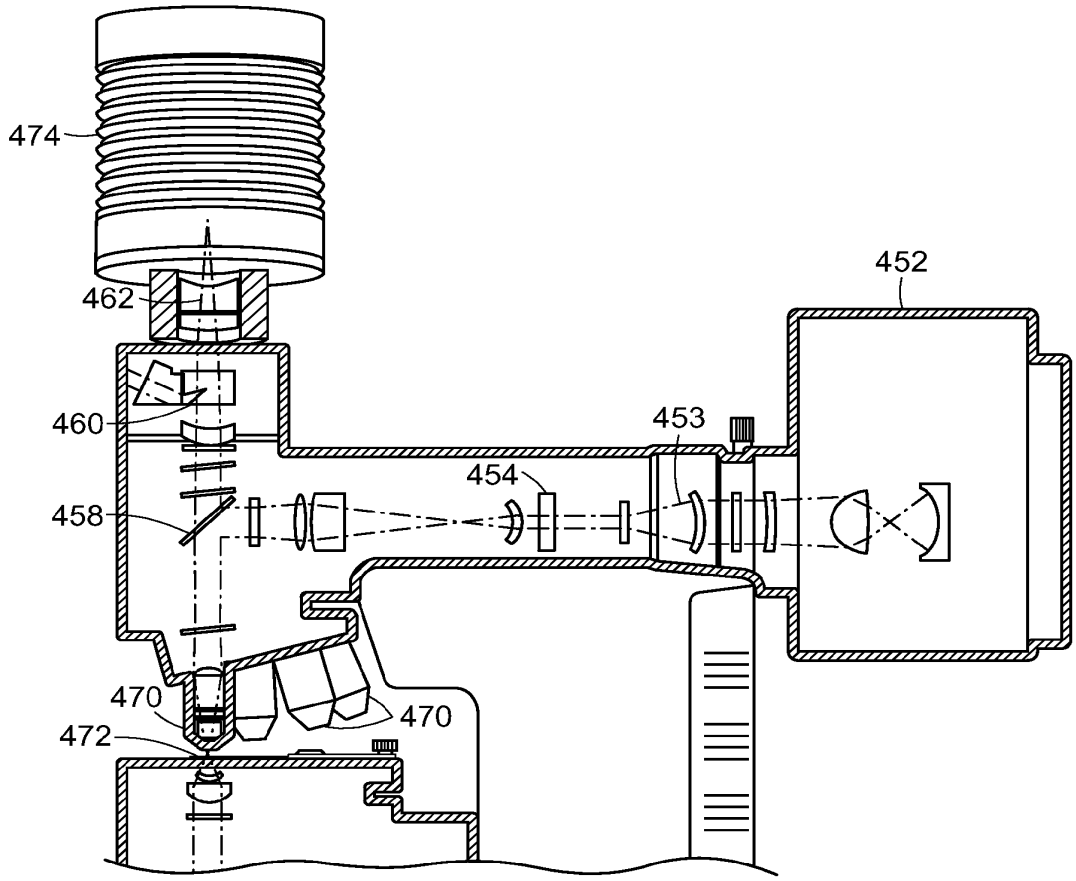


FIG. 11B

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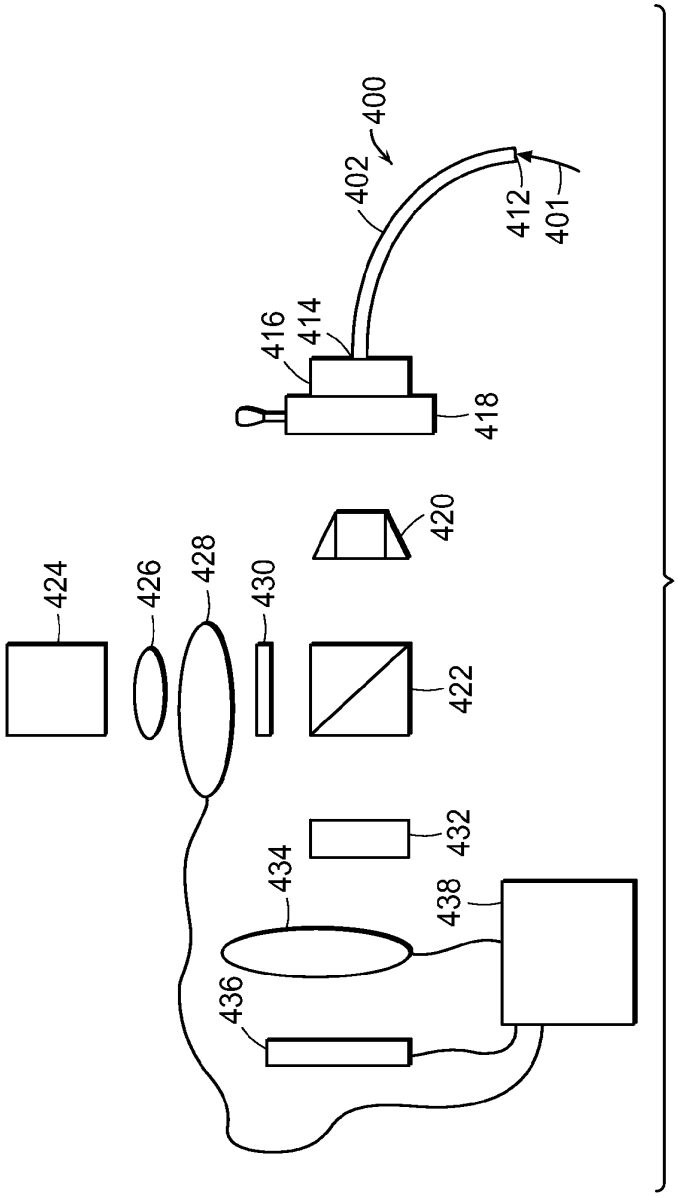

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FIG. 12

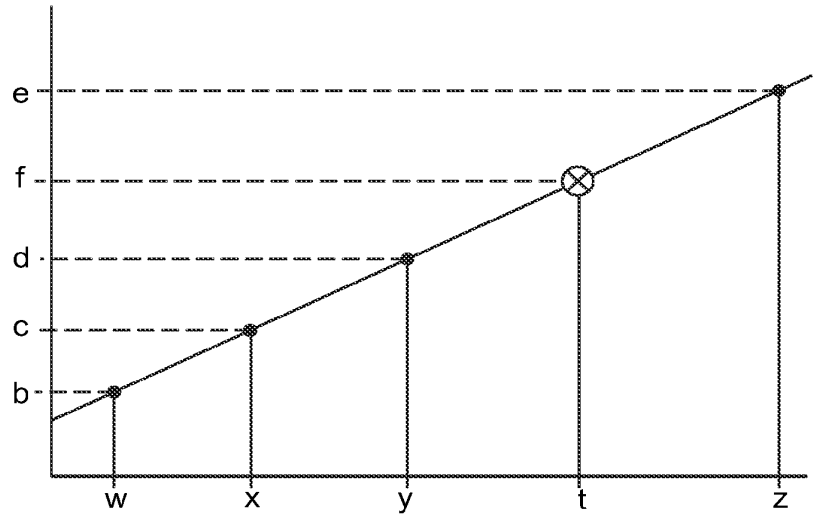


FIG. 13

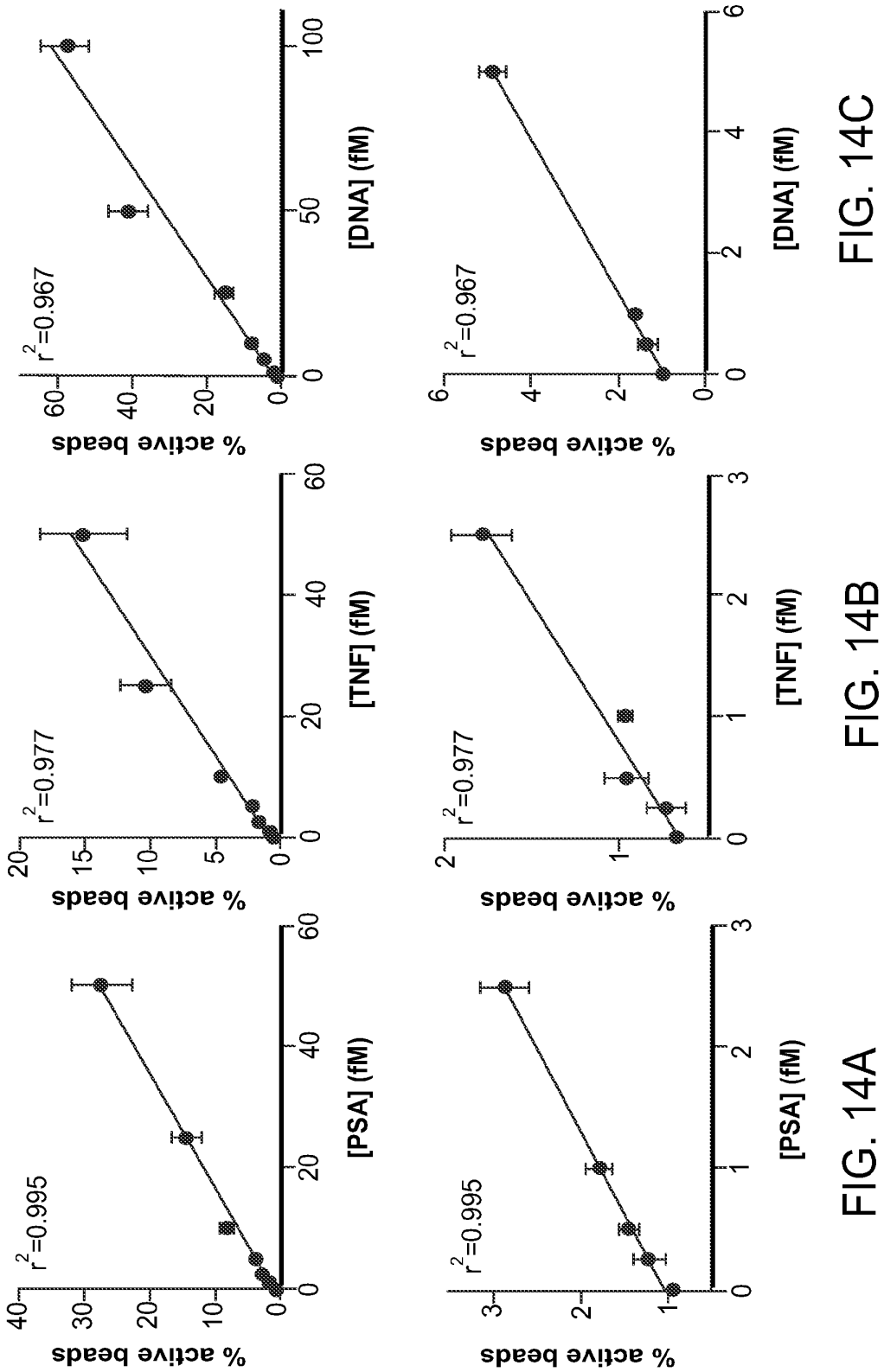


FIG. 14A

FIG. 14B

FIG. 14C

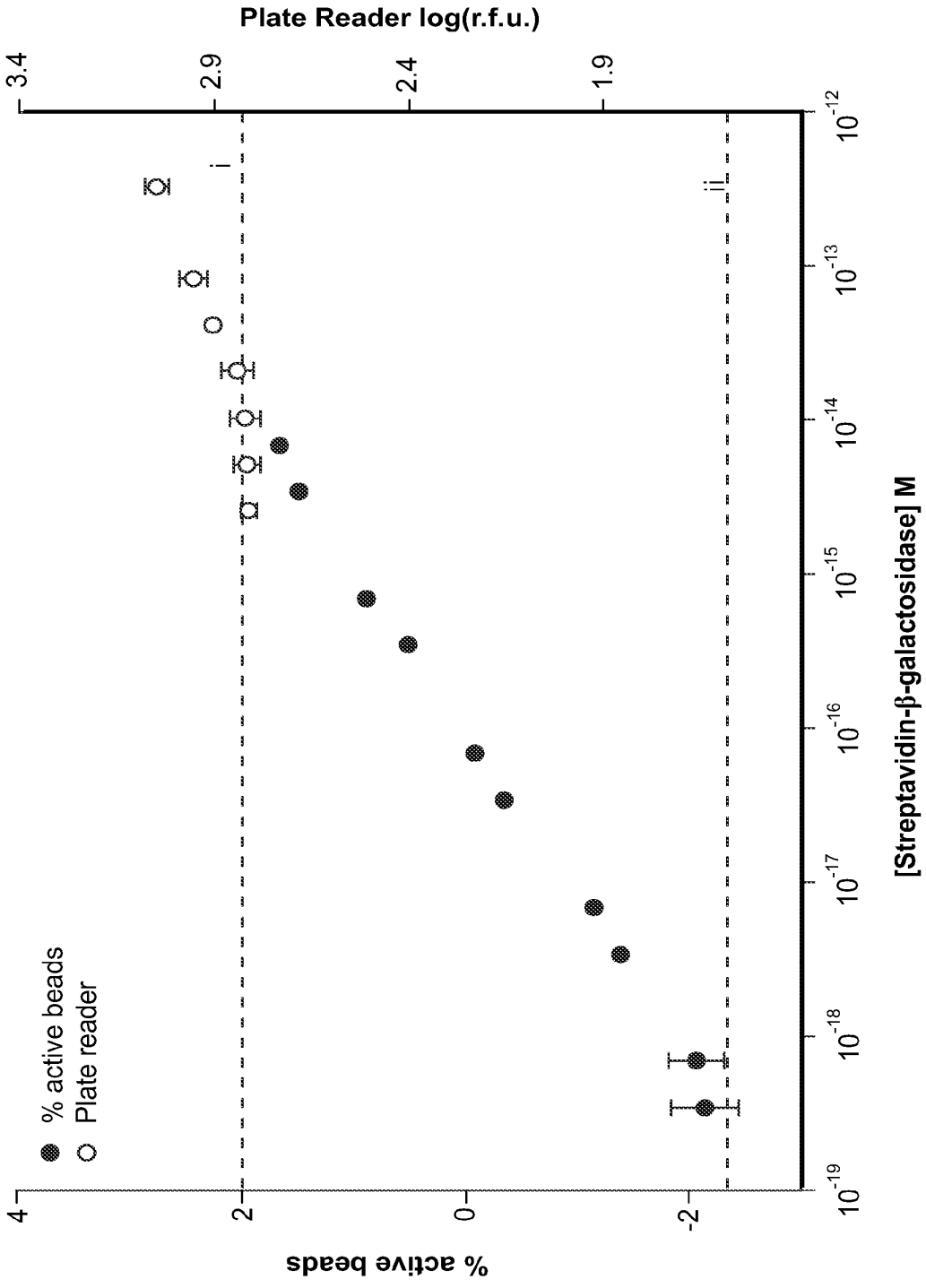


FIG. 15

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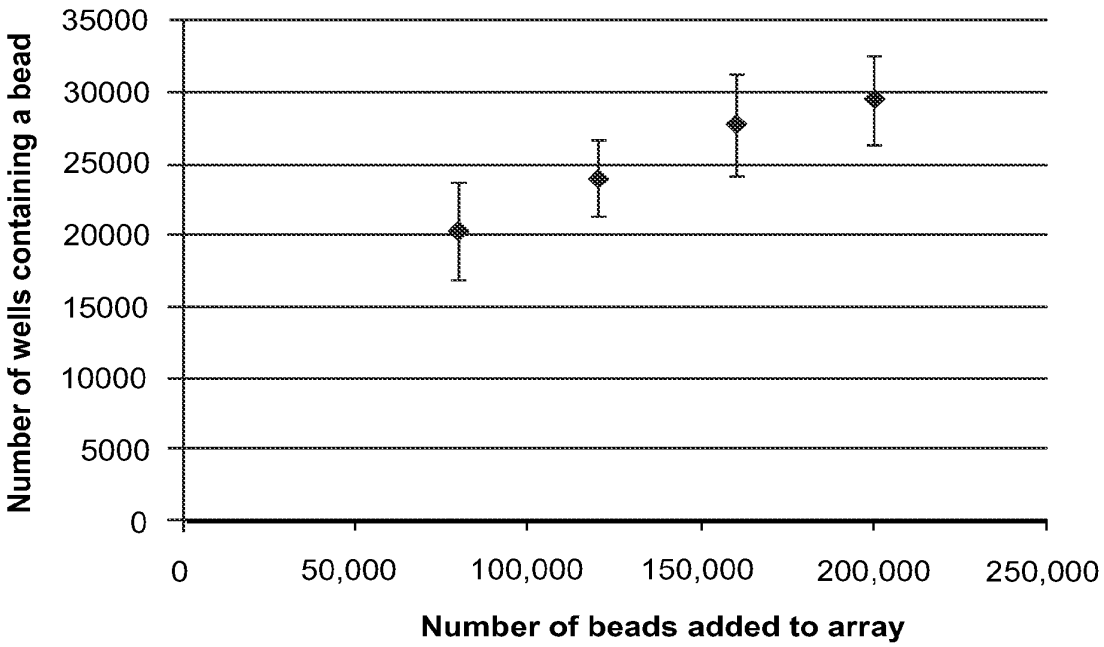


FIG. 16

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FIG. 17A

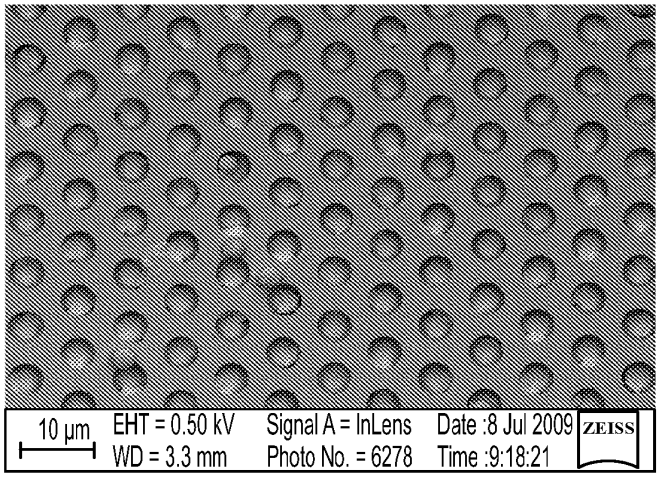


FIG. 17B

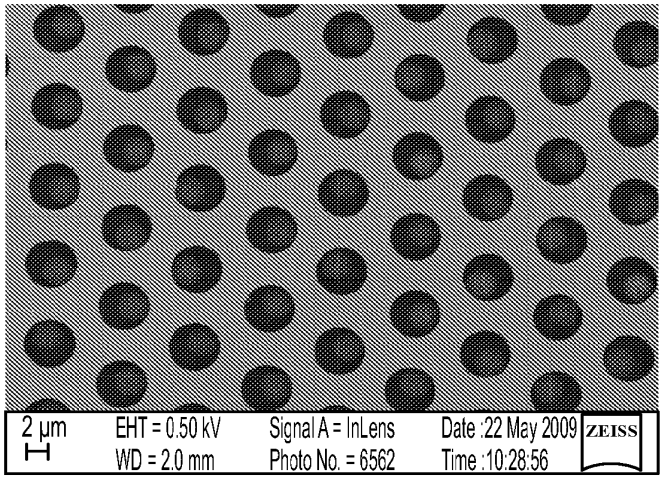
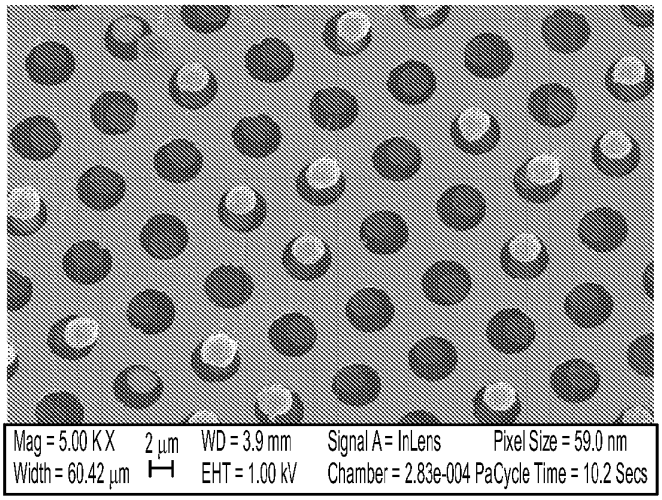


FIG. 17C



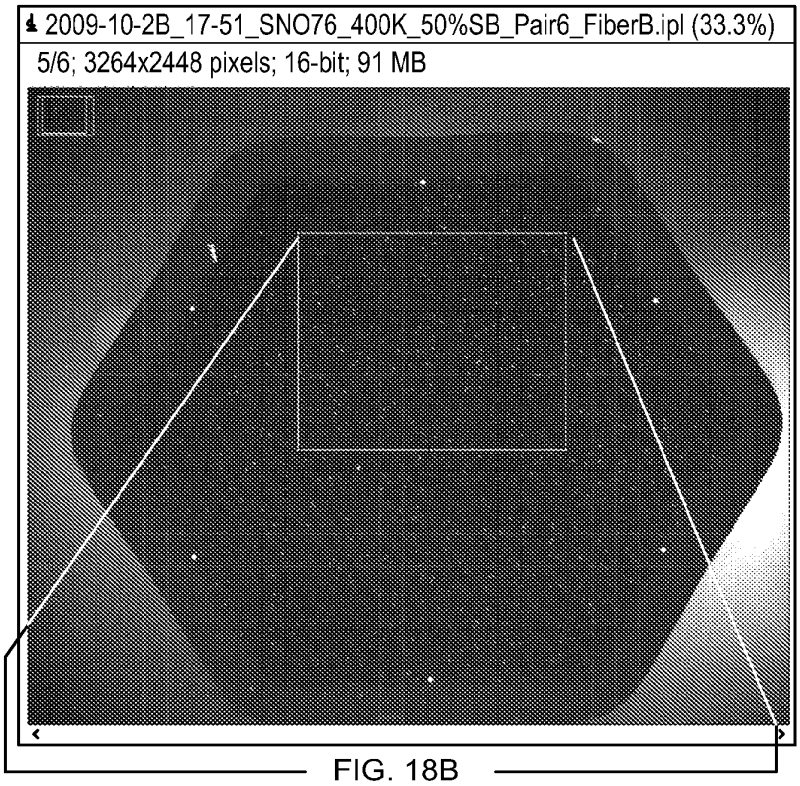


FIG. 18A

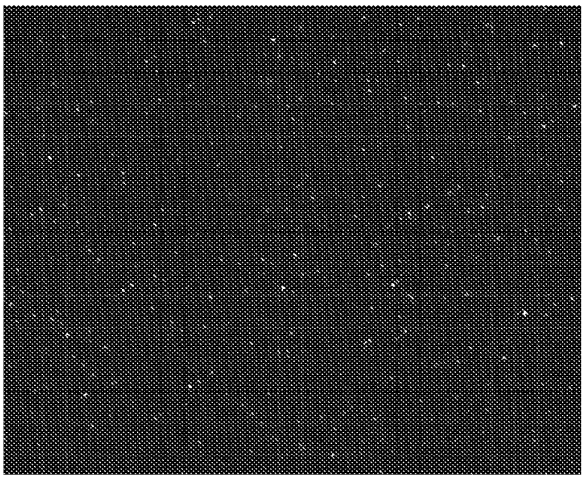


FIG. 18B

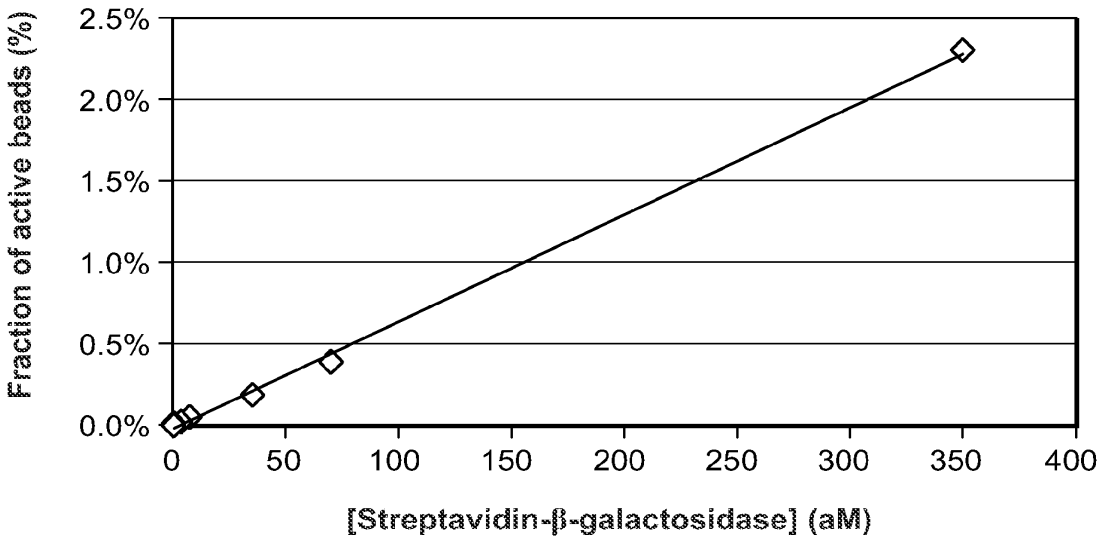


FIG. 19A

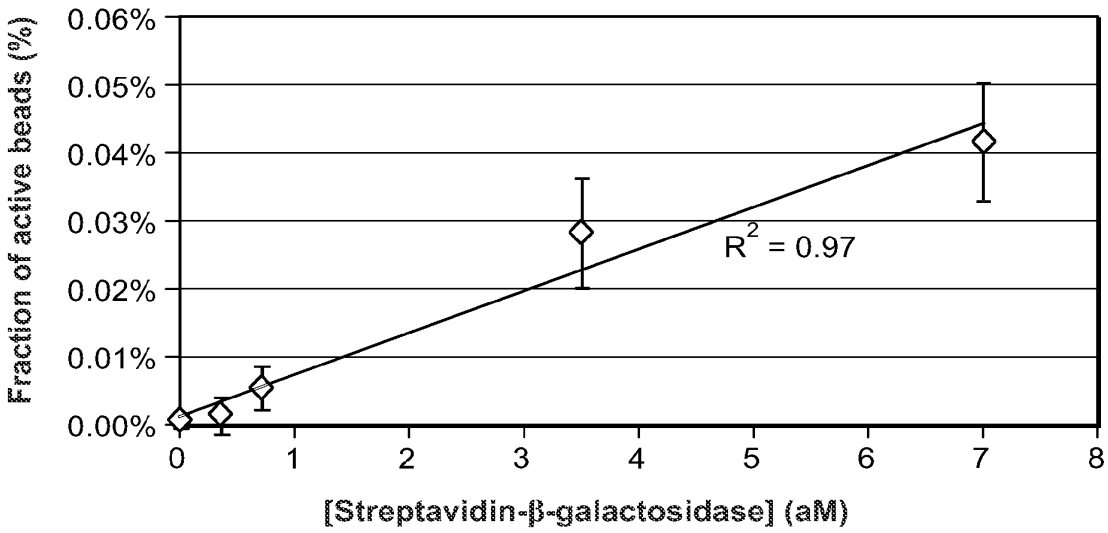


FIG. 19B

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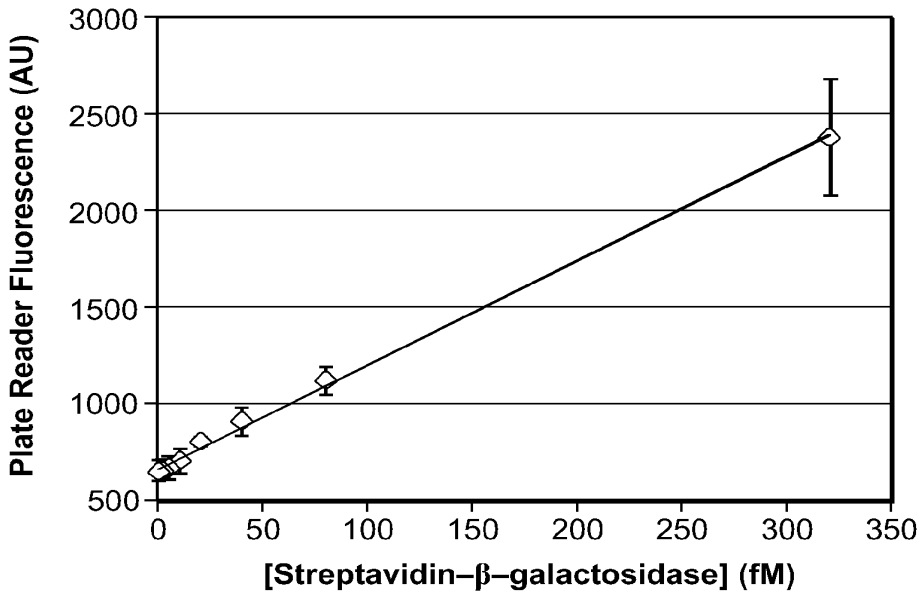


FIG. 19C

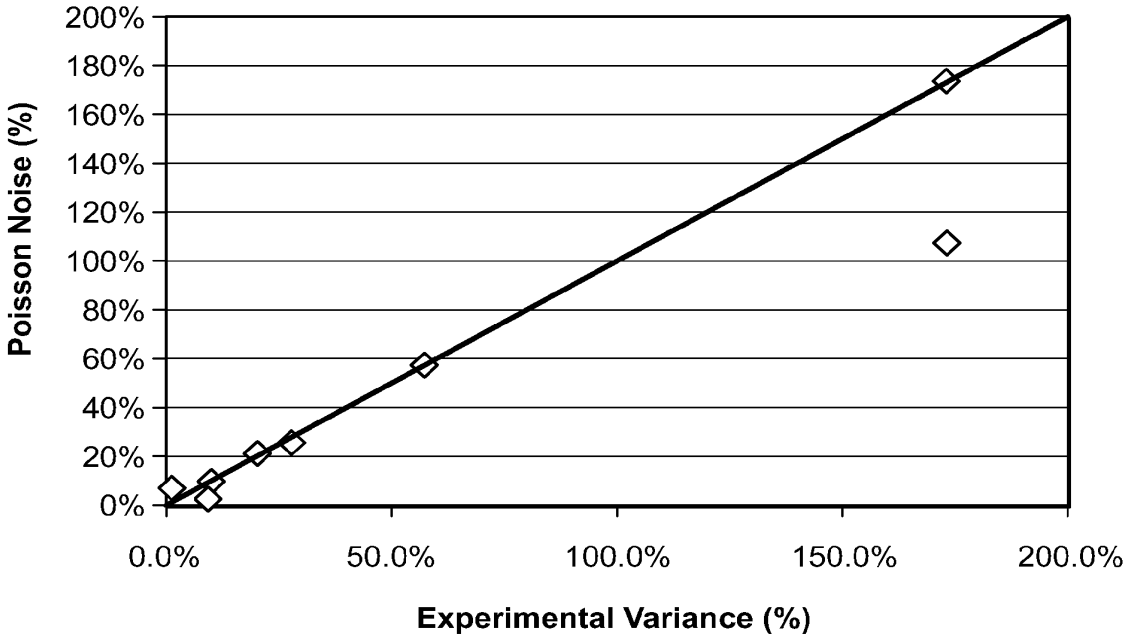


FIG. 20

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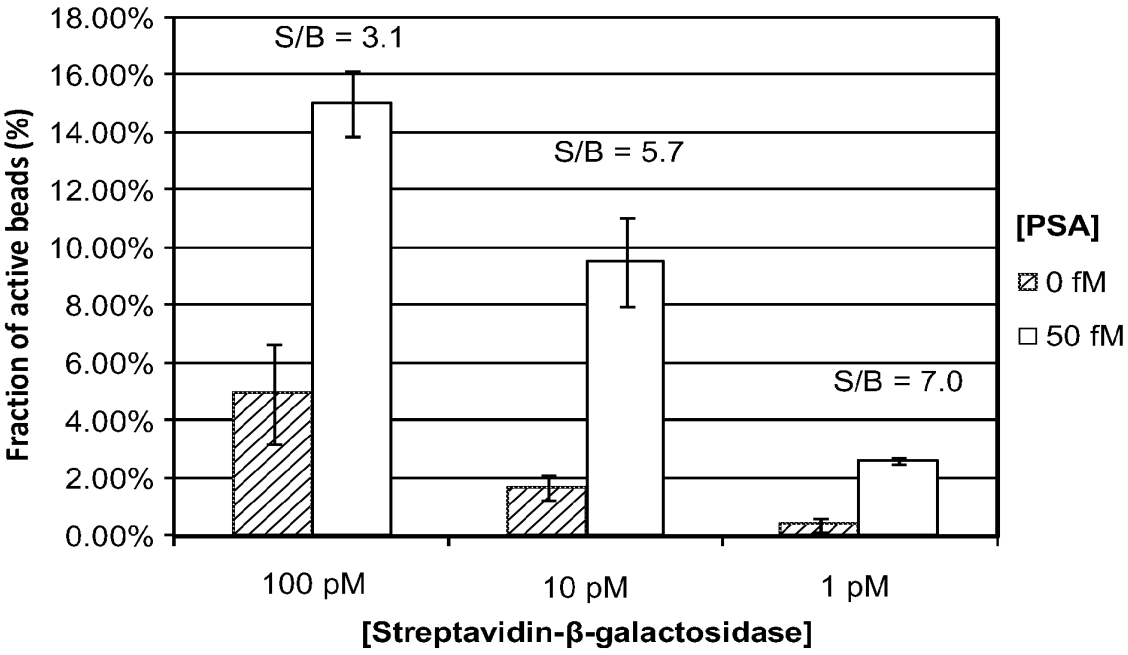


FIG. 21

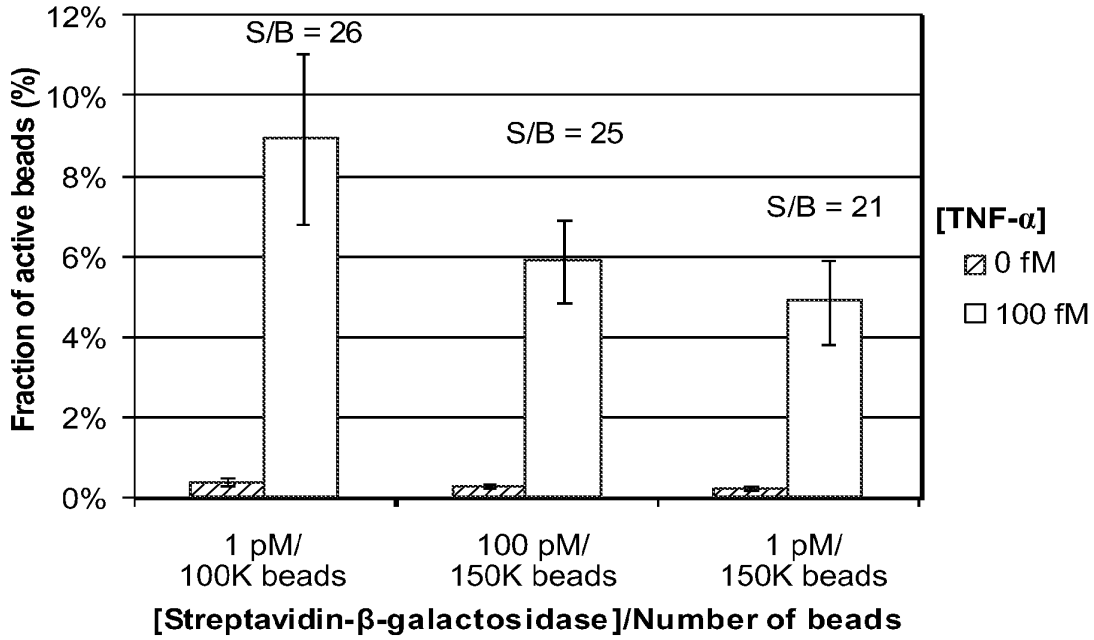


FIG. 22

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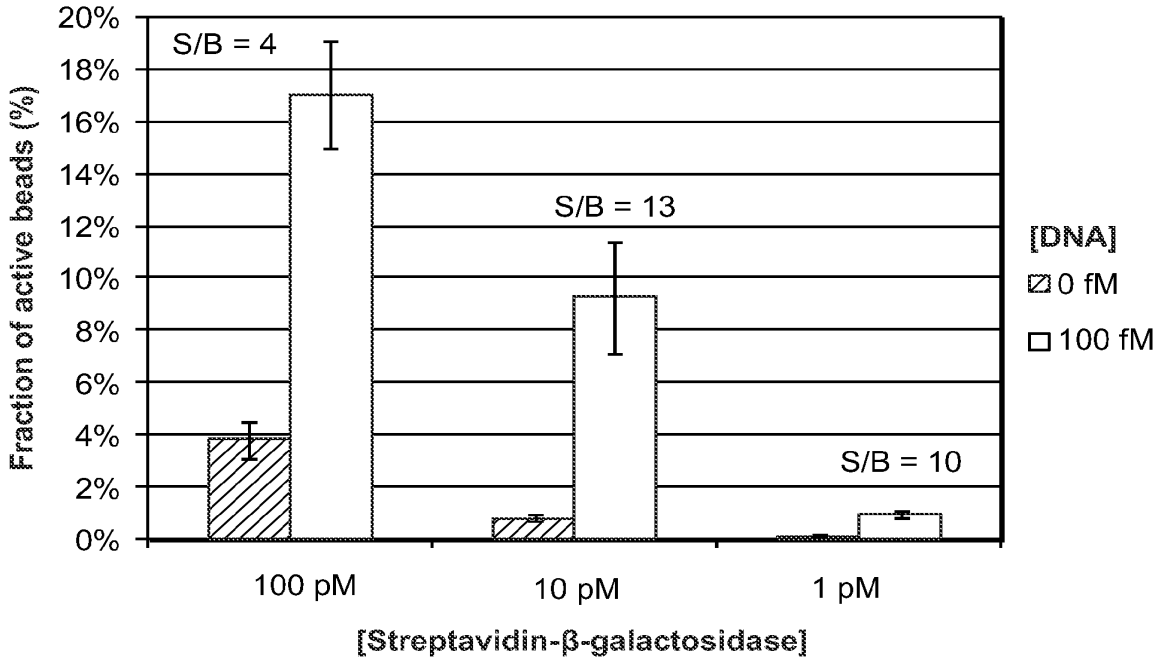


FIG. 23

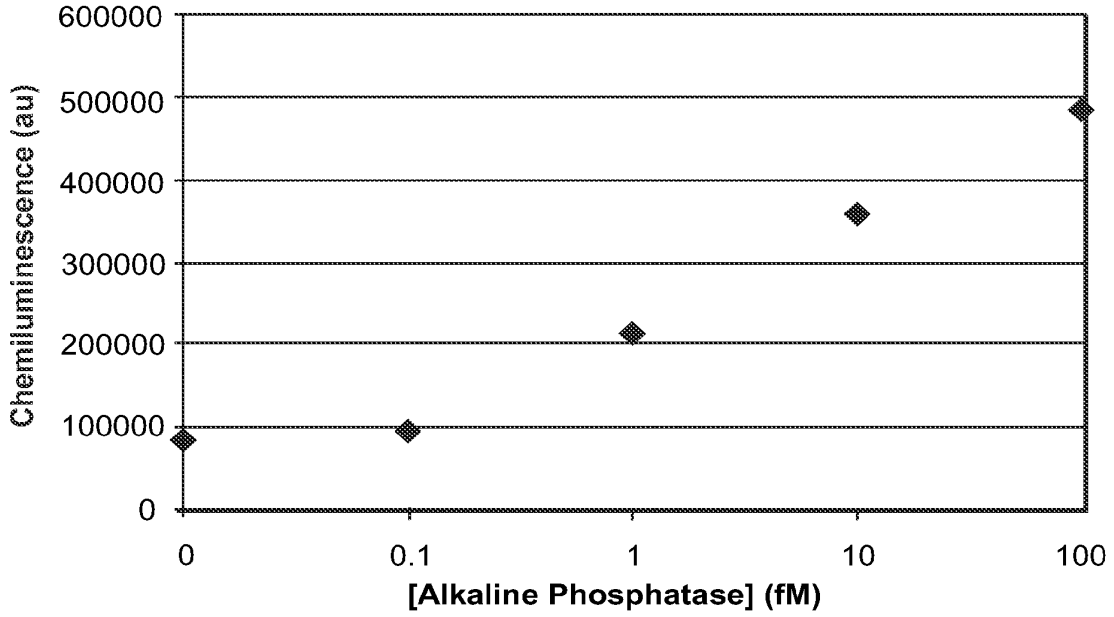


FIG. 24

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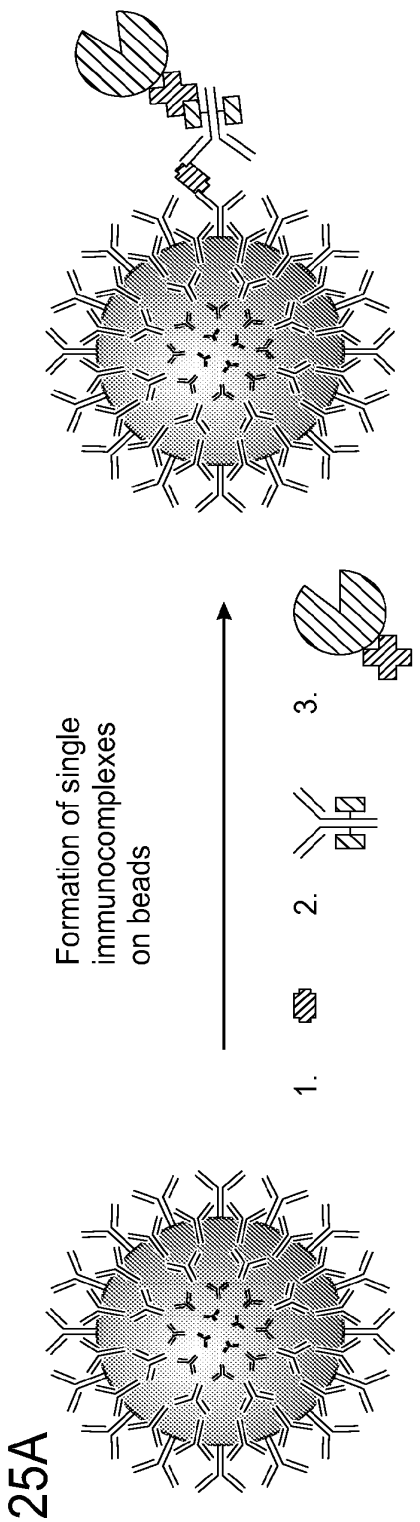


FIG. 25A

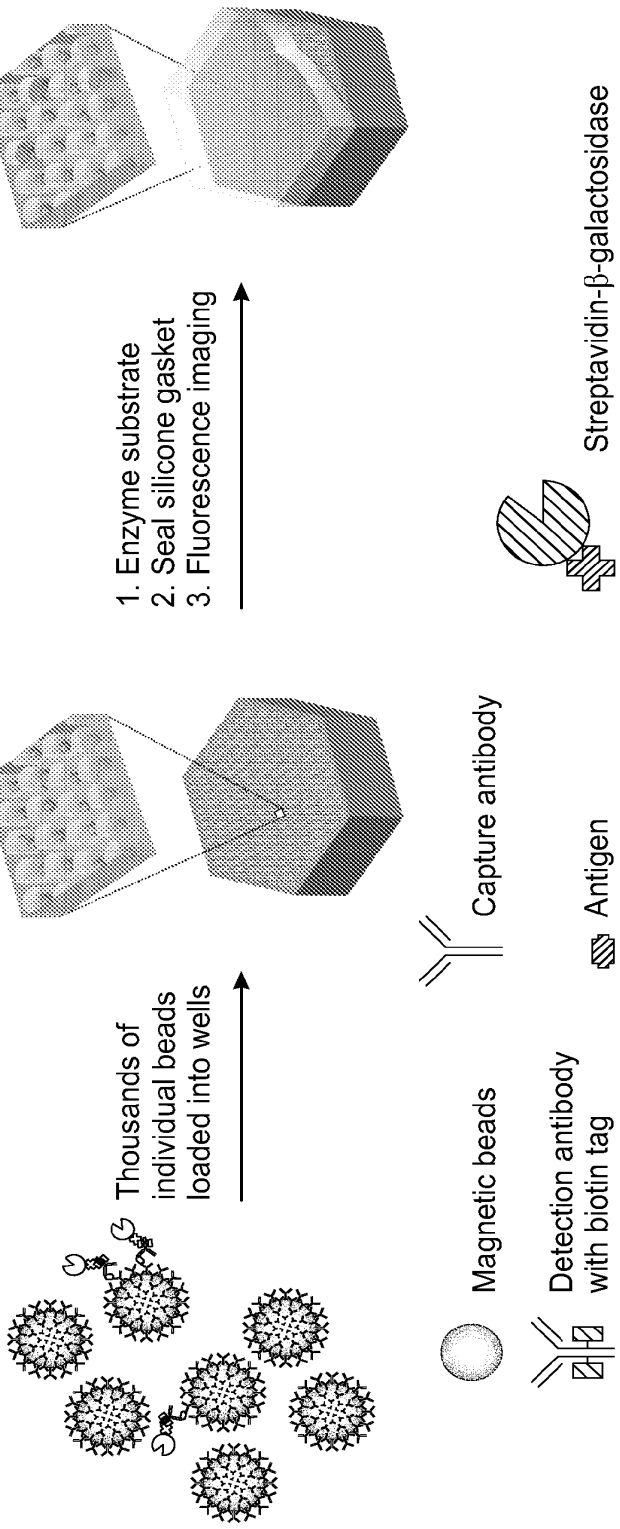


FIG. 25B

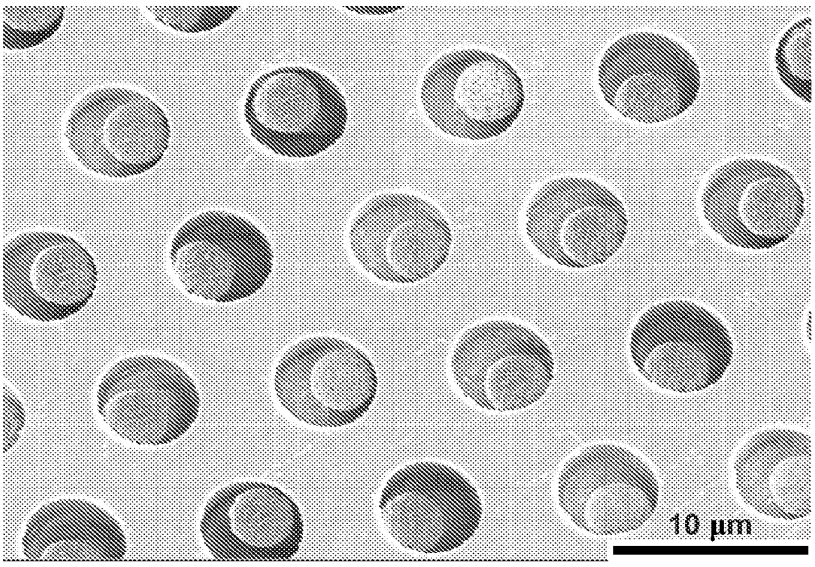


FIG. 25C

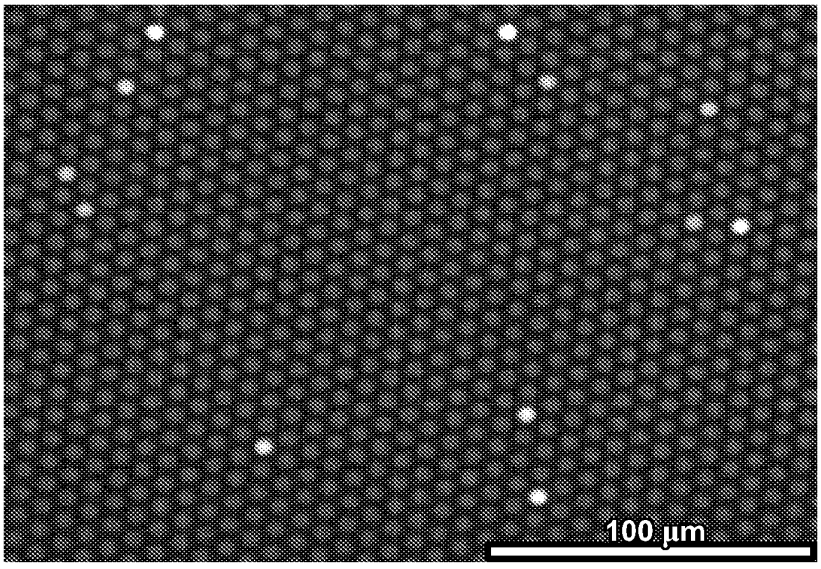


FIG. 25D

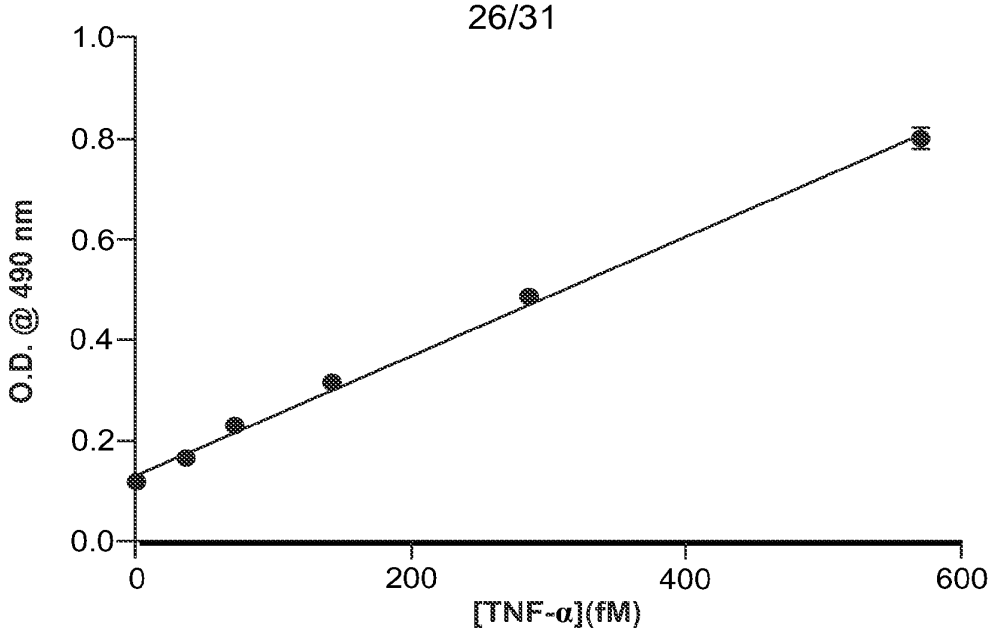

1.0
0.8
0.6
0.4
0.2
0.0
O.D. @ 490 nm
0
200
400
600
[TNF-α](fM)

FIG. 26

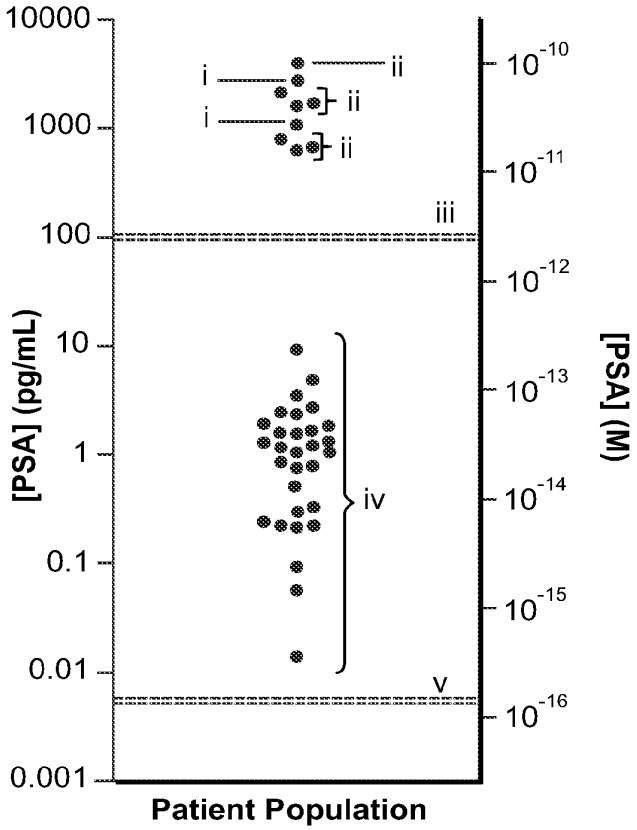

10000
1000
100
10
1
0.1
0.01
0.001
[PSA] (pg/mL)
i
ii
i
ii
ii
iii
iv
v
10^{-10}
10^{-11}
10^{-12}
10^{-13}
10^{-14}
10^{-15}
10^{-16}
[PSA] (M)
Patient Population

FIG. 27

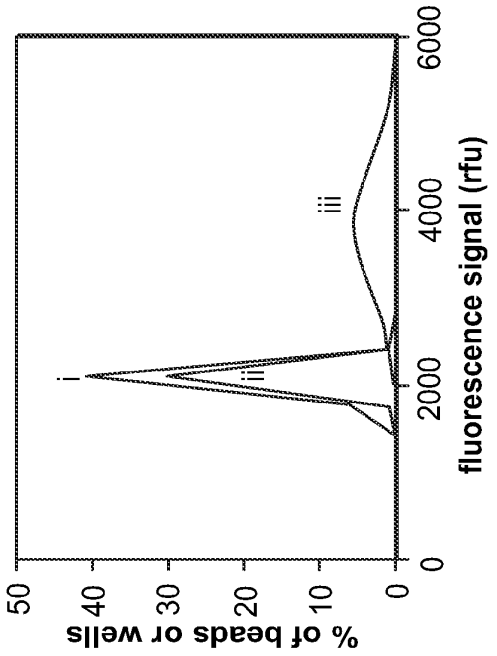


FIG. 28

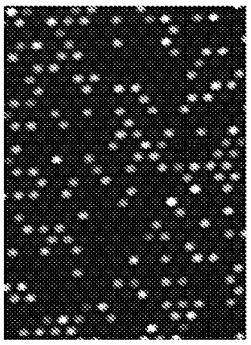


FIG. 29C

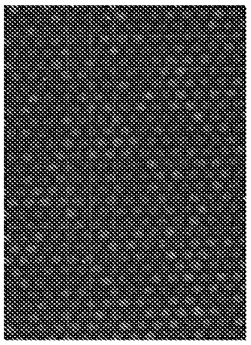


FIG. 29B

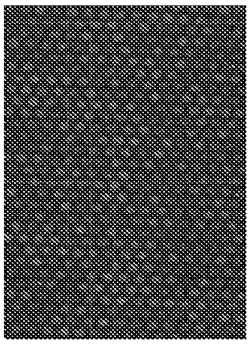


FIG. 29A

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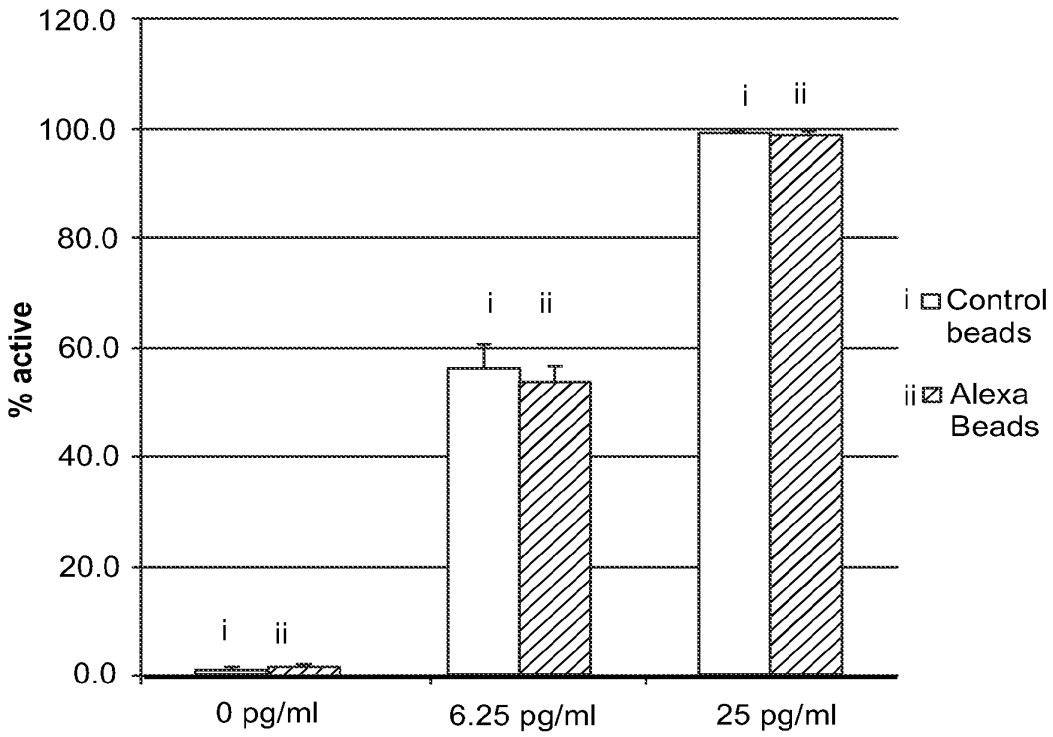


FIG. 29D

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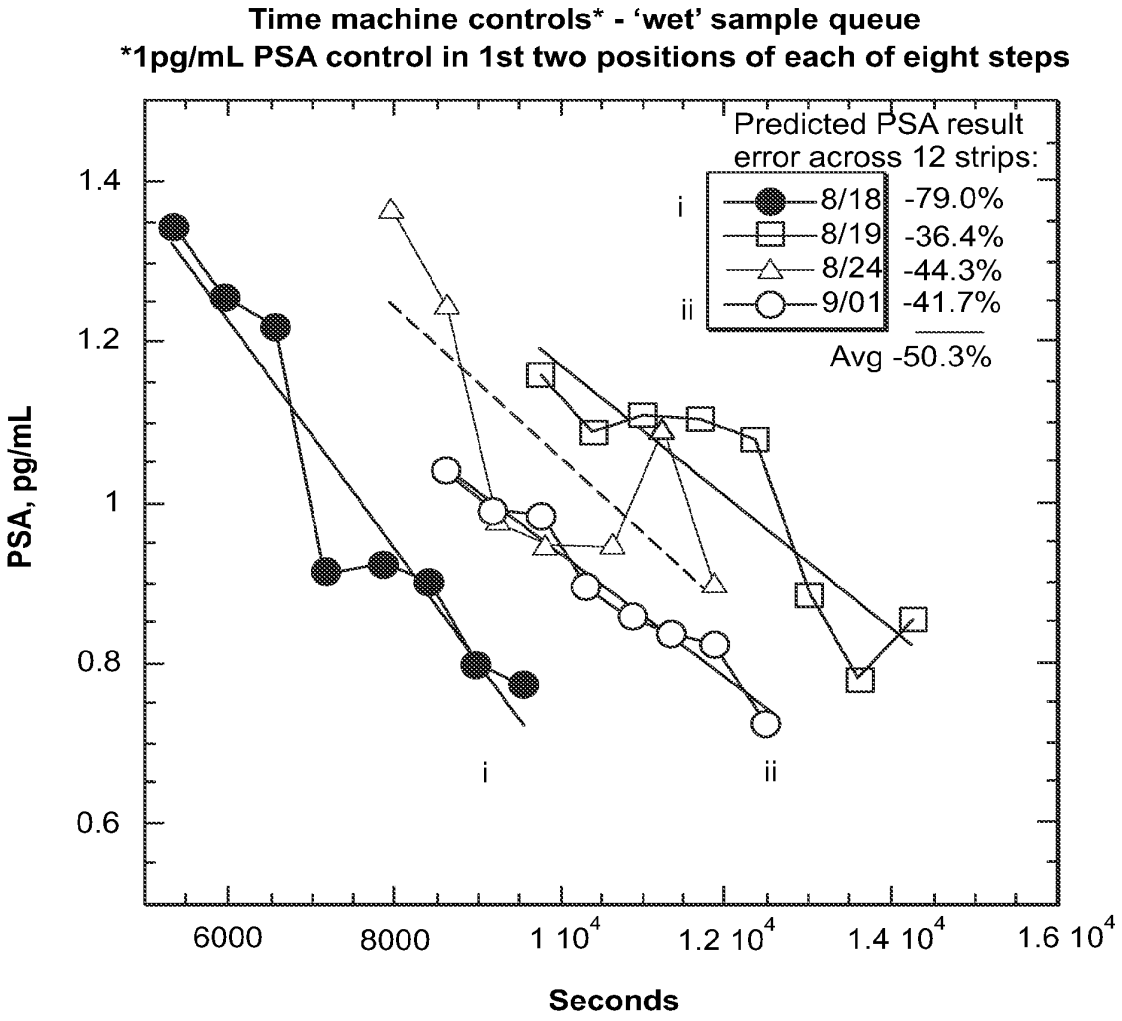


FIG. 30A

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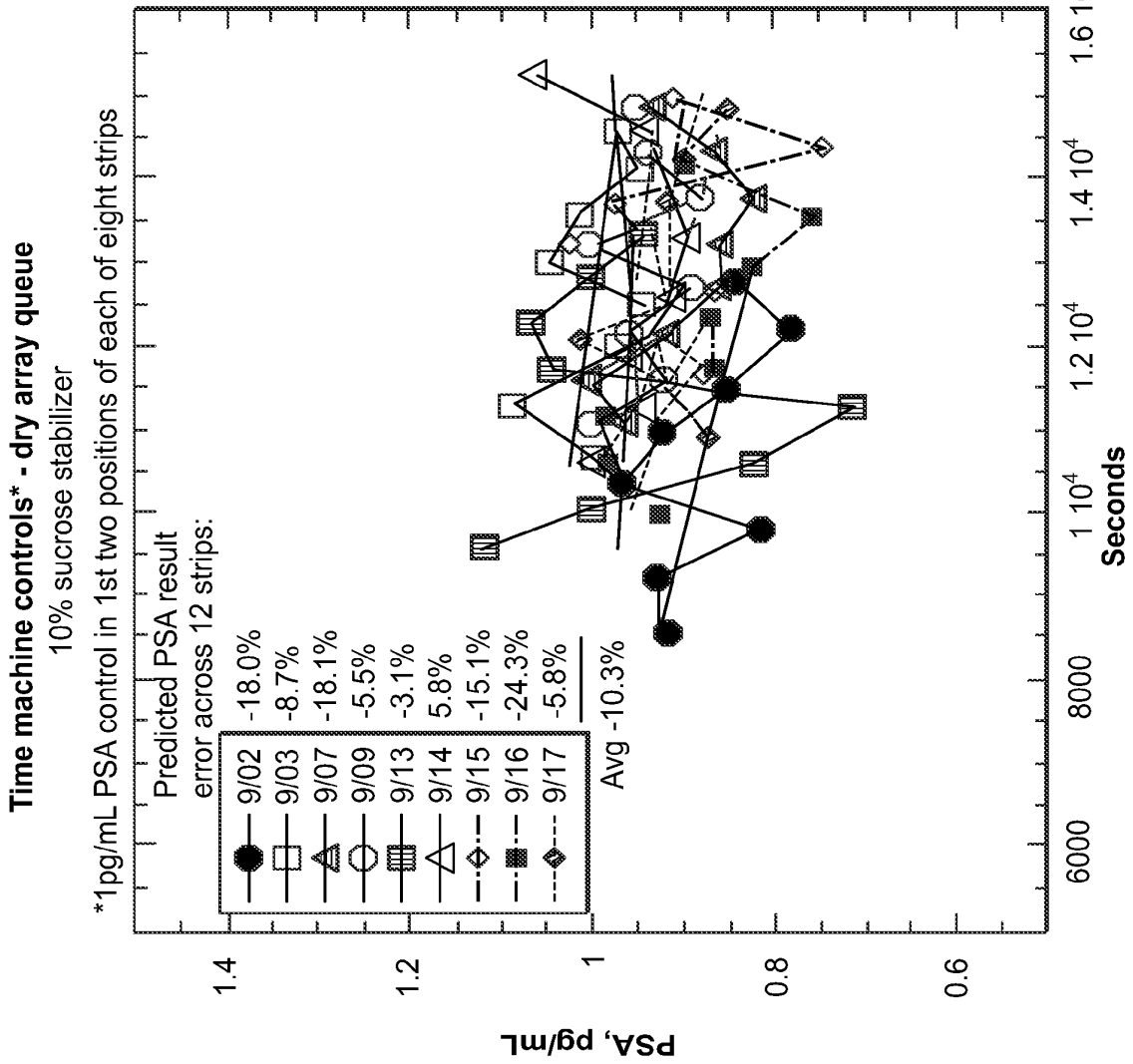


FIG. 30B

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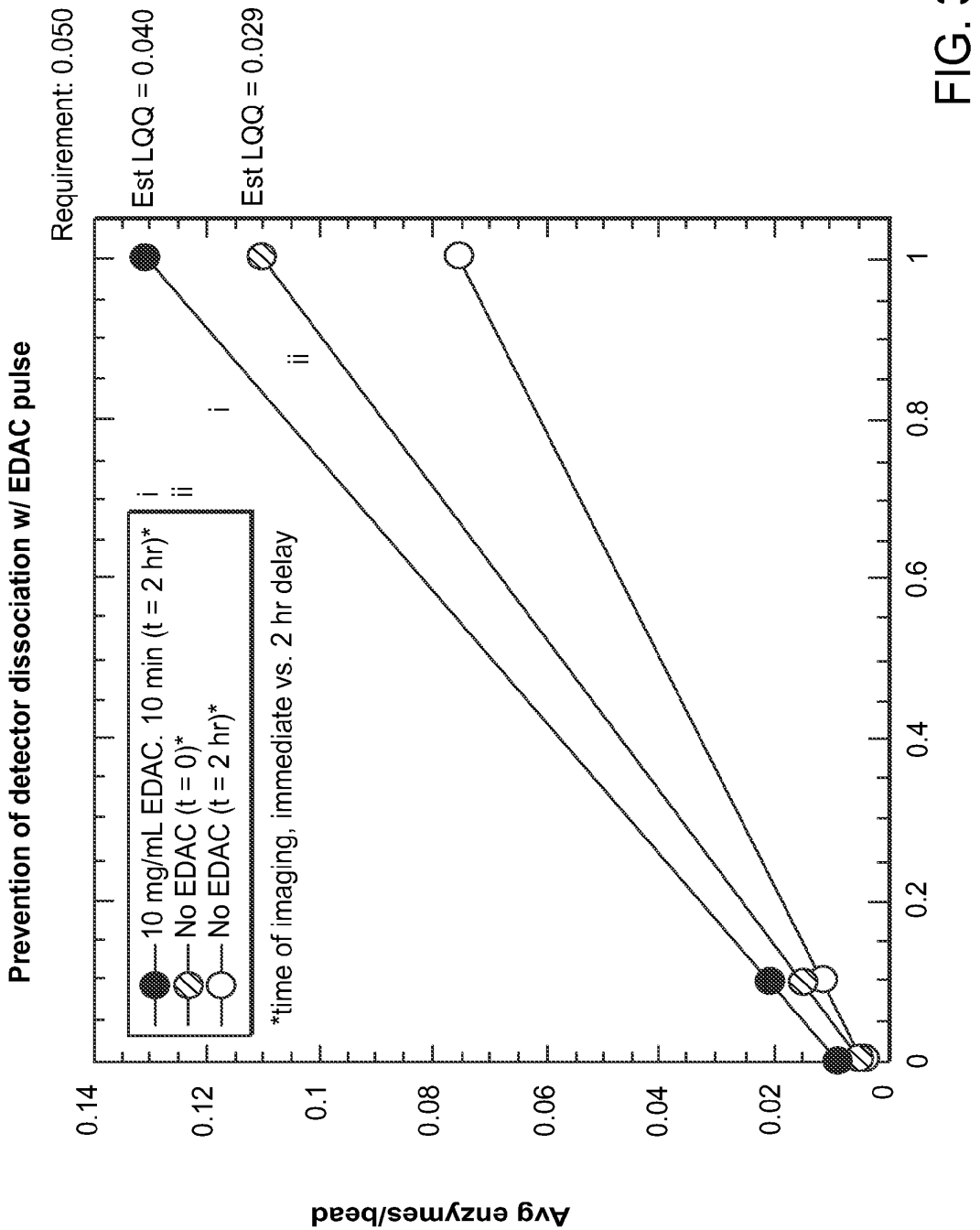

Prevention of detector dissociation w/ EDAC pulse
Requirement: 0.050
Est LQQ = 0.040
Est LQQ = 0.029
10 mg/mL EDAC. 10 min (t = 2 hr)*
No EDAC (t = 0)*
No EDAC (t = 2 hr)*
*time of imaging, immediate vs. 2 hr delay
i
ii
i
ii
Avg enzymes/bead
PSA, pg/mL
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
0.8
1

FIG. 31

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[Continued on next page]

(54) Title: METHODS RELATING TO IMPROVING ACCURACY OF CAPTURE OBJECT-BASED ASSAYS

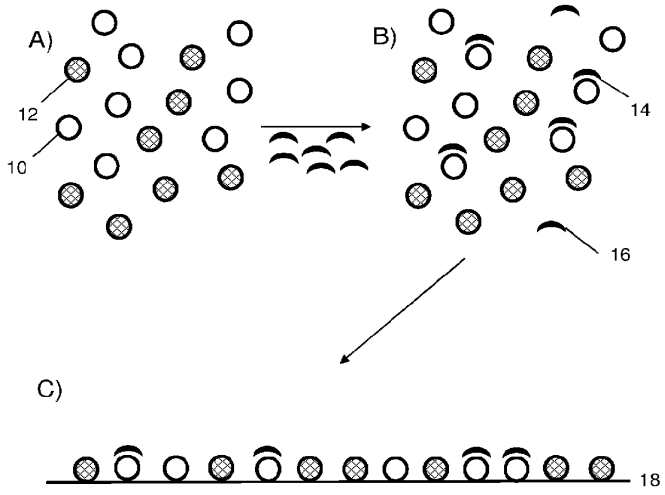


Figure 1

(57) Abstract: Described herein are methods for improving the accuracy of capture object-based assays. In some embodiments, a measure of the number or a measure of the concentration of an analyte molecule or particle in a fluid sample is determined using the capture object-based assay. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

WO 2016/115256 A1

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, KM, ML, MR, NE, SN, TD, TG).

Published:

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METHODS RELATING TO IMPROVING ACCURACY OF CAPTURE OBJECT-BASED ASSAYS

Related Applications

This application claims priority under 35 U.S.C. §119(e) to U.S. provisional application, U.S.S.N. 62/102,818, filed January 13, 2015, entitled "METHODS RELATING TO IMPROVING ACCURACY OF CAPTURE OBJECT-BASED ASSAYS" which is incorporated herein by reference.

Field of the Invention

Described herein are methods for improving the accuracy of capture object-based assays. In some embodiments, a measure of the number and/or a measure of the concentration of an analyte molecule or particle in a fluid sample is determined using the capture object-based assay.

Background of the Invention

The ability to precisely measure target analyte molecules (e.g., proteins) is important in several fields, including clinical diagnostics, testing of blood banks, and the analysis of biochemical pathways. Assays exist for the simultaneous detection of single molecules of target analyte molecules, which may utilize beads or other capture objects (e.g., digital ELISA, see Rissin et al., Nat. Biotechnol. 2010, 28, 595-599, herein incorporated by reference). Certain digital ELISA assays involve capturing proteins on microscopic beads (or other capture objects), labeling the target analytes with an enzyme, isolating the beads in arrays of small wells, and detecting bead-associated enzymatic activity using fluorescence imaging. Spatial localization and/or separation of individual beads, for example in arrays, enables the simultaneous determination of the single molecule signal associated the beads, enabling a measure of the number and/or concentration of the target analyte to be determined at very low values. Various other capture object-based assay have also been developed to determine a measure of the number and/or concentration of analyte molecules in a fluid sample, wherein the analyte molecules are captured on beads or other capture objects. However, there is a continued need for methods and techniques to improve the accuracy and sensitivity of these capture object-based assays.

Summary of the Invention

Described herein are methods for improving the accuracy of capture object-based assays. In some embodiments, a measure of the number and/or the concentration of an analyte molecule or particle in a fluid sample is determined using the capture object-based assay. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample is provided comprising: exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and one or more types of non-targeting capture objects; wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle; wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for any type of analyte molecules or particles contained in or suspected to be contained in the solution; wherein the ratio of the number of first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100; and wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles; spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations; addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object; further determining the number of said locations containing a first type of capture object and a first type of analyte molecule or particle; and determining a measure of the concentration of the first type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object.

In some embodiments, a method for determining a measure of the concentration of a first type of analyte molecules or particles in a fluid sample is provided comprising exposing a plurality of capture objects to a solution containing or suspected of containing the first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and one or more types of non-targeting capture objects; wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte

molecule or particle; wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles; wherein the ratio of the first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100; and wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles; spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations; addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object; further determining the number of said locations containing a first type of capture object and a first type of analyte molecule or particle; and determining a measure of the concentration of only the first type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object.

In some embodiments, a method for determining a measure of the concentration of only a first type and a second type of analyte molecules or particles in a fluid sample is provided comprising exposing a plurality of capture objects to a solution containing or suspected of containing first types of analyte molecules or particles and a second type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object, a second type of capture object, and one or more types of non-targeting capture objects; wherein each of the first type of capture object includes a binding surface having affinity for the first type of analyte molecule or particle; wherein each of the second type of capture object includes a binding surface having affinity for the second type of analyte molecule or particle; and wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having affinity for the first type of analyte molecules or particles or the second type of analyte molecules or particles; wherein the ratio of the first type of capture objects to the total number of capture objects and the ratio of the second type of capture objects to the total number of capture objects are the same or different and are between 1:1.2 and 1:100; wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecule or particle; and wherein at least some of the second type of capture objects associate with at least some of the second type of analyte molecule or particle at least some of the second type of capture objects do not associate with any analyte molecule or particle; spatially separating at least a portion of the capture objects subjected to the exposing steps into a plurality of separate

locations; addressing at least some of the plurality of locations and determining the number of locations containing a first type capture object or a second type of capture object; further determining the number of said locations containing a first type of analyte molecule or particle or a second type of analyte molecule or particle; and determining a measure of the concentration of only the first type of analyte molecules or particles and the second type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object, or based at least in part on the ratio of the number of locations containing a second type of capture object and a second type of analyte molecule and particle, to the number of locations containing a second type of capture object, respectively.

In some embodiments, a method for binding analyte molecules or particles in a fluid sample to capture objects and spatially separating the capture objects is provided comprising exposing a plurality of capture objects to a solution comprising or derived from the fluid sample, wherein the capture objects comprise at least one type of targeting capture objects and at least one type of non-targeting capture objects; wherein each of the at least one type of targeting capture objects includes a binding surface having specific binding affinity for at least one type of target analyte molecule or particle contained in or suspected to be contained in the solution, wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for any of the at least one type of target analyte molecule or particle contained in or suspected to be contained in the solution, wherein the ratio of the number of targeting capture objects to the total number of targeting and non-targeting capture objects is between 1:1.2 and 1:100; spatially separating at least a portion of the plurality of capture objects subjected to the exposing step.

In some embodiments, a method for determining a measure of the concentration of a first type of analyte molecules or particles in a fluid sample is provided comprising exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and a second type of capture object; exposing the plurality of capture objects to a second type of analyte molecules or particles, wherein: each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle; each of the second type of capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles contained in or suspected to be contained in the solution and include at least one binding surface having some affinity for the

second type of analyte molecule or particle; at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles, a statistically significant fraction of the second type of capture objects associate with either zero or one of the second type of analyte molecules or particles, and the ratio of the number of first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100; spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations; addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object; further determining the number of the locations determined to contain a first type of capture object that also contain a first type of analyte molecule or particle; addressing at least some of the plurality of locations and determining which locations contain a second type of capture object and a binding ligand; further determining the average intensity of said locations containing a second type of capture object and a second type of analyte molecule or particle; and determining a measure of the concentration of the first type of analyte molecules or particles in the fluid sample based at least in part on the average intensity of said locations containing a second type of capture object and a second type of analyte molecule or particle.

Brief Description of the Drawings

Figures 1, 2, and 3 illustrate non-limiting examples of methods comprising a plurality of targeting capture objects and a plurality of non-targeting capture objects, according to some embodiments; and

Figure 4 shows plots of the number of beads loaded into a plurality of reaction vessels for a number of samples utilizing A) only targeting beads and b) targeting beads and non-targeting beads.

Other aspects, embodiments, and features of the invention will become apparent from the following detailed description when considered in conjunction with the accompanying drawings. The accompanying figures are schematic and are not intended to be drawn to scale. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. All patent applications and patents mentioned in the text are incorporated by reference in their entirety. In case of conflict between the description contained in the present specification and a document incorporated by reference, the present specification, including definitions, will control.

Detailed Description

Described herein are methods for improving the accuracy of capture object-based assays, wherein a measure of the number and/or a measure of the concentration of analyte molecules or particles in a fluid sample is determined using the capture object-based assays. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles. It should be understood, that while much of the discussion below is directed to assays utilizing beads as the capture objects, this is by way of example only, and other types of capture objects may be employed. In addition, while much of the discussion below is directed to assays utilizing target analyte molecules as the analyte of interest, this is by way of example only, and other types of target analytes may be employed, for example, target analyte particles.

In some embodiments, methods for determining a measure of the number and/or a measure of the concentration of analyte molecules in a fluid sample are provided, wherein the method comprises the steps of associating the analyte molecules or particles with a plurality of capture objects (e.g., beads) and spatially separating at least a portion of the plurality of capture objects. Following spatial separation, a portion of the spatially separated capture objects are addressed to determine a measure of the number of the analyte molecules associated with the portion of the capture objects addressed. In some embodiments, a portion of the spatially separated capture objects are addressed to determine a measure of the number and/or a measure of the concentration of the analyte molecules in the fluid sample.

Without wishing to be bound by theory, the advantages may be caused by a balancing of factors. For examples, in some embodiments, it was determined that the number of capture objects initially added to a fluid sample for target analyte capture could be both advantageous and disadvantageous with respect to changes in sensitivity. For example, when using fewer beads, the ratio of target analyte to capture objects increases, the signal (average target analyte per capture object) increases, and therefore, the assay sensitivity generally increases. However, using fewer capture objects also reduces the number of capture objects that may be spatially separated (e.g., into a plurality of locations), and, if that number drops to a level where Poisson noise becomes significant, then the quantitation of capture objects can become noisy and sensitivity may be decreased. The use of both targeting and non-targeting capture object counters this dilemma by allowing the minimum number of capture objects to be used to increase average target analyte per capture object while keeping the capture loading number as high and/or consistent as possible.

In many embodiments, the methods described herein utilize a combination of targeting capture objects and non-targeting capture objects. For example, in some embodiments, the plurality of capture objects comprise a plurality of types of capture objects, wherein at least one type of capture object is a targeting capture object which includes a binding surface having specific binding affinity for at least one type of target analyte molecule or particle contained in or suspected to be contained in the fluid sample and at least one type of capture object is a non-targeting capture object. In some embodiments, the methods described herein comprising the use of both targeting and non-targeting capture objects provide advantages over previously described methods utilizing only targeting capture objects. For example, in some embodiments, the methods described herein comprising the use of both targeting and non-targeting capture objects may result in a lower coefficient of variation of the number of targeting capture objects determined to be associated with a target analyte as compared to a substantially similar methods utilizing only targeting capture objects.

A non-limiting assay method is depicted in Figure 1. In Step A, a plurality of types of capture objects are provided comprising first type of capture object 10 having specific binding affinity for a first type of target analyte molecule and second type of capture object 12 being non-targeting capture objects. The plurality of capture objects are exposed to a fluid sample comprising the first type of analyte molecule. At least some of the first type of analyte molecules (e.g., 14) associate with a first type of capture object, as shown in Step B. In some embodiments, at least some of the first type of analyte molecules (e.g., 16) do not associate with any capture objects. At least a portion of the capture objects from Step B are then spatially separated, for example, into a plurality of locations. For example, as shown in Step C, a portion of the capture objects are spatially separated by association with surface 18. Other methods for spatially separating the capture objects are described herein. Following spatial separation, a measure of the number of and/or a measure of the concentration of the first type of analyte molecules in the fluid sample may be determined. For example, a portion of the capture objects spatially separated may be addressed to determine the number of the first type of analyte molecule associated with a first type of capture object. In some embodiments, the measure of the concentration of the first type of analyte molecule may be determined at least in part based on the number of the first type of capture object determined to be associated with a first type of analyte molecule and/or the ratio of the number of the first type of capture object associated with a first type of analyte molecule, to the total number of first type of capture objects.

The assay methods described herein may employ a variety of different components, steps, and be used for a variety of different purposes as described herein. Details of certain

exemplary methods and exemplary components utilized by certain of the methods will now be discussed in detail. It should be understood, that none, a portion of, or all of the following steps may be performed at least once during certain exemplary method formats described herein and/or none, a portion, or all of the components may be utilized at least once during certain exemplary method formats described herein. Non-limiting examples of additional steps not described which may be performed include, but are not limited to, washing steps, exposure to additional reagents, and/or various sample/data analysis steps. In some cases, the methods may include the use of at least one binding ligand, as described herein. In some cases, the measure of the concentration of analyte molecules in a fluid sample is based at least in part on comparison of a measured parameter to a calibration curve. In some instances, the calibration curve is formed at least in part by determination at least one calibration factor, as described herein. In some cases, a method may further comprise at least one background signal determination (e.g., and further comprise subtracting the background signal from other determinations).

As used herein, the term targeting capture object refers to a capture object which includes a binding surface having specific binding affinity for at least one type of target analyte molecule or particle contained in or suspected of being contained in a solution to be tested. That is, a targeting capture object is a capture object which includes one or more surfaces which are selected so as to specifically bind a target analyte. The term specific binding affinity, as used herein, refers to the ability of one substance (e.g., capture object) to specifically bind to a first substance (e.g., target analyte) with a high association constant (i.e., $>10^6$ M^{-1}) and with high specificity over other substances (i.e., $<10^6$ M^{-1} association constant other target analytes). In some embodiments, a target analyte may become immobilized with respect to the targeting capture object. As used herein, the term immobilized means captured, attached, bound, or affixed so as to reduce dissociation or loss of the target analyte molecule, but does not require absolute immobility with respect to the capture object.

Those of ordinary skill in the art will be aware of suitable targeting capture objects to be used in the methods described herein. For example, in some cases, the targeting capture object comprises at least one surface comprising a plurality of targeting entities. As used therein, the term "targeting entity" is any molecule or other chemical/biological entity that can be used to impart specific binding affinity for a target molecule or particle (e.g., an analyte molecule), such that the target analyte molecule becomes immobilized with respect to the targeting entity. The immobilization, as described herein, may be caused by the association of an analyte molecule with the targeting entity.

The selection of the targeting entity will depend on the composition of what is being targeted (e.g., the target analyte molecule or particle). Targeting entities for a wide variety of target analyte molecules are known or can be readily found or developed using known techniques. For example, when the target analyte molecule is a protein, the targeting entity may comprise proteins, particularly antibodies or fragments thereof (e.g., antigen-binding fragments (Fabs), Fab' fragments, pepsin fragments, $F(ab')_2$ fragments, full-length polyclonal or monoclonal antibodies, antibody-like fragments, etc.), other native or recombinant proteins, such as receptor proteins, Protein A, Protein G, Protein C, avidin, streptavidin, etc., or small molecules, such as, for example biotin. In some cases, targeting entities for proteins comprise peptides. For example, when the target analyte molecule is an enzyme, suitable targeting entities may include enzyme substrates and/or enzyme inhibitors. In some cases, when the target analyte molecule is a phosphorylated species, the targeting entity may comprise a phosphate-binding agent. In addition, when the target analyte molecule is a single-stranded nucleic acid, the targeting entity may be a complementary nucleic acid. Similarly, the target analyte molecule may be a nucleic acid binding protein and the targeting entity may be a single-stranded or double-stranded nucleic acid; alternatively, the targeting entity may be a nucleic acid-binding protein when the target molecule is a single or double stranded nucleic acid. Also, for example, when the target analyte molecule is a carbohydrate, potentially suitable targeting entity include, for example, antibodies, lectins, and selectins. As will be appreciated by those of ordinary skill in the art, any molecule that can specifically associate with a target analyte molecule of interest may potentially be used as a targeting entity. For certain embodiments, suitable target analyte molecule/targeting entity pairs can include, but are not limited to, antibodies/antigens, antigens/antibodies, receptors/ligands, proteins/nucleic acid, nucleic acids/proteins, nucleic acids/nucleic acids, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins and/or selectins, proteins/proteins, proteins/small molecules; small molecules/small molecules, etc.

In some embodiments, a targeting capture object includes a binding surface having plurality of targeting entities. The portion of the object which comprises a binding surface may be selected or configured based upon the physical shape/characteristics and properties of the objects (e.g., size, shape), and the format of the assay. In some embodiments, substantially all of the outer surfaces of the object comprise a plurality of targeting entities. According to one embodiment, each binding surface of an object comprises a plurality of targeting entities. The plurality of targeting entities, in some cases, may be distributed randomly on the binding surface

like a "lawn." Alternatively, the targeting entities may be spatially separated into distinct region(s) and distributed in any desired fashion or pattern.

As used herein, the term "non-targeting capture object" refers to a capture object which does not include any binding surfaces having specific binding affinity for at least one, some or all of the target analyte molecules or particles contained in or suspected to be contained in a test solution whose amount/concentration is to be determined with an assay that employs such capture objects.. In some embodiments, wherein a first type of analyte molecule is to be detected, the non-targeting capture objects do not include any binding surfaces having specific binding affinity for the first type of target analyte. That is, the non-targeting capture objects do not include any surfaces to which a target analyte would specifically bind, however, such capture objects may include binding surfaces having specific binding affinity for other types of analytes which may be contained or suspected to be contained in the fluid sample. For example, in some embodiments, wherein target molecule B is to be detected, a method may employ non-targeting capture objects A comprising at least one surface comprising targeting entities A which may be utilized to immobilize analyte A. In some embodiments, at least one type of non-targeting capture object does not have specific affinity for a first type of target analyte molecule or particle whose concentration or amount is to be determined with the assay but may include at least one binding surface having specific affinity for a second type of target analyte molecule or particle. In some embodiments, the concentration of the second type of target analyte is not determined. In some embodiments, the second type of target analyte is detected for other reasons (e.g., to calibrate the system, etc.), as described in more detail herein. In other embodiments, wherein a first type of analyte molecule is to be detected, the non-targeting capture objects do not include any binding surfaces having specific binding affinity for any type of analyte molecule or particle contained or suspected of being contained in the fluid sample. For example, in some embodiments, wherein analyte molecules A, B, C, and D are contained or suspected to be contained in the fluid sample, the non-targeting capture objects do not include any binding surfaces having specific binding affinity for analyte molecules A, B, C, or D.

The ratio of the number of each type of targeting capture objects to the total number of targeting and non-targeting capture objects may be any suitable value. In some cases, the ratio may be between 1:1.2 and 1:100, or between 1:2 and 1:100, or between 1:5 and 1:100, or between 1:5 and 1:75, or between 1:5 and 1:50, or between 1:5 and 1:25, or between 1:10 and 1:100, or between 1:10 and 1:75, or between 1:10 and 1:50, or between 1:20 and 1:100, or between 1:25 and 1:100. In embodiments where more than one type of targeting capture object are utilized, the above ratios may apply to each of the types of targeting capture objects. For

example, wherein a first type and a second type of targeting capture objects are utilized, the ratio of the first type of targeting capture object to the total number of capture objects (e.g., the total number of the first type of capture object, the second type of capture object, and all non-targeting capture objects) and the ratio of the second type of targeting to the total number of capture objects may be between 1:1.2 and 1:100, or between 1:2 and 1:100, or between 1:5 and 1:100, or between 1:5 and 1:75, or between 1:5 and 1:50, or between 1:5 and 1:25, or between 1:10 and 1:100, or between 1:10 and 1:75, or between 1:10 and 1:50, or between 1:20 and 1:100, or between 1:25 and 1:100.

Additional non-limiting methods comprising the use of both targeting and non-targeting capture objects will now be described in detail.

In a first non-limiting embodiment, methods for determining a first type of target analyte are provided, wherein the method comprising non-targeting capture objects that do not have any affinity for other molecules in the solution are provided. In some embodiments, a method for determining a measure of the number and/or the concentration of analyte molecules or particles in a fluid sample (or a solution derived from the fluid sample) comprises exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the plurality of capture objects comprise a first type of capture object and one or more types of non-targeting capture objects. In this non-limiting method, each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle and each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for any type of analyte molecules or particles contained in or suspected to be contained in the solution. That is, at least a portion of the capture object contain a binding surface having specific binding affinity to the first type of analyte molecule or particle (e.g., targeting capture objects for the first type of analyte molecule or particle) and at least a portion of the capture objects do not contain any binding surfaces having specific binding affinity for any molecules or particles contained in the fluid sample (e.g., the first type of analyte molecule and any other molecules known or suspected to be contained in the fluid sample). For example, in embodiments where fluid sample contains or is suspected to contain two types of analyte molecules (e.g., analyte molecules A and B), the non-targeting capture objects would not include any binding surfaces having specific binding affinity for either type of analyte molecule (e.g., analyte molecules A and B). The ratio of the first type of capture object to the total number of capture objects (e.g., first type of capture object and any non-targeting capture objects) may be between 1:1.2 to 1:100, or between 1.5 to 1:100, or any other ratio described herein. Following and/or during the

exposing step, at least some of the first type of capture objects associate with at least one analyte molecule or particle while in certain cases at least a some of the first type of capture objects do not associate with any analyte molecules or particles. At least a portion of the capture objects which were subjected to the exposing step may then be spatially separated, for example, into a plurality of separate locations, and analyzed to determine the total number of the first type of capture objects associated with a first type of analyte molecule or particle. In some embodiments, the number of locations containing a first type of capture object (e.g., whether associated or not associated with a first type of capture object) and the number of locations containing a first type of capture object associated with a first type of analyte molecule are both determined. The number of locations which contain a non-targeting capture object may or may not be determined. In embodiments where a measure of the concentration of the first type of target analyte in a fluid sample is to be determined, the measure of the concentration of the first type of target analyte may be determined based at least in part on the ratio of the number of locations containing a first type of capture object associated with a first type of analyte molecule to the total number of locations containing a first type of capture object, as described herein.

This first non-limiting embodiment may be described with reference to Figure 1. In Step A, a plurality of types of capture objects are provided comprising first type of capture object 10 having specific binding affinity of a first type of target analyte molecule and second type of capture object 12 being non-targeting capture objects. Each of first type of capture object 10 includes a binding surface having specific binding affinity for the first type of analyte molecule or particle and each of the second type of capture object 12 does not include any binding surfaces having specific binding affinity for any type of analyte molecules or particles contained in or suspected to be contained in the solution (e.g., the first type of analyte molecule and any other molecules known or suspected to be contained in the fluid sample). The capture objects are exposed to a fluid sample comprising the first type of analyte molecule and at least some of the analyte molecules (e.g., 14) associate with a first type of capture object, as shown in Step B. In some embodiments, at least some of the analyte molecules (e.g., 16) do not associate with any capture objects. At least a portion of the capture objects from Step B are then spatially separated, for example, into a plurality of locations. For example, as shown in Step C, a portion of the capture objects are spatially separated by association with surface 18. Other methods for spatially separating the capture objects are described herein. Following spatial separation, a measure of the number and/or a measure of the concentration of the analyte molecules in the fluid sample may be determined. In some embodiments, at least some of the plurality of capture objects subjected to the spatially separating are addressed to determine the number of locations

containing a first type of capture object (e.g., whether associated or not associated with a first type of analyte) and/or to determine the number of locations containing a first type of capture object associated with a first type of analyte molecule. The number of locations containing a non-targeting capture object may or might not be determined. In embodiments where a measure of the concentration of the first type of target analyte in a fluid sample is to be determined, the measure of the concentration of the first type of target analyte may be determined at least in part on the ratio of the number of locations containing a first type of capture object associated with a first type of analyte molecule to the total number of locations containing a first type of capture object.

It should be understood, that for the first embodiment described above, more than one type of analyte molecule may be determined. For example, a first type and a second type of analyte molecule may be determined. A non-limiting example of such a method is depicted in Figure 2. In Step A, a plurality of types of capture objects are provided comprising a first type of capture object 30 having specific binding affinity for a first type of target analyte molecule, a second type of capture object 32 having specific binding affinity for a second type of target analyte molecule, and a third type of capture object 34 being non-targeting capture objects. Each of the first type of capture object 30 includes a binding surface having specific binding affinity for the first type of analyte molecule or particle, each of the second type of capture object 32 includes a binding surface having specific binding affinity for the second type of analyte molecule or particle, and each of the third type of capture object 34 do not include any binding surfaces having specific binding affinity for the first type or the second type of analyte molecules or particles contained in or suspected to be contained in the solution. The capture objects are exposed to a fluid sample comprising the first type of analyte molecule and the second type of analyte molecule. As shown in Step B, at least some of the first type of analyte molecules (e.g., 36) associate with a first type of capture object and at least some of the second type of analyte molecules (e.g., 38) associate with a second type of capture object. In some embodiments, at least some of the first type and the second type analyte molecules (e.g., 40 and 42, respectively) do not associate with any capture objects. At least a portion of the capture objects from Step B are then spatially separated, for example, in to a plurality of locations. For example, as shown in Step C, a portion of the capture objects are spatially separated by association with surface 44.

Other methods for spatially separating the capture objects are described herein. Following spatial separation, a measure of the number of and/or a measure of the concentration of the first type and/or the second type analyte molecules in the fluid sample may be determined.

In some embodiments, at least some of the plurality of capture objects subjected to the spatial separation step are addressed to determine the number of location containing a first type of capture object (e.g., whether associated or not associated with a first type of target analyte), the number of location containing a second type of capture object (e.g., whether associated or not associated with a second type of target analyte), the number of locations containing a first type of capture object associated with a first type of analyte molecule, and the number of locations containing a second type of capture object associated with a second type of analyte molecule. In embodiments where a measure of the concentration of the first type of target analyte in the fluid sample is to be determined, the measure of the concentration of the first type of target analyte may be determined at least in part on the ratio of the number of locations containing a first type of capture object associated with a first type of analyte molecule to the total number of locations containing a first type of capture object. In embodiments where a measure of the concentration of the second type of target analyte in the fluid sample is to be determined, the measure of the concentration of the second type of target analyte may be determined at least in part on the ratio of the number of locations containing a second type of capture object associated with a second type of analyte molecule to the total number of locations containing a second type of capture object. Those of ordinary skill in the art will be able to apply these teachings to methods for determining more than two types of target analytes, for example, three types, or four types, or more.

In a second non-limiting embodiment, methods for determining only a first type a target analyte are provided, wherein the method comprises non-target capture objects which may or might not have affinity for other molecules known or suspected to be present in the solution. For example, in some embodiments, a method for determining a measure of the concentration of only a first type of analyte molecules or particles in a fluid sample are provided. In some cases, the method comprises exposing a plurality of capture objects to a solution containing or suspected of containing the first type of analyte molecules or particles. In this embodiment, the capture objects comprise a first type of capture object and one or more types of non-targeting capture objects; wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle and each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles. Accordingly, in this embodiment, the one or more types of non-targeting capture objects may optionally have a binding surface which has specific binding affinity for another type of molecule or particles contained or suspected to be contained in the fluid sample, but do not have specific binding

affinity for the first type of analyte molecule being assayed. The ratio of the first type of capture objects to the total number of capture objects may be between 1:1.2 and 1:100, or between 1:5 and 1:100 or any ratio described herein. Following and/or during the exposing step, at least some of the first type of capture objects associate with at least one first type of analyte molecule or particle and at least some of the first type of capture objects may not associate with any of the first type of analyte molecules or particles. Similar steps as described in the previous exemplary embodiment may then be carried out using the capture objects subjected to the exposing step. For example, in some embodiments, at least a portion of the plurality of capture objects subjected to the exposing step are spatially separated, for example, into a plurality of separate locations. At least some of the plurality of locations may be addressed to determine the number of locations containing a first type of capture object and/or the number of locations containing a first type of analyte molecule associated with a first type of capture object. Furthermore, a measure of the concentration of only the first type of analyte molecules or particles in the fluid sample may be determined based at least in part on the ratio of the number of locations containing a first type of capture object associated with a first type of analyte molecule and particle, to the number of locations containing a first type of capture object.

As a specific non-limiting example of the second non-limiting embodiment, methods for determining a first type a target analyte may comprise use of at least one type of non-target capture object which has affinity for a second type of target analyte. In some embodiments, the concentration of the second type of target analyte is not determined. In some embodiments, the second type of target analyte may be detected for other reasons. In some embodiments, the second type of target analyte is a binding ligand. The binding ligand may be utilized to determine the presence or absence of a first type of target analyte. In other embodiments, the second type of target analyte may be a non-target molecule which is known or suspected to be present in the solution, but for which the unknown concentration is not required to be determined. For example, the non-target molecules may be used to calibrate or otherwise provide information to be used in the assay, but the concentration of the non-target molecules need not be determined. In some cases, the method comprises exposing a plurality of capture objects to a solution containing or suspected of containing the first type of analyte molecules or particles. In this non-limiting embodiment, the capture objects comprise a first type of capture object and at least one type of non-targeting capture objects; wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle and at least one type of non-targeting capture object that includes a

binding surface does not have specific binding affinity for the first type of analyte molecules or particles but has specific binding affinity for a non-target molecule. This non-limiting embodiments is depicted in Figure 3. In Step A, a plurality of types of capture objects are provided comprising first type of capture object 10 having specific binding affinity for a first type of target analyte molecule and second type of capture object 12 being non-targeting capture objects having specific affinity for a second type of target analyte. In this embodiment, the second type of target analyte is a binding ligand used in the detection of the first type of analyte molecule. In addition, the concentration of the second target analyte molecule is not determined in this assay, but rather the second target analyte is detected for other purposes. In this non-limiting example, the second type of target analyte is used to assist in or facilitate the concentration determination of the first type of analyte molecule. The plurality of capture objects are exposed to a fluid sample comprising the first type of analyte molecule. At least some of the first type of analyte molecules (e.g., 14) associate with a first type of capture object, as shown in Step B. In some embodiments, at least some of the first type of analyte molecules (e.g., 16) do not associate with any capture objects. The plurality of capture objects are exposed to a fluid sample comprising the second type of analyte molecule, e.g., a binding ligand having affinity for the first type of analyte molecule. At least some of the first type of analyte molecules associated with a first type of capture object also associate with a binding ligand (e.g., 20), at least some of the second type of capture object associate with the binding ligand (e.g., 22), as shown in Step C. In some embodiments, at least some of the second type of analyte molecules (e.g., 24) do not associate with any capture objects. At least a portion of the capture objects from Step C are then spatially separated, for example, into a plurality of locations. For example, as shown in Step D, a portion of the capture objects are spatially separated by association with surface 18. Other methods for spatially separating the capture objects are described herein. Following spatial separation, a measure of the number of and/or a measure of the concentration of the first type of analyte molecules in the fluid sample may be determined. For example, a portion of the capture objects spatially separated may be addressed to determine the number of the first type of analyte molecule associated with a first type of capture object via detection of the binding ligand. In addition, the number of locations containing a second type of capture object and a binding ligand may also be determined. In some cases, the average intensity of those locations may be determined, and the measure of the concentration of the first type of analyte molecule may be determined at least in part based on the average intensity.

As would be understood by one of ordinary skill in the art, and as described herein in more detail elsewhere, the analyte molecules and/or binding ligands may be directly detectable

or indirectly detectable. In some embodiments in which the binding ligand is indirectly detectable, the binding ligand comprises an enzymatic component and/or may be further exposed to a secondary binding ligand comprising an enzymatic component. The binding ligand (or secondary binding ligand) may then be exposed to an precursor labeling agent (e.g., an enzymatic substrated) which is converted to a labeling agent (e.g., a detectable product) upon exposure to the enzymatic component.

Similar to the method described above in the second non-limiting embodiment, described below is a third non-limiting embodiment involving a method for determining a measure of the concentration of only a first type and a second type of analyte molecules or particles in a fluid sample. In this non-limiting embodiment, a plurality of capture objects are exposed to a solution containing or suspected of containing a first type of analyte molecules or particles and a second type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object, a second type of capture object, and one or more types of non-targeting capture objects. Each of the first type of capture object includes a binding surface having affinity for the first type of analyte molecule or particle, each of the second type of capture object includes a binding surface having affinity for the second type of analyte molecule or particle, and each of the one or more types of non-targeting capture objects do not include any binding surfaces having affinity for the first type of analyte molecules or particles or the second type of analyte molecules or particles. Accordingly, in this embodiment, the one or more types of non-targeting capture objects may have a binding surface which optionally has specific binding affinity for another type of analyte molecule or particles contained or suspected to be contained in the fluid sample, but do not have specific binding affinity for the first type or the second type of analyte molecule or particle. For example, at least one of the one or more types of non-targeting capture object may have a binding surface which has specific binding affinity for a non-target molecule (e.g., a molecule for which the concentration of the non-target molecule is not required to be determined for the assay and/or wherein the non-target molecule is used for another purpose (e.g., to calibrate the system/assay, etc.)). The ratio of the first type of capture objects to the total number of capture objects and the ratio of the second type of capture objects to the total number of capture objects are the same or different and may be between 1:1.2 and 1:100, or any ratio described herein. Following and/or during exposure, at least some of the first type of capture objects associate with at least one analyte molecule or particle while at least some of the first type of capture objects may not associate with any analyte molecule or particle, and at least some of the second type of capture objects associate with at least some of the second type of

analyte molecule or particle while at least some of the second type of capture objects may not associate with any analyte molecule or particle. Similar steps as described in the above described two exemplary embodiments may then be carried out using the capture objects subjected to the exposing step. For example, at least a portion of the capture objects subjected to the exposing steps may be spatially separated into a plurality of separate locations. At least a portion of the plurality of locations may be addressed to determine the number of locations containing a first type of capture object, the number of locations containing a first type of capture object and a first type of analyte molecule or particle, the number of locations containing a second type of analyte molecule or particle, and/or the number of locations containing a second type of analyte molecule or particle and a second type of capture object. Optionally, a measure of the concentration of the first type of analyte molecules or particles and the second type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object associated with a first type of analyte molecule and particle, to the number of locations containing a first type of capture object, or based at least in part on the ratio of the number of locations containing a second type of capture object associated with a second type of analyte molecule and particle, to the number of locations containing a second type of capture object, respectively. Those of ordinary skill in the art will be able to apply these teachings to methods for determining more than two types of target analytes, for example, three types, or four types, or more.

Generally, the plurality of capture objects are configured to be able to be spatially separated from each other. In some embodiment, the capture objects may be provided in a form such that the capture objects are capable of being spatially separated into a plurality of locations. For example, the plurality of capture objects may comprise a plurality of beads (which can be of any shape, e.g., sphere-like, disks, rings, cube-like, etc.), a dispersion or suspension of particulates (e.g., a plurality of particles in suspension in a fluid), nanotubes, or the like. In some embodiments, the plurality of capture objects is insoluble or substantially insoluble in the solvent(s) or solution(s) utilized in an assay. In some cases, the capture objects are non-porous solids or substantially non-porous solids (e.g., essentially free of pores); however, in some cases, the plurality of capture objects may be porous or substantially porous, hollow, partially hollow, etc. The plurality of capture objects may be non-absorbent, substantially non-absorbent, substantially absorbent, or absorbent. In some cases, the capture objects may comprise a magnetic material, which may facilitate certain aspect of an assay (e.g., washing step).

The plurality of capture objects may be of any suitable size or shape. Non-limiting examples of suitable shapes include spheres, cubes, ellipsoids, tubes, sheets, and the like. In

certain embodiments, the average diameter (if substantially spherical) or average maximum cross-sectional dimension (for other shapes) of a capture object may be greater than about 0.1 um (micrometer), greater than about 1 um, greater than about 10 um, greater than about 100 um, greater than about 1 mm, or the like. In other embodiments, the average diameter of a capture object or the maximum dimension of a capture object in one dimension may be between about 0.1 um and about 100 um, between about 1 um and about 100 um, between about 10 um and about 100 um, between about 0.1 um and about 1 mm, between about 1 um and about 10 mm, between about 0.1 um and about 10 um, or the like. The "average diameter" or "average maximum cross-sectional dimension" of a plurality of capture objects, as used herein, is the arithmetic number average of the diameters/maximum cross-sectional dimensions of the capture objects. Those of ordinary skill in the art will be able to determine the average diameter/maximum cross-sectional dimension of a population of capture objects, for example, using laser light scattering, microscopy, sieve analysis, the Coulter effect, or other known techniques. For example, in some cases, a Coulter counter may be used to determine the average diameter of a plurality of beads.

In a particular embodiment, the objects comprise a plurality of beads. The beads may each comprise a plurality of targeting entities associated with at least a portion of each bead. In some embodiments, the beads are magnetic. The magnetic property of the beads may help in separating the beads from a solution and/or during washing step(s). Potentially suitable beads, including magnetic beads, are available from a number of commercial suppliers.

The capture objects may be fabricated from one or more suitable materials, for example, plastics or synthetic polymers (e.g., polyethylene, polypropylene, polystyrene, polyamide, polyurethane, phenolic polymers, or nitrocellulose etc.), naturally derived polymers (latex rubber, polysaccharides, polypeptides, etc.), composite materials, ceramics, silica or silica-based materials, carbon, metals or metal compounds (e.g., comprising gold, silver, steel, aluminum, copper, etc.), inorganic glasses, silica, and a variety of other suitable materials.

In some embodiments, each of the types of capture objects may be detectable. This property may be useful in embodiments where the fraction or percentage of capture objects associated with an analyte molecule is to be determined (e.g., when the total number of capture objects interrogated and detected is used to determine the fraction of capture objects associated with an analyte molecule). In a specific embodiment, the capture objects are detectable optically. For example, the location of a capture object may be detected by identifying the optical signature of the object by a conventional optical train and optical detection system. Depending on the optical signature and the operative wavelengths, optical filters designed for a particular

wavelength may be employed for optical interrogation of the locations. For example, a capture object may be characterized as having an emission or absorption spectrum that can be exploited for detection so that capture objects may be interrogated to determine which spatial location contains a capture object. The properties of the emission spectrum (e.g., wavelength(s), intensity, etc.), may be selected such that the emission produced by the capture objects does not substantially alter and/or interfere with any other emission from components used in the assay (e.g., the emission of any labels used to determine the presence or absence of an analyte molecule). In some cases, dye molecules may be associated with a capture object.

Each type of capture object may be encoded to be distinguishable from each other (e.g., to facilitate differentiation upon detection) by including a differing detectable property. For example, each type of capture object may have a differing fluorescence emission, a spectral reflectivity, shape, a spectral absorption, or an FTIR emission or absorption. In a particular embodiment, each type of capture object may comprise one or more dye compounds (e.g., fluorescent dyes) but at varying concentration levels, such that each type of capture object has a distinctive signal (e.g., based on the intensity of the fluorescent emission). Generally, the non-targeting capture objects are distinguishable from the targeting capture objects, and each type of targeting capture object is distinguishable from the other types of targeting capture objects.

Those of ordinary skill in the art will be aware of methods and techniques for exposing a plurality of capture objects to a fluid sample or a solution derived from the fluid sample containing or suspected of containing at least one type of analyte molecule or particle for initial analyte capture. For example, the plurality of capture objects may be added (e.g., as a solid, as a solution) directly to a fluid sample. As another example, the fluid sample may be added to the plurality of capture objects (e.g., in solution, as a solid). In some instances, the solutions may be agitated (e.g., stirred, shaken, etc.).

The plurality of capture objects, subsequent to the exposing step (e.g., at least some capture objects are associated with at least one analyte molecule), may be exposed to one or more additional reagents, prior to spatially separating the plurality of capture objects (e.g., into a plurality of locations). For example, the capture objects may be exposed a plurality of binding ligands, at least some of which may associate with an immobilized analyte molecule. The capture objects may be exposed to more than one type of binding ligand (e.g., a first type of binding ligand and a second, third, etc. type of a binding ligand), as noted above. The association of a binding ligand with an immobilized analyte molecule may aid in the detection of the analyte molecules, as described herein. Additional details are described herein.

As described above, following immobilization of a plurality of analyte molecules with respect to the plurality of capture objects in the analyte capture step, at least a portion of the capture objects may be spatially separated into a plurality of locations, for example on a substrate. For example, each of capture objects of the portion of capture objects which are spatially separated may be positioned in and/or associated with a location (e.g., a spot, region, well, etc. on the surface and/or in the body of a substrate) that spatially distinct from the locations in which each of the other capture objects are located, such that the capture objects and locations can be individually resolved by an analytical detection system employed to address the locations. As an example, each of a portion of the capture objects may be spatially separated into an array of reaction vessels on a substrate, such that statistically only zero or one capture objects are located in at least some of the reaction vessels and in certain cases in essentially each reaction vessel. Each location may be individually addressable relative to the other locations. Additionally, in some embodiments, the locations may be arranged such that a plurality of locations may be addressed substantially simultaneously, as described herein, while still permitting the ability to resolve individual locations and capture objects. While exemplary embodiments for spatially separating a plurality of capture objects into a plurality of locations are described herein, numerous other methods may potentially be employed.

It should be understood, that while much of the discussion herein focusing on locations containing a single capture object, this is by no means limiting, and in some embodiments, more than one capture object may be contained at a single location. In such embodiments, the ratio of capture objects to analyte molecules may be such that following spatial separation of the plurality of capture objects into the plurality of locations, a statistically significant fraction of the locations contain no analyte molecules and a statistically significant fraction of locations contain at least one analyte molecule. That is, while a single location may contain a plurality of capture objects, in some cases, none of the capture objects are associated with any analyte molecules and only a single one of the capture objects in an addressed location is associated with at least one analyte molecule.

In embodiments wherein the plurality of locations comprise a plurality of reaction vessels, the plurality of capture objects may be spatially separated into the plurality of reaction vessels using any of a wide variety of techniques known to those of ordinary skill in the art. In some cases, the plurality of reaction vessels may be exposed to a solution containing the plurality of capture objects. In some embodiments, the plurality of reactions vessels may be exposed to a solution containing the plurality of capture objects using microfluidic techniques (e.g., see U.S. Publication No. 2011/0212848, by Duffy et al., filed March 24, 2010; and

International Publication No. WO2011/109364, by Duffy et al., filed March 1, 2011, herein incorporated by reference). In some cases, time may elapsed following exposure of the reaction vessels to the solution to allow for the capture objects enter the wells (e.g., no applied force, gravity only). In some cases, force may be applied to the solution and/or capture objects, thereby aiding in the spatial separation of the capture objects from the fluid phase and/or the deposition of the capture objects in the vessels. For example, after application of an assay solution containing the capture objects to a substrate containing the reaction vessels, the substrate and solution may be centrifuged to assist in depositing the capture objects in the reaction vessels. In embodiments where the capture objects (e.g., beads) are magnetic, a magnet may be used to aid in containing the capture objects in the reaction vessels. In some cases, when the plurality of reaction vessels is formed on the end of a fiber optic bundle (or another planar surface), a material (e.g., tubing) may be placed around the edges of the surface of the array comprising the plurality of reaction vessel to form a container to hold the solution in place while the capture objects settle in the reaction vessels or are placed into the reaction vessels (e.g., while centrifuging). Following placement of the capture objects into at least some of the reaction vessels, the surrounding material may be removed and the surface of the array may be washed and/or swabbed to remove any excess solution/capture objects.

In some embodiments, the reaction vessels may all have approximately the same volume. In other embodiments, the reaction vessels may have differing volumes. The volume of each individual reaction vessel may be selected to be appropriate to facilitate any particular assay protocol. For example, in one set of embodiments where it is desirable to limit the number of capture objects used for analyte capture contained in each vessel to a small number, the volume of the reaction vessels may range from attoliters or smaller to nanoliters or larger depending upon the nature of the capture objects, the detection technique and equipment employed, the number and density of the wells on the substrate and the expected concentration of capture objects in the fluid applied to the substrate containing the wells. In one embodiment, the size of the reaction vessel may be selected such only a single capture object used for analyte capture can be fully contained within the reaction vessel (see, for example, U.S. Publication No. 2011/0212848, by Duffy et al., filed March 24, 2010; and International Publication No. WO2011/109364, by Duffy et al., filed March 1, 2011, herein incorporated by reference).

In accordance with one embodiment of the present invention, the reaction vessels may have a volume between about 1 femtoliter and about 1 picoliter, between about 1 femtoliters and about 100 femtoliters, between about 10 attoliters and about 100 picoliters, between about 1 picoliter and about 100 picoliters, between about 1 femtoliter and about 1 picoliter, or between

about 30 femtoliters and about 60 femtoliters. In some cases, the reaction vessels have a volume of less than about 1 picoliter, less than about 500 femtoliters, less than about 100 femtoliters, less than about 50 femtoliters, or less than about 1 femtoliter. In some cases, the reaction vessels have a volume of about 10 femtoliters, about 20 femtoliters, about 30 femtoliters, about 40 femtoliters, about 50 femtoliters, about 60 femtoliters, about 70 femtoliters, about 80 femtoliters, about 90 femtoliters, or about 100 femtoliters.

The total number of locations and/or density of the locations employed in an assay (e.g., the number/density of reaction vessels in an array) can depend on the composition and end use of the array. For example, the number of reaction vessels employed may depend on the number of types of analyte molecule and/or binding ligand employed, the suspected concentration range of the assay, the method of detection, the size of the capture objects, the type of detection entity (e.g., free labeling agent in solution, precipitating labeling agent, etc.). Arrays containing from about 2 to many billions of reaction vessels (or total number of reaction vessels) can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple types of analyte molecules to be assayed in parallel. The array may comprise between one thousand and one million reaction vessels per sample to be analyzed. In some cases, the array comprises greater than one million reaction vessels. In some embodiments, the array comprises between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 100,000 and about 500,000, between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000 reaction vessels. In some embodiments, the array comprises about 10,000, about 20,000, about 50,000, about 100,000, about 150,000, about 200,000, about 300,000, about 500,000, about 1,000,000, or more, reaction vessels.

The array of reaction vessels may be arranged on a substantially planar surface or in a non-planar three-dimensional arrangement. The reaction vessels may be arrayed in a regular pattern or may be randomly distributed. In a specific embodiment, the array is a regular pattern of sites on a substantially planar surface permitting the sites to be addressed in the X-Y coordinate plane.

The plurality of locations may be formed using any suitable technique and/or formed from any suitable material. In some embodiments, the plurality of locations comprises a plurality of reaction vessels/wells on a substrate. The reactions vessels, in certain embodiments, may be

configured to receive and contain only a single capture object. In some embodiments, the reaction vessels are formed in a solid material. As will be appreciated by those in the art, the number of potentially suitable materials in which the reaction vessels can be formed is very large, and includes, but is not limited to, glass (including modified and/or functionalized glass), plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), Teflon®, polysaccharides, nylon or nitrocellulose, etc.), elastomers (such as poly(dimethyl siloxane) and poly urethanes), composite materials, ceramics, silica or silica-based materials (including silicon and modified silicon), carbon, metals, optical fiber bundles, or the like. In general, the substrate material may be selected to allow for optical detection without appreciable autofluorescence. In certain embodiments, the reaction vessels may be formed in a flexible material. A reaction vessel in a surface (e.g., substrate or sealing component) may be formed using a variety of techniques known in the art, including, but not limited to, photolithography, stamping techniques, molding techniques, etching techniques, or the like. As will be appreciated by those of the ordinary skill in the art, the technique used can depend on the composition and shape of the supporting material and the size and number of reaction vessels.

In some embodiments, the plurality of analyte molecules may be spatially separated into a plurality of locations such that at least some of the locations contain at least one analyte molecule and a statistically significant fraction of the locations contain no analyte molecules. A statistically significant fraction of reaction vessels that contain at least one analyte molecule (or no analyte molecules) will typically be able to be reproducibly detected and quantified using a particular system of detection and will typically be above the background noise (e.g., non-specific binding) that is determined when carrying out the assay with a sample that does not contain any analyte molecules, divided by the total number of locations addressed. A "statistically significant fraction" as used herein for the present embodiments, may be estimated according to the Equation 1:

$$P_\mu(v) = e^{-\mu}\left(\frac{\mu^v}{v!}\right) \qquad \text{(Eq. 1)}$$

wherein n is the number of determined events for a selected category of events. That is, a statistically significant fraction occurs when the number of events n is greater than three times square root of the number of events. For example, to determine a statistically significant fraction of the locations which contain an analyte molecule or particle, n is the number of locations which contain an analyte molecule. As another example, to determine a statistically significant

fraction of the capture objects associated with a single analyte molecule, n is the number of capture objects associated with a single analyte molecule.

In some embodiments, the statistically significant fraction of locations that contain at least one analyte molecule associated with a targeting capture object to the total number of targeting capture objects is less than about 1:2, less than about 1:3, less than about 1:4, less than about 2:5, less than about 1:5, less than about 1:10, less than about 1:20, less than about 1:100, less than about 1:200, or less than about 1:500. Therefore, in such embodiments, the fraction of targeting capture objects not containing any analyte molecules to the total number of targeting capture objects is at least about 1:100, about 1:50, about 1:20, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, about 5:1, about 10:1, about 20:1, about 50:1, about 100:1, or greater.

Non-limiting methods and techniques for spatially separating a plurality of capture objects into a plurality of locations is described in, for example, U.S. Publication No. 2011/0212848 by Duffy et al., filed March 24, 2010; International Publication No. WO2011/109364 by Duffy et al., filed March 1, 2011; U.S. Publication No. 2007/0259448, by Walt et al., filed February 16, 2007; U.S. Publication No. 2007/0259385, by Walt et al., filed February 16, 2007; U.S. Publication No. 2007/0259381, by Walt et al., filed February 16, 2007; International Publication No. WO2009/029073, by Walt et al., filed August 20, 2007; and International Publication No. WO2010/039179, by Duffy et al., filed September 9, 2009, each herein incorporated by reference.

It should be understood that while many of the embodiments described herein utilize locations comprising reaction vessels, this is by no means limiting, and other types of locations may be utilized. In addition, the locations need not be necessarily detectable substantially simultaneously. For example, in some embodiments, the capture objects subjected to the exposing step may be analyzed using methods and techniques wherein the capture objects are detected sequentially or partially sequentially, for example, using flow cytometry, microfluidics, etc. These and other similar methods and systems will be known to those of ordinary skill in the art. In some cases, a patterned substantially planar surface may be employed, wherein the patterned areas form a plurality of locations. In some cases, the patterned areas may comprise substantially hydrophilic surfaces which are substantially surrounded by substantially hydrophobic surfaces. A plurality of capture objects (e.g., beads) may be substantially surround by a substantially hydrophilic medium (e.g., comprising water), and the beads may be exposed to the pattern surface such that the beads associate in the patterned areas (e.g., the locations), thereby spatially separating the plurality of beads. For example, in one such embodiment, a

substrate may be or include a gel or other material able to provide a sufficient barrier to mass transport (e.g., convective and/or diffusional barrier) to prevent capture objects used for analyte capture and/or precursor labeling agent and/or labeling agent from moving from one location on or in the material to another location so as to cause interference or cross-talk between spatial locations containing different capture objects during the time frame required to address the locations and complete the assay. For example, in one embodiment, a plurality of capture objects is spatially separated by dispersing the capture objects on and/or in a hydrogel material. As still yet another embodiment, the capture objects may be confined in one or more capillaries. In some cases, the plurality of capture objects may be absorbed or localized on a porous or fibrous substrate, for example, filter paper. In some embodiments, the capture objects may be spatially separated on a uniform surface (e.g., a planar surface), and the capture objects may be detected using precursor labeling agents which are converted to substantially insoluble or precipitating labeling agents that remain localized at or near the location of where the corresponding capture object is localized. As another non-limiting embodiment, in some cases, the capture objects may be spatially separated into a plurality of droplets (e.g., using microfluidic techniques).

Following spatial separation of at least a portion of the capture objects into a plurality of locations, at least a portion of the locations may be addressed to determine a measure of the number and/or a measure of the concentration of a target analyte in the fluid sample. In some embodiments, at least a portion of the locations may be addressed and a measure indicative of the number/percentage/fraction of the locations containing at least one target analyte molecule may be made. In some cases, based upon the number/percentage/fraction, a measure of the concentration of the target analyte in the fluid sample may be determined. The measure of the concentration of target analyte molecules in the fluid sample may be determined by a digital analysis method/system optionally employing Poisson distribution adjustment and/or based at least in part on a measured intensity of a signal, as will be known to those of ordinary skill in the art. In some cases, the assay methods and/or systems may be automated. In some embodiments, the capture objects may be detected substantially simultaneously. However, in other embodiments, the capture objects may be detected sequentially.

In some embodiments, the locations addressed may be locations that contain at a certain type of analyte molecule and/or capture object. In other embodiments, the locations addressed may be locations which contain at least one targeting capture object (e.g., either associated with or not associated with any analyte molecules), and thus, in these embodiments, the percentage of locations containing at least one analyte molecule is also the percentage of capture objects associated with at least one analyte molecule (e.g., the percentage "active" beads). For example,

in some embodiments, a measure of the concentration of analyte molecules or particles in the fluid sample may be determined based at least in part on the number/percentage of targeting capture objects associated with at least one analyte molecule or particle.

In certain embodiments, a measure of the concentration of a target analyte molecules in the fluid sample may be determined based on the information received when addressing the locations (e.g., using the information received from the imaging system and/or processed using a computer implemented control system). In some embodiments, the number of locations which contain a targeting capture object are determined. In some embodiments, the number of locations which contain a targeting capture object and an analyte molecule are determined. In some cases, a measure of the concentration may be based at least in part on the number of locations determined to contain a targeting capture object that is or was associated with the corresponding target analyte. In some cases, a measure of the concentration may be based at least in part on the number of locations determined to contain a targeting capture object that is or was associated with the corresponding target analyte. The number of locations which contain non-targeting capture object may or may not be determined. In other cases and/or under differing conditions, a measure of the concentration may be based at least in part on an intensity level of at least one signal indicative of the presence of a plurality of target analyte molecule and/or capture objects associated with a target analyte molecule at one or more of the addressed locations. In some embodiments, the locations may be interrogated optically.

In some embodiments, a measure of the concentration of a first type of target analyte in the fluid sample may be determined at least in part using a calibration curve developed using samples containing known concentrations of the first type of target analyte molecules. In some cases, a measure of the concentration of first type of target analyte in the fluid sample may be determined at least in part by comparison of a measured parameter to a calibration standard. In some cases, a calibration curve may be prepared, wherein the total measured signal is determined for a plurality of samples comprising the first type of target analyte at a known concentration using a substantially similar assay format. For example, the number and/or fraction of locations that comprise a first type of target analyte associated with a target capture object, or alternatively, the average intensity of the capture objects in the array, may be compared to a calibration curve to determine a measure of the concentration of the first type of target analyte in the fluid sample. The calibration curve may be produced by completing the assay with a plurality of standardized samples of known concentration under similar conditions used to analyze test samples with unknown concentrations. A calibration curve may relate the number and/or fraction of the locations determined to contain a target capture object associated

with first type of target analyte with a known concentration of the first type of target analyte. The assay may then be completed on a sample containing the first type of target analyte in an unknown concentration, and number/fraction of locations containing a target capture object associated with a first type of target analyte may be compared to the calibration curve, (or a mathematical equation fitting same) to determine a measure of the concentration of the first type of target analyte in the fluid sample. Similar analysis/calibration may be utilized for additional types of target analytes.

In some embodiments, the non-target capture objects may be used for a number of other purposes alternatively or in addition to those described above including, but not limited to, identification of the orientation of the plurality of locations (e.g., in the case where the plurality of locations is formed as an array of reaction sites, reaction vessels, etc.), to help determine the quality of the assay, and/or to help calibrate the detection system (e.g., optical interrogation system), as described below. It should be understood, when more than one type of non-targeting capture object is utilized, each type of capture object may or might not be utilized for any of these other purposes. For example, each type of capture object may be utilized for the same purpose, or each type of capture object may be used for different purposes, and/or at least one type of capture object may not be used for any of the purposes specifically described. For example, a first type of non-target capture object may be used to determine quality of the assay and a second type of non-target capture object may be used to act as a location marker.

In some cases, the non-targeting capture objects may be used to identify the orientation of the plurality of locations (e.g., reaction vessels, sites, etc.) on an array (e.g., function as location marker(s) for an array). For example, a non-targeting capture object may be randomly or specifically distributed on an array, and may provide one or more reference locations for determining the orientation/position of the array. Such a feature may be useful when comparing multiple images of a portion of the array at different time intervals. That is, the positions of non-targeting capture objects in the array may be used to register the images. In some cases, the non-targeting capture objects may be use to provide reference locations in embodiments where a plurality of images of small overlapping regions are being combined to form a larger image.

In some embodiments, the non-targeting capture objects may be used to provide information regarding the quality of the assay. For example, if a location is found to contain a non-targeting capture object comprising an enzymatic component but no labeling agent is present (e.g., the product of which would be present upon exposure of a non-targeting capture object comprising an enzymatic component to a precursor labeling agent), this gives an indication that some aspect of the assay may not be functioning properly. For example, the

quality of the reagents may be compromised (e.g., concentration of precursor labeling agent is too low, decomposition of the precursor labeling agent has occurred, etc.), and/or perhaps not all of the locations were exposed to the precursor labeling agent.

In some embodiments, the non-targeting capture objects may be used to calibrate the detection system. For example, the non-targeting capture objects may output an optical signal which may be used to calibrate an optical detection system. In some embodiments, the non-targeting capture objects can be characterized and doped with a particular detectable characteristic (e.g., fluorescence, color, absorbance, etc.) which can act as a quality control check for the detection system performance.

In some cases, the non-targeting capture objects may be used to standardize and/or normalize the assay and/or system to account for variations of the performance and/or characteristics of different system components in different assays, over the course of time, etc. (e.g., detection system, arrays, reagents, local environment, etc.), between different portions of an array used in a test, and/or between two different arrays. For example, experimental set-up, parameters, and/or variations may lead to changes the intensity of a signal (e.g., fluorescence signal) produced from the beads in a single array at different time points, or between the beads in at least two arrays at simultaneous or different time points. In addition, in a single array, different portions of the array may produce different background signals. Such variations may lead to changes in calibration signals (e.g., differences in the determined average bead signal) between arrays, portions of and array or at multiple times, which can lead to inaccurate determinations in some cases. Non-limiting examples of parameters that may cause variation include labeling agent concentration, temperature, focus, intensity of detection light, depth and/or size of the locations in an array, reduction in activity of reagents (e.g., enzyme label), etc. To account for the effects of some or all of such variations, in some embodiments, a plurality of non-targeting capture objects may be utilized, wherein each type of capture object may be used to standardize and/or normalize the assay with respect to one or more of the parameters.

In some embodiments, the signals from the non-targeting capture objects may be used to normalize the interrogation values between different arrays, or between different areas of a single array. For example, because the signals from the non-targeting capture objects should be approximately equal between arrays and/or about different areas of a single array, the non-targeting capture object signals may be normalized to an appropriate value and the signals of the targeting capture objects (associated with an analyte molecule may be adjusted accordingly).

In one embodiment, the non-targeting capture objects may comprise a positive control and include an enzymatic component. A precursor labeling agent may be converted to a labeling

agent upon exposure to the enzymatic component. In some cases, the enzymatic component may be the same as the enzymatic component being used to detect the analyte molecules in a fluid sample (e.g., utilized in another component of the assay, for example, an enzymatic component associated with a binding ligand, an analyte molecule, etc.). In such embodiments, the non-targeting capture object may be distinguishable from the targeting capture objects such that the reaction vessels having a positive signal may be analyzed to determine whether the reaction vessel comprises a non-targeting capture object (e.g., having a first detectable signal) or a targeting capture object (e.g., having a second detectable signal distinguishable from the first detectable signal). In other cases, the enzymatic component may be different than an enzymatic component being used to detect the analyte molecules in a fluid sample (e.g., utilized in another component of the assay, for example, an enzymatic component associated with a binding ligand, an analyte molecule, etc.). In such an embodiment, the non-targeting capture object may or may not be distinguishable from the targeting capture objects. Both a first type and a second type of precursor labeling agent may be provided to the reaction vessels, and the first type of precursor labeling agent may be converted to a first type of labeling agent upon exposure to the enzymatic component associated with the non-targeting capture beads and the second type of precursor labeling agent may be converted to a second type of labeling agent upon exposure to the other enzymatic component (e.g., associated with the binding ligand/analyte molecule/etc.). The reaction vessels containing the first type of labeling agent correspond to the reaction vessels containing a non-targeting capture object and reaction vessels containing a second type of labeling agent correspond to the reaction vessels which contain a binding ligand/analyte molecule/etc. The plurality of locations containing a non-targeting capture bead may be analyzed, for example to determine the effectiveness of the enzymatic conversion reaction. In such cases, the targeting and non-targeting capture objects may or might not be distinguishable from each other.

In some embodiments, at least one type of non-targeting capture object may include at least one binding surface having specific affinity for a second type of analyte molecule, wherein the concentration of the second type of analyte molecule need not be determined but which is detected for other reasons (e.g., to calibrate the system, etc.), as described in more detail herein. In some embodiments, the second type of capture object may be a binding ligand (e.g., used in the detection of the first type of analyte molecule or particle) or a non-target analyte molecule. In some embodiments, a non-target capture object may capture a second type of analyte molecule, wherein the second type of analyte molecule may be detected either directly or indirectly. In some embodiments, the second type of analyte molecule may include an enzymatic

component and/or may be exposed to a binding ligand which comprises an enzymatic component or associates with an enzymatic component. Similar to above, a precursor labeling agent may be converted to a labeling agent upon exposure to the enzymatic component. In some cases, the enzymatic component may be the same as the enzymatic component being used to detect the target analyte molecules in a fluid sample. In some embodiments, the second type of analyte molecule may be directly detectable.

In some embodiments, a non-targeting capture object itself may include a surface having a plurality of targeting moieties which can associate with and/or capture the second type of analyte molecule. In some embodiments, the second type of analyte molecule comprises an enzymatic component. For example, the non-targeting capture object may comprises a plurality of biotin molecules, wherein the biotin molecules may be used to capture streptavidin-enzyme conjugates. As another example, the non-targeting capture object may comprises a plurality of at least one type of antibody to beta-galactosidase, wherein the antibodies to beta-galactosidase may be used to capture streptavidin-beta-galactosidase. As another example, the non-targeting capture objects may include a surface which has specific affinity for the binding ligands used in the assay to detect a target analyte. For example, if the assay employs a binding ligand comprising a detection antibody directly conjugated to an enzyme for detection, the non-targeting capture object may include a surface having specific affinity for the detection antibody or the conjugated enzyme (e.g., anti-enzyme antibodies such as anti-beta-galactosidase) or antibodies that bind to the host of the detection antibody (e.g., anti-rabbit antibodies for detection antibodies raised in rabbits).

As will be understood by those of ordinary skill in the art, the amount/quantity of targeting moieties on the surface of the non-targeting capture objects may be selected to allow the desired amount of the second type of analyte molecule to associate with the non-targeting capture object. For example, one of ordinary skill in the art will be able to carry out screening tests to determine an appropriate amount/quantity of biotin or other targeting moieties (e.g., an antibody to beta-galactosidase) to be present on the surface of each of the non-targeting capture objects such that approximately one or zero enzymatic components or other detectable components associate with each non-targeting capture object. In some cases, the amount/quantity of targeting moieties on the surface of reach non-target capture object is selected so that a statistically significant fraction of the non-targeting capture objects associate with a single detectable component (e.g., enzymatic label), while minimizing Poisson noise. In some embodiments, between about 1% and about 20%, or between about 5% and about 20%, or

between about 10% and about 20% of the non-target capture objects associate with a single detectable component (e.g., a second type of binding ligand, an enzymatic component, etc.).

In some embodiments, the non-target capture objects may not include any specific binding surfaces having specific affinity for any target analyte molecules or non-target analyte molecules, however, such surfaces may non-specifically bind to a target analyte or non-target analyte molecule. That is, non-specific binding (NSB) may be used to capture certain molecules for detection. For example, as will be known to those of ordinary skill in the art, certain types of proteins are known to be "sticky" (e.g., fibrinogen) wherein the proteins have a tendency to non-specifically bind a number of components. As another example, hydrophobic proteins may be associated with the surface of the non-targeting capture objects, wherein the hydrophobic proteins aid in non-specific binding of a detectable component. Similar to above, those of ordinary skill in the art will be able to vary the amount/quantity of non-specific targeting moieties (e.g., sticky protein) present on the surface of a non-target capture object such that approximately one or zero second type of analyte molecules associate with each non-targeting capture objection. In some cases, the amount/quantity of non-specific targeting moieties on the surface of each non-target capture object is selected so that a statistically significant fraction of the non-targeting capture objects associate with a second type of target analyte, while minimizing Poisson noise. In some embodiments, between about 1% and about 20%, or between about 5% and about 20%, or between about 10% and about 20% of the non-target capture objects associate with a second type of analyte moleculevia non-specific binding.

As described above, in some embodiments, a method for determining a measure of the concentration of a first type of analyte molecules or particles in a fluid sample comprises exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and a second type of capture object, and exposing the plurality of capture objects to a second type of analyte molecules or particles. Each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle and each of the second type of capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles contained in or suspected to be contained in the solution and include at least one binding surface having some affinity for the second type of analyte molecule or particle. In such embodiments, at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles and a statistically significant fraction of the second type of capture objects associate

with either zero or one of the second type of analyte molecules or particles. The ratio of the number of first type of capture objects to the total number of capture objects may be as described herein (e.g., between 1:1.2 and 1:100). At least a portion of the plurality of capture objects subjected to the exposing step may be spatially separated into a plurality of separate locations, wherein at least some of the locations are addressed to determine the number of locations containing a first type of capture object, the number of locations determined to contain a first type of capture object that also contain a first type of analyte molecule or particle, and the number of locations which contain a second type of capture object and a second type of analyte molecule or particle (e.g. a binding ligand.) The average intensity of the locations containing a second type of capture object and a second type of analyte molecule or particle may be determined, and a measure of the concentration of the first type of analyte molecules or particles in the fluid sample may be determined based at least in part on the average intensity of the locations containing a second type of capture object and a second type of analyte molecule or particle.

As a specific example, in some embodiments, the non-targeting capture objects may be used to determine the value, I_{single}. The term I_{single} is used herein to refer to the average intensity of at least a portion of the locations containing a single analyte molecule. In some embodiments, I_{single} is utilized wherein the analyte molecules are detected using enzymatic components. In some embodiments, the value $I_{single_}$ is used to help normalize variation in the assay conditions which may affect the determination of the measure of the concentration of the target analyte molecule(s). In some embodiments, the measure of the the concentration of a target analyte is determined, in part, via determination of the average-enzyme-per-bead (AEB) for each type of analyte molecule, wherein the AEB for a given analyte molecule is determined by the equation $f_{on} \times I_{bead}/I_{single}$, wherein f_{on} is the fraction of "on" beads for a given type of target analyte molecule, I_{bead} is the average fluorescence intensity value of the active beads, and I_{single} is the average fluorescence intensity generated by a single enzyme. In some embodiments, the I_{single} previously used was based on an average $I_{single_}$ determined in a large number of assays. Such calculations may give rise to errors because variations in the assay (e.g., as described above, including temperature) can vary enzyme activity and thus, I_{single}. Accordingly, using an averaged I_{single} over many assays might not be representative of the value at the time of the present sample measurement. Additional details regarding the determination of the concentration of analyte molecules using AEB are described in U.S. Publication No. 2011/0212537, by Duffy et al., filed March 24, 2010; and U.S. Publication No. 2011/0245097 by Rissin et al., filed March 1, 2011, each herein incorporated by reference.

Accordingly, in some embodiments, instead of using an average $I_{single_}$ determined in a large number of assays, I_{single} may be determined uniquely for each assay utilizing the non-target capture objects (e.g., wherein I_{single} is the average intensity of the locations determined to contain a second type of capture object and a second type of analyte molecule). For example, as described above, the non-target capture objects may associate with either zero or one second type of analyte molecule and I_{single} can be determined for the assay by analyzing the data and determining the average intensity for at least a portion of the locations containing a non-target capture object and a second type of analyte molecule. That is, I_{single} for the assay is determined via analyzing the non-target capture objects which associate with an second type of analyte molecule and averaging the signal detected for those non-target capture objects to provide I_{single}.

In some embodiments, at least one type of non-targeting capture object comprises one or more targeting moieties which have some binding affinity (e.g., specific affinity, or non-specific affinity as described above) for at least one type of binding ligand (e.g., comprising a enzymatic component) which is used to detect the target analyte. For example, in some embodiments, the target analyte molecule may be detected via exposure to a first type of binding ligand comprising biotin, and a second type of binding ligand comprising streptavidin-β-galactosidase, wherein the first binding ligand associates with the target analyte and the second type of binding ligand associates with the first type of binding ligand. In such embodiments, the non-target capture object may comprise a plurality of biotin molecules associated with at least one surface of the non-target capture object, wherein the second type of binding ligand may associate with at least one biotin. In some embodiment, this approach offers a "within array" calibration to account for the many of the variables that can affect I_{single} as described above.

In some embodiments, a plurality of locations may be addressed and/or a plurality of capture objects and/or species/molecules/particles of interest may be detected substantially simultaneously. "Substantially simultaneously" when used in this context, refers to addressing/detection of the locations/capture objects/species/molecules/particles of interest at approximately the same time such that the time periods during which at least two locations/capture objects/species/molecules/particles of interest are addressed/detected overlap, as opposed to being sequentially addressed/detected, where they would not. Simultaneous addressing/detection can be accomplished by using various techniques, including optical techniques (e.g., CCD detector, scanning). Spatially separating capture objects/species/molecules/particles into a plurality of discrete, resolvable locations, according to some embodiments facilitates substantially simultaneous detection by allowing multiple locations to be addressed substantially simultaneously. For example, for embodiments where

individual species/molecules/particles are associated with capture objects that are spatially separated with respect to the other capture objects into a plurality of discrete, separately resolvable locations during detection, substantially simultaneously addressing the plurality of discrete, separately resolvable locations permits individual capture objects, and thus individual species/molecules/particles (e.g., analyte molecules) to be resolved. For example, in certain embodiments, individual molecules/particles of a plurality of molecules/particles are partitioned across a plurality of reaction vessels such that each reaction vessel contains zero or only one species/molecule/particle. In some cases, between about 0.1% and about 50%, or between about 0.1% and about 40%, or between about 0.1% and about 30%, or between about 0.1% and about 20%, or between about 0.1% and about 10%, or between about 0.5% and about 10%, or between about 1% and about 10% of all species/molecules/particles are spatially separated with respect to other species/molecules/particles during detection. A plurality of species/molecules/particles may be detected substantially simultaneously within a time period of less than about 1 second, less than about 500 milliseconds, less than about 100 milliseconds, less than about 50 milliseconds, less than about 10 milliseconds, less than about 1 millisecond, less than about 500 microseconds, less than about 100 microseconds, less than about 50 microseconds, less than about 10 microseconds, less than about 1 microsecond, less than about 0.5 microseconds, less than about 0.1 microseconds, or less than about 0.01 microseconds, less than about 0.001 microseconds, or less. In some embodiments, the plurality of species/molecules/particles may be detected substantially simultaneously within a time period of between about 100 microseconds and about 0.001 microseconds, between about 10 microseconds and about 0.01 microseconds, or less.

In some embodiments, the locations are optically interrogated. The locations exhibiting changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the detected species (e.g., type of fluorescence entity, etc.) and the operative wavelengths, optical filters designed for a particular wavelength may be employed for optical interrogation of the locations. In embodiments where optical interrogation is used, the system may comprise more than one light source and/or a plurality of filters to adjust the wavelength and/or intensity of the light source. In some embodiments, the optical signal from a plurality of locations is captured using a CCD camera.

In some embodiments, the analyte molecules (e.g., optionally associated with a capture objects) may be exposed to at least one reagent. In some cases, the reagent may comprise a plurality of binding ligands which have an affinity for at least one type of analyte molecule (or particle). A "binding ligand," is any molecule, particle, or the like which specifically binds to or

otherwise specifically associates with an analyte molecule to aid in the detection of the analyte molecule. Certain binding ligands can comprise an entity that is able to facilitate detection, either directly (e.g., via a detectable moiety) or indirectly. A component of a binding ligand may be adapted to be directly detected in embodiments where the component comprises a measurable property (e.g., a fluorescence emission, a color, etc.). A component of a binding ligand may facilitate indirect detection, for example, by converting a precursor labeling agent into a labeling agent (e.g., an agent that is detected in an assay). Accordingly, another exemplary reagent is a precursor labeling agent. A "precursor labeling agent" is any molecule, particle, or the like, that can be converted to a labeling agent upon exposure to a suitable converting agent (e.g., an enzymatic component). A "labeling agent" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique. In some embodiments, the binding ligand may comprise an enzymatic component (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase, etc.). A first type of binding ligand may or may not be used in conjunction with additional binding ligands (e.g., second type, etc.).

In some embodiments, the analyte molecules may be directly detected or indirectly detected. In the case of direct detection, the analyte molecule may comprise a molecule or moiety that may be directly interrogated and/or detected (e.g., a fluorescent entity). In the case of indirect detection, an additional component is used for determining the presence of the analyte molecule. In some cases, the analyte molecules may be composed to a precursor labeling agent (e.g., enzymatic substrate) and the enzymatic substrate may be converted to a detectable product (e.g., fluorescent molecule) upon exposure to an analyte molecule. In some cases, the plurality analyte molecules may be exposed to at least one additional reaction component prior to, concurrent with, and/or following spatially separating at least some of the analyte molecules into a plurality of locations. In some cases, a plurality of capture objects at least some associated with at least one analyte molecule may be exposed to a plurality of binding ligands. In certain embodiments, a binding ligand may be adapted to be directly detected (e.g., the binding ligand comprises a detectable molecule or moiety) or may be adapted to be indirectly detected (e.g., including a component that can convert a precursor labeling agent into a labeling agent), as discussed more below. More than one type of binding may be employed in any given assay method, for example, a first type of binding ligand and a second type of binding ligand. In one example, the first type of binding ligand is able to associate with a first type of analyte molecule and the second type of binding ligand is able to associate with the first binding ligand. In another example, both a first type of binding ligand and a second type of binding ligand may associate with the same or different epitopes of a single analyte molecule, as described herein.

In some embodiments, at least one binding ligand comprises an enzymatic component. In some embodiments, the analyte molecule may comprise an enzymatic component. The enzymatic component may convert a precursor labeling agent (e.g., an enzymatic substrate) into a labeling agent (e.g., a detectable product). A measure of the concentration of analyte molecules in the fluid sample can then be determined based at least in part by determining the number of locations containing a labeling agent (e.g., by relating the number of locations containing a labeling agent to the number of locations containing an analyte molecule (or number of capture objects associated with at least one analyte molecule to total number of capture objects)). Non-limiting examples of enzymes or enzymatic components include horseradish peroxidase, beta-galactosidase, and alkaline phosphatase. Other non-limiting examples of systems or methods for detection include embodiments where nucleic acid precursors are replicated into multiple copies or converted to a nucleic acid that can be detected readily, such as the polymerase chain reaction (PCR), rolling circle amplification (RCA), ligation, Loop-Mediated Isothermal Amplification (LAMP), etc. Such systems and methods will be known to those of ordinary skill in the art, for example, as described in "DNA Amplification: Current Technologies and Applications," Vadim Demidov et al., 2004.

In certain embodiments, solubilized, or suspended precursor labeling agents may be employed, wherein the precursor labeling agents are converted to labeling agents which are insoluble in the liquid and/or which become immobilized within/near the location (e.g., within the reaction vessel in which the labeling agent is formed). Such precursor labeling agents and labeling agents and their use is described in commonly owned U.S. Publication No. 2010/0075862, by Duffy et al., filed September 23, 2008, incorporated herein by reference.

In some embodiments, when the plurality of locations comprise a plurality of reaction vessels, the plurality of reaction vessels may be sealed (e.g., after the introduction of the target analyte molecules, binding ligands, etc.), for example, through the mating of the second substrate and a sealing component. Non-limiting examples films that a sealing component may comprise include solid films (e.g., of a compliant material), fluid films (e.g., of fluids substantially immiscible with sample fluid contained in the assay sites), or the like. The sealing of the reaction vessels may be such that the contents of each reaction vessel cannot escape the reaction vessel during the remainder of the assay. In some embodiments, the sealing component may be a fluid. The fluid comprising the sealing component is advantageously substantially immiscible with the fluid contained in the assay sites. As used herein, a "fluid" is given its ordinary meaning, i.e., a liquid or a gas. The fluid may have any suitable viscosity that permits flow. If two or more fluids are present, the fluids may each be substantially miscible or

substantially immiscible. In some cases, the fluid(s) comprising the sealing component can miscible or partially miscible with the assay sample fluid at equilibrium, but may be selected to be substantially immiscible with the assay sample fluid within the time frame of the assay or interaction. Those of ordinary skill in the art can select suitable sealing fluids, such as fluids substantially immiscible with sample fluids, using contact angle measurements or the like, to carry out the techniques of the invention. In some cases, the sample fluid and/or rinsing fluid and/or reagent fluid is an aqueous solution and the sealing component comprises a non-aqueous fluid. Non-limiting examples of potentially suitable non-aqueous fluids include fluorous liquids, oils (e.g., mineral oils, fluorinated oils), ferrofluids, non-aqueous polymer solutions (e.g., thickeners), and the like. In other cases, the sample fluid and/or rinsing fluid and/or reagent fluid is a non-aqueous solution and the sealing component comprising an aqueous fluid. In some cases, the sample fluid is a hydrogel whose viscosity changes upon temperature or other physicochemical triggers.

During the method, one or more wash steps may be carried out using techniques known to those of ordinary skill in the art. A wash step may aid in the removal of any unbound molecules from the solution. A wash step may be performed using any suitable technique known to those of ordinary skill in the art, for example, by incubation of the objects with a wash solution followed by removal of the solution (e.g., in embodiments where small objects are employed such as beads, by centrifuging the solution comprising the objects and decanting off the liquid, or by using filtration techniques). In embodiments where the object is magnetic, the object may be isolated from the bulk solution with aid of a magnet.

In some embodiments, the optical signal may be captured using a CCD camera. Other non-limiting examples of camera imaging types that can be used to capture images include charge injection devices (CIDs), complementary metal oxide semiconductors (CMOSs) devices, scientific CMOS (sCMOS) devices, and time delay integration (TDI) devices, as will be known to those of ordinary skill in the art. The camera may be obtained from a commercial source. CIDs are solid state, two dimensional multi pixel imaging devices similar to CCDs, but differ in how the image is captured and read. For examples of CIDs, see U.S. Patent No. 3,521,244 and U.S. Patent No. 4,016,550. CMOS devices are also two dimensional, solid state imaging devices but differ from standard CCD arrays in how the charge is collected and read out. The pixels are built into a semiconductor technology platform that manufactures CMOS transistors thus allowing a significant gain in signal from substantial readout electronics and significant correction electronics built onto the device. For example, see U.S. Patent No. 5,883,830. CMOS devices comprise CMOS imaging technology with certain technological improvements that

allows excellent sensitivity and dynamic range. TDI devices employ a CCD device that allows columns of pixels to be shifted into and adjacent column and allowed to continue gathering light. This type of device is typically used in such a manner that the shifting of the column of pixels is synchronous with the motion of the image being gathered such that a moving image can be integrated for a significant amount of time and is not blurred by the relative motion of the image on the camera. In some embodiments, a scanning mirror system coupled with a photodiode or photomultiplier tube (PMT) could be used to for imaging.

The capture objects described herein may find use in a variety of applications. That is, wherein the application makes use of a plurality of types of objects, wherein each type of object is uniquely identifiable (e.g., via association with a unique type of reporter molecule or unique of reporter molecule amount) and uniquely targeted (e.g., via association of a unique target moiety, each unique targeting moiety being associated with a unique type or amount of reporter molecule). In some cases, the objects may comprise a plurality of beads, and the objects may be employed in the methods and systems described in those described in U.S. Publication No. 2007/0259448, by Walt et al., filed February 16, 2007; U.S. Publication No. 2007/0259385, by Walt et al., filed February 16, 2007; U.S. Publication No. 2007/0259381, by Walt et al., filed February 16, 2007; International Publication No. WO2009/029073, by Walt et al., filed August 30, 2007; U.S. Publication No. 2010/0075862, by Duffy et al., filed September 23, 2008; U.S. Publication No. 2010/0075407, by Duffy et al., filed September 23, 2008; U.S. Publication No. 2010/0075439, by Duffy et al., filed September 23, 2008; U.S. Publication No. 2010/0075355, by Duffy et al., filed September 23, 2008; U.S. Publication No. 2011/0212848, by Duffy et al., filed March 24, 2010; U.S. Publication No. 2011/0212462, by Duffy et al., filed March 24, 2010; U.S. Publication No. 2011/0212537, by Duffy et al., filed March 24, 2010; U.S. Publication No. 2012/0196774 by Fournier et at., filed Feb. 25, 2011; and U.S. Publication No. 2011/0245097 by Rissin et al., filed March 1, 2011, each herein incorporated by reference. In some embodiments, the methods described herein may be conducted using the systems and methods described in U.S. Publication No. 2012/0196774 by Fournier et at., filed Feb. 25, 2011, herein incorporated by reference.

As will be appreciated by those in the art, a large number of analyte molecules and particles may be detected and, optionally, quantified using methods and systems of the present invention; basically, any analyte molecule that is able to be made to become immobilized with respect to a capture object can be potentially investigated using the invention. Certain more specific targets of potential interest that may comprise an analyte molecule are mentioned below. The list below is exemplary and non-limiting.

In some embodiments, the analyte molecule may be an enzyme. Non-limiting examples of enzymes include, an oxidoreductase, transferase, kinase, hydrolase, lyase, isomerase, ligase, and the like. Additional examples of enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When a system/method of the present invention is used to detect the presence of viral or bacterial agents, appropriate target enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases, or the like.

In other embodiments, the analyte molecule may comprise an enzymatic component. For example, the analyte particle can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte particle is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below. Non-limiting example of enzymatic components are horseradish peroxidase, beta-galactosidase, and alkaline phosphatase.

In yet other embodiments, the analyte molecule may be a biomolecule. Non-limiting examples of biomolecules include hormones, antibodies, cytokines, proteins, nucleic acids, lipids, carbohydrates, lipids cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, or combinations thereof. Non-limiting embodiments of proteins include peptides, polypeptides, protein fragments, protein complexes, fusion proteins, recombinant proteins, phosphoproteins, glycoproteins, lipoproteins, or the like. As will be appreciated by those in the art, there are a large number of possible proteinaceous analyte molecules that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analyte molecules include, but are not limited to, immunoglobulins, hormones, growth factors, cytokines (many of which serve as ligands for cellular receptors), cancer markers, etc. Non-limiting examples of biomolecules include PSA, TNF-alpha, troponin, and p24.

In certain embodiments, the analyte molecule may be a host-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with capture components comprising a multiplicity of specific antibodies and then the captured proteins may be further bound to a binding ligand comprising a secondary antibody with specificity to a post-translational modification. Alternatively, modified proteins may be captured with capture components comprising an antibody specific for a post-translational modification

and then the captured proteins may be further bound to binding ligands comprising antibodies specific to each modified protein.

In another embodiment, the analyte molecule is a nucleic acid. A nucleic acid may be captured with a complementary nucleic acid fragment (e.g., an oligonucleotide) and then optionally subsequently labeled with a binding ligand comprising a different complementary oligonucleotide.

Suitable analyte molecules and particles include, but are not limited to small molecules (including organic compounds and inorganic compounds), environmental pollutants (including pesticides, insecticides, toxins, etc.), therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.), biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc.), whole cells (including prokaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells), viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.), spores, etc.

The fluid sample containing or suspected of containing an analyte molecule may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, fluid suspension of solid particles, supercritical fluid, and/or gas. In some cases, the analyte molecule may be separated or purified from its source prior to determination; however, in certain embodiments, an untreated sample containing the analyte molecule may be tested directly. The source of the analyte molecule may be synthetic (e.g., produced in a laboratory), the environment (e.g., air, soil, etc.), a mammal, an animal, a plant, or any combination thereof. In a particular example, the source of an analyte molecule is a human bodily substance (e.g., blood, serum, plasma, urine, saliva, stool, tissue, organ, or the like). The volume of the fluid sample analyzed may potentially be any amount within a wide range of volumes, depending on a number of factors such as, for example, the number of capture objects used/available, the number of locations us/available, etc. In a few particular exemplary embodiments, the sample volume may be about 0.01 ul, about 0.1 uL, about 1 uL, about 5 uL, about 10 uL, about 100 uL, about 1 mL, about 5 mL, about 10 mL, or the like. In some cases, the volume of the fluid sample is between about 0.01 uL and about 10 mL, between about 0.01 uL and about 1 mL, between about 0.01 uL and about 100 uL, or between about 0.1 uL and about 10 uL.

In some cases, the fluid sample may be diluted prior to use in an assay. Therefore, the method may utilized a solution derived from the fluid sample. For example, in embodiments where the source of an analyte molecule is a human body fluid (e.g., blood, serum), the fluid

may be diluted with an appropriate solvent (e.g., a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, or greater, prior to use. The sample may be added to a solution comprising the plurality of capture objects, or the plurality of capture objects may be added directly to or as a solution to the sample.

U.S.S.N. 62/102,818, filed January 13, 2015, entitled "METHODS RELATING TO IMPROVING ACCURACY OF CAPTURE OBJECT-BASED ASSAYS" is incorporated herein by reference.

The following examples are included to demonstrate various features of the invention. Those of ordinary skill in the art should, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments which are disclosed while still obtaining a like or similar result without departing from the scope of the invention as defined by the appended claims. Accordingly, the following examples are intended only to illustrate certain features of the present invention, but do not necessarily exemplify the full scope of the invention

Example 1

The following example describes a non-limiting method of determining a measure of the number of analyte molecules in a fluid sample using both targeting and non-targeting capture objects (e.g., beads). As described below, the accuracy of the assay improved with the use of both targeting and non-targeting capture objects, as compare to a substantially similar method using only targeting capture objects.

General description of method and reagents:

Materials: 2.7 um (micrometer) diameter carboxyl-functionalized paramagnetic beads were purchased from Agilent Technologies. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Thermo Scientific. Bovine serum albumin (BSA), Dimethyl Sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), 2-(N-morpholino)ethanesulfonic acid (MES), Tween20, and were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) was purchased from Amresco. Alexa Fluor 488 hydrazide and Resorufin-B-D-galactopyranoside (RGP) were purchased from Life Technologies. Cy5 Mono Hydrazide was purchased from GE Healthcare. Hilyte Fluor 750 Hydrazide was purchased from Anaspec. Antibodies and proteins standards were all purchased from commercial vendors. Detection antibodies were biotinylated using standard methods as described previously in Rissin et. al. Anal Chem, 2011, 83, 2279-2285. Streptavidin-β-Galactopyranoside (RGP) was conjugated in house using methods described previously, the average number of enzyme and

streptavidin molecules per conjugate were 1.2 and 2.7, respectively, (e.g., see Rissin et. al. Anal Chem, 2011, 83, 2279-2285, herein incorporated by reference). Simoa™ discs comprised of 24 arrays of femtoliter size wells molded into cyclic olefin copolymer (COC) and bonded to a microfluidic manifold were obtained from Sony DADC. Fluorocarbon oil (Krytox®) was obtained from DuPont.

Preparation of populations of fluorescently-labeled and Ab-coated paramagnetic beads: A stock solution of paramagnetic beads (2.3 x 10^9 beads/mL) was vortexed for 5 s three times, then placed on a rotary mixer for 15 minutes. 521 µL of bead solution (1.2 x 10^9 beads/mL) was pipetted into a 1.7 mL polypropylene tube. The beads were separated on a magnet and washed three times with 1 mL PBS + 0.1% Tween 20, and twice with 1 mL PBS. The beads were resuspended in 1 mL of PBS and transferred into a 1.7 mL polypropylene tube. 1 mg. of the dye-hydrazide was dissolved in 100 µL DMSO. A solution of 40 mg mL-1 EDC in MES buffer pH 6.2 was prepared. Sufficient PBS was first added to the tube to make the total reaction volume 1 mL. 9.4 – 62.1 µL of dye hydrazide solution was then added depending on the fluorescence dye choice and a level required, and 250 µL of 40 mg/mL EDC was added to the bead/dye suspension. The tube was capped, vortexed intermittently for 10 s, and placed on a rotating mixer with custom light-protective cover for 30 min. After separating the beads on a magnet, the beads were washed three times with 1 mL PBS + 0.1% Tween 20, resuspended in 1 mL PBS + 0.1% Tween 20, and placed on a rotating mixer for 1 h. After separating the beads from the magnet, the PBS + 0.1% Tween 20 solution was removed, and the beads were resuspended in 1 mL of 100 mM sodium bicarbonate buffer and placed on a rotating mixer for 1 h. The beads were stored in 100 mM sodium bicarbonate buffer, pH 9.3 at 2-8° C in an opaque container until conjugation with antibody. To conjugate the antibody to the dye-encoded beads, 500 µL of encoded bead stock (1.2 x 10^9 beads/mL = 0.600 x 10^9 beads) was pipetted into a 1.7 mL polypropylene tube. The beads were separated and washed 3 times with PBS + 0.1% Tween 20, followed by twice with 50 mM MES buffer pH 6.2. A solution of 0.526 mg/mL capture antibody in 50 mM MES pH 6.2 was prepared. The beads were pelleted on a magnet, the buffer was aspirated, and 0.475 mL of 0.526 mg/mL antibody solution was added to the beads. The mixture of beads and solution of antibody was vortexed, and incubated on a rotation mixer for 30 min in a custom light-protective cover. A solution containing 1 mg/mL EDC in cold 25 mM MES pH 6.0 was prepared, and 0.025 mL of this solution was added to the bead/antibody solution. This mixture was vortexed and incubated on the rotational mixer for 30 min with custom light-protective cover. After separating the beads on a magnet, the beads were washed twice with 0.5 mL PBS + 0.1% Tween 20. 1 mL of 1% BSA in PBS was added to the beads and incubated for

60 min on the rotation mixer with custom light-protective cover. The beads were then washed twice with PBS + 0.1 % Tween 20, and stored at 2-8° C in a buffer containing 500 mM Tris + 1% BSA + 0.1% Tween 20 + 0.15% Proclin300 antimicrobial in an opaque container until ready for use.

Capture of multiple proteins on subpopulations of magnetic beads and formation of enzyme-labeled immunocomplexes: 100 000 beads of each of the six subpopulations presenting antibodies to the six proteins were mixed, pelleted and the supernatant was aspirated. Test solutions (100 µL) were added to the mixture of the 600 000 magnetic bead beads and incubated for 35 minutes at 23° C. The beads were then separated and washed three times in 5 x PBS and 0.1% Tween 20. The beads were resuspended and incubated with solutions containing mixtures of biotinylated detection antibodies (anti-IL-6 at 0.125 ug/mL; anti-TNF-a at 0.4 ug/mL; anti-GM-CSF at 0.1 ug/mL; anti-IL-10 at 0.1 ug/mL; anti-IL-1β at 0.1 ug/mL; anti-IL-1α at 0.3 ug/mL) for 5 minutes at 23° C. The beads were then separated and washed three times in 5 x PBS and 0.1% Tween 20. The beads were incubated with solutions containing SβG (50 pM) for 5 min at 23° C, separated, washed seven times in 5 x PBS and 0.1% Tween 20, and washed once in PBS. 600 000 beads were then resuspended in 25 µL of 100 µM RGP in PBS, and 15 µL of this bead solution was loaded into a Simoa™ disc. The bead manipulation steps and incubations were performed on a Simoa HD-1 analyzer™.

Loading and sealing of beads in femtoliter-volume well arrays: A single molecule array disc composed of 24, 3 mm x 4 mm arrays of ~216,000 femtoliter wells and individually addressable microfluidic manifolds was loaded onto the deck of a Simoa HD-1 Analyzer™ (commercially available from Quanterix, Inc.). The disc was used in the fully automated load, seal, and imaging of the subpopulations of magnetic beads and enzyme-labeled immunocomplexes and RGP in the arrays. For each sample analyzed, 15 µL of the solution containing the mixture of bead subpopulations and RGP was pipetted by the Simoa HD-1 Analyzer™ into the inlet port of the disc. Vacuum pressure was then applied to the outlet port and drew the bead solution over the arrays of femtoliter wells. The beads were allowed to settle via gravity into the wells of the array for 90 seconds. After the beads had settled, 40 µL of fluorocarbon oil was automatically dispensed by the Simoa HD-1 Analyzer™ in the inlet port, and vacuum was simultaneously applied to the outlet port to pull the oil over the array. The oil pushed the aqueous solution and the beads that were not in the wells off of the array surface, and formed a liquid-tight seal over the wells containing beads and enzyme substrate.

Imaging of single molecules and fluorescent beads in femtoliter-volume well arrays on the Simoa HD1 Analyzer™: Once the wells were sealed using the automated load, seal, and

image process, the instrument performed the imaging steps necessary for identifying which bead types were in which well, and whether enzyme activity was associated with the beads. The fluorescence-based optical system was composed of: a white light illumination source; a custom, 12-element, wide field of view (3 x 4 mm object) microscope lens system; a CCD camera (Allied Vision, Prosilica GT3300 8 Mp). The imaging process took 45 s in total for each array, and was composed of the following sequential steps. Initially, the array is indexed to the appropriate array and held against a reference plane to which the rest of the optical system is aligned. Next, the imaging system automatically focuses to the array by taking successive images at different focus positions using "dark field" images of the array by using the 622 nm/615 nm excitation/emission filters (exposure time = 0.3 ms) and setting focus to the highest contrast image, which sets up the system for the five step image acquisition process. First, a "dark field" image of the array was acquired by using the 622 nm/615 nm excitation/emission filters (exposure time = 0.3 ms). Second, an image at 574 nm/ 615 nm excitation/emission (exposure time = 3 s) was acquired; this image is the t = 0 image (F1) of the single molecule resorufin signal. Third, an image at excitation/emission of 740 nm/800 nm (exposure time = 3s) was acquired to identify beads labelled with the HF-750 dye. Fourth, an image at excitation/emission of 680 nm/720 nm (exposure time = 3 s) was acquired; this image was not used in this work. Fifth, an image at excitation/emission of 574 nm/615 nm excitation/emission (exposure time = 3 s) was acquired 30 s after the image F1; this image is the t = 30 s image (F2) of the single molecule resorufin signal. Finally, an image at excitation/emission of 490 nm/530 nm (exposure time = 2 s) was acquired to identify beads labelled with the AF-488 dye. Images were saved as a single IPL file.

Analysis of Images: A custom image analysis software program was used to determine the enzyme activity associated with each bead within each subpopulation from the captured images. An algorithm first identified and removed occlusions (such as bubbles and dust) from the images. A masking method was then applied to the dark field image to define the locations and boundaries of the wells. The resulting well mask was then applied to each of the fluorescence images to determine the presence of beads and enzymes within the wells. For the bead fluorescence images, histograms of fluorescence intensity were generated for the well population. Peaks in the histograms were identified automatically and used to determine the populations of empty wells (low fluorescence), and populations of single beads at a particular fluorescence level for each fluorescence wavelength. The well mask was also applied to the difference between the second and first frame at the resorufin wavelengths, i.e., F2–F1. Wells that had been classified as containing a single bead from a particular bead subpopulation were

classified as: a) associated with enzyme activity (''on'' or active), if the fluorescence from resorufin within that well increased beyond a known threshold, or; b) not associated with enzyme activity (''off'' or inactive), if the fluorescence from resorufin within that well did not increase beyond a known threshold. For each ''on'' bead the intensity increase was determined. For each bead subpopulation, the fraction of ''on'' beads (fon) was determined. In the digital range ($f_{on} < 0.7$), f_{on} was converted to average number of enzymes per bead (AEB) using the Poisson distribution equation as described previously. In the analog range ($f_{on} > 0.7$), AEB was determined from the average increase in fluorescence of all the beads in an array. During classification of beaded wells and determination of enzyme activity, the fluorescence and location of wells were corrected for the following: optical blurring and scattering, background non-uniformity, intra-well bead settling locations, wavelength-dependent refraction differences in the lens assembly, and bleed of fluorescence of dyes outside their dominant wavelengths.

Results and Discussion: In the non-limiting method described in this example, it was determined that the amount of beads initially added to the sample for target analyte capture can be both advantageous and disadvantageous with respect to changes in sensitivity. For example, when using fewer beads, the ratio of target analyte to beads increases, the signal (average enzyme (e.g., target analyte) per bead or AEB) increases, and therefore, the assay sensitivity increases. However, using fewer beads also reduces the number of beads that may be loaded into the single molecule array and, if that number drops to a level where Poisson noise becomes significant, then the quantitation of beads becomes noisy and sensitivity can be decreased. The use of both targeting and non-targeting capture object counters this by allowing the minimum number of beads to be used to increase AEB while keeping the bead loading number as high and consistent as possible.

In this example, the number of target beads for a particular analyte loaded into the arrays was held at a fixed number of input beads (e.g., 100,000), and the number of non-targeting beads was varied (e.g., 0 to 500,000). The assay provided more consistent results in the presence of the non-targeting beads as compared to in the absence of the non-targeting beads. The targeting beads were distinguished from the non-targeting beads by use of fluorescence dyes.

In a first non-limiting example wherein the target analyte was IL-1alpha, 100,000 IL-1alpha targeting beads per assay sample were utilized with no other beads added (e.g., with no non-targeting beads), the average number of targeting beads detected was 2,622 per sample with a coefficient of variation (CV) of 37%. Furthermore, 6 samples did not have sufficient beads loaded to allow determination of bead number. Using 100,000 targeting IL-1alpha beads and 500,000 non-targeting beads (e.g., 100,000 each of beads specific to IL-6, TNF-alpha, GM-CSF,

IL-10, IL-1beta) that could be distinguished by fluorescent the average number of targeting beads detected was 3,127 with a CV of 10%. Alexa Fluor 488 hydrazide (AF-488), cyanine 5 hydrazide (cy5), and Hilyte Fluor 750 hydrazide (HF-750) dyes were used to encode bead types for multiplexed digital ELISA. By precisely controlling the ratio of encoding dye molecules to beads, discrete encoding levels for each dye were prepared, yielding subpopulations of beads that can be distinguished on the Simoa HD1 Analyzer™. An algorithm first identified and removed occlusions (such as bubbles and dust) from the images. A masking method was then applied to the dark field image to define the locations and boundaries of the wells. The resulting well mask was then applied to each of the fluorescence images to determine the presence of beads and enzymes within the wells. All samples had sufficient beads loaded in this case. The 3-fold improvement in the precision of the targeting bead loaded greatly improves the reliability of quantifying AEB at these low bead numbers (100,000 per sample). See Table 1 for tabulated data.

Table 1. Comparison of number of targeting beads loaded for 24 samples each of 100,000 beads coated with anti-IL-1alpha alone and with 500,000 of other non-targeting bead types present.

Sample	100,000 IL-1alpha beads alone	100,000 IL-1alpha beads + 500,000 of non-targeting beads
1		3297
2	2580	3435
3	3971	3361
4	2542	3195
5	2792	3395
6	3153	3210
7	2275	2921
8	1210	2876
9		2916
10	3336	2629
11	2784	3278
12		2951
13	1697	3631
14	711	3362
15	2836	3200
16	4293	2706
17	3064	3507
18	2806	2414
19	3711	3626
20		3246
21	2348	2978
22		2949
23	1089	2869
24		3100

Average number of beads	**2622**	**3127**
CV	**37%**	**10%**

As a second non-limiting example, wherein the target analyte was TnI, target beads coated in a specific antibody to troponin I (TnI) were utilized. Using 300,000 target beads per sample, the average number of targeting beads detected was 11,284 per sample with a coefficient of variation (CV) of 33%. Using 300,000 target beads plus 300,000 of non-targeting beads that did not present any antibodies but could be distinguished from the target beads by a dye that fluoresces at 647 nm, the average number of targeting beads detected was 12,664 with a CV of 11% (see Figure 4 and Table 2). The plots of average number of targeting beads detected in the two cases shows the dramatic improvement in the variability of bead loading by inclusion of the non-targeting beads.

In Figure 4: Comparison of the average number of targeting beads detected over 48 samples using: a) 300,000 targeting TnI beads (left); and b) 300,000 targeting TnI beads + 300,000 647 nm-labeled non-targeting beads.

Table 2. Comparison of signals (AEB) and imprecision (CV) as a function of TnI concentration for targeting TnI beads alone and combined with non-targeting beads.

	300,000 targeting TnI beads alone		300,000 targeting TnI beads + 300,000 non-targeting 647 nm beads	
[TnI] (pg/mL)	AEB	CV	AEB	CV
0	0.00903314	25%	0.009094454	8%
0.1	0.01774402	6%	0.017927884	13%
0.3	0.03279242	17%	0.034686352	10%
1	0.10172504	20%	0.10539009	11%
3	0.22942951	39%	0.275594076	9%
10	0.89510673	15%	0.947662845	13%
30	2.88942545	5%	2.66543617	7%
100	8.37164458	8%	8.409316094	8%

Example 2

In this example, non-target capture beads were used to improve the reproducibility of bead fill and AEB in an assay for troponin I (TnI). Single molecule assays were running on three Simoa HD-1 Analyzers™ (VU 15, 16, and 18) using 300,000 target capture TnI-specific beads with and without 300,000 non-target dye-labeled beads. As can be seen from Table 3and the associated plots of bead fill for each instrument, the precision of bead fill (quantified by CV of bead fill) was improved on all three instruments by using non-target capture beads. As a result of

more reliable bead fill, the instrument-to-instrument variability of AEB values of TnI at various concentrations in a calibration curve was reduced.

Table 3:

	Ex. 1		Ex. 2		Ex. 3	
	w/o non-target beads	w/ non-target beads	w/o non-target beads	w/ non-target beads	w/o non-target beads	w/ non-target beads
Beads fill% average	7.7	9.0	8.9	9.4	10.0	9.3
Beads fill CV	32%	11%	19%	10%	12%	10%

Example 3

In this example, non-targeting capture objects (e.g., non-targeting beads) were used to provide signal normalization. That is, an array specific measurement of I_{single} using the non-targeting beads was obtained to account for assay variations, including but not limited to temperature, labeling reagent concentrations, enzyme activity, and well depth. As noted herein, I_{single} calculation for every array may be useful and more accurate as compared to using average I_{single} values from previously analyzed arrays, e.g., stored calibration curves. As noted, any changes in the environment that affect the velocity of substrate turnover may yield inaccurate results for analog samples (f_{on} of > 0.7)) that are processed at a different time than the previously analyzed arrays used to determine the average I_{single} value, e.g., from a calibration curve. The reason for this potential inaccuracy may be caused because those analog samples are converted to AEB from the I_{single} value calculated from the stored calibration curve that may have been generated under different environmental conditions.

Method Overview: A two-bead assay format was utilized, wherein the first type of bead was an assay-specific bead used to target the target analyte, and the second type of bead was a non-targeting bead used to determine I_{single} for every sample/array. Each of the two types of beads were encoded with a unique dye barcode so image analysis was used to determine which type of bead was in each location. The non-target beads were used to bind a statistically significant number of single molecules to enable accurate and precise calculation of I_{single}, but not too many single molecules such that the f_{on} value would be so high that the I_{single} would be inaccurately determined. In order to assure that an optimal number of single molecules were bound to the non-target beads, the non-target beads were associated with a low concentration of biotin molecules after the dye encoding process. Experiments were conducted to determine the amount of biotin that the non-target beads should be functionalized with to yield $f_{(on)}$ between 0.05 and 0.20 (see below). The protocols outlined below yielded 488 nm-encoded non-target beads. These non-target beads can be combined with assay beads encoded with other encoding dyes or combination of dyes, for example, that are decoded with the 647 nm, 700 nm, and 750

nm optical channels. It should be understood that the non-target beads could also be encoded with any dye and at any dye level, but generally, the non-target beads are distinguishable from the target beads.

Preparation of 488 encoded beads. 488-glycine amide dye was dissolved in DMSO to 10 mg/mL and diluted in phosphate buffered saline (PBS) to 1 mg/mL. 1.2 x 10^9 2.7-micron diameter paramagnetic beads/mL was pipetting into the solution. The beads were washed three times in PBS and 0.1% Tween 20. The beads were then washed three times in PBS. The beads were resuspended in 971.76 uL PBS per mL of the batch was added. Added 3.24 uL/mL of the batch size of 1 mg/mL dye stock solution, which was then incubated on a mixer for 5 min. 25ul/mL of the batch size was added to 40 mg/mL EDC. This was vortexed ten times and placed on mixer for 30 min. The beads were washed three times in PBS and 0.1% Tween 20 and then incubated for 1 hour. 1 ml/mL Sodium Bicarbonate Buffer pH 9.3 was added to the solution.

Preparation of biotin-functionalized 488-nm encoded beads. Hydrazide biotin was dissolved in DMSO to 10 mg/mL. 1.2 x 10^9 2.7-micron diameter paramagnetic beads/mL were added. The beads were washed three times in PBS and 0.1% Tween 20. The beads were then washed three times PBS. The beads were resuspended in 962.2 uL PBS per mL of the batch size. 12.8 uL of 1:10,000 dilution of 10 mg/mL biotin/DMSO solution was added. This was incubated on mixer for 30 min. Added 25 uL/mL batch size of 40 mg/mL EDC dissolved in 50 mM MES pH 6.2, which was then vortexed ten times and placed on mixer for 30 min. The beads were then washed three times PBS and 0.1% Tween 20. 1 mL 1% bovine serum albumin (BSA) per mL of the batch size was added, followed by incubation for 1 hour. The beads were washed three times in PBS and 0.1% Tween 20. 1 mL of bead storage buffer was added per 1Ml of the batch size for storage.

Protocol for preparing the two-bead solution and running the assay: 4 x 10^6 beads/mL of 750-nm encoded PSA beads and 4 x 10^6/mL of 488-nm encoded non-target capture normalization beads were prepared in bead diluent. The resulting number of beads per cuvette was 400,000 PSA beads and 400,000 non-target beads. The assay was performed using the standard PSA assay reagents and conditions, with the only difference being the bead reagent containing the non-target capture normalization beads. The beads were incubated with 100-µL samples containing known concentrations of PSA (shown in Table 3) for 15 min. The beads were washed 3 times in wash buffer and then incubated for 5 min with 100 µL of anti-PSA detection antibody (0.328 µg/mL). The beads were washed 3 times in wash buffer and then incubated for 5 min with 100 µL of streptavidin-beta-galactosidase (50 pM). The beads were washed 6 times with wash buffer, resuspended in RGP substrate, load, sealed and imaged on the

Simoa HD-1 Analyzer™. Table 4 shows results from the same experiment analyzed, either with or without using the non-targeting capture beads to determine I_{single}.

In this example, AEB (digital) was determined for arrays with $f_{on} < 0.76$, and AEB (analog) was determined for arrays with $f_{on} > 0.76$, for 7 concentrations of PSA in quadruplicate. Table 5 summarizes the average AEB (both digital and analog) and standard deviation and coefficient of variation

Table 4:

[PSA] (pg/mL)	I(bead)	f(on)	Isingle of target capture beads in all arrays with fon < 0.2 in batch	Isingle of within array non-target capture beads with fon < 0.2	AEB (digital)	AEB (analog) using target beads batch Isingle	AEB (analog) using within-array non-target beads Isingle
0.1	123.6	0.037	91.3	95.9	0.038		
0.1	103.3	0.037	91.3	89.2	0.038		
0.1	104.3	0.039	91.3	84.5	0.040		
0.1	91.9	0.035	91.3	85.7	0.036		
0.3	93.6	0.084	91.3	78.8	0.088		
0.3	91.0	0.090	91.3	85.3	0.094		
0.3	86.3	0.089	91.3	78.3	0.094		
0.3	89.6	0.085	91.3	83.9	0.089		
1	95.1	0.224	91.3	79.2	0.254		
1	99.1	0.223	91.3	83.2	0.252		
1	96.6	0.215	91.3	83.0	0.241		
1	96.9	0.225	91.3	82.2	0.255		
3	119.7	0.509	91.3	83.4	0.711		
3	124.0	0.528	91.3	81.7	0.751		
3	125.1	0.520	91.3	83.0	0.735		
3	123.5	0.511	91.3	86.5	0.715		
10	233.0	0.872	91.3	84.8		2.227	2.396
10	233.9	0.884	91.3	83.7		2.266	2.472
10	234.2	0.896	91.3	90.5		2.299	2.320
10	235.9	0.897	91.3	84.7		2.318	2.499
30	597.0	0.975	91.3	87.7		6.379	6.638
30	650.8	0.991	91.3	92.4		7.065	6.978
30	601.0	0.963	91.3	87.1		6.339	6.641
30	633.1	0.989	91.3	89.6		6.863	6.994
100	1425.9	0.999	91.3	95.8		15.614	14.869
100	1359.5	0.998	91.3	94.8		14.864	14.314
100	1368.7	0.999	91.3	94.6		14.987	14.463
100	1404.2	0.991	91.3	96.0		15.243	14.488

Table 5:

	Based on I_{single} from batch I_{single} of target beads in arrays with f(on) < 0.2			Based on I_{single} from within-array non-target beads with f(on) < 0.2		
[PSA] (pg/mL)	AEB	Standard Deviation	CV (%)	AEB	Standard Deviation	CV (%)
0.1	0.038	0.002	4.8%	0.038	0.002	4.8%
0.3	0.091	0.003	3.4%	0.091	0.003	3.4%
1	0.251	0.006	2.5%	0.251	0.006	2.5%
3	0.728	0.018	2.5%	0.728	0.018	2.5%
10	2.277	0.040	1.8%	2.422	0.081	3.3%
30	6.661	0.359	5.4%	6.813	0.200	2.9%
100	15.177	0.331	2.2%	14.534	0.236	1.6%

Example 4

The following example utilizes substantially similar procedures as described in Example 3, except that instead of using biotin-presenting non-targeting capture objects, non-targeting capture objects were prepared that were coated with an antibody to beta-galactosidase (anti-beta-gal). The loading of anti-beta-gal on these beads was optimized to ensure that the f_{on} of these beads in the presence of streptavidin-beta-galactosidase was < 0.2, so that single molecules predominated on the non-target capture beads and precise I_{single} values could be determined within each array. These experiments were performed in a laboratory held at a temperature of 25 degrees C, and four concentrations of PSA (0, 0.1, 30, and 100 pg/mL) were tested. Table 6 shows the I_{single} values determined from the target capture beads. Table 7 shows the AEB values in these experiments, where the analog values of AEB (>1.2) were calculated either using the batch I_{single} from target capture beads or the I_{single} from within array non-target capture beads.

Table 6:

	Isingle from target capture beads with f(on) < 0.2		Isingle of within array non-target capture beads with f(on) < 0.2							
			0 pg/mL		0.1 pg/mL		30 pg/mL		100 pg/mL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
25°C	276.7	16.6	267.2	15.9	271.4	17.2	275.9	9.5	274.5	1.8

Table 7:

	25°C			
	AEB(analog) calculated using non- target capture beads Isingle		AEB(analog) calculated using target beads batch Isingle	
[PSA] (pg/mL)	Mean	SD	Mean	SD
0	0.026242	0.00921	0.026242	0.00921
0.1	0.049648	0.011267	0.049648	0.011267
30	2.489554	0.568742	2.479028	0.566931
100	8.43896	1.595863	8.394636	1.691394

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the

transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively.

Claims

What is claimed:

1. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and one or more types of non-targeting capture objects,

wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle;

wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for any type of analyte molecules or particles contained in or suspected to be contained in the solution;

wherein the ratio of the number of first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100; and

wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles;

spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object;

further determining the number of said locations containing a first type of capture object and a first type of analyte molecule or particle; and

determining a measure of the concentration of the first type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object.

2. A method for determining a measure of the concentration of a first type of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects to a solution containing or suspected of containing the first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and one or more types of non-targeting capture objects,

wherein each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle;

wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles;

wherein the ratio of the first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100; and

wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles;

spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object;

further determining the number of said locations containing a first type of capture object and a first type of analyte molecule or particle; and

determining a measure of the concentration of only the first type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object.

3. A method for determining a measure of the concentration of only a first type and a second type of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects to a solution containing or suspected of containing first types of analyte molecules or particles and a second type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object, a second type of capture object, and one or more types of non-targeting capture objects,

wherein each of the first type of capture object includes a binding surface having affinity for the first type of analyte molecule or particle;

wherein each of the second type of capture object includes a binding surface having affinity for the second type of analyte molecule or particle; and

wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having affinity for the first type of analyte molecules or particles or the second type of analyte molecules or particles;

wherein the ratio of the first type of capture objects to the total number of capture objects and the ratio of the second type of capture objects to the total number of capture objects are the same or different and are between 1:1.2 and 1:100;

wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecule or particle; and

wherein at least some of the second type of capture objects associate with at least some of the second type of analyte molecule or particle at least some of the second type of capture objects do not associate with any analyte molecule or particle;

spatially separating at least a portion of the capture objects subjected to the exposing steps into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a first type capture object or a second type of capture object;

further determining the number of said locations containing a first type of analyte molecule or particle or a second type of analyte molecule or particle; and

determining a measure of the concentration of only the first type of analyte molecules or particles and the second type of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a first type of capture object and a first type of analyte molecule and particle, to the number of locations containing a first type of capture object, or based at least in part on the ratio of the number of locations containing a second type of capture object and a second type of analyte molecule and particle, to the number of locations containing a second type of capture object, respectively.

4. A method for binding analyte molecules or particles in a fluid sample to capture objects and spatially separating the capture objects, comprising:

exposing a plurality of capture objects to a solution comprising or derived from the fluid sample, wherein the capture objects comprise at least one type of targeting capture objects and at least one type of non-targeting capture objects,

wherein each of the at least one type of targeting capture objects includes a binding surface having specific binding affinity for at least one type of target analyte molecule or particle contained in or suspected to be contained in the solution,

wherein each of the one or more types of non-targeting capture objects do not include any binding surfaces having specific binding affinity for any of the at least one type of target analyte molecule or particle contained in or suspected to be contained in the solution,

wherein the ratio of the number of targeting capture objects to the total number of targeting and non-targeting capture objects is between 1:1.2 and 1:100;

spatially separating at least a portion of the plurality of capture objects subjected to the exposing step.

5. The method of claim 4, further comprising:

addressing at least some of the spatially separated capture objects and determining the number of the at least one type of analyte molecules associated with a capture objects.

6. A method for determining a measure of the concentration of a first type of analyte molecules or particles in a fluid sample, comprising:

exposing a plurality of capture objects to a solution containing or suspected of containing a first type of analyte molecules or particles, wherein the capture objects comprise a first type of capture object and a second type of capture object;

exposing the plurality of capture objects to a second type of analyte molecules or particles,

wherein:

each of the first type of capture object includes a binding surface having specific binding affinity for the first type of analyte molecule or particle;

each of the second type of capture objects do not include any binding surfaces having specific binding affinity for the first type of analyte molecules or particles contained in or suspected to be contained in the solution and include at least one binding surface having some affinity for the second type of analyte molecule or particle;

at least some of the first type of capture objects associate with at least one analyte molecule or particle and at least some of the first type of capture objects do not associate with any analyte molecules or particles,

a statistically significant fraction of the second type of capture objects associate with either zero or one of the second type of analyte molecules or particles, and

the ratio of the number of first type of capture objects to the total number of capture objects is between 1:1.2 and 1:100;

spatially separating at least a portion of the plurality of capture objects subjected to the exposing step into a plurality of separate locations;

addressing at least some of the plurality of locations and determining the number of locations containing a first type of capture object;

further determining the number of the locations determined to contain a first type of capture object that also contain a first type of analyte molecule or particle;

addressing at least some of the plurality of locations and determining which locations contain a second type of capture object and a binding ligand;

further determining the average intensity of said locations containing a second type of capture object and a second type of analyte molecule or particle; and

determining a measure of the concentration of the first type of analyte molecules or particles in the fluid sample based at least in part on the average intensity of said locations containing a second type of capture object and a second type of analyte molecule or particle.

7. The method of claim 6, wherein the second type of capture objects include at least one binding surface having specific affinity for the second type of analyte molecule.

8. The method of claim 6 or 7, wherein the second type of analyte molecule is a binding ligand used in the detection of the first type of analyte molecule.

9. The method of claim 8, wherein the binding ligand may be detected directly or indirectly.

10. The method of claim 6 or 7, wherein the second type of analyte molecule is different than the first type of analyte molecule and is added to the fluid sample in a known concentration.

11. The method of claim 10, wherein the second type of analyte molecule is detected indirectly.

12. The method of any preceding claim, wherein at least a portion of the capture objects subjected to the exposing step are spatially separating into a plurality of locations.

13. The method of any preceding claim, wherein the plurality of capture objects comprises a plurality of beads.

14. The method of any preceding claim, wherein the average diameter of the plurality of capture objects is between about 0.1 micrometer and about 100 micrometers.

15. The method of any preceding claim, wherein the average diameter of the plurality of capture objects is between about 1 micrometer and about 10 micrometers.

16. The method of any preceding claim, further comprising performing at least one wash step.

17. The method of any preceding claim, wherein the measure of the concentration of the at least one type of analyte molecule or particle, or the first type or analyte molecule or particle, and/or the second type of analyte molecule or particle is determined at least in part by comparison of a measured parameter to a calibration standard.

18. The method of any preceding claim, wherein the plurality of locations comprising a plurality of reaction vessels.

19. The method of any preceding claim, further comprising sealing the plurality of reaction vessels.

20. The method of any preceding claim, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters, or between about 1 femtoliter and about 100 femtoliters, or between about 1 femtoliter and about 1 picoliter, or between about 1 micrometer and about 10 micrometers.

21. The method of any preceding claim, wherein the plurality of locations is addressed using optical techniques.

22. The method of any preceding claim, wherein the at least one type of analyte molecule or particle, or the first type or analyte molecule or particle, or the second type of analyte molecule or particle are proteins or nucleic acids.

23. The method of any preceding claim, wherein the number of said locations containing the at least one type of analyte molecule or particle, or the first type or analyte molecule or particle, or the second type of analyte molecule or particle are determined using optical techniques.

24. The method of any preceding claim, wherein the ratio of the first type of capture object to the total number of capture objects is between 1:2 and 1:100, or between 1:5 and 1:100, or between 1:5 and 1:75, or between 1:5 and 1:50, or between 1:5 and 1:25, or between 1:10 and 1:100, or between 1:10 and 1:75, or between 1:10 and 1:50, or between 1:20 and 1:100, or between 1:25 and 1:100.

25. The method of any preceding claim, wherein the ratio of the second type of capture object to the total number of capture objects is between 1:2 and 1:100, or between 1:5 and 1:100, or between 1:5 and 1:75, or between 1:5 and 1:50, or between 1:5 and 1:25, or between 1:10 and 1:100, or between 1:10 and 1:75, or between 1:10 and 1:50, or between 1:20 and 1:100, or between 1:25 and 1:100.

26. The method of any preceding claim, wherein at least some of the first type of capture objects associate with at least one analyte molecule or particle and a statistically significant fraction of the first type of capture objects do not associate with any analyte molecules or particles.

27. The method of any preceding claim, wherein at least some of the second type of capture objects associate with at a second type of analyte molecule or particle and a statistically significant fraction of the second type of capture objects do not associate with any analyte molecules or particles.

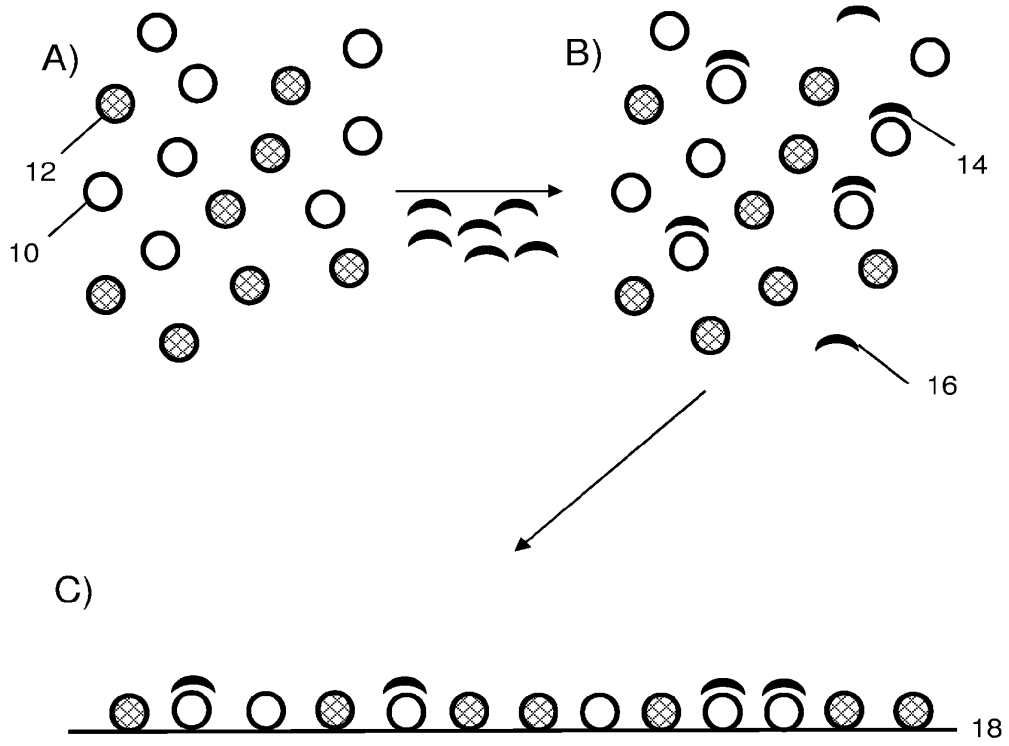


Figure 1

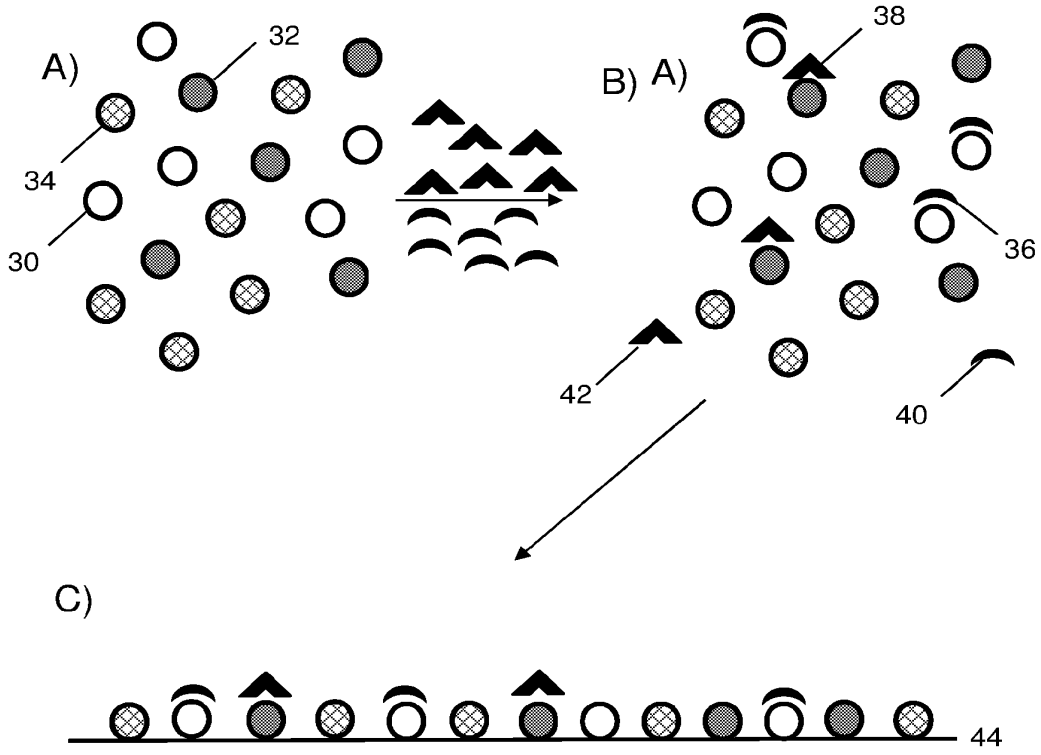


Figure 2

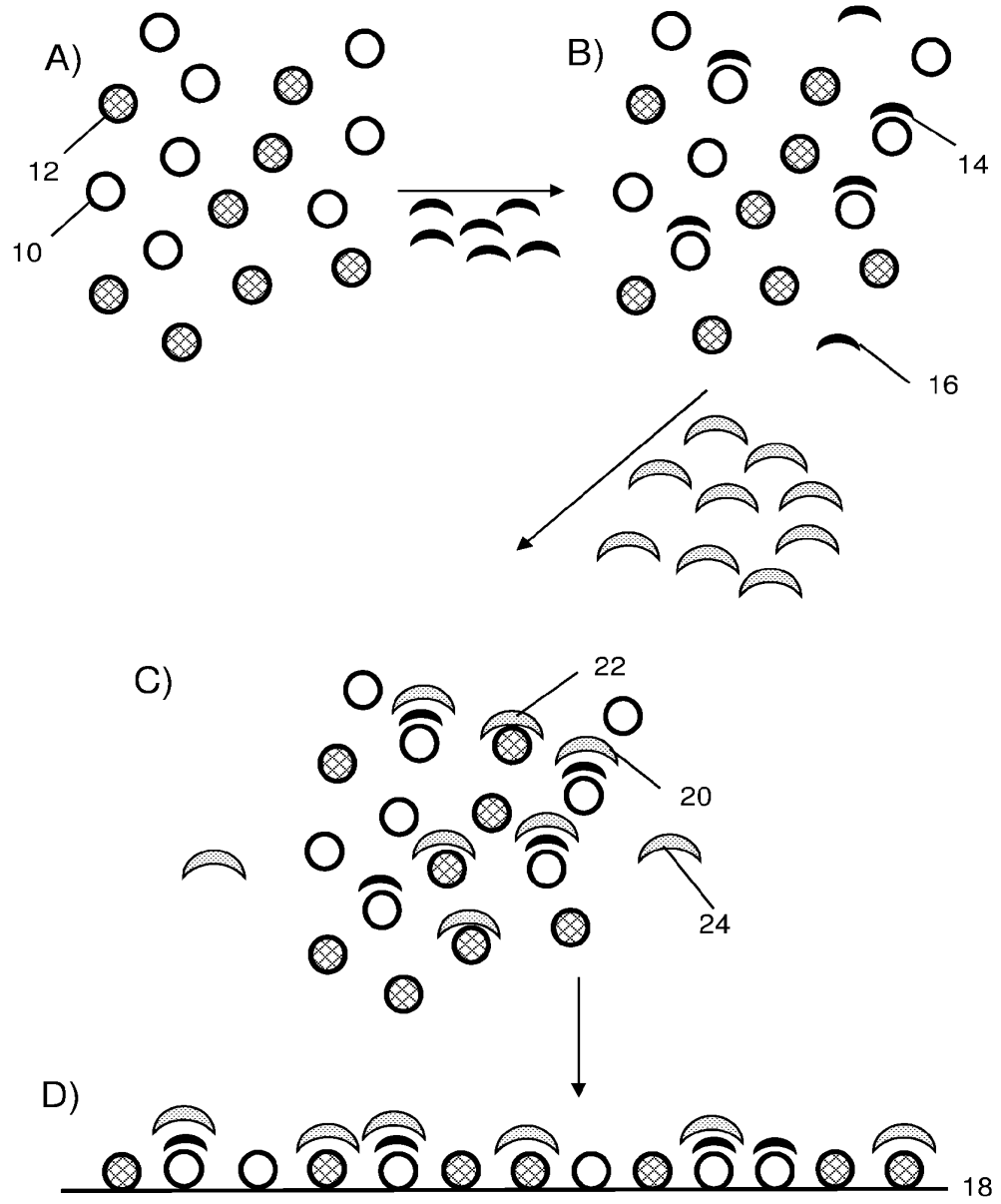


Figure 3

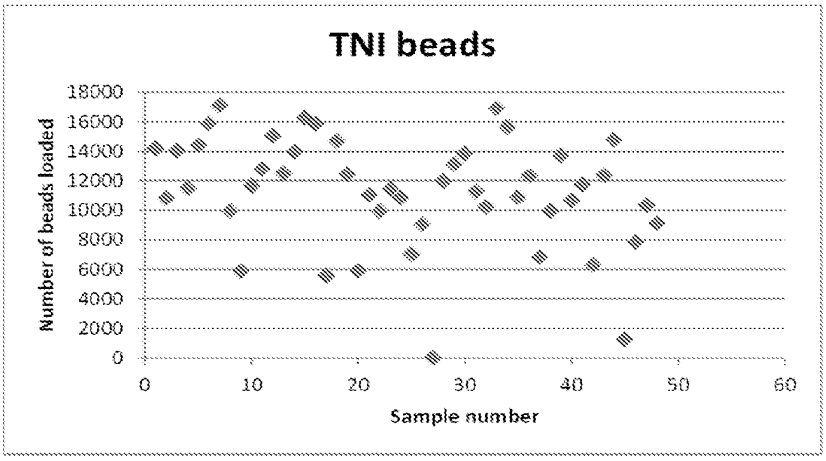


Figure 4A

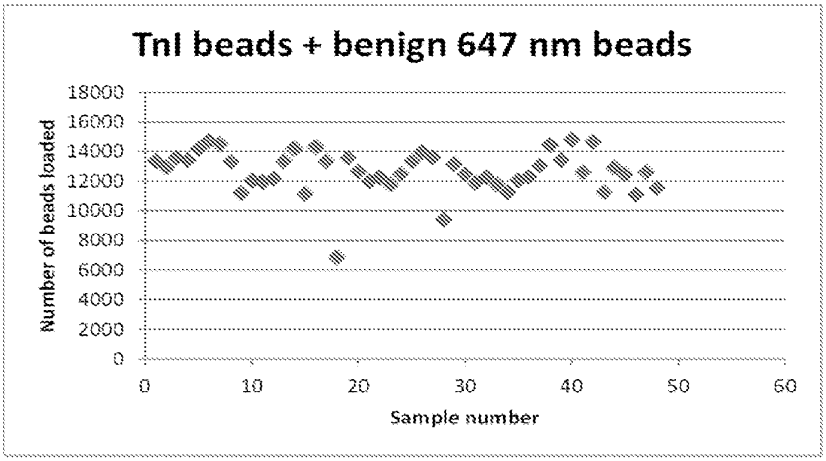


Figure 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/013255

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/00 (2016.01)
CPC - C12Q 1/00 (2016.02)
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)
IPC(8) - C12Q 1/00; G01N 33/50, 33/543 (2016.01)
CPC - C12Q 1/00; G01N 33/50, 33/53, 33/54313 (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 422/68.1; 436/518; 506/9 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed.

Search terms used: concentration analyte capture number location ratio

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0165342 A1 (QUANTERIX CORPORATION) 27 June 2013 (27.06.2013) entire document	1-5
A	US 2012/0289428 A1 (DUFFY et al) 15 November 2012 (15.11.2012) entire document	1-11
A	WO 2014/183096 A1 (QUANTERIX CORPORATION) 13 November 2014 (13.11.2014) entire document	1-11
A	WO 2014/113502 A1 (QUANTERIX CORPORATION) 24 July 2014 (24.07.2014) entire document	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"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
07 March 2016	17 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/013255

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

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

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

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

3. ☒ Claims Nos.: 12-27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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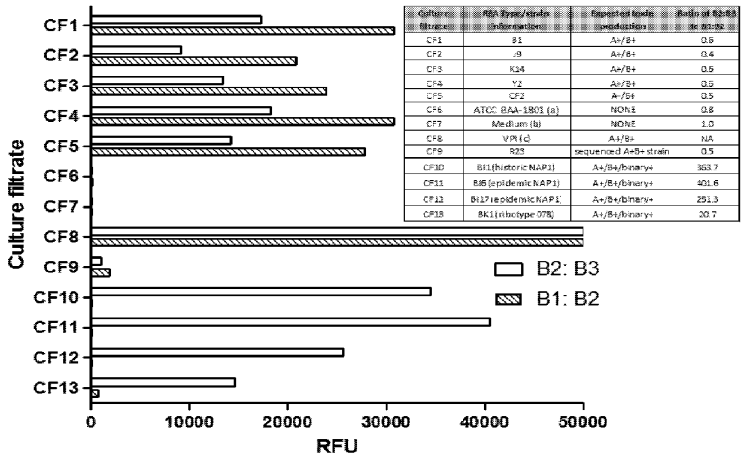
(74) Agent: **CHEN, Yahua**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

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(54) Title: IMMUNOASSAYS FOR DIFFRENTIAL DETECTION OF CLOSTRIDIUM DIFFICILE

Fig. 1A



Culture filtrate	PCR Ribo/strain information	Expected toxin production	Ratio of B2:B3 or B1:B2
CF1	B1	A+/B+	0.6
CF2	9	A+/B+	0.4
CF3	K14	A+/B+	0.5
CF4	Y2	A+/B+	0.5
CF5	CF2	A+/B+	0.5
CF6	ATCC BAA-1803 (a)	NONE	0.8
CF7	Medium (b)	NONE	1.0
CF8	VPI (c)	A+/B+	NA
CF9	R20	sequenced A+B+ strain	0.5
CF10	B11(historic NAP1)	A+/B+/binary+	353.7
CF11	B16 (epidemic NAP1)	A+/B+/binary+	401.6
CF12	B12 (epidemic NAP1)	A+/B+/binary+	251.3
CF13	B41 (ribotype 078)	A+/B+/binary+	20.7

(57) Abstract: Ultrasensitive and quantitative assays for detecting toxins of *Clostridium difficile*, which may involve digital ELISA, are provided. Also provided herein are differential detection assays that allow for distinguishing toxin B of highly virulent *Clostridium difficile* strains from toxin B of less virulent strains.

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Published:

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

Immunoassays For Differential Detection of *Clostridium Difficile*

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/116,073, filed February 13, 2015, the contents of which are incorporated by reference herein in their entirety.

GOVERNMENT SUPPORT

This invention was made with government support under Grant No. 5 R21AI103612-02, awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

C. difficile is a significant nosocomial and community-acquired pathogen, associated with disease ranging from mild diarrhea to fatal pseudomembranous colitis. Cohen, et al., *Infect Control Hosp Epidemiol*, 2010. **31**(5): p. 431-55. Since the turn of the millennium, rates of *C. difficile* infection (CDI) have increased globally, concomitant with increased rates of severe clinical presentations and worsened clinical outcomes. Cohen et al., 2010 and Burnham et al., *Clin Microbiol Rev*, 2013. **26**(3): p. 604-30. CDI results from the production of two large protein exotoxins: toxins A (~308 kDa) and B (~270 kDa). Nontoxigenic strains are not pathogenic. While most strains produce both toxins A and B, a minority produce toxin B only. Toxins A and B are the primary virulence factors contributing to the pathogenesis of CDI, and the genes for these toxins (tcdA and tcdB) are co-located in a pathogenicity locus in toxigenic strains. Burnham et al., 2013 and Rupnik, *FEMS Microbiol Rev*, 2008. **32**(3): p. 541-55.

Accurate diagnosis is critical for the effective management of *C. difficile* infection, but currently available diagnostics have major limitations. Despite mounting evidence that toxin detection is paramount for CDI diagnosis, conventional toxin immunoassays are insufficiently sensitive and cytotoxicity assays too complex. Assays that detect toxigenic

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organisms, including toxigenic culture (TC) and nucleic acid amplification testing (NAAT), are confounded by asymptomatic colonization by toxigenic *C. difficile*.

SUMMARY OF THE INVENTION

In aspect, the present disclosure provides a method for differentially detecting *C. difficile* based on virulence, the method comprising: (i) obtaining a sample comprising a *C. difficile* strain (e.g., obtained from a subject having or suspected of having C. difficile infection); (ii) performing a first immunoassay to detect toxin B of *C. difficile* in the sample, wherein the first immunoassay involves a first antibody and a second antibody, both of which bind toxin B of both highly virulent and less virulent *C. difficile* strains; (iii) performing a second immunoassay to detect toxin B of *C. difficile* in the sample, wherein the second immunoassay involves the second antibody and a third antibody, wherein the third antibody differentially binds toxin B of highly virulent *C. difficile* strains as relative to toxin B of less virulent *C. difficile* strains; (iv) comparing results obtained from step (ii) and step (iii); and (v) determining whether the sample comprises a highly virulent *C. difficile* strain or a less virulent *C. difficile* strain based on the comparing result of step (iv).

In some examples, the first immunoassay, the second immunoassay, or both are digital ELISA assays. Alternatively or in addition, either the first antibody or the second antibody in step (ii) may be conjugated to a label (*e.g.*, biotin) and the other antibody may be immobilized on a solid support. The first immunoassay may further involves (a) a conjugate of streptavidin and an enzyme, and (b) a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal, such as a fluorescent signal.

In any of the differential detection methods described herein, the comparing step (iv) can be performed by calculating a ratio between a first value obtained from step (ii) and a second value obtained from step (iii), the first value representing the level of the toxin B detected in the first immunoassay and the second value representing the level of the toxin B detected in the second immunoassay.

In some embodiments, either the second antibody or the third antibody in step (iii) is conjugated to a label (*e.g.*, biotin) and the other antibody is immobilized on a solid support. Alternatively or in addition, the second immunoassay may further involve (a) a conjugate of

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streptavidin and an enzyme, and (b) a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal, such as a fluorescent signal.

In any of the differential detection methods described herein, the third antibody is specific to toxin B of highly virulent *C. difficile* strains. In some examples, detection of toxin B in both the first immunoassay and the second immunoassay indicates that the sample comprises a highly virulent *C. difficile* strain; and detection of toxin B in the first immunoassay but not in the second immunoassay indicates that the sample comprises a less virulent *C. difficile* strain.

Alternatively, the third antibody is specific to toxin B of less virulent *C. difficile* strains. In some examples, detection of toxin B in the first immunoassay but not in the second immunoassay indicates that the sample comprises a highly virulent *C difficile* strain, and detection of toxin B in both the first immunoassay and the second immunoassay indicates that the sample comprises a less virulent *C. difficile* strain.

In some embodiments, the highly virulent strain is a BI/NAP-1/027 strain, a ribotype 078/BK strain, or a ribotype 244/AF strain.

In some embodiments, the sample (e.g., a stool sample) is obtained from a subject having or suspected of having *C. difficile* infection. In some examples, the method may further comprise determining whether or not the subject is infected with a highly virulent *C difficile* strain.

In another aspect, the present disclosure provides a kit for differentially detecting *C. difficile* based on virulence, the kit comprising: (i) a first pair of anti-toxin B antibodies including a first antibody and a second antibody, wherein the first antibody and the second antibody bind toxin B of both highly virulent and less virulent *C. difficile* strains; and (ii) a second pair of anti-toxin B antibodies, including the second antibody and a third antibody, which differentially binds toxin B of highly virulent *C. difficile* strains as relative to toxin B of less virulent *C. difficile* strains. In the first pair of anti-toxin B antibodies, either the first antibody or the second antibody may be conjugated to a label (e.g., biotin) and the other antibody may be immobilized on a solid support. Alternatively or in addition, in the second pair of anti-toxin B antibodies, either the second antibody or the third antibody may be

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conjugated to a label (e.g., biotin) and the other antibody may be immobilized on a solid support.

In some examples, the kit may further comprise a conjugate of streptavidin and an enzyme, and optionally a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal, e.g., a fluorescent signal.

Any of the kits described herein may further comprise instructions for carrying out a method set forth in any of the differential detection methods described herein.

In yet another aspect, the present disclosure provides a method for detecting *C. difficile*, comprising: (i) providing a sample suspected of having a *C difficile* strain; (ii) detecting toxin B of the *C difficile* by digital ELISA; (iii) detecting toxin A of the *C difficile* by digital ELISA; and (iv) assessing presence of a toxigenic *C difficile* strain and/or a toxin thereof in the sample based on the result of step (ii), the result of step (iii), or a combination thereof. In some embodiments, the level of toxin B detected in step (ii) higher than 20 pg/ml indicates that the sample contains a toxigenic *C. difficile* strain or a toxin thereof.

Also within the scope of the present disclosure is a method for diagnosing *C. difficile* infection in a subject, comprising: (i) examining whether the subject manifests a symptom associated with *C. difficile* infection (*e.g.*, diarrhea); (ii) detecting the presence of toxin A, toxin B, or both in a sample obtained from the subject; and (iii) determining whether the subject has *C. difficile* infection. The method may further comprise detecting the presence of a nucleic acid that encodes toxin A, a nucleic acid that encodes toxin B, or both, in a sample obtained from the subject.

In some embodiments, step (ii), step (iii), or both are performed by a digital ELISA. Alternatively or in addition, step (ii) is performed by a differential detection method to determine whether the sample contains a highly virulent strain or a less virulent strain.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a chart showing the comparison of toxin B levels in culture filtrates prepared from a panel of *C. difficile* isolates (table inset), as measured by conventional ELISA using the B2/B3 antibody pair (white bars) vs the B1/B2 antibody pair (hatched bars). Ratios presented for each culture filtrate are ratios of the signals measured using the two different assays. CF, culture filtrate; REA, Restriction Endonuclease Analysis.

Fig. 1B is a chart showing the comparison of toxin B levels in clinical stool samples, which in toxigenic culture, yielded isolates with REA types shown (table inset), as measured by digital ELISA using the B2/B3 antibody pair (white bars) vs the B1/B2 antibody pair (hatched bars).

Fig. 1C is a diagram showing a comparison of ratios of signals from B2/B3 vs B1/B2 digital ELISAs, as measured in clinical stool samples yielding the isolates (via toxigenic culture) in the following three groups: BI/NAP-1/027 strains (n=10), "non-specified REA" strains including one from Group AF (n=3), and all other strains recovered (noted here as "standard virulence," as none of the types have been particularly associated with hypervirulence). Horizontal bars indicate the mean for each group.

Fig. 2A is a diagram showing a graph of the digital ELISA results for groups of samples testing positive vs. negative on other assays for toxin A. One of the 80 "true negative" samples was excluded from the cutoff calculation because it was an extreme outlier in the toxin A assay. Mean signals in each group are indicated by horizontal lines. The calculated clinical cutoffs for each digital ELISA (29.4 pg/mL for the toxin A assay) are shown as dotted lines spanning the figure. The arrow indicates a sample that was excluded from calculation of the cutoff because it was an extreme outlier and substantially distorted the mean for that assay. NAAT, nucleic acid amplification testing; TC, toxigenic culture.

Fig. 2B is a graph showing the digital ELISA results for groups of samples testing positive vs. negative with other assays for toxin B. Mean signals in each group are indicated by horizontal lines. The calculated clinical cutoffs for each digital ELISA (23.3 pg/mL for the toxin B assay) are shown as dotted lines spanning each figure. NAAT, nucleic acid amplification testing; TC, toxigenic culture.

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Fig. 3 is a diagram showing representative calibration curves for both toxin A and B assays. The LOD, defined as a concentration corresponding to the AEB from zero calibrator plus 3 SD, was 0.028 pg/ml for toxin A and 0.061 pg/ml for toxin B from the presented curves respectively, using a 10% CV for zero calibrator.

Fig. 4 is a schematic illustration showing a representative multiplex assay for differentially detecting a toxin such as toxin B from a highly virulent *C. difficile* strain (NAP 1) relative to the toxin from a less virulent *C. difficile* strain.

Fig. 5 is a schematic illustration showing a representative simplified assay with a single detector for differentially detecting a toxin such as toxin B from a highly virulent *C. difficile* strain (e.g., NAP 1) relative to the toxin from a less virulent *C. difficile* strain.

Fig. 6 is a schematic illustration showing a representative simplified assay with a single detector and capture switch for differentially detecting a toxin such as toxin B from a highly virulent *C. difficile* strain (e.g., NAP 1) relative to the toxin from a less virulent *C. difficile* strain.

DETAILED DESCRIPTION OF THE INVENTION

The recent increase in global incidence and severity of Clostridium difficile infection (CDI) [1-3] is of major concern. CDI, ranging from mild antibiotic-associated diarrhea to lethal pseudomembranous colitis, consumes an increasing proportion of resources for diagnosis, treatment, and infection control in both adult and pediatric populations [2, 4]. A recent US prevalence survey of health care-associated (HCA) infections [5] found that C. difficile was the most commonly reported pathogen, causing 12.1% of HCA infections. Despite available therapies, treatment failure and relapse are common [1, 2, 6]. The emergence of epidemic strains capable of toxin hyper-production and increased disease severity/mortality [2, 7, 8] has further increased the urgency of improving methods for diagnosis and treatment. Accurate diagnosis remains the cornerstone of effective management.

Disease caused by C. difficile is due to production of two large protein exotoxins: toxins A and B [9, 10]; nontoxigenic strains are not considered to be pathogenic [11]. These high molecular weight protein exotoxins (308 and 270 kDa, respectively) are immunologically and biologically distinct. Most strains produce both toxins A and B, though

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there are a minority of strains that produce B only (e.g.[12, 13]); the genes for these toxins (tcdA and tcdB) are co-located in a pathogenicity locus in toxigenic strains [11, 14].

While the presence of toxin is necessary for disease, the optimal method for diagnosis of CDI remains controversial. The classic gold standard stool assay, toxigenic culture (TC), is complex, lengthy, and unstandardized; furthermore, it is increasingly recognized that detection of a toxigenic organism, rather than the toxin itself, is suboptimal. Despite arguments that detection of toxin in stool has highest clinical specificity and predictive value [2, 15, 16], the field has rapidly moved towards nucleic acid amplification testing (NAAT), for ultrasensitive detection of toxigenic organisms. However, studies have repeatedly reported NAAT-positive individuals who clinically did not have CDI [17-20], and the field is increasingly recognizing that the utility of NAAT (like TC) is confounded by asymptomatic colonization by toxigenic C. difficile [19, 21-23]. Furthermore, the presence of the toxin genes also does not necessarily correlate to gene expression [14, 24]. Thus, by detecting C. difficile organisms (potentially including even transient environmental spores), rather than toxins A and B which are the known effectors of disease, NAAT lacks specificity for clinical disease [2].

Despite mounting evidence that toxin detection is paramount for CDI diagnosis, current methods for toxin detection remain inadequate. While cytotoxicity assay can have good analytical sensitivity for toxin B [limit of detection (LOD) 1-10 pg/mL for toxin spiked into buffer [25]], this assay (like TC) is laborious, slow, and unstandardized. Conventional qualitative toxin immunoassays are widely used [2], but have high LODs (e.g. ~0.8-2.5 ng/mL) [26, 27] and poor sensitivity (52-75% vs TC [28, 29]). Given these limitations, the field is poised for a simple toxin detection test that combines high analytical sensitivity with the clinical specificity of toxin detection. The fact that disease severity has been correlated to toxin levels in the host in preliminary studies [30, 31] further suggests that the ability to simply and reliably quantify toxin levels in stool could be clinically valuable to predict disease and treatment outcomes, and in identifying those who need aggressive therapy.

The present disclosure provides highly sensitive and/or quantitative methods for detection of toxin A, toxin B, or both of *C. difficile,* including methods that allow for differential detection of toxin B polypeptides from *C. difficile* strains having different leveled

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virulence (*e.g.*, highly virulent or less virulent). Such assay methods may involve digital ELISA based on single molecule array (Simoa) technology. [Rissin, et al., Nat Biotechnol, 28(6): p. 595-9 (2010); Rissin, et al., Anal Chem, 83(6): p. 2279-85 (2011); and Kan, et al., Lab Chip, 2012. 12(5): p. 977-85.] These detection methods can be applied in clinical settings for diagnosing *C. difficile*, either alone or in combination with one or more other diagnostic approaches known in the art or described herein. The assays described herein have high analytical sensitivity and specificity, and detect toxin directly, thereby providing higher diagnostic accuracy over existing assays. Further, the results obtained from such assays, for example, the differential detection assay for identifying highly virulent strains versus less virulent strains, can help determine suitable approaches for treating *C. difficile* infection based on the level of virulence of the involved *C. difficile* strain.

Any one of the detection assays described herein can be performed using a suitable detecting system or device, such as Simoa™ and Simoa HD-1 Analyzer™ provided by Quanterix.

Differential Detection Assays

One aspect of the present disclosure provides differential detection assays, which allows for distinguishing highly virulent *C. difficile* strains from those that are less virulent.

There are at least 25 *C. difficile* "toxinotypes" (*i.e.*, groups of strains defined by changes in the genetic locus (PaLoc) encoding toxins A and B), including a reference strain (VPI 10463, or "toxinotype 0") [Rupnik., *FEMS Microbiol Rev*, **32**(3): p. 541-55 (2008)]. There are two types of "variant" toxinotypes: those with variations in the toxin genes themselves (as defined by RFLP-PCR), and those with variations in toxin production (*i.e.*, producing only toxin B, or producing binary toxin (an additional toxin with unclear function)). Variant toxins may differ from wild-type toxins in size, substrate specificity, *in vitro* and *in vivo* activity, and immunoreactivity. [Rupnik, *FEMS Microbiol Rev*, **32**(3): p. 541-55 (2008)]. The sensitivity of EIA tests [vs. toxigenic culture (TC)] performed on stool has been reported to vary with *C. difficile* ribotype [Tenover, et al., *J Clin Microbiol*, **48**(10): p. 3719-24 (2010)], whereas sensitivity of PCR testing in the same study did not vary with ribotype.

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Different *C. difficile* strains vary in toxin production with respect to type, level, or both. Multiple studies suggested a correlation between stool toxin concentrations and disease severity [e.g., Burnham, et al., *Clin Microbiol Rev*, **26**(3): p. 604-30 (2013); and Planche, et al., *Lancet Infect Dis*, **13**(11): p. 936-45 (2013)]. Consistent with this finding, a recently emerged epidemic strain, BI/NAP-1/027 (toxinotype IIIb) (henceforth "BI") is notable for its higher toxin yields *in vitro* (about 20-fold higher than toxinotype 0 strains [Warny, et al., *Lancet*, **366**(9491): p. 1079-84 (2005)]), deletions in a putative negative regulator for toxins A and B (*tcdC*), and its ability to cause unusually severe and recurrent disease *[Cohen, S.H. et al., Infect Control Hosp Epidemiol*, **31**(5): p. 431-55 (2010); Warny, M. et al., *Lancet*, **366**(9491): p. 1079-84 (2005); Loo, V.G. et al., *N Engl J Med*, **353**(23): p. 2442-9 (2005)].

In addition to BI/NAP-1/027 strains, other emergent strains have been associated with particularly severe disease, including the ribotype 078/BK group [Goorhuis, et al., *Clin Infect Dis*, **47**(9): p. 1162-70 (2008)] and the ribotype 244/AF group [Lim, et al., *Clin Infect Dis*, (2014)].

Identifying or excluding highly virulent strains is relevant to therapeutic decision-making; two Phase III clinical trials demonstrated that fidaxomicin (vs. vancomycin) achieved significantly lower recurrence rates for CDI associated with non-BI strains [Louie, et al., *N Engl J Med*, **364**(5): p. 422-31 (2011)]). However, other than molecular testing, the highly virulent BI strains are not easily identified by routine laboratory testing [Rupnik, M., *FEMS Microbiol Rev*, **32**(3): p. 541-55 (2008)].

The differential detection methods described herein may involve one or more immunoassays to detect the presence or determine the level (quantity) of toxin B or toxin A of *C. difficile* in a sample having or suspected of containing *C. difficile* cells using at least one antibody that differentially binds a toxin B or toxin A polypeptide from a highly virulent *C. difficile* strain as relative to a less virulent strain. Highly virulent *C. difficile* strains (also known as hypervirulent strains) are those that are capable of inducing severe clinical courses, increased mortality, and recurrent infections. Examples include, but are not limited to, BI/NAP-1/027 strains, ribotype 078/BK group strains, and ribotype 244/AF group strains. Less virulent *C. difficile* strains are those that may cause clinical courses at a moderate level. Non-virulent *C. difficile* strains are those that do not have pathogenic effects. Examples of

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less virulent strains include, but are not limited to, strains from the CF group, Y group, DH group, and J group. See Fig. 1B. The level of virulence of a *C. difficile* strain can be determined by clinical studies associating the particular *C. difficile* strain with disease severity following routine medical practice. Disease severity may be defined by death, ICU admission, or colectomy within 40 days of stool testing.

(I) Antibodies having differential binding specificities to toxin B or toxin A

An antibody (interchangeably used in plural form) is an immunoglobulin molecule capable of specific binding to a target, such as toxin A or toxin B of a *C. difficile* strain, through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term "antibody" encompasses not only intact (*i.e.*, full-length) polyclonal or monoclonal antibodies, but also antigen-binding fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, chimeric antibodies, diabodies, linear antibodies, single chain antibodies, multispecific antibodies (*e.g.*, bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies. An antibody includes an antibody of any class, such as IgD, IgE, IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant domain of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. The antibodies described herein can be murine, rat, human, or any other origin (including chimeric or humanized antibodies).

In some embodiments, the anti-toxin B antibodies described herein (or anti-toxin A antibodies described below) have a suitable binding affinity to the antigen. As used herein, "binding affinity" refers to the apparent association constant or K_A. The K_A is the reciprocal

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of the dissociation constant (K_D). The antibody described herein may have a binding affinity (K_D) of at least 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M, or lower. An increased binding affinity corresponds to a decreased K_D. Higher affinity binding of an antibody to a first target relative to a second target can be indicated by a higher K_A (or a smaller numerical value K_D) for binding the first target than the K_A (or numerical value K_D) for binding the second target. In such cases, the antibody has specificity for the first target (*e.g.*, a protein in a first conformation or mimic thereof) relative to the second target (*e.g.*, the same protein in a second conformation or mimic thereof; or a second protein). Differences in binding affinity (*e.g.*, for specificity or other comparisons) can be at least 1.5, 2, 3, 4, 5, 10, 15, 20, 37.5, 50, 70, 80, 91, 100, 500, 1000, 10,000 or 10^5 fold.

Binding affinity can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (*e.g.*, using a fluorescence assay). Exemplary conditions for evaluating binding affinity are in HBS-P buffer (10 mM HEPES pH7.4, 150 mM NaCl, 0.005% (v/v) Surfactant P20). These techniques can be used to measure the concentration of bound binding protein as a function of target protein concentration. The concentration of bound binding protein ([Bound]) is related to the concentration of free target protein ([Free]) and the concentration of binding sites for the binding protein on the target where (N) is the number of binding sites per target molecule by the following equation:

[Bound] = [N][Free]/(Kd+[Free])

It is not always necessary to make an exact determination of K_A, though, since sometimes it is sufficient to obtain a quantitative measurement of affinity, *e.g.*, determined using a method such as ELISA or FACS analysis, which is proportional to K_A, and thus can be used for comparisons, such as determining whether a higher affinity is, *e.g.*, 2-fold higher, to obtain a qualitative measurement of affinity, or to obtain an inference of affinity, *e.g.*, by activity in a functional assay, *e.g.*, an in vitro or in vivo assay.

In some embodiments, the antibodies used in the detection assays described herein differentially bind toxin B or toxin A of highly virulent *C. difficile* strains as compared with toxin B (or toxin A) of less virulent *C. difficile* strains. An antibody that "differentially binds" to a first target or a first epitope as relative to a second target or a second epitope

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refers to an antibody that has different binding affinities to the first and second targets or different binding affinities to the first and second epitopes. In some examples, such an antibody may have a much higher binding affinity to the first target/epitope as relative to the second target/epitope, or *vice versa*, *e.g.*, at least 2-fold, 5-fold, 10-fold, 50-fold, 100-fold, 200-fold, 500-fold, 1,000-fold, 10,000-fold higher. In other examples, the antibody may have a much lower binding affinity to the first target/epitope as relative to the second target/epitope, or *vice versa*, *e.g.*, at least 2-fold, 5-fold, 10-fold, 50-fold, 100-fold, 200-fold, 500-fold, 1,000-fold, 10,000-fold lower.

In specific examples, the antibodies used in the detection assays described herein specifically bind toxin B or toxin A of a highly virulent *C. difficile* strain. Alternatively, the antibodies specifically bind toxin B or toxin A of a less virulent *C. difficile* strain. An antibody "specifically binds" to a target antigen if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that specifically (or preferentially) binds to toxin B or toxin A of highly virulent *C. difficile* strains is an antibody that binds this target antigen with greater affinity, avidity, more readily, and/or with greater duration than it binds to other antigens, such as toxin B or toxin A of less virulent *C. difficile* strains. It is also understood by reading this definition that, for example, an antibody that specifically binds to a first target antigen may or may not specifically or preferentially bind to a second target antigen. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding. In some examples, the antibodies used in the assay methods described herein bind toxin B or toxin A of a highly virulent *C. difficile* strain but not toxin B or toxin A of less virulent *C. difficile* strains, *e.g.*, binding to an epitope that is distinct in toxin B or toxin A of highly virulent *C. difficile* strains. In other examples, the antibodies used in the assay methods described herein bind toxin B or toxin A of a less virulent *C. difficile* strain but not toxin B or toxin A of highly virulent *C. difficile* strains, *e.g.*, binding to an epitope that is distinct in toxin B or toxin A of less virulent *C. difficile* stains.

The detection methods described herein may also involve antibodies that bind toxin B or toxin A of a broad spectrum of *C. difficile*, regardless of virulence levels.

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Antibodies capable of binding toxin B or toxin A of *C. difficile* strains with different virulence levels can be made by any method known in the art. See, for example, Harlow and Lane, (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.

In some embodiments, antibodies differentially binding (*e.g.*, specific to) a target antigen (*e.g.*, toxin B/toxin A of a highly virulent *C. difficile* strain or toxin B/toxin A of a less virulent *C. difficile* strain) can be made by the conventional hybridoma technology, using a suitable antigen. Sequences of toxin B or toxin A of different *C. difficile* strains are known in the art. Some examples are provided in the tables 1 and 2 below.

Table 1: Toxin B of Exemplary *C. difficile* strains

C. difficile Strains	GenBank Accession Number for Toxin B
BI1	YP_006197785.1
F253	WP_021407033.1
P59	WP_021426110.1
CD160	WP_021383384.1
F314	WP_021407773.1
CD69	WP_021374582.1
E19	CCL40218.1
CD45	WP_021371861.1
70-100-2010, CD41, CD104, CD206, CD 212, E12	WP_003434531.1
DA00211	WP_021402343.1
P48, P70, P73, P 75	WP_021424365.1
P13, P46, P68	WP_021418418.1
T6	CCL51870.1
630	WP_009902069.1
P11	WP_021418106.1
P7	WP_021417070.1
CD8, CD9, P5, P37	WP_021360236.1
NAP07, T5, T20, E1	WP_003418170.1
DA00305	WP_021405616.1
DA00129, CD86	WP_021377046.1
CD129	WP_021380256.1
DA00216	WP_021403734.1
CD43, CD131, CD149, CD166, CD200, F152, P8, P69	WP_021370125.1

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CD113	WP_021378956.1
050-P50-2011, 002-P50-2011	WP_003426838.1
Y184	EQI47082.1
T14	CCL93631.1
CD40	EQE46098.1
840	WP_021391274.1
P3	EQI94941.1
DA00232	EQH37808.1
CD201	EQF71112.1
CD201	EQF71113.1
CD196, R20291	WP_009892729.1
P13	WP_021418419.1
Y41	WP_021411551.1
CD181	WP_021388614.1
655	WP_021390804.1
DA00114	WP_021393724.1
630	WP_011860904.1
DA0023, DA00261	WP_021401850.1
DA00197	WP_021398198.1
P25, DA00244	WP_021404260.1
P5	EQJ00853.1
CD9	WP_021361364.1
P1	WP_021415976.1
DA00129, F152	WP_021394767.1
P59	WP_021426113.1
Y266	WP_021413482.1
CD42	WP_021369285.1
CD17	WP_021362272.1
CD70	WP_021376831.1
CD159	WP_021381998.1
P32	WP_021422924.1
P69	WP_021427085.1
CD111, DA00128	WP_021378690.1
DA00191	WP_021400350.1
P31	WP_021422850.1
P73	WP_021427542.1
Y247	WP_021413325.1
CD90, DA00167, 842	WP_021377621.1
CD166	WP_021386968.1
CD8	EQE15594.1

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P72	WP_021427279.1

Table 2: Toxin A of Exemplary *C. difficile* strains

C. difficile Strains	GenBank Accession Number for Toxin A
ATCC 4325/VPI 10463	P16154
ST37	AGG91507
JF09	AFN52237
BI1	YP_006197787
R20291	YP_003217088

The full-length target antigen or a fragment thereof, optionally coupled to a carrier protein such as KLH, can be used to immunize a host animal for generating antibodies binding to that antigen. The route and schedule of immunization of the host animal are generally in keeping with established and conventional techniques for antibody stimulation and production, as further described herein. General techniques for production of mouse, humanized, and human antibodies are known in the art and are described herein. It is contemplated that any mammalian subject including humans or antibody producing cells therefrom can be manipulated to serve as the basis for production of mammalian, including human hybridoma cell lines. Typically, the host animal is inoculated intraperitoneally, intramuscularly, orally, subcutaneously, intraplantar, and/or intradermally with an amount of immunogen, including as described herein.

Hybridomas can be prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C. (1975) Nature 256:495-497 or as modified by Buck, D. W., et al., In Vitro, 18:377-381 (1982). Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. Generally, the technique involves fusing myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium, to eliminate unhybridized parent cells. Any of the media described herein, supplemented with or without

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serum, can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells may be used to produce the anti-toxin B or anti-toxin A monoclonal antibodies described herein. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (*e.g.*, radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that may be used as source of antibodies encompass all derivatives, progeny cells of the parent hybridomas that produce monoclonal antibodies capable of binding to toxin B or toxin A. Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity if present, can be removed, for example, by running the preparation over adsorbents made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen. Immunization of a host animal with a target antigen or a fragment containing the target amino acid sequence conjugated to a protein that is immunogenic in the species to be immunized, *e.g.,* keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl, or R1N=C=NR, where R and R1 are different alkyl groups, can yield a population of antibodies (*e.g.*, monoclonal antibodies).

If desired, an antibody (monoclonal or polyclonal) of interest (*e.g.*, produced by a hybridoma) may be sequenced and the polynucleotide sequence may then be cloned into a vector for expression or propagation. The sequence encoding the antibody of interest may be maintained in a vector in a host cell and the host cell can then be expanded and frozen for future use. In an alternative, the polynucleotide sequence may be used for genetic manipulation to improve the affinity (affinity maturation), or other characteristics of the antibody. It may be desirable to genetically manipulate the antibody sequence to obtain greater affinity to the target antigen as relative to other antigens. It will be apparent to one of

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skill in the art that one or more polynucleotide changes can be made to the antibody and still maintain its binding specificity to the target antigen.

Antigen-binding fragments of an intact antibody (full-length antibody) can be prepared via routine methods. For example, F(ab')2 fragments can be produced by pepsin digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')2 fragments. Genetically engineered antibodies, such as humanized antibodies, chimeric antibodies, single-chain antibodies, and bi-specific antibodies, can be produced via, *e.g.*, conventional recombinant technology.

In some embodiments, the full-length of toxin B or toxin A of a highly virulent *C. difficile* strain or toxin B or toxin A of a less virulent *C. difficile* strain, or a fragment thereof, can be used as the antigen for preparing antibodies binding to such, following methods known in the art or described herein. Antibodies thus obtained can be characterized using methods well known in the art, for example, to confirm its binding activity to the antigen. Such antibodies can then be characterized for their binding activities to toxin B or toxin A of a different *C. difficile* strain, *e.g.*, a strain having a different virulence level. For example, if toxin B or toxin A of a highly virulent *C. difficile* strain is used as the antigen for antibody production, the antibodies thus obtained that bind the toxin B or toxin A of a highly virulent *C. difficile* strain can be characterized for their binding activity/affinity to toxin B or toxin A of less virulent *C. difficile* strains. Alternatively, if toxin B or toxin A of a less virulent *C. difficile* strain is used as the antigen for antibody production, the antibodies thus obtained that bind the toxin B or toxin A can be characterized for their binding activity/affinity to toxin B or toxin A of highly virulent *C. difficile* strains. Antibodies that differentially bind toxin B or toxin A of highly virulent *C. difficile* strains as relative to toxin B or toxin A of less virulent *C. difficile* strains can thus be identified.

Alternatively, a fragment that comprises an epitope that presents only in toxin B or toxin A of highly virulent *C. difficile* strains or an epitope that presents only in toxin B or toxin A of less virulent *C. difficile* strains can be used as the antigen for antibody production. Antibodies thus produced can be characterized to confirm their differential binding activity to toxin B or toxin A of highly virulent *C. difficile* strains versus toxin B or toxin A of less virulent *C. difficile* strains. Such highly virulent strain-specific or less virulent strain-

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specific epitopes can be identified by comparing the amino acid sequences of toxin B or toxin A polypeptides from different *C. difficile* strains, *e.g.*, those listed in Table 1 above, by conventional methods (*e.g.*, computational analysis).

Any of the antibodies described herein may be prepared by culturing a hybridoma cell line producing such and purifying the antibodies from the hybridoma culture. Alternatively, they may be produced by recombinant technology.

As a further alternative, antibodies may be produced that bind toxins (e.g., toxin A or toxin B) from a broad range of *C. difficile* strains, including both highly virulent and less virulent strains. For example, fragments containing an epitope present in a toxin of both highly virulent and less virulent strains may be used to produce antibodies. Antibodies capable of binding such can be selected via routine methods. Such antibodies can be further confirmed for their binding activity to toxins of both highly virulent and less virulent strains.

In one example, DNA encoding a monoclonal antibody specific to a target antigen can be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into one or more expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. See, *e.g.*, PCT Publication No. WO 87/04462.

A single-chain antibody can be prepared via recombinant technology by linking a nucleotide sequence coding for a heavy chain variable region and a nucleotide sequence coding for a light chain variable region. Preferably, a flexible linker is incorporated between the two variable regions. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent Nos. 4,946,778 and 4,704,692) can be adapted to produce a phage or yeast scFv library. scFv clones specific to toxin B of a highly virulent *C. difficile* strain or toxin B of a less virulent *C. difficile* strain can be identified from the library following routine procedures. Positive clones can be subjected to further screening to identify those that differentially bind toxin B of highly virulent strains as relative to toxin B

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of less virulent strains.

Antibodies obtained following a method known in the art or described herein can be characterized using methods also known in the art or described herein. For example, one method is to identify the epitope to which the antigen binds, also referred to as "epitope mapping." There are many methods known in the art for mapping and characterizing the location of epitopes on proteins, including solving the crystal structure of an antibody-antigen complex, competition assays, gene fragment expression assays, and synthetic peptide-based assays, as described, for example, in Chapter 11 of Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999. As an additional example, epitope mapping can be used to determine the sequence to which an antibody binds. The epitope can be a linear epitope, *i.e.*, contained in a single stretch of amino acids, or a conformational epitope formed by a three-dimensional interaction of amino acids that may not necessarily be contained in a single stretch (primary structure linear sequence). Peptides of varying lengths (*e.g.*, at least 4-6 amino acids long) can be isolated or synthesized (*e.g.*, recombinantly) and used for binding assays with an antibody. In another example, the epitope to which the antibody binds can be determined in a systematic screening by using overlapping peptides derived from the target antigen sequence and determining binding by the antibody. According to the gene fragment expression assays, the open reading frame encoding the target antigen is fragmented either randomly or by specific genetic constructions and the reactivity of the expressed fragments of the antigen with the antibody to be tested is determined. The gene fragments may, for example, be produced by PCR and then transcribed and translated into protein in vitro, in the presence of radioactive amino acids. The binding of the antibody to the radioactively labeled antigen fragments is then determined by immunoprecipitation and gel electrophoresis. Certain epitopes can also be identified by using large libraries of random peptide sequences displayed on the surface of phage particles (phage libraries). Alternatively, a defined library of overlapping peptide fragments can be tested for binding to the test antibody in simple binding assays. As an additional example, mutagenesis of an antigen binding domain, domain swapping experiments and alanine scanning mutagenesis can be performed to identify residues required, sufficient, and/or necessary for epitope binding. For example, domain swapping

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experiments can be performed using a mutant of a target antigen in which various fragments of the toxin B (or toxin A) polypeptide have been replaced (swapped) with sequences from a closely related, but antigenically distinct protein (such as a toxin B of a different *C. difficile* strain). By assessing binding of the antibody to the mutant toxin B, the importance of the particular antigen fragment to antibody binding can be assessed.

Alternatively, competition assays can be performed using other antibodies known to bind to the same antigen to determine whether an antibody binds to the same epitope as the other antibodies. Competition assays are well known to those of skill in the art.

Any of the suitable methods known in the art, *e.g.*, the epitope mapping methods as described herein, can be applied to determine whether the antibody binds one or more of the specific residues/segments in a toxin B or toxin A polypeptide as described herein. Further, the interaction of the antibody with one or more of those defined residues in the antigen can be determined by routine technology.

(II) Immunoassays

One or more immunoassays can be performed, using any of the antibodies described herein that differentially binds toxin B or toxin A of highly virulent *C. difficile* strains as relative to toxin of less virulent *C. difficile* strains, to determine the presence of highly virulent *C. difficile* or less virulent *C. difficile* in a sample, *e.g.*, a biosample obtained from a subject such as a human patient having or suspected of having *C. difficile* infection.

An immunoassay is a biochemical test that measures the presence or concentration of a molecule of interest (*e.g.*, a macromolecule such as a protein) in a sample through the use of an antibody or immunoglobulin. Typically, an antibody specific to the molecule of interest interacts with the molecule in an immunoassay. The antibody can be labeled, directly or indirectly such that those bound to the molecule could release a detectable signal. Presence or concentration of the molecule of interest can be determined based on the level of the detectable signal. The immunoassay described herein can involve the use of different types of labels, including enzymes, radioactive isotopes, DNA reporters, fluorogenic reporters, electrochemiluminescent tags, all of which are well known in the art. In some instances, the immunoassay can involve a catalyst such as an enzyme to amplify the signal. Alternatively, the immunoassay described herein can be performed without the use of a label.

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The "immunoassay" used to detect an antigen of interest (*e.g.*, toxin B or toxin A) according to the present disclosure may be based on standard techniques known in the art. The immunoassay as described herein may be in various formats, *e.g.*, competitive, one-site non-competitive, or two-site, noncompetitive (Sandwich assays). In some examples, it may comprise multiple steps with reagents being added and washed away or separated at different points in the assay. Such an assay is known as a heterogeneous immunoassay. In other examples, the immunoassay may be carried out simply by mixing the reagents and sample and making a physical measurement. Such assays are known as homogenous immunoassays.

In a preferred embodiment, the immunoassay may be an ELISA, which may be performed based on single molecule array (Simoa) technology as described herein. See also Rissin, et al., Nat Biotechnol, 28(6): p. 595-9 (2010); Rissin, et al., Anal Chem, 83(6): p. 2279-85 (2011); Kan, et al., Lab Chip, 2012. 12(5): p. 977-85. In digital ELISAs, single protein molecules are captured on paramagnetic beads, labeled with an enzyme, and detected in arrays of femtoliter-sized wells. Sensitivities of digital ELISAs are typically 1000-fold greater than conventional ELISAs. In applying Simoa to the development of digital immunoassays for toxins A and B, the differential binding of toxin B of BI/NAP-1/027 and BK/078 strains by specific pairs of candidate monoclonal antibodies against toxin B was observed. It is shown that the disclosed immunoassays can differentiate toxin Bs of highly virulent strains from those of other *C. difficile* isolates, and thus can provide information that is highly relevant to clinical prognosis.

ELISAs are generally well known in the art. The ELISA assay used in the detection methods described herein can be in any format known in the art, including direct ELISA, Sandwich ELISA, competitive ELISA, and multiple and ready-to-use ELISA. In a typical "indirect" ELISA, an antibody having specificity for the antigen of interest is immobilized on a solid surface (*e.g.*, the wells of a standard microtiter assay plate, or the surface of a microbead or a microarray) and a sample comprising, *e.g.*, bodily fluid or substances extracted from stool samples, to be tested for the presence of the antigen is brought into contact with the immobilized antibody. Any antigen of interest in the sample will bind to the immobilized antibody. The bound antibody/antigen complexes may then be detected using any suitable method. In one embodiment, a second antibody, which specifically recognizes

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an epitope of the antigen, which may be different from the epitope recognized by the immobilized antibody, is used to detect the antibody/antigen complexes. The second antibody is usually labelled with a detectable marker (directly or indirectly). In some examples, the maker can be an enzyme such as peroxidase, alkaline phosphatase, or galactosidase, allowing quantitative detection by the addition of a substrate for the enzyme which generates a detectable product, for example a colored, chemiluminescent or fluorescent product. Other types of detectable labels known in the art may be used with equivalent effect. In other examples, the second antibody may be labeled with a member of a receptor/ligand pair, for example, biotin. An enzyme conjugate comprising an enzyme conjugated with the other member of the receptor/ligand pair, *e.g.*, streptavidin, can be brought into contact with the second antibody. A substrate of the enzyme is then added to produce a product that releases a detectable signal.

In some embodiments, a differential detection method as described herein involves two immunoassays, one or both of which may be digital ELISAs. The first immunoassay may comprise a pair of antibodies capable of binding to *C. difficile* toxin B or toxin A, one being a capture antibody and the other being a detection antibody. Both the capture and detection antibodies may bind to toxin B or toxin A of a broad spectrum of *C. difficile* strains such that toxin B or toxin A of both highly virulent strains and less virulent strains can be detected/quantified in the first immunoassay. In a preferred example, the capture and detection antibodies bind to two separate epitopes of the toxin B or toxin A.

The second immunoassay may comprise a pair of antibodies, one of which differentially binds to toxin B or toxin A of highly virulent *C. difficile* strains as relative to toxin B or toxin A of less virulent strains. The other antibody may bind to toxin B or toxin A of a broad spectrum of *C. difficile* strains, regardless of their virulence levels. In some instances, this antibody may be identical to the capture or detection antibody used in the first immunoassay. The differential binding antibody can be used as the detection antibody and the other antibody can be used as the capture antibody, or *vice versa*. In some examples, the differential binding antibody is specific to toxin B of highly virulent *C. difficile* strains (*e.g.*, it has a higher binding affinity to toxin B or toxin A of highly virulent *C. difficile* strains as compared with its binding affinity to toxin B or toxin A of less virulent strains or it does not

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bind toxin B or toxin A of less virulent *C. difficile* strains) and the second immunoassay allows for detection or quantification of toxin B or toxin A of highly virulent *C. difficile* strains. In other examples, the differential binding antibody is specific to toxin B or toxin A of less virulent *C. difficile* strains (*e.g.*, it has a higher binding affinity to toxin B or toxin A of less virulent *C. difficile* strains as compared with its binding affinity to toxin B or toxin A of highly virulent strains or it does not bind to toxin B or toxin A of highly virulent *C. difficile* strains) and the second immunoassay allows for detection or quantification of toxin B or toxin A of less virulent *C. difficile* strains.

By comparing the results obtained from the first and second immunoassays, the presence of a highly virulent *C. difficile* strain or a less virulent *C. difficile* strain can be determined. For example, if an antibody specific to toxin B of highly virulent strains is used in the second immunoassay, positive results from both the first immunoassay and the second immunoassay indicate that the sample contains a highly virulent strain, while positive results from the first immunoassay and negative results from the second immunoassay indicate that the sample contains a less virulent strain. Alternatively, if an antibody specific to toxin B of less virulent strains is used in the second immunoassay, positive results from both the first immunoassay and the second immunoassay indicate that the sample contains a less virulent strain, while positive results from the first immunoassay and negative results from the second immunoassay indicate that the sample contains a highly virulent strain. If negative results are observed in both the first and second immunoassays, it indicates that the sample may contain no *C. difficile* or a non-virulent strain.

In some examples, a ratio between a first value obtained from the first immunoassay and a second value obtained from the second immunoassay can be calculated. The first value (*e.g.*, relative fluorescent units) represents the level of toxin B or toxin A measured in the first immunoassay and the second value (*e.g.*, relative fluorescent units) represents the level of toxin B or toxin A measured in the second immunoassay. This ratio can be compared with a predetermined value to determine the presence of a highly virulent *C. difficile* strain or a less virulent *C. difficile* strain in a sample that is analyzed by the differential detection method described herein. The predetermined value represents a cutoff value, which can be

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determined by performing the same assay on samples containing a standard highly virulent strain and/or samples containing a standard less virulent strain.

The differential detection method described herein that involves two immunoassays can be of any suitable format. In some instances, the differential detection method is a multiplex assay, in which different capture antibodies can be used in the first and second immunoassays. Fig. 4. In other instances, the differential detection method is a simplified assay with a single detector, in which the same antibody can be used as the capture antibody in both the first and second immunoassays. Fig. 5 and 6. Preferably, in each immunoassay, an antibody that is capable of binding to a toxin (e.g., toxin B) of a broad spectrum of *C. difficile* strains, and the antibody that conjugates to a label (e.g., a dye) can be an antibody that differentially binds to the toxin (e.g., toxin B) of a highly virulent *C. difficile* strain as compared to the toxin of a less virulent *C. difficile* strain. In some examples, the labels (e.g., a dye) used in the first and second immunoassays are identical. In other examples, different labels are used in the first and second immunoassays.

Those of ordinary skill in the art will be aware of a variety of assay methods and systems that may be used in connection with the methods described herein. Generally, the methods employed have low limits of detection and/or limits of quantification as compared to bulk analysis techniques (*e.g.*, ELISA methods). The use of assay methods that have low limits of detection and/or limits of quantification allows for correlations to be made between the various parameters discussed above and a method of treatment and/or diagnostic indication that may otherwise not be determinable and/or apparent.

The terms "limit of detection" (or LOD) and "limit of quantification" (or LOQ) are given their ordinary meaning in the art. The LOD refers to the lowest analyte concentration likely to be reliably distinguished from background noise and at which detection is feasible. The LOD as used herein is defined as three standard deviations (SD) above background noise. The LOQ refers to the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. Generally, as is used herein, the LOQ refers to the lowest concentration above the LOD wherein the coefficient of variation (CV) of the measured concentrations is less than about 20%.

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In some embodiments, an assay method employs a step of spatially segregating biomarker molecules into a plurality of locations to facilitate detection/quantification, such that each location comprises/contains either zero or one or more biomarker molecules. Additionally, in some embodiments, the locations may be configured in a manner such that each location can be individually addressed. In some embodiments, a measure of the concentration of biomarker molecules in a fluid sample may be determined by detecting biomarker molecules immobilized with respect to a binding surface having affinity for at least one type of biomarker molecule. In certain embodiments the binding surface may form (*e.g.*, a surface of a well/reaction vessel on a substrate) or be contained within (*e.g.*, a surface of a capture object, such as a bead, contained within a well) one of a plurality of locations (*e.g.*, a plurality of wells/reaction vessels) on a substrate (*e.g.*, plate, dish, chip, optical fiber end, *etc*). At least a portion of the locations may be addressed and a measure indicative of the number/percentage/fraction of the locations containing at least one biomarker molecule may be made. In some cases, based upon the number/percentage/fraction, a measure of the concentration of biomarker molecules in the fluid sample may be determined. The measure of the concentration of biomarker molecules in the fluid sample may be determined by a digital analysis method/system optionally employing Poisson distribution adjustment and/or based at least in part on a measured intensity of a signal, as will be known to those of ordinary skill in the art. In some cases, the assay methods and/or systems may be automated.

Additional details of exemplary, non-limiting assay methods which comprise one or more steps of spatially segregating biomarker molecules will now be described. In certain embodiments, a method for detection and/or quantifying biomarker molecules in a sample comprises immobilizing a plurality of biomarker molecules with respect to a plurality of capture objects (*e.g.*, beads) that each include a binding surface having affinity for at least one type of biomarker. For example, the capture objects may comprise a plurality of beads comprising a plurality of capture components (*e.g.*, an antibody as described herein). At least some of the capture objects may be spatially separated/segregated into a plurality of locations, and at least some of the locations may be addressed/interrogated (*e.g.*, using an imaging system). A measure of the concentration of biomarker molecules in the fluid sample may be determined based on the information received when addressing the locations (*e.g.*, using the

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information received from the imaging system and/or processed using a computer implemented control system). In some cases, a measure of the concentration may be based at least in part on the number of locations determined to contain a capture object that is or was associated with at least one biomarker molecule. In other cases and/or under differing conditions, a measure of the concentration may be based at least in part on an intensity level of at least one signal indicative of the presence of a plurality of biomarker molecules and/or capture objects associated with a biomarker molecule at one or more of the addressed locations.

In some embodiments, the number/percentage/fraction of locations containing a capture object but not containing a biomarker molecule may also be determined and/or the number/percentage/fraction of locations not containing any capture object may also be determined. In such embodiments, a measure of the concentration of biomarker molecules in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the total number of locations determined to contain a capture object not associated with a biomarker molecule, and/or a measure of the concentration of biomarker molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the number of locations determined to not contain any capture objects, and/or a measure of the concentration of biomarker molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the number of locations determined to contain a capture object. In yet other embodiments, a measure of the concentration of biomarker molecules in a fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object and a biomarker molecule to the total number of locations addressed and/or analyzed.

In certain embodiments, at least some of the plurality of capture objects (*e.g.*, at least some associated with at least one biomarker molecule) are spatially separated into a plurality of locations, for example, a plurality of reaction vessels in an array format. The plurality of reaction vessels may be formed in, on and/or of any suitable material, and in some cases, the reaction vessels can be sealed or may be formed upon the mating of a substrate with a sealing

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component, as discussed in more detail below. In certain embodiments, especially where quantization of the capture objects associated with at least one biomarker molecule is desired, the partitioning of the capture objects can be performed such that at least some (*e.g.*, a statistically significant fraction; e.g., as described in WO 2011/109364, by Duffy et al., the relevant disclosures of which are incorporated by reference herein) of the reaction vessels comprise at least one or, in certain cases, only one capture object associated with at least one biomarker molecule and at least some (*e.g.*, a statistically significant fraction) of the reaction vessels comprise an capture object not associated with any biomarker molecules. The capture objects associated with at least one biomarker molecule may be quantified in certain embodiments, thereby allowing for the detection and/or quantification of biomarker molecules in the fluid sample by techniques described in more detail herein.

An exemplary assay method may proceed as follows. A sample fluid containing or suspected of containing biomarker molecules is provided. An assay consumable comprising a plurality of assay sites is exposed to the sample fluid. In some cases, the biomarker molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the assay sites contain a single biomarker molecule and a statistically significant fraction of the assay sites do not contain any biomarker molecules. The assay sites may optionally be exposed to a variety of reagents (*e.g.*, using a reagent loader) and or rinsed. The assay sites may then optionally be sealed and imaged (see, for example, US 2012-0196774 ," by Fournier et al., the relevant disclosures of which are incorporated by reference herein.). The images are then analyzed (e.g., using a computer implemented control system) such that a measure of the concentration of the biomarker molecules in the fluid sample may be obtained, based at least in part, by determination of the number/fraction/percentage of assay sites which contain a biomarker molecule and/or the number/fraction/percentage of sites which do not contain any biomarkers molecules. In some cases, the biomarker molecules are provided in a manner (*e.g.*, at a concentration) such that at least some assay sites comprise more than one biomarker molecule. In such embodiments, a measure of the concentration of biomarker molecules in the fluid sample may be obtained at least in part on an intensity level of at least one signal indicative of the presence of a plurality of biomarkers molecules at one or more of the assay sites

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In some cases, the methods optionally comprise exposing the fluid sample to a plurality of capture objects, for example, beads. At least some of the biomarker molecules are immobilized with respect to a bead. In some cases, the biomarker molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single biomarker molecule and a statistically significant fraction of the beads do not associate with any biomarker molecules. At least some of the plurality of beads (*e.g.*, those associated with a single biomarker molecule or not associated with any biomarker molecules) may then be spatially separated/segregated into a plurality of assay sites (*e.g.*, of an assay consumable). The assay sites may optionally be exposed to a variety of reagents and/or rinsed. At least some of the assay sites may then be addressed to determine the number of assay sites containing a biomarker molecule. In some cases, the number of assay sites containing a bead not associated with a biomarker molecule, the number of assay sites not containing a bead and/or the total number of assay sites addressed may also be determined. Such determination(s) may then be used to determine a measure of the concentration of biomarker molecules in the fluid sample. In some cases, more than one biomarker molecule may associate with a bead and/or more than one bead may be present in an assay site. In some cases, the plurality biomarker molecules may be exposed to at least one additional reaction component prior to, concurrent with, and/or following spatially separating at least some of the biomarker molecules into a plurality of locations.

The biomarker molecules (e.g., toxin A or toxin B of *C. difficile*) may be directly detected or indirectly detected. In the case of direct detection, a biomarker molecule may comprise a molecule or moiety that may be directly interrogated and/or detected (*e.g.*, a fluorescent entity). In the case of indirect detection, an additional component is used for determining the presence of the biomarker molecule. For example, the biomarker molecules (e.g., optionally associated with a bead) may be exposed to at least one type of binding ligand. A "binding ligand," is any molecule, particle, or the like which specifically binds to or otherwise specifically associates with a biomarker molecule to aid in the detection of the biomarker molecule. In certain embodiments, a binding ligand may be adapted to be directly detected (e.g., the binding ligand comprises a detectable molecule or moiety) or may be adapted to be indirectly detected (e.g., including a component that can convert a precursor

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labeling agent into a labeling agent). A component of a binding ligand may be adapted to be directly detected in embodiments where the component comprises a measurable property (e.g., a fluorescence emission, a color, etc.). A component of a binding ligand may facilitate indirect detection, for example, by converting a precursor labeling agent into a labeling agent (e.g., an agent that is detected in an assay). A "precursor labeling agent" is any molecule, particle, or the like, that can be converted to a labeling agent upon exposure to a suitable converting agent (e.g., an enzymatic component). A "labeling agent" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique. In some embodiments, the binding ligand may comprise an enzymatic component (*e.g.*, horseradish peroxidase, beta-galactosidase, alkaline phosphatase, etc.). A first type of binding ligand may or may not be used in conjunction with additional binding ligands (*e.g.*, second type, etc.).

More than one type of binding may be employed in any given assay method, for example, a first type of binding ligand and a second type of binding ligand. In one example, the first type of binding ligand is able to associate with a first type of biomarker molecule and the second type of binding ligand is able to associate with the first binding ligand. In another example, both a first type of binding ligand and a second type of binding ligand may associate with the same or different epitopes of a single biomarker molecule, as described herein. In some embodiments, at least one binding ligand comprises an enzymatic component.

In some embodiments, a binding ligand and/or a biomarker may comprise an enzymatic component. The enzymatic component may convert a precursor labeling agent (*e.g.*, an enzymatic substrate) into a labeling agent (*e.g.*, a detectable product). A measure of the concentration of biomarker molecules in the fluid sample can then be determined based at least in part by determining the number of locations containing a labeling agent (*e.g.*, by relating the number of locations containing a labeling agent to the number of locations containing a biomarker molecule (or number of capture objects associated with at least one biomarker molecule to total number of capture objects)). Non-limiting examples of enzymes or enzymatic components include horseradish peroxidase, beta-galactosidase, and alkaline phosphatase. Other non-limiting examples of systems or methods for detection include embodiments where nucleic acid precursors are replicated into multiple copies or converted to

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a nucleic acid that can be detected readily, such as the polymerase chain reaction (PCR), rolling circle amplification (RCA), ligation, Loop-Mediated Isothermal Amplification (LAMP), etc. Such systems and methods will be known to those of ordinary skill in the art, for example, as described in "DNA Amplification: Current Technologies and Applications," Vadim Demidov et al., 2004, the relevant disclosures of which are incorporated by reference herein.

Another exemplary embodiment of indirect detection is as follows. In some cases, the biomarker molecules may be exposed to a precursor labeling agent (*e.g.*, an enzymatic substrate) and the enzymatic substrate may be converted to a detectable product (*e.g.*, fluorescent molecule) upon exposure to a biomarker molecule.

The assay methods and systems may employ a variety of different components, steps, and/or other aspects that will be known and understood by those of ordinary skill in the art. For example, a method may further comprise determining at least one background signal determination (*e.g.*, and further comprising subtracting the background signal from other determinations), wash steps, and the like. In some cases, the assays or systems may include the use of at least one binding ligand, as described herein. In some cases, the measure of the concentration of biomarker molecules in a fluid sample is based at least in part on comparison of a measured parameter to a calibration curve. In some instances, the calibration curve is formed at least in part by determination at least one calibration factor, as described above.

In certain embodiments, solubilized, or suspended precursor labeling agents may be employed, wherein the precursor labeling agents are converted to labeling agents which are insoluble in the liquid and/or which become immobilized within/near the location (e.g., within the reaction vessel in which the labeling agent is formed). Such precursor labeling agents and labeling agents and their use is described in US-2010-0075862, by Duffy et al., the relevant disclosures thereof are incorporated by reference herein.

Certain methods and systems suitable for performing any of the detection assays described herein are known in the art. See, e.g., US20070259448 (by Walt et al.); US20070259385 (by Walt et al.); US20070259381 (by Walt et al.); WO2009029073 (by Walt et al.); US20100075862 (by Duffy et al.); US201000754072 (by Duffy et al.); US20100075439 (by Duffy et al.); WO2010/039179 (by Duffy et al.); US20100075355 (by Duffy et al.);

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US20110212848 (by Duffy et al.); WO 2011/109364 (by Duffy et al.); WO 2011/109372 (by Duffy et al.); US20110212462 (by Duffy et al.); WO 2011/109379 (by Rissin et al.); US20110212537 (by Duffy et al.); US20120196774 (by Fournier et al.); US 2011-0245097 (by Rissin et al.). The relevant disclosures of each of those are incorporated by reference herein.

Detection of Both Toxin A and Toxin B

Another aspect of the present disclosure provides an ultrasensitive and quantitative assay method for diagnosing *C. difficile* infection, which involves detecting or quantifying both toxin A and toxin B. In some embodiments, the assay method includes two immunoassays, one for detecting toxin A of *C. difficile* and the other for detecting toxin B of *C. difficile*. Antibodies specific to toxin A or toxin B can be used in the two immunoassays. The anti-toxin A antibody can be an antibody that is capable of binding to toxin A of a broad spectrum of *C. difficile* strains, including highly virulent strains and less virulent strains. Alternatively or in addition, the anti-toxin B antibody can be an antibody that is capable of binding to toxin B of a broad spectrum of *C. difficile* strains, including highly virulent strains and less virulent strains. Such an anti-toxin A antibody or an anti-toxin B antibody may bind to an epitope common to toxin A or toxin B of the various strains, which can be identified by comparing the amino acid sequences of toxin A or toxin B as known in the art. Such an assay method would allow for detecting the presence of various *C. difficile* strains, regardless of their virulence levels.

The immunoassays can be of any format known in the art, *e.g.*, those disclosed herein. In some examples, one or both of the immunoassays are Sandwich assays, which involve the use of two anti-toxin A antibodies and/or two anti-toxin B antibodies, one being a capture antibody and the other being a detection antibody. The capture antibody can be immobilized on a solid support, which can be a bead or a multi-well plate. The detection antibody can be conjugated to a label, *e.g.*, those described herein. In a preferred example, the label is a catalyst, such as an enzyme, so that the detecting signal can be amplified.

In one example, both of the immunoassays for detecting toxin A and toxin B are digital ELISA assays as described herein. Such assays can be performed using systems and devices known in the art or disclosed herein.

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In some cases, an assay method as described herein has a limit of detection of about 0.3 pg/mL (*e.g.*, 0.4 pg/mL, 0.5 pg/mL, 0.8 pg/mL, 1 pg/mL, 5 pg/mL, or 10 pg/mL) for toxin A and about 1 pg/mL for toxin B (*e.g.*, 1.2 pg/mL, 1.5 pg/mL, 1.8 pg/mL, 2 pg/mL, 5 pg/mL, 10 pg/mL, 15 pg/mL, 20 pg/mL, or 30 pg/mL).

Clinical Applications

Any one of the detection methods described herein, including the differential detection methods and the toxin A/toxin B detection method, can be applied in clinical settings for diagnosing *C. difficile* infection. To diagnose *C. difficile* in a subject having or suspected of having *C. difficile* infection, such as a human patient, a biological sample can be obtained from the subject. The biological sample can be a body fluid sample (*e.g.*, blood or urine). Alternatively the biological sample can be a stool sample, which can be processed before applying a detection method as described herein.

In some embodiments, a biological sample can be analyzed by the method described herein to detect or quantify both toxin A and toxin B in the sample. Presence of toxin-producing *C. difficile* in the sample can be determined if the concentration of toxin A in the sample, the concentration of toxin B in the sample, or both, are above a cutoff value(s). For example, if the concentration of toxin B is higher than about 20 pg/ml as determined by a digital ELISA method described herein, it can be determined that the sample contains *C. difficile* cells producing toxin B. If the subject is diagnosed as infected by *C. difficile*, a suitable treatment can be determined for treating the subject. Also, since the level of toxin A and/or toxin B appears to correlate with disease severity, the results obtained from the detection method can be relied upon to predict disease course and/or treatment outcome. Detection of the presence and/or concentration of a toxin (e.g., toxin A or toxin B) has higher clinical specificity than detection of the organism per se. This is one of the advantages of the present method over detection methods known in the art, such as NAAT and culture-based methods.

In other embodiments, a biological sample can be analyzed by applying a differential detection assay as described herein to detect or quantify toxin B of either highly virulent *C. difficile* strains or toxin B of less virulent *C. difficile* strains. Based on the results, the

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presence of highly virulent *C. difficile* strains or less virulent *C. difficile* strains in the sample can be determined. If the subject is diagnosed as being infected by a highly virulent *C. difficile* strain, the subject can be treated by a suitable anti-*C. difficile* drug, *e.g.*, an antibiotic such as metronidazole (Flagyl), Dificid (fidaxomicin), or vancomycin. Presence of highly virulent stains also provides clinically relevant prognostic information.

Any one of the detection assays described herein can be combined with one or more additional diagnosing approaches for better assessing *C. difficile* infection. For example, a subject (*e.g.*, human patient) can be examined to determine one or more symptoms associated with *C. difficile* infection, *e.g.*, diarrhea. In addition, a detection assay as described herein can be performed to detect the presence of toxin A, toxin B, or both in a biological sample from the subject (*e.g.*, a stool sample). Further, a genetic assay (*e.g.*, NAAT) can be performed to determine whether the subject is infected with a *C. difficile* strain that carries a gene encoding toxin A or toxin B. In some embodiments, the subject can be diagnosed as having *C. difficile* infection based on observation of a symptom associated with *C. difficile* infection (*e.g.*, diarrhea), and the presence of toxin A, toxin B, or both in a biological sample of the subject. Such a diagnostic method may further comprise detecting the presence of a gene encoding toxin A or toxin B.

Kit for Performing the Detection Methods

Also within the scope of this disclosure are kits for use in performing any one of the detection assays described herein for diagnosing *C. difficile* infection.

Such a kit may comprise two pairs of anti-toxin B antibodies. The first pair includes two antibodies capable of binding to toxin B of a broad spectrum of *C. difficile* strains, including both highly virulent stains and less virulent stains. These two antibodies may bind to different epitopes of a toxin B polypeptide. The second pair includes one antibody that is capable of binding to toxin B of a broad spectrum of *C. difficile* strains (*e.g.*, identical to one of the two antibodies in the first pair) and the other antibody differentially binds to toxin B of highly virulent *C. difficile* strains as relative to toxin B of less virulent *C. difficile* strains (*e.g.*, specific to toxin B of a highly virulent *C. difficile* strain or specific to toxin B of a less virulent *C. difficile* strain) as described herein.

One of the two antibodies in the first pair, the second pair, or both may be

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immobilized on a solid support such as a bead or a multi-well plate. The other antibody may be conjugated with a label, *e.g.*, an enzyme, a radioactive isotope, a DNA reporter, a fluorogenic reporter, or an electrochemiluminescent tag, all of which are well known in the art. In some examples, the antibody is conjugated with a catalyst such as an enzyme (*e.g.*, those described herein) for signal amplification. Alternatively, this antibody may be conjugated with a member of a receptor/ligand pair and the kit may further comprise a conjugate comprising the other member of the receptor/ligand pair and any of the labels described herein (*e.g.*, an enzyme). In one example, the antibody is labeled with biotin and the kit further comprises a streptavidin-enzyme conjugate.

In another embodiment, the kit may comprise two pairs of antibodies, the first pair including two antibodies binding to toxin B and the second pair including antibodies binding to toxin A. Antibodies of the first pair may be capable of binding to toxin B of a broad spectrum of *C. difficile* strains, including both highly virulent and less virulent strains. Alternatively or in addition, antibodies of the second pair may be capable of binding to toxin A of a broad spectrum of *C. difficile* strains, including both highly virulent and less virulent strains. One antibody of the first pair, the second pair, or both can be immobilized on a solid support and the other antibody may be conjugated with a label as described herein.

Any of the kits described here may further comprise one or more components for performing the detection assays (*e.g.*, immunoassays). For example, when an enzyme is used as the label, a substrate of the enzyme can be included in the kit. Further, the kit may also comprise an instruction manual providing guidance for using the kit to perform any one of the detection assay provided herein, such as any one of the differential detection methods by, *e.g.*, digital ELISA, or any one of the methods for detecting both toxin A and toxin B of *C. difficile* by, *e.g.*, digital ELISA.

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

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Example 1: Differential immunodetection of toxin B of highly virulent *C. difficile* BI/NAP-1/027

METHODS

Conventional Bead-Based ELISA

Mouse monoclonal antibodies against toxin B (B1, B2, and B3) were provided by bioMerieux (Lyon, France). Paramagnetic beads *($5x10^6$/ml)* coated in capture antibodies were incubated with culture filtrates (CF; see Example 2 below) in the wells of a microtiter plate for 2 h at room temperature. Captured toxin proteins were labeled with a biotinylated detection antibody *(0.1 μg/ml)* and an enzyme conjugate *(0.5 nM)*. Following washes, enzyme substrate was added to the microtiter plate wells, and fluorescent signals were measured using a Tecan plate reader.

Digital ELISA

Details of the Simoa technology have been described previously [Rissin, D.M. et al., *Nat Biotechnol*, **28**(6): p. 595-9 (2010); Rissin, D.M. et al., *Anal Chem*, **83**(6): p. 2279-85 (2011)], and assays were performed on the Simoa HD-1 Analyzer (Quanterix Corporation). Kan et al., *Lab Chip*, 2011. Briefly, paramagnetic beads coated in capture antibodies were incubated with stool samples after dilution and filtration; captured toxin proteins were labeled with a biotinylated detection antibody and an enzyme conjugate (streptavidin-beta-galactosidase). Following the addition of an enzyme substrate, beads were loaded into arrays of femtoliter-sized wells for isolation and detection of bound molecules. Digital ELISAs were developed with two separate pairs of antibodies (B1 capture/B2 detector, and B2 capture/B3 detector). Signals from the digital ELISAs were calibrated by spiking known concentrations of purified native toxin B (prepared from strain VPI using established methods) into either buffer or toxin-negative stool samples.

Specimen Collection and Testing

A panel of REA-typed clinical strains were each cultured in chopped meat broth for 24 hours. Culture filtrates (CF) were prepared by 3000 x g centrifugation to obtain supernatant, followed by passage through 0.2 μm syringe filter. CF were tested by conventional ELISA as described above.

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In all, 149 clinical stool specimens (each <72 hours old) that had been tested for toxigenic *C. difficile* by NAAT (Meridian Illumigene) were aliquoted and frozen at −80 °C; this study was approved by the BIDMC IRB. Aliquots were subsequently tested by digital ELISA (Quanterix, Lexington, MA) and by toxigenic culture (TC) (Gerding laboratory, Hines VA Hospital, IL) *[Tenover, F.C. et al., J Clin Microbiol*, **49**(5): p. 1831-7 (2011)]. All *C. difficile* isolates recovered from TC were typed by restriction endonuclease analysis (REA; Gerding laboratory) [Clabots, C.R. et al., *J Clin Microbiol,*. **31**(7): p. 1870-5 (1993)].

RESULTS

Detection of Toxin B Produced in vitro by Divergent Clinical Isolates

The ability of the antibody pairs to detect native toxin B of CF prepared from a panel of 12 clinical *C. difficile* isolates representing major strains in circulation as well as key control strains was evaluated first (Figure 1A). The high abundance of toxin *in vitro* allowed the use of conventional bead-based ELISA for these experiments. Relative amounts of toxin B in each sample as measured with each antibody pair, expressed in relative fluorescent units (RFU), were compared. Signal below the analytical limit of detection (defined as 3 SD above assay background, RFU = 157) for each assay was interpreted as "not detected" (ND); the signal was considered saturated at 50,000 RFU. Two toxin B assays as described above differentially detected toxin B as follows: the B1/B2 pair (B1 capture, B2 detection) detected toxin B of all B-producing strains except all three BI/NAP-1/027 strains (CF10, 11, and 12; Figure 1A), while the B2/B3 pair (B2 capture, B3 detection) detected toxin B of all B-producing strains (Figure 1A). For detection of toxin B of the BK1/078 strain (CF13, Figure 1A), the signal detected using the B2/B3 pair was ~21-fold more than that using the B1/B2 pair.

To confirm that the differential toxin detection observed was specifically due to differential binding of the B1 vs. B3 antibodies to toxin B, the CFs were retested via conventional ELISA using the B2 antibody as the capture antibody in combination with B1 vs. B3 as the detecting antibody, and obtained exactly the same results.

Detection of Toxin B Produced in vivo by Divergent Clinical Isolates

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Two digital ELISA assays were used to directly detect toxin B in clinical stool samples, and the results of toxin detection were correlated with the results of REA typing of the corresponding *C. difficile* isolates from TC. The lower abundance of toxin in *in vivo* samples necessitated the use of a single molecule immunoassay. Similar to results obtained with CF, differential detection of toxin B (by the two digital ELISAs) was observed in stool samples from which BI/NAP-1/027 isolates were recovered. Specifically, for all stool samples from which BI/NAP-1/027 isolates were recovered (n= 10), levels of toxin B as measured by the B2/B3 digital ELISA were >20-fold higher than levels of toxin B as measured by the B1/B2 pair (Figure 1B). Differential toxin detection was also observed in three other stool samples, which yielded *C. difficile* isolates with the following characteristics: two (sample IDs v26 and v122) were from separate but undefined REA groups, and the third (sample ID v60) was a member of the REA Group AF. Notably, Group AF includes PCR ribotype 244, a strain which has been shown to have genetic similarity to BI/NAP-1/027 strains and to similarly be associated with more severe disease [Lim, et al., *Clin Infect Dis*, (2014)]. For all other stool samples yielding toxigenic isolates (n= 53), the ratio of the toxin B level as measured by the B2/B3 assay to that measured by the B1/B2 assay ranged from 0.1-12.3 (mean of 1.8); representative examples are presented in Figure 1B (v46, v98, v11, v32, and v90).

CONCLUSIONS

In this study, two monoclonal antibodies were found to exhibit differential binding to *C. difficile* toxin B of highly virulent strains (BI/NAP-1/027 and BK/078) compared to other strain types. This indicates antigenic similarities between toxin B of BI/NAP-1/027 and BK/078 strains that are not present in toxin B of other strain types. This serendipitous observation was exploited to develop a simple, quantitative, and sensitive immunoassay (digital ELISA, based on Simoa technology) to differentially detect toxin B of these highly virulent strains. The differentiating capacity of the digital ELISA is important for two reasons. First, while toxins from different strain types have been hypothesized to be antigenically distinct, this has not been definitively demonstrated for the BI/NAP-1/027, BK/078, or AF group/ribotype 244 strains (nor for most toxinotypes [Rupnik, *FEMS Microbiol Rev*, **32**(3): p. 541-55 (2008)]). Second, because the digital ELISAs can

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differentially distinguish toxins from these highly virulent strains in clinical stool samples, the digital ELISA represents a sensitive stool immunoassay that can simultaneously identify the presence of toxigenic *C. difficile*, confirm *in vivo* toxin production, quantify stool toxin concentrations, and report the presence of highly virulent strains (particularly BI/NAP-1/027), thereby providing clinically relevant prognostic information.

Example 2: Preclinical and clinical validation of digital ELISAs for ultrasensitive detection and quantification of *C. difficile* toxins A and B in stool

Described herein is the development and validation of ultrasensitive and quantitative "digital ELISA" assays for toxins A and B based on single molecule array (Simoa) technology [32, 33]). Simoa technology is based on the high efficiency capture and labeling of single protein molecules on paramagnetic beads, and their detection in arrays of femtoliter-sized wells [32, 33]. The ability to isolate and detect single protein molecules leads to dramatic improvements in sensitivity such that fg/mL concentrations of proteins can be detected, typically 1000-fold more sensitive than conventional ELISA. Digital ELISAs have been shown to be highly robust and reproducible for detection of low levels of proteins in highly complex matrices including cell culture supernatant, serum, plasma, and cerebral spinal fluid (CSF) [32-37]. The assays described herein represent the first application of Simoa to measurement of proteins in stool specimens. We submit that our new assays may provide clinical sensitivity equal to that of NAAT and TC, with higher clinical specificity, and thus offer the potential for a new approach to the diagnosis and management of CDI.

This study relates to ultrasensitive "digital ELISA" assays for toxins A and B using single molecule array technology and validated using a) culture filtrates from a panel of clinical C. difficile isolates and b) 149 adult stool specimens already tested by NAAT per clinical routine. This rapid, simple tool allows diagnosis of CDI with both high analytical sensitivity and high clinical specificity.

MATERIALS AND METHODS

Antibody Conjugation and Biotinylation

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Antibody-coated capture beads were prepared as follows: paramagnetic particles functionalized with COOH groups (Agilent) were washed three times with 1x PBS + 1% Tween 20, followed by two washes with 50 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 6. Beads were subsequently activated with 0.5 mg/ml freshly prepared EDAC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) in cold 50mM MES buffer (pH 6.0) for 30 minutes at room temperature. After activation, beads were immediately washed with cold MES buffer (pH 6.0), and incubated with 0.5 mg/ml of antibody diluted in MES buffer (pH 6.0) for 2 hours at room temperature for conjugation. Antibody-coated beads were then washed twice with 1x PBS + 1% Tween 20 and blocked with 1x PBS + 1% BSA for 30 minutes. After blocking, beads were washed and stored in 50mM Tris buffer with 1% BSA, pH 7.8 at 4^0C for future use. Detection antibody was biotinylated in-house using EZ-link NHS-PEG_4-Biotin (Thermo Scientific) following the manufacturer's instructions. Briefly, antibody was first buffer- exchanged into phosphate buffer and then reacted with NHS-PEG_4-Biotin at optimized concentrations for 30 minutes. After biotinylation, antibody was purified to remove excess free biotin using centrifuge filters and stored at 4^0C for future use.

Conventional ELISA

Conventional plate- and bead-based ELISA were used to screen antibodies for assay development and to test culture filtrates (CF). We ultimately selected one pair of monoclonal antibodies against toxin A (Meridian Life Sciences, Memphis, TN) and another pair against toxin B (bioMerieux, Lyon, France). For bead-based ELISA, paramagnetic beads (5x106/ml) coated in capture antibodies were incubated with calibration buffer containing purified native toxins A or B (prepared from strain VPI10463 in C.P.K's laboratory using established methods, [38]) or CF in the wells of a microtiter plate for 2 h at room temperature. The calibration buffer (pH 7.4) contains 50 mM Tris-HCL, 150 mM NaCl, 0.5% BSA, 0.5% new born half serum (NBCS), 0.1% Tween 20, and 0.15% Proclin 300. Captured toxin proteins were labeled with a biotinylated detection antibody (0.1 µg/ml) and an enzyme conjugate (streptavidin-beta-galactosidase, 0.5 nM). Following washes, enzyme substrate was added to the microtiter plate wells, and fluorescent signals were measured using a Tecan plate reader.

Simoa assays (Digital ELISAs)

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Frozen aliquots of stool specimens were completely thawed at room temperature and mixed thoroughly either by vortexing or using a wooden applicator stick. Stool samples were then diluted and filtered to remove particulates before testing by digital ELISA.

Details of the Simoa technology used to develop and perform digital ELISA have been described previously [32, 33]. Antibody-coated capture beads and biotinylated detection antibodies were prepared using standard methods [32, 33]). Digital ELISAs were performed on the Simoa HD-1 Analyzer (Quanterix Corporation) [39] by automating the following steps. Capture beads (2.5x106/ml, for both toxin A and B assays) were incubated with diluted stool samples for 15 min at 23 □C. Beads were washed three times with 5x PBS + 0.1% Tween 20. Captured toxin proteins were labeled with a biotinylated detection antibody (0.4 µg/mL for the A assay, 0.2 µg/mL for the B assay) and an enzyme conjugate (streptavidin-beta-galactosidase, 250 pM). Following addition of enzyme substrate, beads were loaded into arrays of femtoliter-sized wells for isolation and detection of bound molecules. Total assay time was 69 min.

Simoa signals were quantified as average enzymes per bead, or AEB [33]. Signals from the digital ELISAs were calibrated by spiking a series of known concentrations of purified native toxin A or B (prepared as previously described [38]) into either buffer or NAAT-negative stool samples processed as described above. Calibration curves were used to calculate toxin concentrations (pg/mL) in stool specimens. For each digital ELISA, all calibrators and samples were assayed in triplicate, and average AEB, SD, and CV% were calculated for measured toxin concentrations.

To evaluate assay accuracy, NAAT-negative stool samples were spiked (after dilution) with or without a known concentration of purified native toxin A or B, processed as described above, and tested by digital ELISA. Recovery of spiked toxins was calculated as follows: % recovery = (measured concentration in spiked stool – measured concentration in non-spiked stool)/ expected concentration of spiked toxins).

For each digital ELISA, all calibrators and samples were assayed in triplicate, and average AEB, SD, and CV% were calculated as well as for measured toxin concentrations. To evaluate assay accuracy, NAAT-negative stool samples were spiked (after dilution) with or without a known concentration of purified native toxin A or B, centrifuged and filtered as

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described above, and tested by digital ELISA. Recovery of spiked toxins was calculated as follows: % recovery = (measured concentration in spiked stool – measured concentration in non-spiked stool)/ expected concentration of spiked toxins).

Interfering factor analysis

A panel of potential interfering substances was evaluated to determine the effect of their presence in stool specimens on detection of toxin A or B using digital ELISA. Briefly, each of the potential interferents was individually spiked into diluted stool samples at targeted concentrations. These stool samples were then processed and concentrations of toxin A or B were measured and compared to controls without added interferent. The tested substances included (concentrations noted are maximum final concentrations tested) vancomycin (100 mg/mL), metronidazole (40 mg/mL), loperamide HCl, salicylate, bismuth subsalicylate, Imodium (tablets or liquid), pepto-bismol (tablets or liquid), barium sulfate, and 50% (v/v) whole blood.

Stool Sample Processing

Frozen aliquots of stool specimens were completely thawed at room temperature and mixed thoroughly either by vortexing or using a wooden applicator stick. Stool samples were then diluted 1:20 in sample diluent (50 mM Tris-HCl, 150 mM NaCl, 0.5% NBCS, 0.1% Tween 20, and 0.15% Proclin 300, pH 7.4) as follows: 3.8 mL of sample diluent was first added to a graduated centrifuge tube. For liquid stools, 200 µL sample was added to the diluent using a disposable transfer pipet; for semi-solid or solid stools, the specimen was carefully transferred (using a plastic disposable spatula) into the diluent until the level of liquid reached 4 mL. Diluted stool samples were then vortexed thoroughly and centrifuged at 9,500 g for 15 minutes. Supernatants were filtered using a 0.45 µm vacuum filter (EMD Millipore) and then assayed by digital ELISA.

An alternative sample diluent that contained more concentrated NBCS (50 mM Tris-HCl, 150 mM NaCl, 25% NBCS, 0.1% Tween 20, and 0.15% Proclin 300, pH 7.4) was applied to some (approximately 6%) stool samples to prevent bead aggregation during toxin capture. In such cases, a corresponding alternative calibration buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% BSA, 25% NBCS, 0.1% Tween 20, and 0.15% Proclin 300, pH 7.4) was

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used to generate calibration curves for determination of toxin concentrations in these stool samples.

Sample Collection and Testing

(i) Culture filtrates

A panel of clinical strains previously typed by restriction endonuclease analysis (REA) (provided by D.N.G.) were each cultured in chopped meat broth for 24 hours. CF were prepared by 3000 x g centrifugation to obtain supernatant, followed by passage through an 0.2 μm syringe filter. CF were tested by conventional bead-based ELISA as described above.

(ii) Clinical Stool Specimens

Clinical stool specimens (each <72 hours old) that had been tested for toxigenic *C. difficile* by NAAT (Meridian Illumigene) were aliquoted and frozen at −80 °C; this study was approved by the BIDMC IRB. 52 samples (11 NAAT-positive, 41 NAAT-negative) were used for assay development, and 149 additional samples (65 NAAT-positive, 84 NAAT-negative) were collected for assay validation. Separate aliquots of each validation specimen were subsequently thawed and tested by digital ELISA, cytotoxicity assay, and toxigenic culture (TC). Relevant clinical and laboratory data corresponding to each sample was collected by chart review. The presence or absence of diarrhea as documented at the time of sample collection was determined using the following definition: three or more unformed bowel movements during any 24 hour period during the 48 hours before the time of stool collection, OR diarrhea specifically mentioned in MD/RN progress notes as being present in the 48 hours before stool collection.

(iii) Cytotoxicity Assay

Cytotoxicity assays were performed using standard methods [25] using stool samples diluted 1:100 in PBS, added to NIH 3T3 fibroblast cell monolayers and incubated for 24h. A positive result required a typical cell rounding effect in >50% of cells that was inhibited using specific anti-serum to toxin A and B.

(iv)Toxigenic Culture (TC)

C. difficile (CD) culture was performed using standard, well-established methods [40] in D.N.G.'s laboratory. Stool samples were directly inoculated onto selective TCCFA plates

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and streaked for isolation. If initial TCCFA culture failed to yield colonies with characteristic CD morphology, an alcohol shock protocol was performed on the stool sample to enhance recovery of CD spores. All C. difficile isolates were evaluated for their ability to produce toxin in vitro by culturing in BHI media for 48-72 hours and testing supernatants in the Bartel's cell cytotoxicity assay (MarDx Diagnostics, Carlsbad, CA). Samples that yielded a C. difficile isolate that did not produce toxin were scored as TC negative. All C. difficile isolates recovered from CD culture were REA typed in D.N.G.'s laboratory using established methods [41, 42].

RESULTS

Digital ELISA Development and Limit of Detection (LOD) in Buffer

A panel of antibodies were screened from commercial and academic sources by plate- and bead-based ELISA to find pairs that detected purified native toxin A or toxin B with highest analytical sensitivity and specificity (optimal signal/background). One pair of monoclonal antibodies were ultimately selected for each toxin assay (see Methods above; B2 and B3 disclosed in Example 1). As part of the screening process, culture filtrates (CF) prepared (Methods) from a panel of 12 clinical *C. difficile* isolates representing the major strains in circulation were also screened. It was observed that toxin A and B antibody pairs were able to detect their respective toxins in CF from all toxin-A and toxin B-producing strains in the panel. See Table 3 below.

Table 3. Detection of *C. difficile* Toxins A and B by Conventional Bead-Based ELISA in Culture Filtrates Prepared from a Panel of *C. difficile* Isolates.

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Culture filtrate	REA Type/strain information	Expected toxin production	Toxin A detection by bead-based ELISA	Toxin B detection by bead-based ELISA
CF1	B1	A+/B+	+	+
CF2	J9	A+/B+	+	+
CF3	K14	A+/B+	+	+
CF4	Y2	A+/B+	+	+
CF5	CF2	A-/B+	ND	+
CF6	ATCC BAA-1801	NONE	ND	ND
CF7	Medium	NONE	ND	ND
CF8	VPI	A+/B+	+	+
CF9	R23	sequenced A+B+ strain	+	+
CF10	BI1 (historic NAP1)	A+/B+/binary+	+	+
CF11	BI6 (epidemic NAP1)	A+/B+/binary+	+	+
CF12	BI17 (epidemic NAP1)	A+/B+/binary+	+	+
CF13	BK1 (ribotype 078)	A+/B+/binary+	+	+

CF, culture filtrate; REA, Restriction Endonuclease Analysis; ND, not detected.

After optimization of the digital ELISAs, the typical LOD for native toxin spiked into calibration buffer was 0.028 pg/mL for the toxin A assay and 0.061 pg/mL for the toxin B assay (CVs ranged from 1-13%). See Fig. 3.

LOD in Stool

To measure LOD in stool, native, purified toxins A and B were spiked into clinical stool samples that had tested negative by NAAT (Meridian Illumigene) during routine clinical testing. Stool samples were diluted prior to spiking and then processed as described (Methods). LOD in stool was defined as an interpolated concentration corresponding to an average AEB from NAAT-negative stools plus 3 SD, and corrected for the dilution factor. Our assays detected native toxins in stool with LODs of 0.45 pg/mL (toxin A) and 1.50 pg/mL (toxin B), respectively. % recovery ranged from 50-125% (average 84%). The mean % CV of Simoa signal (AEB) was 8%; this imprecision translated into a mean imprecision of

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15% in measured toxin concentrations for all stool specimens analyzed during this study, including those close to the LOD that have intrinsically higher imprecision. Of note, in ~6% of samples we observed aggregation of the paramagnetic beads (after incubation in the stool samples) that prevented measurement using Simoa. This limitation was overcome by using a modified dilution buffer for those samples that caused aggregation.

Interfering Factors

A panel of potential interferents [at concentrations at or above those tested for commercial EIAs (e.g.[43, 44])] was tested in our assay (Methods). There was no evidence of interference from supraphysiologic levels of vancomycin, metronidazole, loperamide HCl, salicylate, bismuth subsalicylate, Imodium (tablets or liquid), pepto-bismol (tablets or liquid), or barium sulfate, nor from 50% (v/v) whole blood.

Clinical Validation

149 clinical specimens (<72 hours old) that had tested either positive (n=65) or negative (n= 84) by NAAT (Meridian Illumigene) during routine clinical testing were tested with the two digital ELISAs (each run in triplicate and averaged), cytotoxicity assay, and TC (latter followed by REA typing of all *C. difficile* isolates).

A clinical cutoff for positive for each digital ELISA was calculated by averaging the Simoa signal for "true negative" stool samples (n= 80; negative by NAAT, TC, and cytotoxicity assay; Figure 2A and B), plus 3 standard deviations (SD) of that mean. One of the 80 "true negative" samples was excluded from the calculation of the cutoff because it was an extreme outlier on the toxin A assay (Figure 2A, marked with arrow) and substantially distorted the mean for that assay. Using the remaining 79 true negatives, the calculated cutoff for positive was 29.4 pg/mL for the toxin A assay and 23.3 pg/mL for the toxin B assay; with these cutoffs, the toxin A and B digital ELISA specificities in the true negative group were 96% and 98%, respectively.

Plots of A and B digital ELISA results for groups of samples testing positive vs negative on other assays (i.e. NAAT+ vs NAAT-, TC+ vs TC-, and cytotoxicity+ vs cytotoxicity-) are shown in Figure 2A and B; mean signals in each group are indicated by horizontal lines, and the calculated cutoffs for each digital ELISA are shown as dotted lines

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spanning the entire figure. As expected, for the toxin B digital ELISA, 16/65 (25%) samples that were positive by NAAT and 14/63 (22%) samples which were positive by TC were negative by the toxin B digital ELISA, consistent with the presence of organism (but minimal or no toxin) in those samples (Figure 2B). In contrast, 34/34 (100%) samples positive by cytotoxicity assay were positive by the toxin B digital ELISA.

As expected based on LODs, we noted some overlap between toxin B digital ELISA signals in cytotoxicity+ vs cytotoxicity- samples in the range of ~102-2078 pg/mL (Figure 1B), indicating that in this range the cytotoxicity assay was variably sensitive. Results from the toxin A digital ELISA (Figure 2A) revealed four samples for which cytotoxicity assay and TC were positive but toxin A digital ELISA was negative. REA typing of isolates obtained from TC confirmed that all four isolates were REA type CF, a group of isolates that produce toxin B but not toxin A. TC and cytotoxicity assay were used as alternative laboratory gold standards for calculations of digital ELISA sensitivity and specificity (Table 2); for comparison, we also calculated sensitivity and specificity of each digital ELISA vs NAAT (Table 4). Toxin concentrations (pg/mL) measured by digital ELISA spanned a >4 log dynamic range (Figure 2A,B).

Table 4. Sensitivity and Specificity of the Toxin A and B Digital ELISAs as Compared to Three Alternative Reference Standards by Nucleic Acid Amplification Testing (NAAT).

	Sensitivity vs. reference standard shown			Specificity vs. reference standard shown		
	Toxigenic culture (%)	Cytotoxicity assay (%)	NAAT (%)	Toxigenic culture (%)	Cytotoxicity assay (%)	NAAT (%)
Toxin A digital ELISA	75	88	71	95	84	94
Toxin B digital ELISA	78	100	75	97	87	96

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Correlation of Toxin Detection with Disease and Disease Severity

Among the 73 patients whose stool tested positive by any of the assays used (toxin A and B digital ELISAs, NAAT, TC, or cytotoxicity assay), there were 8 subjects who had a severe outcome potentially attributable to CDI, as defined by death, ICU admission, or colectomy within 40 days of stool testing. For those 8 subjects, detailed chart review (by clinicians blinded to stool assay results, other than NAAT) indicated that 5/8 severe outcomes were likely attributable to CDI. Mean toxin levels (toxin [A], toxin [B], or toxin [A+B]) in the 5 subjects with CDI-attributable severe outcomes were higher (1.7-fold, 1.5-fold, and 1.6-fold, respectively) than mean toxin levels in the 68 subjects without CDI-attributable severe outcomes, though this was not statistically significant ($p = 0.099$, 0.182, and 0.084, respectively).

In sum, the digital ELISAs detected toxins A and B in stool with limits of detection of 0.45 and 1.5 pg/mL respectively, quantified toxins across a 4-log dynamic range, and detected toxins from all major clinical strains studied. Using specimens negative by cytotoxicity assay/TC/NAAT, clinical cutoffs were set at 29.4 pg/mL (A) and 23.3 pg/mL (B); resulting clinical specificities were 96% and 98%, respectively. The toxin B digital ELISA was 100% sensitive vs cytotoxicity assay. 25% and 22% of samples positive by NAAT and TC, respectively, were negative by the toxin B digital ELISA, consistent with the presence of organism (but minimal or no toxin) in those samples. Mean toxin levels by digital ELISA were 1.5-1.7-fold higher in patients with CDI-attributable severe outcomes, vs. those without.

DISCUSSIONS

Within the complex and rapidly shifting C. difficile diagnostic landscape, one major theme is emerging: that detection of toxin, rather than of bacteria capable of producing that toxin, is the key to accurate diagnosis of CDI [2, 15, 16, 46]. Qualitative enzyme immunoassays (EIAs) that detect *C. difficile* toxins in stool were for many years the mainstay of diagnosis, used by more than 90% of laboratories in the US [2]. When compared to TC, however, these assays showed limited sensitivity [52-75% [28, 29]]; consistent with this finding, the analytical LODs for some of the highest-performing EIAs [29] range from 0.8-2.5 ng/mL in stool [26, 27], i.e., ~1 ng/mL. Ryder et al [31] have described a cell-based

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assay for quantification of toxin in stool and calculated stool toxin concentrations down to as low as 30 pg/mL. Their data indicated that almost half of the toxin-positive specimens would not be detected by EIA with LODs of ~1 ng/mL. However, EIAs have been shown to have high specificity vs TC (96-98%; [28, 29]), and high clinical specificity [46, 47].

Cell culture cytotoxicity assay for direct detection of toxin in stool is re-emerging as a reference assay favored over TC. Cytotoxicity assays can have analytical LODs far below that of EIA (e.g. 1.5 pg/mL for toxin B in buffer [25]), but are complex and lengthy, making them unsuitable for routine diagnostic use. Moreover, in contrast to its low LOD in buffer, it was observed that the assay appeared to be variably sensitive for detection of toxin B in stool (see Figure 2). One large comparison study found cytotoxicity assay to be approximately 86% sensitive compared to TC [29], and the question has been raised as to whether this difference is due to lower sensitivity of the cytotoxicity assay or the detection, by TC, of *C. difficile* organisms in the absence of toxin production [15]. Cytotoxicity assay primarily detects toxin B, which is far more potent than toxin A in this assay [48].

Given the limitations of existing toxin detection assays, over the past few years the field has made a dramatic shift towards clinical diagnosis by NAAT for the tcdA and tcdB genes, with its potential for high sensitivity and rapid turn-around time (despite higher expense). Frustratingly, despite relatively high analytical sensitivity and specificity (90-95%/94-96%, respectively, vs TC [49-51]), NAAT testing is confounded by its inability to distinguish disease from colonization or even transient contamination with environmental spores [15]. Positive predictive values for the commercial NAATs (even vs TC) are low (71-79% for prevalence <20% [51, 52]), in contrast to their high negative predictive values (98-99% in the same studies). The problem remains that like TC, NAAT indicates the presence of *C. difficile* organisms capable of producing toxin—not whether they are actually producing it (nor at what levels) in vivo [18, 48, 53, 54].

Until now, no highly sensitive assay existed that could rapidly detect and quantify both toxins A and B in stool samples at the time of diagnosis. The assays described herein have high analytical sensitivity and specificity, and given that we are detecting toxin directly, high clinical specificity—cumulative advantages that arguably may provide higher diagnostic, and even prognostic, accuracy over existing assays. As expected, the analytical

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sensitivity (~1 pg/mL, determined using NAAT-negative samples with uniformly low background) of the assays described herein was lower than our final clinical cutoffs (~20 pg/mL, determined using a large number of NAAT-negative samples with varied background, i.e. more representative of "real-life" sample quality). With the calculated clinical cutoff described herein, the toxin B assay has 100% sensitivity vs cytotoxicity assay, 97% specificity vs TC, and 98% specificity in samples negative by NAAT, TC, and cytotoxicity assay. As expected, 22-25% of NAAT+ and TC+ samples were negative for toxin B by the ultrasensitive digital ELISA described herein, suggesting that these sample contained toxigenic organism, but minimal or no toxin.

A final advantage of the assays described herein over existing tools is the ability to separately detect and quantify both toxins A and B. While each of the two toxins has been shown to be independently capable of causing disease, the relative contributions of the two toxin proteins to disease remains unclear (e.g. [9, 10, 55, 56]). The results provided herein suggest that patients with higher stool toxin levels are more likely to have severe CDI outcomes.

In conclusion, an ultrasensitive and quantitative digital ELISAs for *C. difficile* toxins A and B has successfully developed. These assays detect native toxins A and B in stool with analytical LODs that are ~1000-fold more sensitive than current EIAs, can quantify toxin across a 4-log range, and detect toxins from all major clinical strains tested. These assays represent the first application of Simoa to measurement of toxin A and/or toxin B of *C. difficile* in stool specimens. Many complex and important questions remain regarding these toxins and the overall pathogenesis of CDI, and the instant assays provide a new tool with which to address these questions. Moreover, the assays described herein offer the potential for a future paradigm shift in how *C. difficile* disease is diagnosed and managed.

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OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

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While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of examples only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements

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specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one,

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optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

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What is claimed is:

1. A method for differentially detecting *C. difficile* based on virulence, the method comprising:

(i) obtaining a sample comprising a *C. difficile* strain and/or a toxin thereof,

(ii) performing a first immunoassay to detect toxin B of *C. difficile* in the sample, wherein the first immunoassay involves a first antibody and a second antibody, both of which bind toxin B of both highly virulent and less virulent *C. difficile* strains;

(iii) performing a second immunoassay to detect toxin B of *C. difficile* in the sample, wherein the second immunoassay involves the second antibody and a third antibody, wherein the third antibody differentially binds toxin B of highly virulent *C. difficile* strains as relative to toxin B of less virulent *C. difficile* strains;

(iv) comparing results obtained from step (ii) and step (iii); and

(v) determining whether the sample comprises a highly virulent *C. difficile* strain or a less virulent *C. difficile* strain based on the comparing result of step (iv).

2. The method of claim 1, wherein the first immunoassay, the second immunoassay, or both are digital ELISA assays.

3. The method of claim 1 or claim 2, wherein the comparing step (iv) is performed by calculating a ratio between a first value obtained from step (ii) and a second value obtained from step (iii), the first value representing the level of the toxin B detected in the first immunoassay and the second value representing the level of the toxin B detected in the second immunoassay.

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4. The method of any one of claims 1-3, wherein in step (ii), either the first antibody or the second antibody is conjugated to a label and the other antibody is immobilized on a solid support.

5. The method of claim 4, wherein the label is biotin.

6. The method of claim 5, wherein the first immunoassay further involves (a) a conjugate of streptavidin and an enzyme, and (b) a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal.

7. The method of claim 6, wherein the signal is a fluorescent signal.

8. The method of any one of claims 1-7, wherein in step (iii), either the second antibody or the third antibody is conjugated to a label and the other antibody is immobilized on a solid support.

9. The method of claim 8, wherein the label is biotin.

10. The method of claim 9, wherein the second immunoassay further involves (a) a conjugate of streptavidin and an enzyme, and (b) a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal.

11. The method of claim 10, wherein the signal is a fluorescent signal.

12. The method of any one of claims 1-11, wherein the third antibody is specific to toxin B of highly virulent *C. difficile* strains.

13. The method of claim 12, wherein detection of toxin B in both the first immunoassay and the second immunoassay indicates that the sample comprises a highly

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virulent *C. difficile* strain; and detection of toxin B in the first immunoassay but not in the second immunoassay indicates that the sample comprises a less virulent *C. difficile* strain.

14. The method of any one of claims 1-11, wherein the third antibody is specific to toxin B of less virulent *C. difficile* strains.

15. The method of claim 14, wherein detection of toxin B in the first immunoassay but not in the second immunoassay indicates that the sample comprises a highly virulent *C difficile* strain, and detection of toxin B in both the first immunoassay and the second immunoassay indicates that the sample comprises a less virulent *C. difficile* strain.

16. The method of any one of claims 1-15, wherein the highly virulent strain is a BI/NAP-1/027 strain, a ribotype 078/BK strain, or a ribotype 244/AF strain.

17. The method of any of claims 1-16, wherein the sample is obtained from a subject having or suspected of having *C. difficile* infection.

18. The method of claim 17, wherein the sample is a stool sample.

19. The method of claim 17 or claim 18, further comprising determining whether or not the subject is infected with a highly virulent *C difficile* strain.

20. A kit for differentially detecting *C. difficile* based on virulence, the kit comprising:

(i) a first pair of anti-toxin B antibodies including a first antibody and a second antibody, wherein the first antibody and the second antibody bind toxin B of both highly virulent and less virulent *C. difficile* strains; and

(ii) a second pair of anti-toxin B antibodies, including the second antibody and a third antibody, which differentially binds toxin B of highly virulent *C. difficile* strains as relative to toxin B of less virulent *C. difficile* strains.

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21. The kit of claim 20, wherein in the first pair of anti-toxin B antibodies, either the first antibody or the second antibody is conjugated to a label and the other antibody is immobilized on a solid support.

22. The kit of claim 20 or claim 21, wherein in the second pair of anti-toxin B antibodies, either the second antibody or the third antibody is conjugated to a label and the other antibody is immobilized on a solid support.

23. The kit of claim 21 or claim 22, wherein the label is biotin.

24. The kit of claim 23, wherein the kit further comprises a conjugate of streptavidin and an enzyme.

25. The kit of claim 24, wherein the kit further comprises a substrate of the enzyme, wherein the enzyme converts the substrate to a molecule that releases a detectable signal.

26. The kit of claim 25, wherein the signal is a fluorescent signal.

27. The kit of any one of claims 20-26, wherein the highly virulent strain is a BI/NAP-1/027 strain, a ribotype 078/BK strain, or a ribotype 244/AF strain.

28. The kit of any one of claims 20-27, further comprising instructions for carrying out a method set forth in any one of claims 1-19.

29. A method for detecting *C. difficile*, comprising:

(i) providing a sample suspected of containing a *C difficile* strain and/or a toxin thereof,

(ii) detecting toxin B of the *C difficile* by digital ELISA,

(iii) detecting toxin A of the *C difficile* by digital ELISA, and

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(iv) assessing presence of a toxin of *C difficile* in the sample based on the result of step (ii), the result of step (iii), or a combination thereof.

30. The method of claim 29, wherein the level of toxin B detected in step (ii) higher than 20 pg/ml indicates that the sample contains a toxigenic *C. difficile* strain and/or a toxin thereof.

31. A method for diagnosing *C. difficile* infection in a subject, comprising:

(i) examining whether the subject manifests a symptom associated with *C. difficile* infection;

(ii) detecting the presence of toxin A, toxin B, or both in a sample obtained from the subject; and

(iii) determining whether the subject has *C. difficile* infection.

32. The method of claim 31, wherein the method further comprises detecting the presence of a nucleic acid that encodes toxin A, a nucleic acid that encodes toxin B, or both, in a sample obtained from the subject.

33. The method of claim 31 or claim 32, wherein the level of toxin B detected is higher than 20 pg/ml indicates that the subject has *C. difficile* infection.

34. The method of any one of claims 31-33, wherein the symptom is diarrhea.

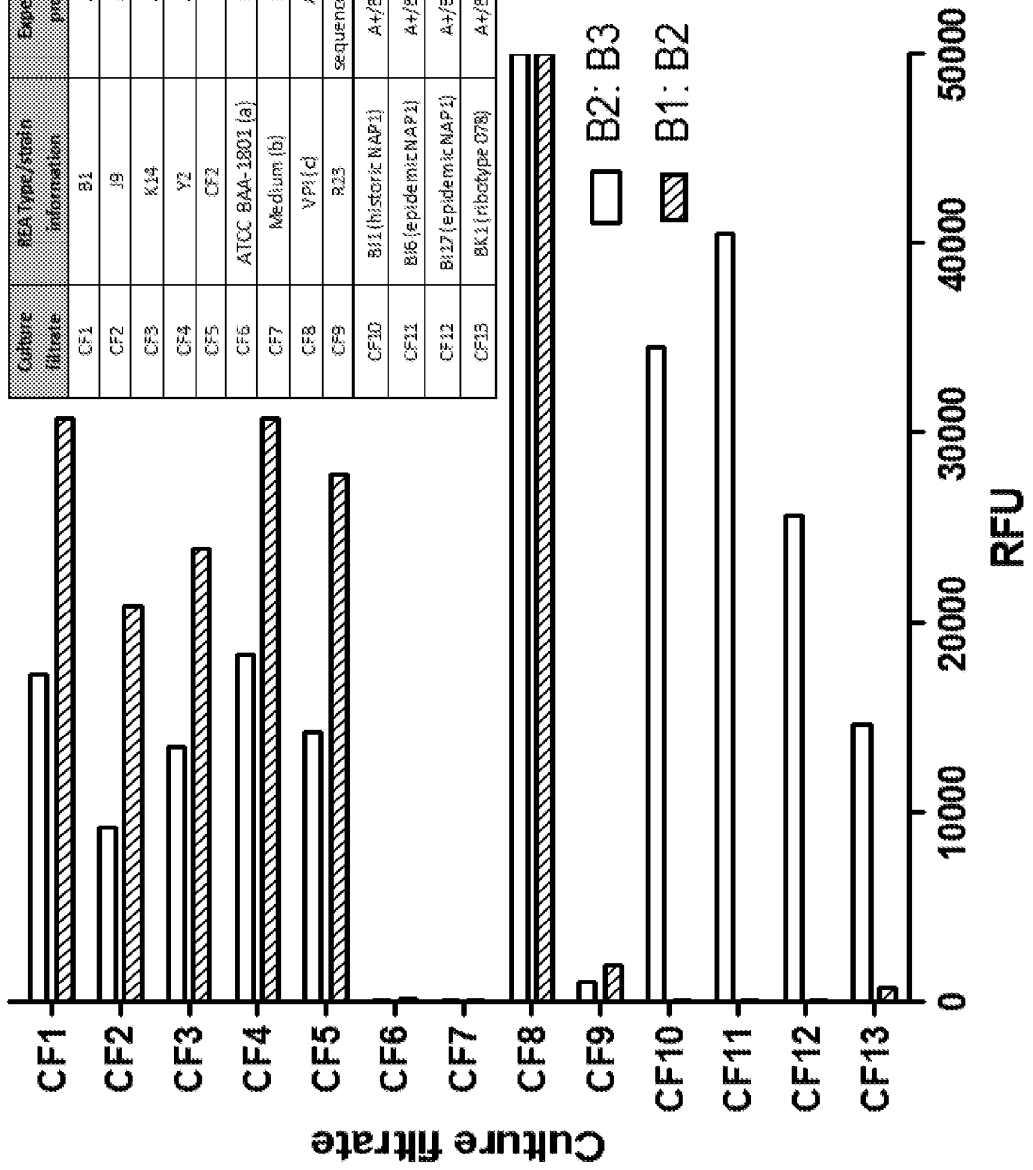
35. The method of any one of claims 31-34, wherein step (ii) is performed by a digital ELISA.

36. The method of any one of claims 31-35, wherein step (ii) is performed by a differential detection method to determine whether the sample contains a highly virulent strain or a less virulent strain.

Fujirebio Diagnostics, Inc. v. Quanterix Corp. EX-1044
690

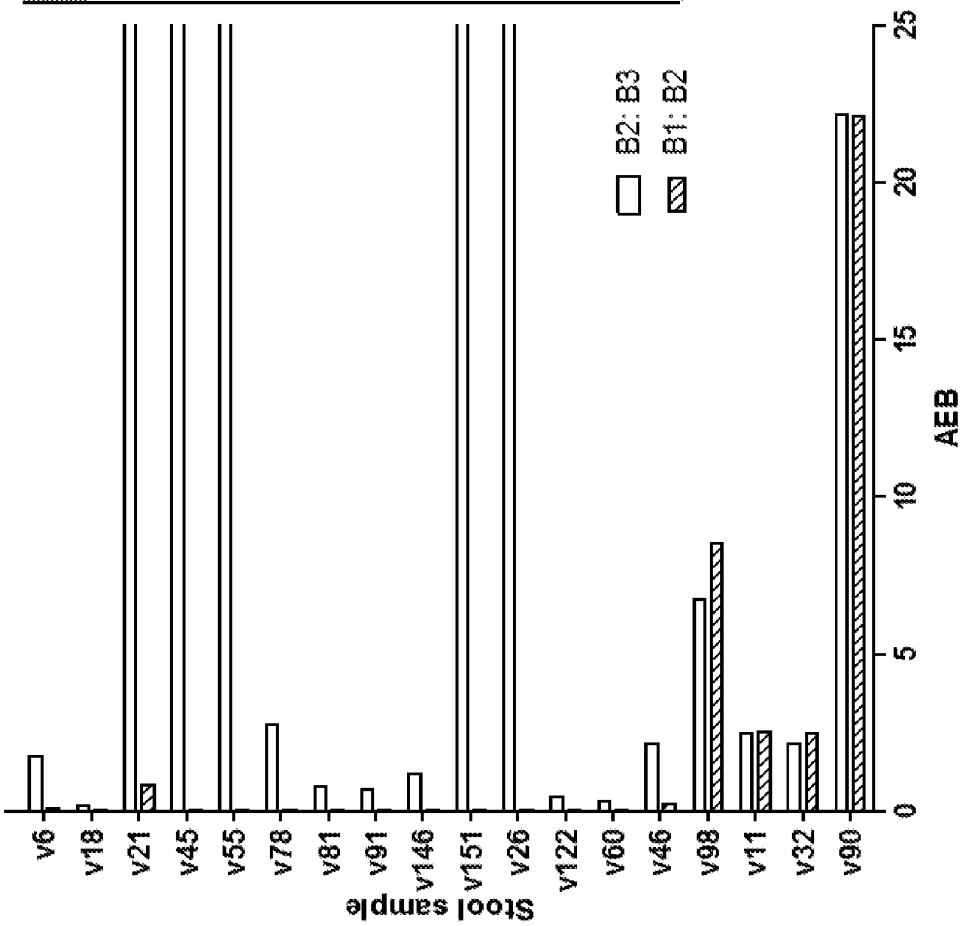
Fig. 1A

Culture filtrate	REA Type/strain information	Expected toxin production	Ratio of B2:B3 to B1:B2
CF1	B1	A+/B+	0.6
CF2	J9	A+/B+	0.4
CF3	K14	A+/B+	0.6
CF4	Y2	A+/B+	0.6
CF5	CF2	A-/B+	0.5
CF6	ATCC BAA-1801 (a)	NONE	0.8
CF7	Medium (b)	NONE	1.0
CF8	VP1 (c)	A+/B+	NA
CF9	R23	sequenced A+B+ strain	0.5
CF10	B31 (historic NAP1)	A+/B+/binary+	363.7
CF11	B6 (epidemic NAP1)	A+/B+/binary+	401.6
CF12	B127 (epidemic NAP1)	A+/B+/binary+	251.3
CF13	BK1 (ribotype 078)	A+/B+/binary+	20.7



Stool Sample	REA Type/strain information	Ratio of B2-B3 to B1-B2
v6	B1 group	23.8
v18	B1 group	48.5
v21	B1 group	21.5
v45	B1 group	2771.0
v55	B1 group	2871.0
v78	B1 group	133.0
v81	B1 group	22.3
v91	B1 group	72.3
v146	B1 group	42.1
v151	B1 group	3008.0
v26	Non-sp. REA	1683.0
v122	Non-sp. REA	40.1
v60	AF group	30.0
v46	CF group	12.3
v98	CF group	1.1
v11	Y group	1.0
v32	DH group	0.8
v90	J group	1.0

Fig. 1B



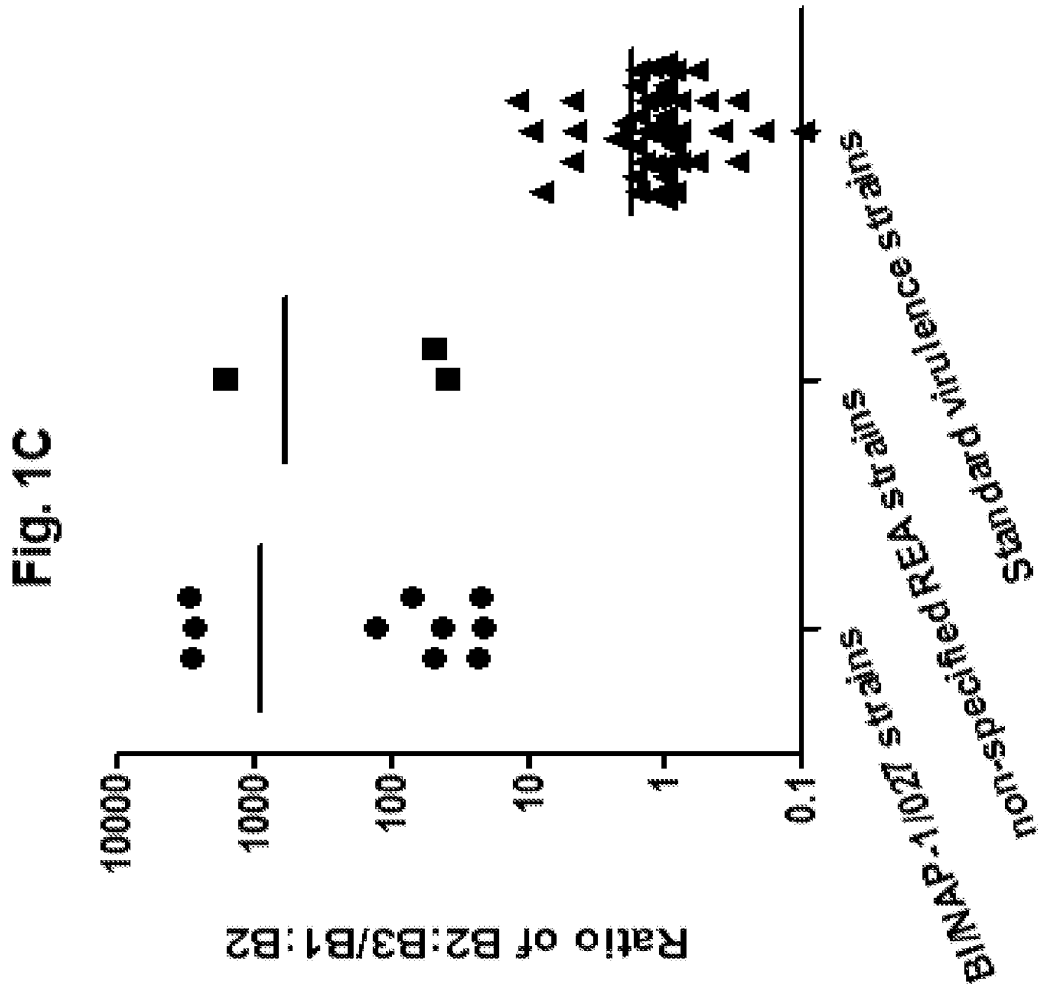
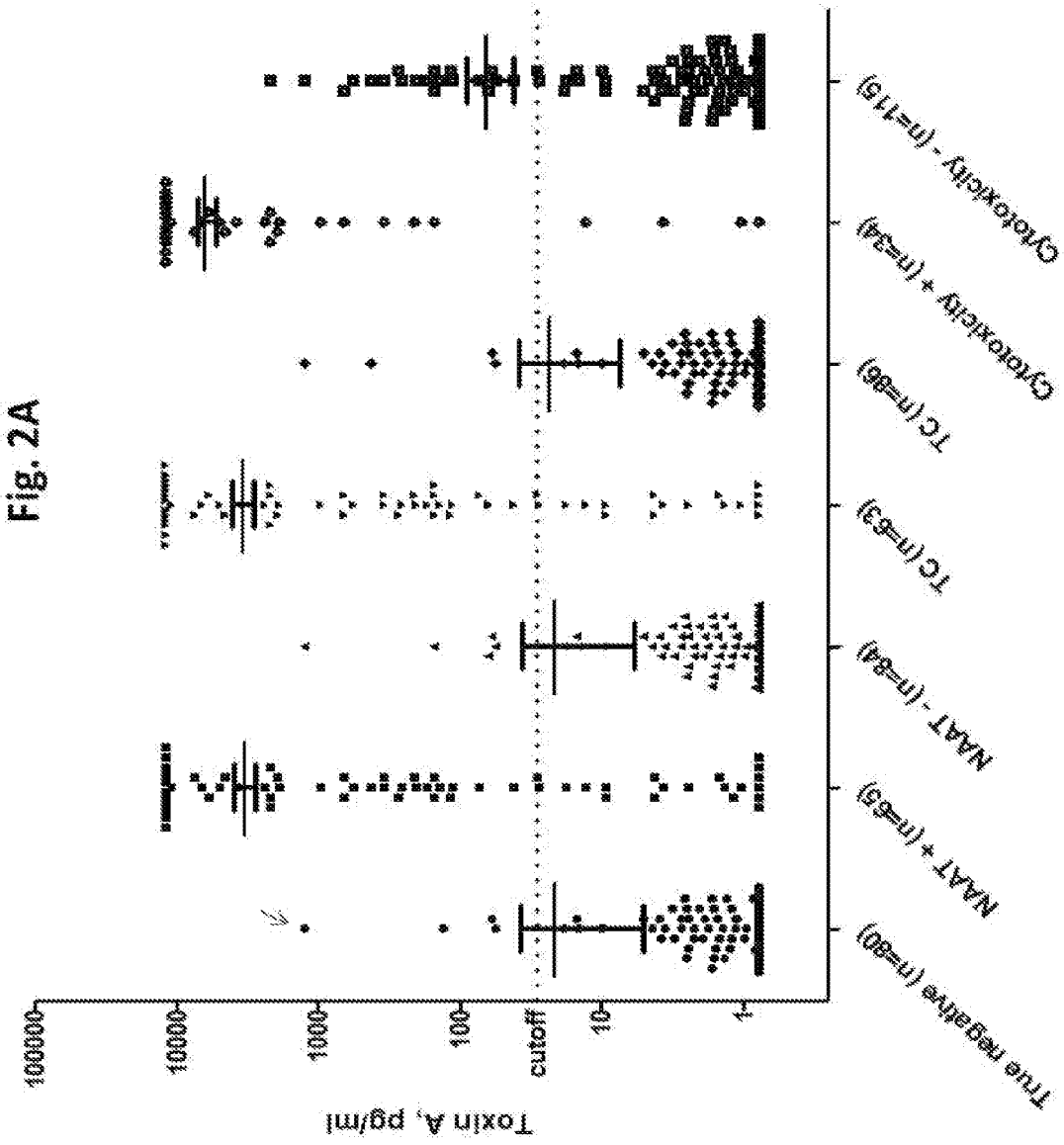

Ratio of B2:B3/B1:B2
10000
1000
100
10
1
0.1
BlNAP-1/027 strains
non-specified REA strains
Standard virulence strains

Fig. 1C

Fig. 2A


Toxin A, pg/ml
100000
10000
1000
100
cutoff
10
1
True negatives (n=80)
NAAT + (n=63)
NAAT - (n=84)
TC (n=63)
TC (n=86)
Cytotoxicity + (n=24)
Cytotoxicity - (n=115)

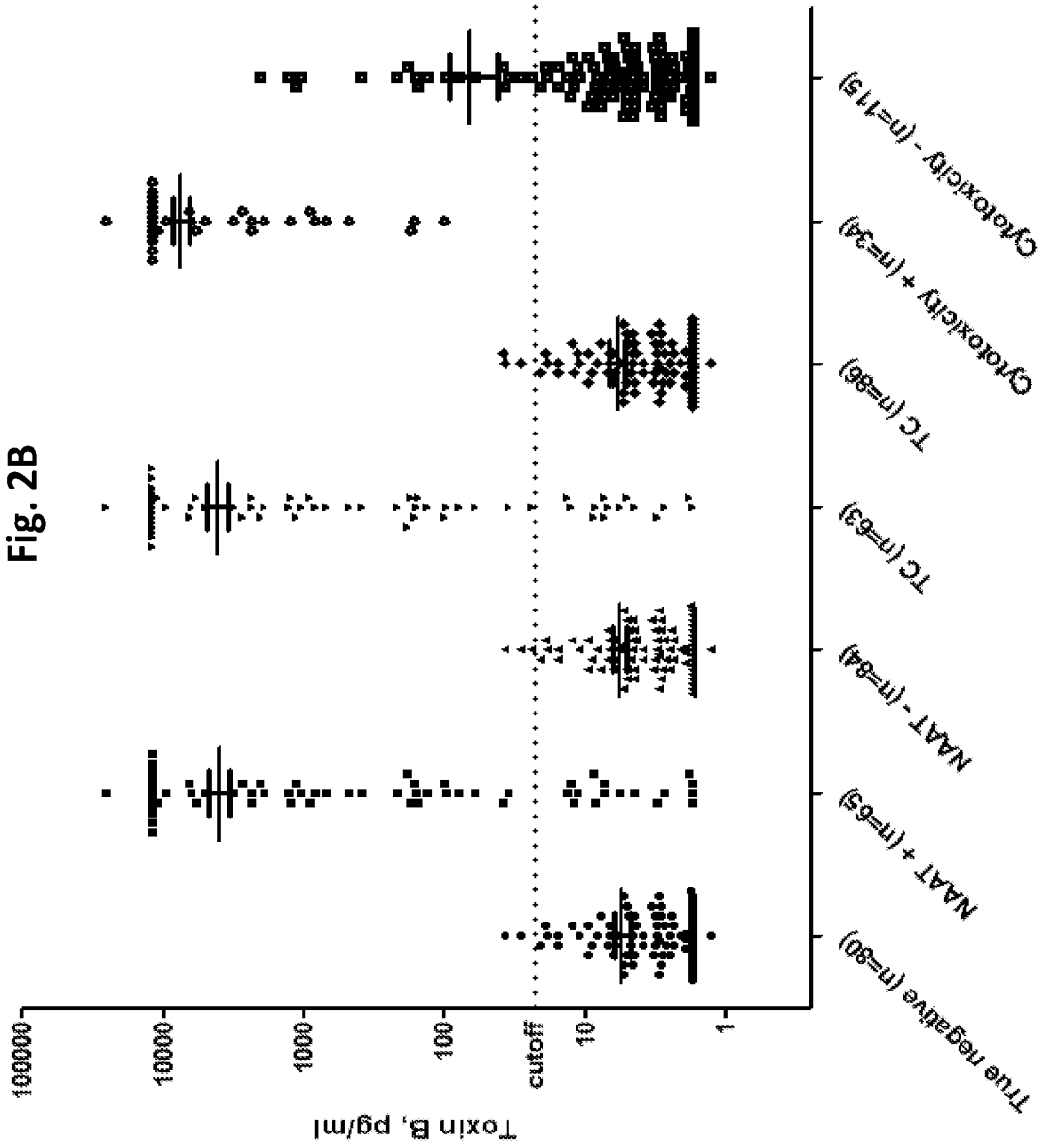

Fig. 2B
Toxin B, pg/ml
100000
10000
1000
100
cutoff
10
1
True negative (n=80)
NAAT + (n=65)
NAAT - (n=84)
TC (n=63)
TC (n=86)
Cytotoxicity + (n=24)
Cytotoxicity - (n=115)

Fig. 3

toxin A, pg/ml	Ave of AEB	SD	CV
0	0.007	0.001	9%
0.1	0.014	0.001	10%
1	0.077	0.003	4%
10	0.680	0.039	6%
100	6.670	0.236	4%
500	23.079	1.187	5%
3xSD + background at 10% CV	0.0086	LOD, pg/ml	0.028

toxin B, pg/ml	Ave of AEB	SD	CV
0	0.014	0.001	8%
0.1	0.022	0.003	15%
1	0.062	0.001	2%
10	0.470	0.021	4%
100	4.100	0.049	1%
500	17.247	2.486	14%
3xSD + background at 10% CV	0.0177	LOD, pg/ml	0.061

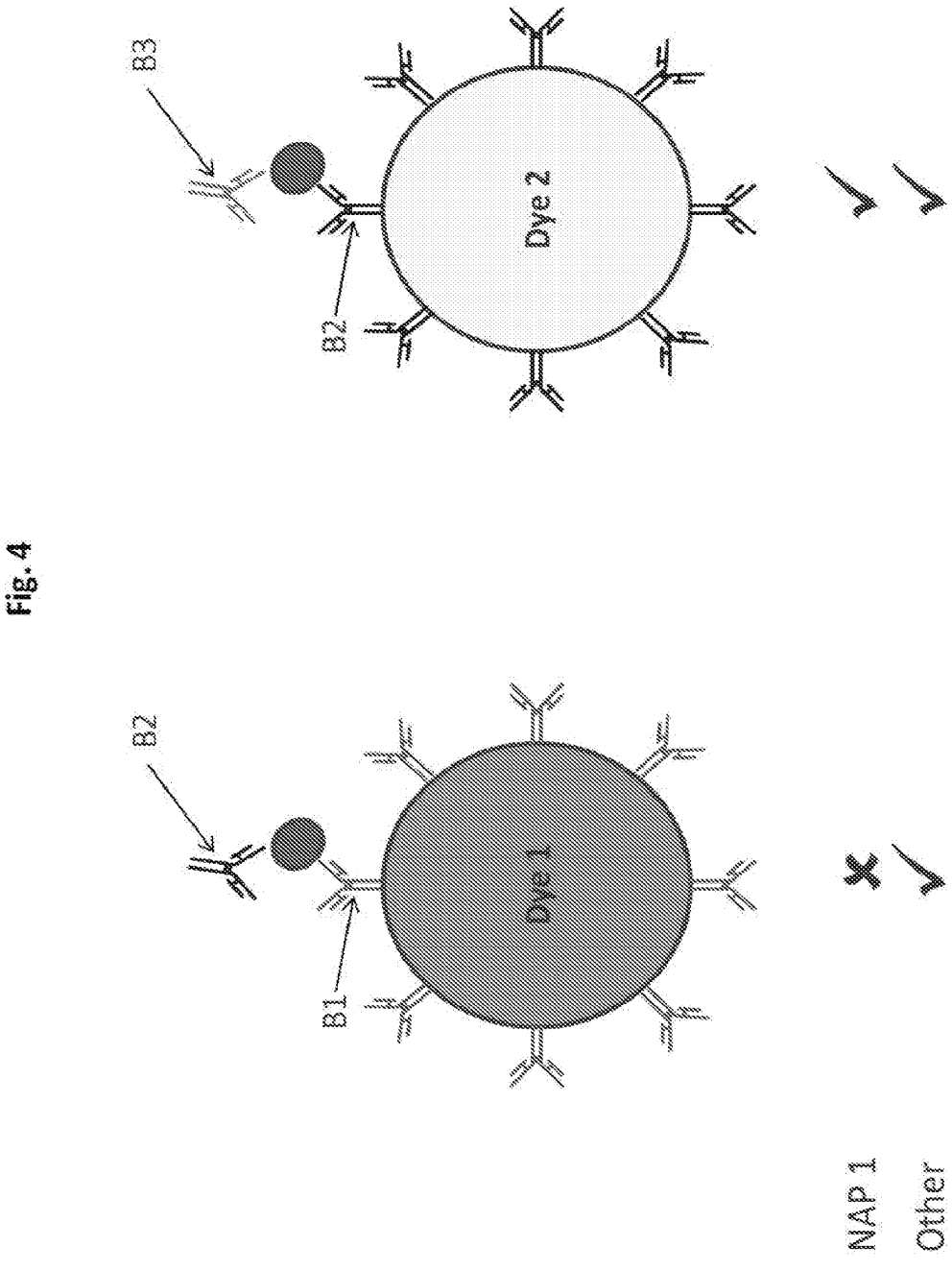
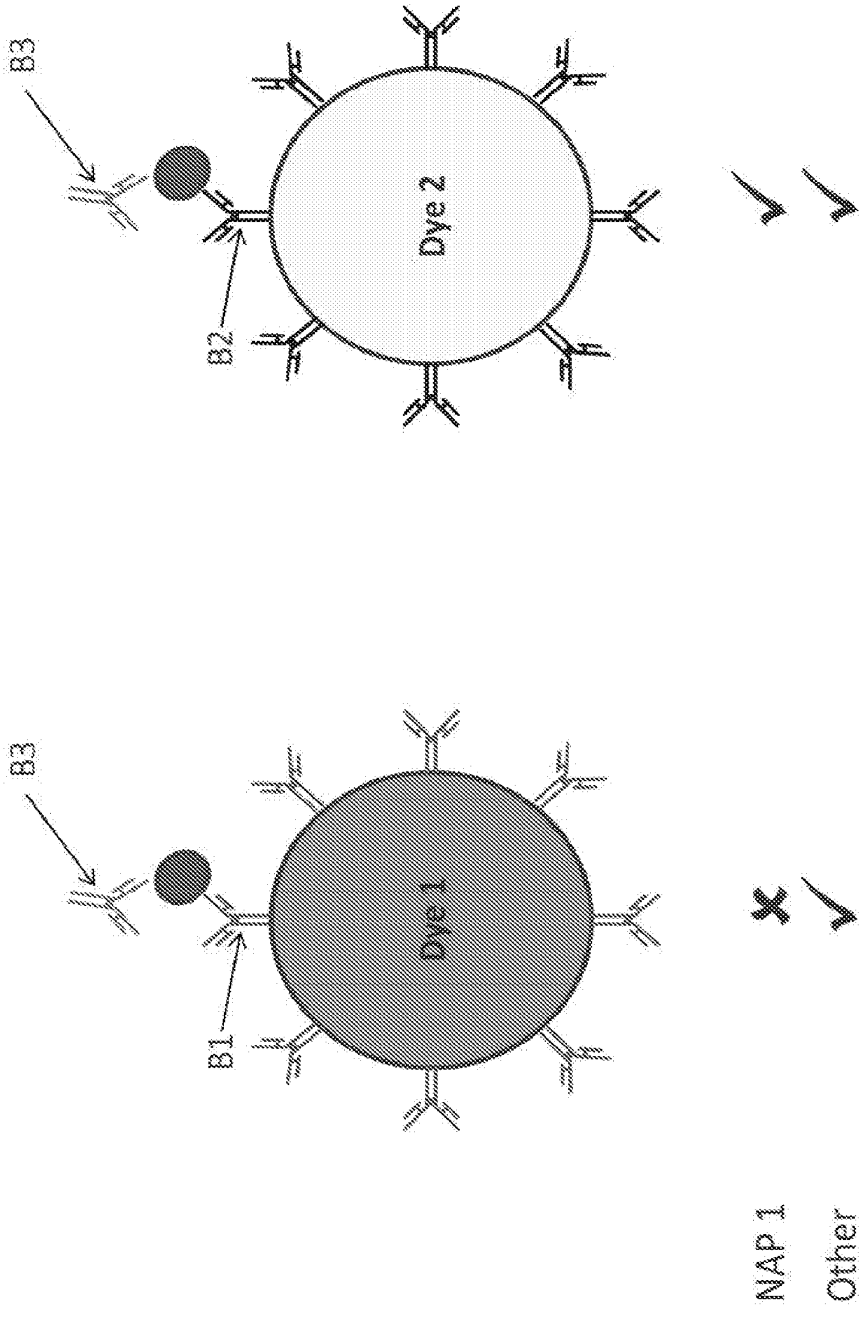


Fig. 4

Fig. 5



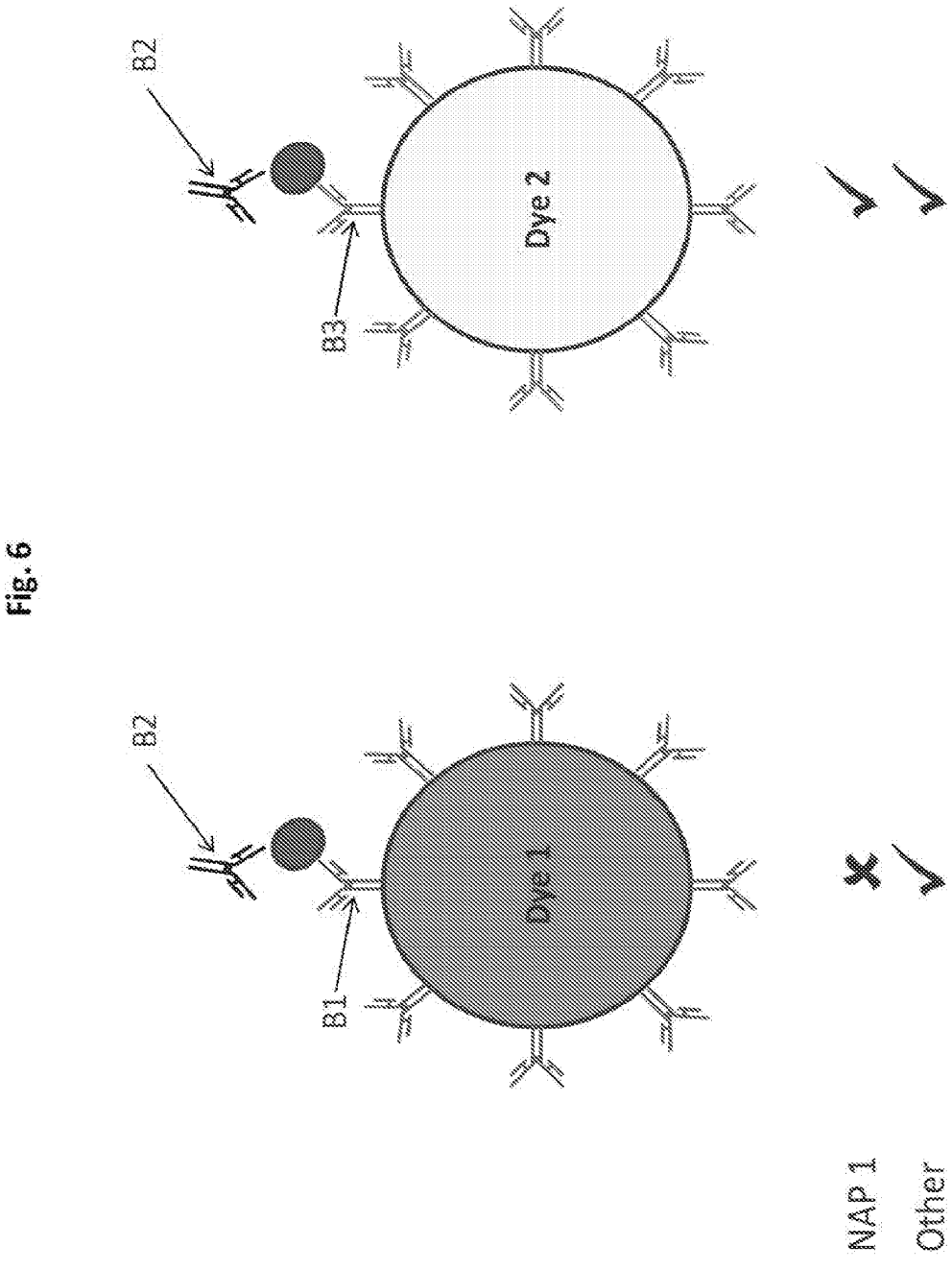


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/17758

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/569; C07K 14/33, 16/12 (2016.01)
CPC - G01N 33/56911; C07K 14/33, 16/1282
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)
IPC(8): G01N 33/569; C07K 14/33, 16/12; C12N 15/09, 1/21; A61K 39/02, 39/08; A61P 31/04; C12Q 1/68 (2016.01)
CPC: G01N 33/56911; C07K 14/33, 16/1282, 16/40; C12Q 1/689; A61K 2039/505, 39/08

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)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar; Pubmed; EBSCO
Keywords: Clostridium difficile, infection, disease, subject, patient, virulence, toxin A, toxin B, virulence, immunoassay, ELISA, digital, cytotoxicity, concentration

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	CA 2768528A1 (BIODICS GMBH) 27 July 2009; abstract; page 1, lines 19-25; page 3, lines 11-12, lines 28-30; page 4, lines 1-5, lines 21-24; page 5, line 8, lines 22-26; page 7, line 31-page 8, line 5; page 8, lines 10-11; page 9, lines 11-12; page 9, line 23 – page 10, line 26; page 22, lines 8-10	1, 31-32 --- 2, 3/1-2, 20-21, 22/20-21, 29-30, 33/31-32
Y	CHANG, L et al. Single Molecule Enzyme-Linked Immunosorbent Assays: Theoretical Considerations. J Immunol Methods. 30 April 2012; Vol. 378, No. 1-2; pages 1-28; abstract; page 2, paragraph 2; DOI: 10.1016/j.jim.2012.02.011	2, 3/2, 29-30
Y	NGUYEN, VK et al. Enzyme Immunoassay (ELISA) for Detection of Clostridium difficile Toxin B in Specimens of Faeces. J Med Microbiol. April 1990; Vol. 31, No. 4; pages 251-257; page 252, column 2, paragraph 4; page 253, column 1, paragraphs 1-2; page 254, column 1, paragraph 1	3/1-2, 21, 22/20-21
Y	US 2014/0038184 A1 (JIN, PJ et al) 6 February 2014; abstract; paragraph [0123]	20-21, 22/20-21
Y	US 2013/0330371 A1 (WYETH LLC) 12 December 2013; paragraph [0571]	30, 33/31-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"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
31 March 2016 (31.03.2016)	03 MAY 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/17758

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

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

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

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

3. ☒ Claims Nos.: 4-19, 23-28 and 34-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	A. M. Weidner
Attorney Docket Number	Q0052.70018US02

ENCLOSURES (*Check all that apply*)

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [x] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [x] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

-FORM PTO-1449
-Copies of cited references

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Andrea J. Gabert/		
Printed name	Andrea J. Gabert, PhD		
Date	September 27, 2017	Reg. No.	68,904

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 27, 2017 Electronic Signature for: /Eileen M. MacKenzie/

5769772.1

FEE TRANSMITTAL

Complete if known	
Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Examiner Name	A. M. Weidner
Art Unit	1649
Practitioner Docket No.	Q0052.70018US02

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)1,580.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☐ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	280	140*	70	600	300	150	720	360	180	
Design	180	90	45	120	60	30	460	230	115	
Plant	180	90	45	380	190	95	580	290	145	
Reissue	280	140	70	600	300	150	2,160	1,080	540	
Provisional	260	130	65	0	0	0	0	0	0	

* The $140 small entity status filing fee for a utility application is further reduced to $70 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	80	40	20
Each independent claim over 3 (including Reissues)	420	210	105
Multiple dependent claims	780	390	195

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 20 or HP =	_____ x	_____ =	_____

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**
_____	_____

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 3 or HP =	_____ x	_____ =	_____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____ - 100 =	_____ /50 =	_____ (round **up** to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1253 Extension for response within third month	1,400.00
1806 Submission of an Information Disclosure Statement	180.00

SUBMITTED BY

Signature	/Andrea J. Gabert/	Registration No. (Attorney/Agent)	68,904	Telephone	617.646.8000
Name (Print/Type)	Andrea J. Gabert, PhD			Date	September 27, 2017

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 27, 2017 Electronic Signature for: /Eileen M. MacKenzie/

5769771.1

Electronic Patent Application Fee Transmittal

Application Number:	15269142
Filing Date:	19-Sep-2016
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Andrea J. Gabert/Eileen MacKenzie
Attorney Docket Number:	Q0052.70018US02

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**1580**

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/269,142	Filing Date 09/19/2016	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)		
	09/27/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0	X $80 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)		
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
EMORY LANE

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): Q0052.70018US02

Application Number	15/269,142-Conf. #6662	Filed	September 19, 2016

For: METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Art Unit	1649	Examiner	A. M. Weidner

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
X	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1,400.00
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

X Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

X The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

X Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

X attorney or agent of record. Registration number 68,904 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Andrea J. Gabert/	September 27, 2017
Signature	Date
Andrea J. Gabert, PhD	617.646.8000
Typed or printed name	Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 27, 2017 Electronic Signature for: /Eileen M. MacKenzie/

5769773.1

Electronic Patent Application Fee Transmittal

Application Number:	15269142
Filing Date:	19-Sep-2016
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Andrea J. Gabert/Eileen MacKenzie
Attorney Docket Number:	Q0052.70018US02

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**1580**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/269,142	09/19/2016	David Wilson	Q0052.70018US02	6662

23628 7590 12/14/2017

WOLF GREENFIELD & SACKS, P.C.
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EXAMINER
WEIDNER, ADAM M

ART UNIT	PAPER NUMBER
1649	

NOTIFICATION DATE	DELIVERY MODE
12/14/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 15/269,142	Applicant(s) Wilson et al.	
	Examiner ADAM M WEIDNER	Art Unit 1649	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 27 September 2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☑ This action is **FINAL.** 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 12-13,21-24 and 92-105 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 12-13,21-24 and 92-105 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

DETAILED ACTION

This action is in response to claim amendments filed 9/27/17. Claims 12-13, 21-24, and 92-105 are pending and under examination.

Withdrawn Rejections

The objections/rejections of cancelled claims are moot.

The rejections under §112, second paragraph are withdrawn. Applicant points to the methods and citations across the specification pages 22-33 as support for assays which convey to the artisan possession of the breadth of assays which measure tau at less than about 0.2 pg/mL. There is no evidence that these methods do not possess the required sensitivity and therefore the evidence supports a conclusion that Applicant has sufficiently described the genus to meet the requirements under §112.

The rejections under §102 and 103 are withdrawn. The prior art clearly measures tau at time periods within 12 hours; however, Mortberg does not detect any appreciable tau at 12 hours or earlier (figure 18) and clearly excludes this data when determining the ROC (figure 19) while Wunderlich measures tau at these time periods but notes no difference between a favorable vs a poor outcome (e.g., figure 2). While changes in result-effective variables are generally obvious, instant figure 2 demonstrates that those with a poor outcome have a significant peak at this time period and thus this would contribute to the ROC measurement and thus the prognosis in a way considered non-obvious over the teachings of the prior art. Therefore, the rejection over claims requiring the samples taken at 12 h or less be included in the data analysis are allowable over the prior art. Note that new claim 105 does not contain these limitations and is rejected as below.

The double patenting rejection over 14/111326 is withdrawn as the '326 application has been abandoned.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 12-13, 21-24, and 92-105 are rejected under 35 U.S.C. § 101 because the claims as a whole are not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, these claims do not recite something significantly more than a judicial exception. This rejection has been modified solely to address the amendments. The rationale for this determination is explained below:

Guidance with respect to patent eligibility under 35 U.S.C. § 101 has been provided, effective 16 December 2014 and published in the Federal Register V 79 No 241 (please visit http://www.uspto.gov/patents/law/exam/interim_guidance_subject_matter_eligibility.jsp for the published guidance). This guidance has been provided in light of recent court decisions such as *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. __, 133 S. Ct. 2107, 2116, 106, USPQ2d 1972 (2013), *Mayo Collaborative Services v. Prometheus Laboratories, Inc.*, 566 U.S. __, 132 S. Ct. 1289, 101 USPQ2d 1961 (2012), and *Alice Corporation PTY. LTD v CLS Bank International*.

The first step under this guidance is determining if the claim is directed to one of the four statutory categories (process, machine, manufacture, or composition of matter). In this case, the claims are methods (a process). The second step is determining if the claims recite or involve judicial exception(s), such as laws of nature, natural phenomena, natural products, or an abstract idea. In this case, the claims are drawn to judicial exceptions: "determining a prognosis…based on the area under the curve determined in step (b)".

Thus, it must be determined if the claim as a whole recites something significantly more than the judicial exceptions.

Where a claim describes a judicial exception, such a claim "requires closer scrutiny for eligibility because of the risk that it will 'tie-up' the excepted subject matter and pre-empt others from using [the judicial exception]" (federal register, p.74622, C1). While all inventions to some degree involve natural laws, products, and other judicial exceptions, the new guidance regarding patent eligibility makes clear that a practical application of these exceptions is necessary, offering "significantly more" than the exception itself. Limitations that were found not to be enough to qualify as "significantly more" include:

- Mere instructions to implement an abstract idea on a computer
- Adding generic instructions that the judicial exception should be used ("apply it")
- Simply appending well-understood, routine and conventional activities previously known to the industry, specified at a high level of generality
- Adding insignificant extrasolution activity to the exception ("mere data gathering")
- Generally linking the use of the exception to a particular technological environment or field of use

Independent claim 12 introduce the judicial exception of calling out the natural correlation between the area under the curve value of tau protein samples taken over time and determining a treatment protocol/prognosis. Such limitations follow the pattern of Julitis claim 2, which is ineligible. This is also similar to the fact pattern of the claims at issue in *Mayo*, where a drug was administered, the natural metabolites were measured, and a conclusion was made about treatment efficacy; this was deemed ineligible by the Supreme Court.

In Mayo, a level of the metabolite "less than about 230 pmol per $8x10^8$ red blood cells [indicated] a need to increase the amount of said drug". Such claim limitations were found to be a description of the correlation itself and not "significantly more". It was also found that steps prior to this constituted routine data gathering, instructing the artisan to engage in conventional, well-known practices and then "warning" the user of the implications of the data; this was not eligible in Mayo.

"Determining a prognosis" is the same as "warning" the user of the implications of the area under the curve determined in step b, i.e., this portion of the claim tells the user the AOC of tau over this time period is correlated to the prognosis without more ("apply it").

Claim 12 has been amended to include the step of determining an area under the curve using the collected tau data. However, this represents no more than a necessary data gathering step (analysis) required to use the correlation, i.e., the correlation is between this gathered data and the prognosis. Data gathering is explicitly discussed in the guidelines for an analysis under §101 as "insignificant extrasolution activity". Regardless of how simple or complex the natural correlation is, it remains a description of the correlation itself and does not provide significantly more. See also the decision in Alice v CLS Bank in which the Court held that merely requiring generic computer implementation fails to transform an abstract idea into a patent-eligible invention.

The step of "determining" (mental) as well as the correlation between tau levels to prognosis/treatment protocol are judicial exceptions which must be set aside to determine if the claims are significantly more than a recitation of these exceptions.

For claim 12, the remaining elements of the claim are directed to a method of measuring (observing) the natural phenomenon. As above, "mere data gathering" is both necessary to utilize the judicial exception as well as insignificant extrasolution activity. Limitations such as obtaining the samples within 12/48 hours or that the relevant tau concentrations require a sensitivity of less than about 0.2 pg/mL are merely a description of the natural correlation itself, informing the artisan of the ranges in which the correlation is observable. In another light, it is generic instructions to use assays of high sensitivity; using sensitive assays to detect proteins represents no more than conventional wisdom in the art and such descriptions do not offer significantly more.

The same is true of newly added limitations of spatially segregating and detecting single tau protein molecules. As discussed previously, such techniques were known. See for example Rissin ("single molecule enzyme linked immunosorbent assay detects serum proteins as subfemtomolar concentrations"; previously cited). Here, Rissin describes antibodies attached to beads, which then bind a target antigen, followed by binding the antigen with a biotin-labeled antibody, where only some of the beads bind the biotin-antibody, dividing these beads into wells, and imaging them to determine the number of locations containing the biotin-antibody by exposure to a streptavidin-ß-gal (streptavidin-enzyme) conjugate in order to determine the concentration of the antigen (e.g., figure 1). While Rissin does not explicitly use the technique to measure tau, Rissin teaches the technology is broadly applicable

to detecting single protein molecules in blood for sensitive diagnostic assays (abstract). The instant claims do not require any particular, novel antibody or other unique reagent. Thus, once informed of the relevant correlation, the "digital ELISA" technique would have been a well-known choice using conventional, commercially available monoclonal antibodies in order to measure tau concentration at the required sensitivity (such as by single molecule detection). Thus, such an assay does not provide significantly more than instructions to a relevant audience to use known techniques to observe a natural phenomenon and to "apply it". This is not patent eligible.

See also (previously cited):

- Harma 2000 *Clinical Chemistry*
- Gorris 2010 "analytical chemistry on the femtoliter scale"
- Ekins 2010 "*Clinical Chemistry*
- Duffy 2011 "immunoassays with broad dynamic ranges based on combining digital and digitally enhanced analog detection of enzyme labels"
- *Chen 2009 *AIChE*
- Xie 2009 *JACS*
- Burton 2010 *Lab Chip*
- Agrawal 2007 *J Agric Food Chem1*
- Quaternix 2010 *IQT Technology Focus Day*
- 2011 "Quanterix discovers link between heart attack-induced hypoxia suspected Alzheimer's disease pathway"
- US 20110245097
- US 20100075407
- US 20110212537
- US 20110212848

This list is not exhaustive, but illustrates that at the time of or before the instant invention, an assay meeting the criteria of the instantly claimed assay was already publicly known and had been disclosed as measuring numerous different proteins from cancer antigens to Aß to tau. For example, the Quaternix

document above ("single molecule arrays for digital detection in complex samples" *IQT technology focus day* presented 25 March 2010, more than one year before the instant priority date of 12 April 2011) describes using antibodies bound to beads to capture a "target protein", followed by a biotin-labeled detection antibody, performed in a plurality of wells (p.13), suggests its use relative to neurological diseases (p.19), and explicitly states that the method was used to measure tau in blood of AD patients and age-matched controls (p.22). The document also teaches measuring tau in relation to TBI (p.29).

Thus, taken as a whole, instant claim 12 does not provide significantly more than the recitation of the judicial exception and is thus not patent eligible.

Claims 13 and 21-24 simply describe the population on which to perform this method. This is a further description of the correlation (where the correlation exists) and must likewise be set aside and cannot offer "significantly more".

Claim 92 further requires the user "determine a method of treatment" based at least in part on the AOC determined in step b. This is recognition of another judicial exception—the AOC of tau samples collected in the first 48 hours are correlated to treatment decisions—and does not confer eligibility for the same reason. Note that this is also similar to *Mayo* in which the collected data informed some treatment decision but was deemed ineligible.

Claim 93 further describes the method of collecting the data. As above, gathering the data is required to utilize the exception and the limitations herein do not represent a novel methodology of measuring proteins; see citations above including Rissen which states at p.2 "98.5% [of beads] would have zero protein molecules".

Claims 94-98 are not eligible for the reasons above, particularly in light of the prior art documents such as Rissen describing such technology for highly sensitive assays.

Claims 99-100 are directed to further characterizing the judicial exception and thus cannot add significantly more.

Claims 101-103 also further inform the user of the implications of the data; the area under the curve of the required tau measurements "is correlated" with the prognosis. This is not significantly more for the reasons above.

Claims 104-105 are more generic versions of claim 12 while still being drawn to the same judicial exception and are not eligible for the reasons above.

In summary, the above analysis conducted in line with all current guidance issued by the USPTO sets forth the rationale for deeming the above claims patent ineligible. It is appreciated that all inventions at some level embody, use, reflect, rest upon, or apply a law of nature, natural phenomenon, or abstract idea. However, the instant claims do not improve another technology, apply the judicial exception with any specific machine, transform the gathered data into a different state or thing, add a limitation that is anything more than what is well-understood, conventional, or routine in the field, or any other meaningful limitation beyond generally linking the use of the exception to a particular technological environment. In other words, the claims as a whole instruct a user to observe tau using conventional samples and a known methodology and then informs this relevant audience about a correlation that exists between the gathered data and a prognosis/treatment protocol. The added steps of determining the prognosis "based on" the gathered data is no more than instructions to "apply it".

Therefore, claims 12-13, 21-24, and 92-105 are not patent eligible.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the

examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claim 105 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix "single molecule arrays for digital detection in complex samples" *IQT technology Focus Day* (hereinafter Quaternix 2010a; previously cited) in view of Wunderlich (previously cited). This is a new rejection necessitated by amendment.

Quaternix 2010a describes the bead-based SiMoA (single molecule array) immunoassay (p.13). Quaternix 2010a teaches a monomeric bead attached to an antibody (see bead at left of page 13) exposed to blood plasma ("100 µl of serum or plasma as test sample") to form a bead comprising at least one immobilized protein molecule (second bead image on page 13). Quaternix 2010a then discloses exposing (mixing) the bead to an antibody/biotin conjugate followed by exposure to an antibody-enzyme complex. This is followed by using the enzyme to determine the concentration of the protein by spatially segregating the reporter. This can also be seen on page 5 where samples are partitioned into a plurality of wells and each reporter is individually counted (where a statistically significant number of well are not associated), page 12 where the reporters are spatially separated and on page 14 where the number of "active wells" is measured. Quaternix 2010a teaches using this technology for diagnostic use in neurological diseases such as Alzheimer's (p.19) and explicitly uses this assay to measure tau in blood of AD patients and age matched controls (p.22). Quaternix 2010a states "You want a nice serum marker for TBI, you do it through tau" (p.29). While Quaternix 2010a suggests the use of tau as a diagnostic marker for TBI, Quaternix 2010a follows this with "you may have a use" and "you may be able to get".

Wunderlich measures tau in multiple samples from the same patient (66 such patients) over the course of 120 hours after stroke, eight out of nine measurements being made at 96 hours or less after stroke onset. Wunderlich teaches that tau protein values were significantly correlated with functional outcome at 3 months, is associated with clinical deficits and infarct volume, and can be used as an additional predictor of the early course of stroke and functional outcomes (abstract).

Regarding claim 105, Wunderlich teaches that tau serum levels are correlated with stroke prognosis. Thus, one of ordinary skill in the art at the time of the invention would have found it obvious to

utilize such tau concentrations for determining a patient's prognosis. Obtaining those values using the methodology of Quaternix 2010a would have been obvious because Quaternix 2010a discloses the high specificity of the assay when measuring proteins in blood. In the reverse, Quaternix 2010a suggests that tau is correlated to stroke and Wunderlich provides the experimental evidence to support that conclusion, thus providing the motivation to the ordinary artisan to reasonably expect the method of Quaternix 2010a to provide useful information when determining a patient's prognosis after brain injury.

Therefore, claim 105 would have been obvious over Quaternix 2010a and Wunderlich.

Claim 105 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix 2010a in view of Mortberg (previously cited). This is a new rejection necessitated by amendment.

Quaternix 2010a describes the bead-based SiMoA (single molecule array) immunoassay (p.13). Quaternix 2010a teaches a monomeric bead attached to an antibody (see bead at left of page 13) exposed to blood plasma ("100 µl of serum or plasma as test sample") to form a bead comprising at least one immobilized protein molecule (second bead image on page 13). Quaternix 2010a then discloses exposing (mixing) the bead to an antibody/biotin conjugate followed by exposure to an antibody-enzyme complex. This is followed by using the enzyme to determine the concentration of the protein by spatially segregating the reporter. This can also be seen on page 5 where samples are partitioned into a plurality of wells and each reporter is individually counted (where a statistically significant number of well are not associated), page 12 where the reporters are spatially separated and on page 14 where the number of "active wells" is measured. Quaternix 2010a teaches using this technology for diagnostic use in neurological diseases such as Alzheimer's (p.19) and explicitly uses this assay to measure tau in blood of AD patients and age matched controls (p.22). Quaternix 2010a states "You want a nice serum marker for TBI, you do it through tau" (p.29). While Quaternix 2010a suggests the use of tau as a diagnostic marker for TBI, Quaternix 2010a follows this with "you may have a use" and "you may be able to get".

Mortberg teaches measuring tau in plasma after cardiac arrest at 2, 6, 12, 24, 48, and 96 hours (p.28). Mortberg teaches there is a significant difference in tau levels between patients with a good outcome versus a poor outcome (p.48). Note that Mortberg is concerned with cerebral ischemic/reperfusion injury (a brain injury) following said cardiac arrest.

Thus, one of ordinary skill in the art at the time of the invention would have found it obvious to utilize such tau concentrations for determining a patient's prognosis as Mortberg teaches tau levels are correlated with prognosis. Obtaining those values using the methodology of Quaternix 2010a would have been obvious because Quaternix 2010a discloses the high specificity of the assay when measuring proteins in blood, thus providing the motivation to the ordinary artisan to reasonably expect the method of Quaternix 2010a to provide useful information when determining a patient's prognosis after cerebral ischemic/reperfusion injury following cardiac arrest.

Therefore, claim 105 would have been obvious over Quaternix 2010a and Mortberg.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-15 and 20-24 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1-11, 15, and 23-30 of copending Application No. 14111331 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the reference application is directed to the same methodology:

- detection limit less than 0.2 pg/mL, e.g., claim 1
- plurality of samples within 96 hours, e.g., claim 9
- same samples, e.g., claims 6,7

The difference is in the reference application being drawn to beta-amyloid rather than tau. However, in turning to the specification of the reference application for support of this limitation, the specification clearly indicates tau is an alternate biomarker (p.12), making such a method an obvious variation of the instant method.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Response to Arguments

Applicant's arguments filed 9/27/17 have been fully considered but they are not persuasive.

Applicant has not argued the merits of the double patenting rejection and it is maintained for reasons of record.

Regarding the §103 rejection, Applicant's arguments are directed to limitations included in instant claim 12, which are persuasive as noted above. However, these limitations are not present in claim 105 and thus this rejection is appropriate.

Regarding the §101 rejection, Applicant argues the steps "involve an application of the judicial exception in a way that was unconventional and unprecedented at the time the invention was made" and argues "the link between the measured tau levels in blood or serum or plasma and the prognosis was not known at the time the invention was made". While this has been considered, such novelty of the judicial exception (the "link") does not weigh in favor of eligibility. The Court has made it clear that an analysis under §101 is distinct from any analysis under §102/103. Rather than determine if the exception is novel or non-obvious, the question must be asked that, once informed of this exception, does the claim add anything significant beyond the exception. This is clearly seen in the decision in *Ariosa v Sequenom*, where the sample was clearly unconventional (maternal plasma had routinely been disregarded as medical waste) but when provided with information regarding the natural relationship between cffDNA in maternal plasma and a diagnosis, the remaining portion of the claim was drawn to no more than "known laboratory techniques". Thus, the correlation itself must be set aside when determining eligibility.

Applicant argues that the data gathering steps should be considered significantly more as the recited steps were not a well-known choice in the context of measuring tau. Applicant also notes the May 2016 guidance in which eligibility was conferred to a claim as a whole given the fact that a certain technique (SNOM) was known but not generally used in the field of DNA hybridization.
This has been fully considered but is not persuasive. The facts in example 31 of the May 2016 guidance state that the SNOM assay was "not commonly or routinely" used in DNA hybridization. In contrast, numerous documents have been cited in the rejection supporting a conclusion that assays meeting the instant limitations—so called single-molecule detection assays or "digital ELISA"—were actively being used to study protein concentrations. Thus, other than Applicant's assertion that the claimed techniques were not well-known, Applicant does not offer evidence to support this position. Applicant is reminded that "arguments of counsel cannot take the place of factually supported objective evidence" (MPEP§2415). In this case, the artisan is informed that in order to utilize the judicial exception, tau must be detected at low levels early after the injury. As noted previously and reiterated above, there were many documents that

did not merely hypothesize about using such assays for the sensitive detection of proteins, but actually did so. Rissen, Quaternix, Duffy, and others were using methods meeting the instant limitations for detecting proteins. Thus, the evidence supports the conclusion that given knowledge that observation requires assays with high specificity such assays would have been well-known to the artisan and thus does not add significantly more.

Conclusion

No claim is allowed.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ADAM M WEIDNER whose telephone number is (571)272-3045. The examiner can normally be reached on M-F 9-16.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeffrey Stucker can be reached on 571-272-0911. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Adam Weidner/
Primary Examiner, Art Unit 1649

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Examiner's Initials #	Cite No.	U.S. Patent Document Number	U.S. Patent Document Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
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	**	TODD et al., Ultrasensitive flow-based immunoassays using single-molecule counting. Clin Chem. 2007 Nov; 53(11):1990-5. Epub 2007 Sep 21.	
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	**	VO-DINH et al., Phase-resolved fiber-optics fluoroimmunosensor. Applied Spectroscopy. 1990; 44(1):128-132.	
	**	VOGELSTEIN et al., Digital PCR. Proc Natl Acad Sci U S A. 1999 Aug 3;96(16):9236-41.	
	**	WALT et al., Biosensing with live cells using a high-density optical fiber array. Radiation Research. 2001; 156(4):442.	

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

**a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 14/111,326, filed June 24, 2014, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /AMW/

FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 15/269,142	ATTY. DOCKET NO.: Q0052.70018US02
				FILING DATE: September 19, 2016	CONFIRMATION NO.: 6662
				FIRST NAMED INVENTOR: David Wilson	
Sheet	21	of	22	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

	**	WALT et al., Microsensor arrays for saliva diagnostics. Ann N Y Acad Sci. 2007 Mar; 1098:389-400.	
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	**	WU et al., Development and preliminary clinical validation of a high sensitivity assay for cardiac troponin using a capillary flow (single molecule) fluorescence detector. Clin Chem. 2006 Nov; 52(11):2157-9.	

EXAMINER:	DATE CONSIDERED:

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FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 15/269,142	ATTY. DOCKET NO.: Q0052.70018US02
				FILING DATE: September 19, 2016	CONFIRMATION NO.: 6662
				FIRST NAMED INVENTOR: David Wilson	
Sheet	22	of	22	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

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[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/Adam Weidner/	12/11/2017

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /AMW/

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L4	9	L3 and tau.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/12/11 14:05
L3	7356	L1 or L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/12/11 14:05
L2	7332	((wilson near2 david) or (john near2 zetterber) or (kaj near2 blennow) or (jeffrey near2 randall)).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/12/11 14:05
L1	34	quanterix.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/12/11 14:05

12/11/2017 2:06:31 PM
C:\Users\aweidner\Documents\EAST\Workspaces\15269142.wsp

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/269,142	Wilson et al.
	Examiner	Art Unit
	ADAM M WEIDNER	1649

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor/assignee east	3/17/17	amw
text/claims east/internet	3/17/17	amw
inventor/assignee east update	12/11/2017	amw

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

NOTICE OF APPEAL FROM THE EXAMINER TO THE PATENT TRIAL AND APPEAL BOARD	Docket Number (Optional) Q0052.70018US02

In re Application of	David Wilson et al.
Application Number	15/269,142-Conf. #6662
Filed	September 19, 2016
For	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Art Unit	1649
Examiner	A. M. Weidner

Applicant hereby **appeals** to the Patent Trial and Appeal Board from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 800.00

☐ Applicant asserts small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by 50%, and the resulting fee is: $ _____

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Therefore, the fee shown above is reduced by 75%, and the resulting fee is: $ _____

Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached~~.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 23/2825 .

☒ Payment made via EFS-Web.

☒ A petition for an extension of time under 37 CFR 1.136(a) (PTO/AIA/22 or equivalent) is enclosed. For extensions of time in reexamination proceedings, see 37 CFR 1.550.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant ☒ attorney or agent of record Registration number 46,190 ☐ attorney or agent acting under 37 CFR 1.34 Registration number _____

Signature /Michael J. Pomianek/

Typed or printed name Michael J. Pomianek

Telephone Number 617.646.8000

Date June 14, 2018

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

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Dated: June 14, 2018 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): Q0052.70018US02

Application Number	15/269,142-Conf. #6662	Filed	September 19, 2016

For: METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Art Unit	1649	Examiner	A. M. Weidner

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☒ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1,400.00
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached~~.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☒ Payment made via EFS-Web.

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I am the

☐ applicant.

☒ attorney or agent of record. Registration number 46,190 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Michael J. Pomianek/ — Signature
June 14, 2018 — Date

Michael J. Pomianek — Typed or printed name
617.646.8000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

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Dated: June 14, 2018 — Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

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Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	A. M. Weidner
Attorney Docket Number	Q0052.70018US02

ENCLOSURES (*Check all that apply*)

- [X] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [X] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [X] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [ ] Other Enclosure(s) (please Identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Michael J. Pomianek/		
Printed name	Michael J. Pomianek		
Date	June 14, 2018	Reg. No.	46,190

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FEE TRANSMITTAL

Complete if known	
Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Examiner Name	A. M. Weidner
Art Unit	1649
Practitioner Docket No.	Q0052.70018US02

[ ] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT	($)2,200.00

METHOD OF PAYMENT (check all that apply)

[ ] Check [X] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[X] Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

[ ] Charge fee(s) indicated below

[ ] Charge fee(s) indicated below, **except for the filing fee**

[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

[ ] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

[X] Credit any overpayment of fee(s)

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- or HP =	_____	x _____	=	_____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- or HP =	_____	x _____	=	_____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**
_____	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____	- 100 =	_____	/50 =	_____ (round **up** to a whole number)	x _____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1253 Extension for response within third month	1,400.00
1401 Notice of appeal	800.00

SUBMITTED BY

Signature	/Michael J. Pomianek/	Registration No. (Attorney/Agent)	46,190	Telephone	617.646.8000
Name (Print/Type)	Michael J. Pomianek			Date	June 14, 2018

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6376682.1

Electronic Patent Application Fee Transmittal

Application Number:	15269142
Filing Date:	19-Sep-2016
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Michael Pomianek/Ellen Paul
Attorney Docket Number:	Q0052.70018US02

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
NOTICE OF APPEAL	1401	1	800	800
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
		Total in USD ($)		**2200**

Electronic Acknowledgement Receipt

EFS ID:	32899047
Application Number:	15269142
International Application Number:	
Confirmation Number:	6662
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Michael Pomianek/Ellen Paul
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US02
Receipt Date:	14-JUN-2018
Filing Date:	19-SEP-2016
Time Stamp:	14:24:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2200
RAM confirmation Number	061518INTEFSW14253000
Deposit Account	232825
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.17 (Patent application and reexamination processing fees) 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	Q005270018US02-TRN-MJP.pdf	24822 17e61f893348aa63744cc41dd08575306e1a487a	no	1
Warnings:					
Information:					
2	Extension of Time	Q005270018US02-EXT-MJP.pdf	23682 16de52bd05d33a9ad6794550bc9934bff84bcec2	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	Q005270018US02-FEE-MJP.pdf	31695 d1f79bed607fb28f45dcf580d065809bc63d5e60	no	1
Warnings:					
Information:					
4	Notice of Appeal Filed	Q005270018US02-NAPL-MJP.pdf	20849 3ae3678139c309447c17aa590dec65438814c235	no	1
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	33003 7451c51f1d3fb139d19adeec3cf9bb1f66827094	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	134051		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	A. M. Weidner
Attorney Docket Number	Q0052.70018US02

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C 371, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO on page 2.)

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. [ ] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. [ ] Other _____

   b. [X] Enclosed

      i. [X] Amendment/Reply

      ii. [ ] Affidavit(s)/ Declaration(s)

      iii. [X] Information Disclosure Statement (IDS)

      iv. [X] Other -Request for a Three-Month Extension of Time -Fee Transmittal -Form PTO-1449

2. Miscellaneous

   a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. [ ] Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. [ ] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 23/2825 .

      i. [X] RCE fee required under 37 CFR 1.17(e)

      ii. [X] Extension of time fee (37 CFR 1.136 and 1.17)

      iii. [X] Other Excess claims fees

   b. [ ] Check in the amount of $ _____ enclosed

   c. [X] Payment by credit card ~~(Form PTO-2038 enclosed)~~

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Michael J. Pomianek/	Date	November 14, 2018
Name (Print/Type)	Michael J. Pomianek	Registration No.	46,190

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

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Dated: November 14, 2018 Electronic Signature for: /Eileen M. MacKenzie/

6769099.1

Docket No.: Q0052.70018US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	A. M. Weidner
Art Unit:	1649

Certificate of Electronic Filing under 37 CFR §1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: November 14, 2018 Electronic Signature for: /Eileen M. MacKenzie/

RESPONSE AFTER FINAL ACTION UNDER 37 C.F.R. § 1.116

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Final Office Action dated December 14, 2017, please enter the following in the above-identified U.S. patent application:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 10 of this paper.

AMENDMENTS TO THE CLAIMS

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

1-11. (Cancelled)

12. (Currently Amended) A method of determining a prognosis of a patient's recovery from a brain injury comprising:

(a) performing an assay on each of a plurality of blood samples obtained from the patient following the brain injury and/or plasma and/or serum derived from each of the blood samples to determine a measure of the tau protein molecule concentration in each sample,

wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours,

wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury,

wherein the assay ~~comprises spatially segregating and detecting single tau protein molecules, and~~

~~wherein~~ is characterized by a ~~the~~ limit of detection of tau protein molecules ~~of the assay~~ that is less than about 0.2 pg/mL; and comprises:

i. exposing a plurality of capture objects that each include a binding surface having affinity for tau protein molecules to the blood sample;

ii. immobilizing at least a portion of the tau protein molecules with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one tau protein molecule and a statistically significant fraction of the capture objects do not associate with any tau protein molecules;

iii. determining a proportion of the plurality of capture objects that associated with a tau protein molecule in step ii; and

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iv. determining a measure of tau protein molecule concentration in the blood sample based at least in part on the proportion determined in step iii;

(b) determining an area under a curve of a graph of the measure of the tau protein molecule concentration in the plurality of samples versus time, wherein the area is determined for the total period of time and/or a second peak in the tau protein molecule concentration; and

(c) determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based on the area under the curve determined in step (b).

13. (Original) The method of claim 12, wherein the brain injury results from a hypoxic event.

14-20. (Cancelled)

21. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by cardiac arrest.

22. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by stroke.

23. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by an ischemic event.

24. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by a thrombosis.

25- 91. (Cancelled)

92. (Currently Amended) The method of claim 12, further comprising ~~determine~~ determining a method of treatment based at least in part on the area under the curve determined in step (b).

93. (Previously presented) The method of claim 12, wherein tau protein molecules are spatially segregated into a plurality of locations, wherein a statistically significant fraction of the locations contain a single tau protein molecule and a statistically significant fraction of the locations do not contain any tau protein molecules

94. (Previously presented) The method of claim 93, wherein the plurality of locations comprises a plurality of reaction vessels.

95. (Previously presented) The method of claim 93, wherein the plurality of locations comprises plurality of droplets.

96. (Canceled)

97. (Previously presented) The method of claim 12, wherein the plurality of capture objects comprises a plurality of beads.

98. (Previously presented) The method of claim 12, further comprising plotting the measure of the tau protein molecule concentration versus time on a graph to determine the area under the curve.

99. (Previously presented) The method of claim 12, wherein each of the tau protein molecule is selected from the group consisting of tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and tau 40 (441, 2N4R).

100. (Previously presented) The method of claim 99, wherein at least a portion of the tau protein molecules are phosphorylated.

101. (Previously presented) The method of claim 12, wherein the patient's prognosis is correlated with a Cerebral Performance Category (CPC) score including classifications of good or poor,

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wherein a good CPC score indicates a prognosis of the patient being conscious and alert with normal function or only slight disability or conscious and alert with moderate disability after a selected period of time, and

wherein a poor CPC score indicates a prognosis of the patient being conscious with the patient having a severe disability, being comatose or in a persistent vegetative state, or being brain dead or suffering death from other causes after a selected period of time.

102. (Previously presented) The method of claim 101, wherein the prognosis is good for the recovery from the brain injury if the area under the curve is less than about 800.

103. (Previously presented) The method of claim 101, wherein the prognosis is poor for the recovery from the brain injury if the area under the curve is greater than about 800.

104. (Canceled)

105. (Currently Amended) A method of determining a prognosis of a patient's recovery from a brain injury comprising:

(a) performing an assay on a blood sample obtained from the patient following the brain injury and/or plasma and/or serum derived from the blood sample to determine a measure of the tau protein molecule concentration in the sample,

wherein the assay comprises spatially segregating and detecting single tau protein molecules,

wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury,

wherein the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL; and

(b) determining a prognosis of the patient's recovery from the brain injury based on the measure of the tau protein molecule concentration in the sample determined in step (a).

106. (New) The method of claim 12, wherein the assay comprises, between step ii and step iii, spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of locations.

107. (New) The method of claim 106, further comprising addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a tau protein molecule.

108. (New) The method of claim 12, wherein the plurality of capture objects comprises a plurality of paramagnetic beads covalently attached to anti-tau antibodies.

109. (New) The method of claim 106, wherein the plurality of locations comprises a plurality of reactions vessels.

110. (New) The method of claim 109, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters, or between about 1 femtoliter and about 1 picoliter.

111. (New) A method of treating a patient following a brain injury, comprising:

administering to the patient a therapeutic intervention selected from the group consisting of: administering a therapeutic agent, a blood thinner, a steroid, an anti-oxidant, and/or hypothermia at a dosage or under conditions suitable for a patient who has a poor prognosis for recovery according to a Cerebral Performance Category score, if an area under a curve of a graph of a measure of tau protein molecule concentration versus time in a plurality of samples obtained from the patient is greater than or equal to 500, wherein the area under the curve is determined by:

(a) performing an assay on each of a plurality of test samples comprising or derived from a plurality of blood samples obtained from the patient following the brain injury, of said test samples comprising at least one of whole blood, plasma, or serum derived from corresponding

blood samples to determine a measure of the tau protein molecule concentration in each blood sample,

wherein the plurality of blood samples are obtained from the patient over a period of time of at least about 48 hours,

wherein at least one of the plurality of blood samples is obtained within 12 hours of the brain injury,

wherein the assay comprises

i. detecting tau protein molecules in each test sample with single molecule resolution such that the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL; and

ii. determining the measure of tau protein molecule concentration in each blood sample based on the results of the detecting step; and

(b) determining the area under the curve of the graph of the measure of the tau protein molecule concentration in the plurality of blood samples versus time, wherein the area is determined for the total period of time and/or a second peak in the tau protein molecule concentration.

112. (New) The method of claim 111, wherein the administering step is performed if the area under the curve of the graph of a measure of tau protein molecule concentration in a plurality of samples obtained from the patient versus time is greater than or equal to 800.

113. (New) The method of claim 111, wherein the therapeutic agent is a neuroprotective drug.

114. (New) A method for determining a measure of tau protein from a plurality of blood or blood derived samples obtained from a patient following a brain injury, comprising:

(a) performing an assay on each of the plurality of samples obtained from the patient following the brain injury and/or plasma and/or serum derived from each of the samples to determine a measure of the tau protein molecule concentration in each sample,

wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours,

wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury,

wherein the assay comprises

i. detecting tau protein molecules in each sample with single molecule resolution such that the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL; and

ii. determining the measure of tau protein molecule concentration in each blood sample based on the results of the detecting step; and

(b) determining an area under a curve of a graph of the measure of the tau protein molecule concentration in the plurality of samples versus time, wherein the area is determined for the total period of time and/or a second peak in the tau protein molecule concentration.

115. (New) The method of claim 114, wherein the assay further comprises:

a) exposing a plurality of capture objects that each include a binding surface having affinity for tau protein molecules to the sample;

b) immobilizing at least a portion of the tau protein molecules with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one tau protein molecule and a statistically significant fraction of the capture objects do not associate with any tau protein molecules;

c) determining a proportion of the plurality of capture objects that associated with a tau protein molecule in step b; and

d) determining a measure of tau protein molecule concentration in the blood sample based at least in part on the proportion determined in step c.

116. (New) The method of claim 115, wherein the assay comprises, between step b and step c, spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of locations.

117. (New) The method of claim 116, further comprising addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a tau protein molecule.

118. (New) The method of claim 115, wherein the plurality of capture objects comprises a plurality of paramagnetic beads covalently attached to anti-tau antibodies.

119. (New) The method of claim 116, wherein the plurality of locations comprises a plurality of reactions vessels.

120. (New) The method of claim 119, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters, or between about 1 femtoliter and about 1 picoliter.

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REMARKS

Applicant respectfully requests reconsideration. Claims 12-13, 21-24 and 92-105 were previously pending in this application. By this amendment, Applicant is canceling claim 96 and 104 without prejudice or disclaimer. Claims 12, 92, and 105 have been amended. The previously presented versions of claims 12 and 104 had recited identical methods except with regard to whether the area under the curve was determined for the total period of time (claim 12) or a second peak in the tau protein molecule concentration (claim 104), as well as claim 104 reciting determining a prognosis of the patient's recovery from the brain injury *and/or a method of treatment*. Claim 12 has been amended to incorporate these features of claim 104 as alternatives, and now recites "…wherein the area is determined for the total period of time and/or a second peak in the tau protein molecule concentration…", as well as "…determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment…". This amendment of claim 12 and cancelation of claim 104 has been made solely for the purpose of reducing the number of independent claims, and not for any reason of patentability, and is supported in the specification at least by lines 18-30 of page 3 of the Application as filed.

New claims 106-120 have been added, with claims 111 and 114 being independent. As a result, claims 12-13, 21-24, 92-95, 97-103, and 105-120 are pending for examination with claims 12, 105, 111, and 114 being independent claims. No new matter has been added.

Rejections Under 35 U.S.C. §101

Claims 12-13, 21-24, and 92-105 were rejected under 35 U.S.C. § 101 on the basis that the claims as a whole are not directed to patent eligible subject matter. It is alleged in the Office Action that the claims are directed to a judicial exception and, considering the claim as a whole, these claims do not recite something significantly more than the judicial exception.

The 2014 Interim Guidance on Patent Subject Matter Eligibility ("Guidelines") released by the Patent Office on December 16, 2014 set forth a two-step test for analyzing subject-matter eligibility of a claim under §101. The first step of the two-part test is whether the claims is directed to a judicial exception such as an abstract idea (Step 2A), for example, a "law of nature". If so, the second step involves determining whether the claims recite additional elements that amount to

significantly more than the judicial exception (Step 2B). In the Office Action, the Examiner alleges that these claims are directed to the following judicial exception to patent eligibility – the correlation of measured tau protein concentration to patient prognosis, which, presumably, the Examiner characterizes as a "natural phenomenon" or "law of nature" – and that the remainder of the claims does not recite "something significantly more than the judicial exception(s)," thus rendering the claims ineligible for patent protection.

As an initial matter, with regard to Step 2A of the two-step test, Applicant disagrees that claim 12 as a whole is directed to a natural phenomenon, law of nature, or any other judicial exception for reasons noted below. Furthermore, even if it were, for the sake of argument which is not conceded, the claim as a whole, on the basis of at least the above amendments and remarks below, recites features that amount to something significantly more than the judicial exception. With respect to whether claim 12 is "directed to" a natural phenomenon or law of nature," Applicant points out that claim 12 does not simply claim the correlation between a tau protein molecule concentration and patient outcome following a traumatic brain injury. Instead, claim 12, as a whole, is directed to the new and improved technique for achieving a useful clinical and therapeutic result involving prognosis of a patient's recovery from a brain injury via using blood samples or blood derived samples as opposed to CSF [cerebrospinal fluid] as is conventional. The technique also involves performing and incorporating measurements during an unconventional time window (e.g., with at least one sample having been obtained within 12 hours). The direction of the claim is thus a new assay utilizing an unconventional sample source. Like the freeze-thaw method found eligible in *Rapid Litig. Mgmt. v. CellzDirect, Inc.,* 827 F.3d 1042, 1048-49, 119 USPQ2d 1370 (Fed. Cir. 2016) ("Rapid Litigation"), which was held to be directed to a new and useful cell storage and lysis method that while involving the natural phenomenon of the cell's natural resistance to viability loss over multiple freeze-thaw cycles, the present claim 12, while it may exploit and involve a correlation between tau level and prognosis, is similarly directed to an improved practical method of using the much more conveniently collected blood or blood-derived samples (versus CSF) for a practical tau assay to inform patient prognosis. Additionally, the new and improved assay employs the unconventional step of using blood samples collected within 12 hours, contrary to the teachings of the art at the time of the invention and earliest effective filing date (see below). As such, like the

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claims in Rapid Litigation, claim 12 is not "directed to a natural phenomenon" and thus is patent eligible and compliant with Section 101 for this reason alone. Supporting this contention, the specification as filed states that "[in] some cases, it is advantageous to determine the level of a biomarker in the blood of a patient as compared to CSF, as blood sampling is generally less invasive and may result in fewer complications as compared to CSF sampling. However, many of the biomarkers that are present in the CSF have a slow rate of transmission across and/or a high barrier of transportation across the blood-brain barrier (BBB) and thus, the concentration of the biomarker in the patient's blood may be sufficient lowered as compared to the concentration in CSF to make it difficult or impossible to accurately determine using typically employed conventional immunoassays." (See page 6 line 28 to page 7 line 6). Claim 12, rather than being directed to a correlation, is in fact directed to a practical assay for advantageously achieving the specific and useful result of enabling use of blood or blood-derived samples to measure tau, in a specific timeframe, to inform a patient diagnosis.

Moreover, even if claim 12 were for the sake of argument considered to be "directed to" a judicial exception under Step 2A, as mentioned above, amended claim 12 is patent-eligible at least because the steps of claim 12, when taken as a whole, clearly recite something "significantly more" than the judicial exception, and thus render the claim eligible subject matter under Step 2B of the test.

As one example, amended claim 12 recites determining an area under a curve of a graph of the measure of a tau protein molecule concentration in a plurality of samples versus time, with the samples referring to blood samples and/or plasma and/or serum derived from each of the blood samples. Additionally, the measure of the tau protein molecule concentration is determined using an assay characterized by a limit of detection of tau protein molecules that is less than about 0.2 pg/mL. The Office Action states that the step of determining the area under the curve of a graph of the measure of tau protein concentration "represents no more than a necessary data gathering step (analysis) required to use the correlation," and that such data gathering amounts to "insignificant extrasolution activity." (See Office Action at page 5). The Applicant disagrees. Performing such a combination of steps goes beyond mere insignificant extra-solution activity and is more than a restatement of the natural phenomenon, at least because it represents an unconventional approach to

monitoring neural biomarkers such as tau, which were, at the time of the invention and earliest effective filing date of the Application, conventionally measured from samples taken from cerebrospinal fluid, not blood. Moreover, the use of an area under the curve technique to analyze the concentrations of neural biomarkers from blood samples at the level of detection recited in the claim was unconventional and non-routine. No evidence of record establishes that it would have been well-known to a person of ordinary skill that neural biomarkers such as a tau could be reliably measured from blood samples, let alone reliably measured at a level of detection and statistical significance necessary for an area under the curve method to be a reasonable choice for use in a method of determining a prognosis. For example, the specification as filed states that "[t]au is known to be elevated in the cerebrospinal fluid (CSF) of patients with neurodegenerative disease and head injuries. However, since such biomarkers must diffuse across the blood brain barrier, they may be present in the blood in proportion in extremely low concentrations that are not reliably measurable by typical *conventional* immunoassays" (see page 1, lines 24-27). The Application goes on to teach methods of reliable measurement of tau protein in blood that enable the use of an area under the curve technique, stating that "in the method described above which correlates the total area under the curve and/or the area under the curve of a second peak of tau protein concentration to a prognostic indication of brain injury, the limits of detection, and/or limits of quantification needs to be substantially lower than the LOD and/or LOQ provided by common ELISA techniques." (See page 19, lines 21-25). Finally, the Application states that "[u]se of peripheral tau protein measurements as an indicator of the presence of brain injury has remained elusive, in large part due to the lack of adequate sensitivity for reliable measurement of the protein *in serum or plasma* using common technologies," (emphasis added, see page 38, lines 24-27), which clearly indicates that any method that involves reliably measuring tau protein in blood (e.g., or serum or plasma derived from blood) would be at best *uncommon* and thus certainly not "conventional." Furthermore, the step of determining of an area under a curve of the tau protein molecule concentrations in claim 12 represents a *transformation* of what the Examiner asserts is "gathered data" (e.g., the measured tau protein molecule concentrations from the plurality of blood samples) into a different state or thing – that is, it transforms the "gathered data" into a useful, specific, and improved metric for the prognosis of a patient following a brain injury. As the Examiner admits, transformations of

gathered data into different state or thing is an important consideration with regard to the patent-eligibility of a claim.

Additionally, as mentioned above, claim 12 requires that at least one of the plurality of samples is obtained within 12 hours of the brain injury. As the Examiner admitted in the Office Action, the use of measurements of tau protein molecules from samples collected within 12 hours of a brain injury in determining a prognosis was not only *not* disclosed in the prior art, but in fact such measurements were previously *excluded* from data analyses (see Mortberg et al., below) and considered to be *non-indicative* of future prognoses of patients (see Wunderlich et al., below). As such, the express inclusion of blood samples collected within 12 hours of a brain injury, as recited in claim 12, would have been not only unconventional and non-routine, but in fact would have been counter-intuitive in view of the teachings of the prior art. The Examiner asserts that obtaining the samples within 12/48 hours (or that the relevant tau concentrations require a sensitivity of less than about 0.2 pg/mL) are merely a description of the natural correlation itself. First of all, obtaining the samples within the claimed timeframe at the claimed sensitivity describes actively performed process *improvements* (analogously to the improved cell processing techniques in Rapid Litigation). As such, the 12 hour limitation of claim 12 is not a description of the natural law itself, but is rather an unconventional active manipulative component of an unconventional technique and analysis.

While the passages cited above establish the factual basis for the unconventionality and non-routine nature of determining an area under a curve of a graph of a measure of the concentration of neural biomarkers such as tau in non-CSF samples (e.g. blood or blood-derived samples) based on measurements from an assay characterized by a level of detection of 0.2 pg/mL, even without this support the present rejection is unsupportable in that the Patent Office has failed to establish a factual basis for such a feature of the claimed method being conventional, well-known, and routine. As explained in the USPTO Memorandum of April 19, 2018 titled: "*Changes in Examination Procedure Pertaining to Subject Matter Eligibility, Recent Subject Matter Eligibility Decision (Berkheimer v. HP, Inc.)*," the conclusion that an element (or combination of elements) is widely prevalent or in common use in an industry (which is necessary for a determination that the element(s) represents well-understood, routine, conventional activity) ***must*** be based upon a factual determination that is supported by 1) a citation to an express statement in the specification or to a

statement made by the applicant during prosecution that demonstrates the well-understood, routine, conventional nature of the additional element(s), 2) a citation to one or more court decisions discussed in MPEP §2106.05(d)(II), 3) a citation to a publication that demonstrates the well-understood, routine, conventional nature of the additional element(s), with the description in the publication demonstrating that the additional elements are widely prevalent or in common use in the relevant field, or 4) a statement of the Examiner taking official notice of the well-understood, routine, conventional nature of the additional elements(s). The Patent Office has yet to provide any such citation or factual support or statement of official notice with respect to determining an area under a curve of a graph of a measure of the concentration of neural biomarkers such as tau in blood samples based on measurements from an assay characterized by a level of detection of less than about 0.2 pg/mL.

For at least the reasons stated above, the claimed method, to the extent it could be considered directed to any judicial exception, when taken as a whole, represents something significantly more than any such judicial exception, and is therefore compliant with 35 U.S.C. § 101 for this additional reason. However, without acceding to the merits of the rejection and in order to expedite prosecution, claim 12 has also been amended to recite specific steps of the assay that is characterized by a limit of detection of tau protein molecules that is less than about 0.2 pg/mL to further distinguish the claim from any assay that is conventionally used in the context of the claimed application – namely, an assay that comprises "i. exposing a plurality of capture objects that each include a binding surface having affinity for tau protein molecules to the blood sample; ii. immobilizing at least a portion of the tau protein with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one tau protein molecule and a statistically significant fraction of the capture objects do not associate with any tau protein molecules; iii. determining a proportion of the plurality of capture objects that associated with a tau protein molecule in step ii; and iv. determining a measure of tau protein molecule concentration in the blood sample based at least in part on the proportion determined in step iii." The Office Action alleges that claim 12 followed the pattern of *Julitis* claim 2, which is ineligible because the additional limitations of the claim beyond that which was directed to the natural phenomenon were stated at *a high level of generality*. Claim 12, in contrast to *Julitis* claim 2, describes a specific,

unconventional assay in the claimed context. The assay described in amended claim 12 was not well-known, routine, or conventional for measuring neural biomarkers such as tau protein, particularly from blood samples or samples derived from blood, and even more particularly within the claimed time frame of collection (e.g., with at least one sample collected within 12 hours). In the Office Action, the Examiner posits that the use of highly sensitive assays for measuring proteins represents no more than conventional wisdom in the art, and that assays such as digital ELISA assays do not provide significantly more than instructions to a relevant audience to use *known techniques* to observe a natural phenomenon and to "apply it". However, even assuming the accuracy of the Examiner's contention, the mere fact that assays having a high sensitivity (such as a digital ELISA assay) may have been publicly known does not mean that such assays were widely prevalent, well-known, and routine in the art, let alone in the claimed context of the measurement of neural biomarkers from blood samples or samples derived from blood. *Berkheimer v. HP Inc., 881 F.3d* at 1369 states that "[w]hether a particular technology is well-understood, routine, and conventional goes beyond what was simply known in the prior art. The mere fact that something is disclosed in a piece of prior art, for example, does not mean it was well-understood, routine, and conventional." (emphasis added). The Patent Office has not made a factual determination that an assay comprising the features of claim 12 was indeed well-understood, routine, or conventional for measuring neural biomarkers such as tau in blood samples or samples derived from blood, in the context described in claim 12.

Indeed, and as has been previously mentioned by the Applicant in reference to Example 31 of the USPTO's May 2016 "*Subject Matter Eligibility Examples: Life Sciences,*" the mere description of an assay procedure generally in the prior art does not alone render it "a well-known choice" if it has not been previously engaged in by researchers in the field in the context of the invention as claimed (e.g., the field of neural biomarker monitoring in blood samples). As explained in the commentary related to patent-eligibility of claim 70 of Example 31, which describes the application of a previously-known "SNOM" technique to DNA hybridization, "[a]t the time the invention was made and the application was filed, the use of SNOM to study DNA hybridization had been discussed in several articles in widely-read scientific journals. However, scientists were not *commonly or routinely* using SNOM to study DNA hybridization at the time the

Fujirebio Diagnostics, Inc. v. Quanterix Corp. EX-1044
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invention was made and the application was filed." (emphasis added). Again, the Patent Office has not pointed to any factual evidence that the features of the assay of claim 12, even if previously "well known" for other purposes or applications, which is not conceded, were *commonly or routinely* used in the field of monitoring neural biomarkers. Furthermore, it should be understood that even isolated nearly contemporaneous references speculating about the development of an assay for the sensitive detection of neural biomarkers in blood is not indicative of, but in fact is the contrary of such an assay being common or routine in the context of the later development and use of that assay in the speculated context.

Thus, for at least the reasons above, claim 12 and its dependents are patent-eligible. Accordingly, withdrawal of the claim objections is respectfully requested.

Independent claim 105 recites a method involving an assay performed on a plurality of samples, at least one of which is collected within 12 hours of a brain injury, with the assay comprising spatially segregating and detecting single tau molecules. The method of claim 105 is patent-eligible for at least the reasons outlined above for claim 12 with respect to the unconventionality and non-routine nature of assays for detecting tau molecule concentration in blood samples during timeframes involving at least one sample acquisition within 12 hours. Specifically, it was unconventional and non-routine at the time of the invention and earliest effective filing date to measure the concentration of neural biomarkers such as tau in blood by spatially segregating single biomarker molecules. The Patent Office has not pointed to any factual evidence that such an assay was *conventional and routine* in this context. Additionally, as mentioned above, collection of blood samples within 12 hours of a brain injury and incorporating measurements from such samples in determining a patient's prognosis was non-routine, unconventional, and even contrary to the teachings of the prior art. Thus, for at least the reasons above, claim 105 is patent-eligible. Accordingly, withdrawal of the claim objection is respectfully requested.

Fujirebio Diagnostics, Inc. v. Quanterix Corp. EX-1044
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Rejections Under 35 U.S.C. §103

Claim 105 was rejected under pre-AIA 35 U.S.C. § 103(a) as being unpatentable over Quaternix " Single Molecule Arrays for Digital Detection in Complex Samples " *IQT Technology Focus Day* (hereinafter Quaternix 2010a; previously cited) in view of Wunderlich (previously cited).

Without acceding to the merits of the rejection and in order to expedite allowance, independent claim 105 has been amended to recite that at least one of the plurality of samples is obtained within 12 hours of the brain injury. In the Office Action, the Examiner indicated that claims requiring that samples taken at 12 hours or less be included in the data analysis are allowable over the prior art. Furthermore, nowhere does Quanterix teach or suggest this feature, and nowhere does Wunderlich cure the deficiencies of Quanterix. Wunderlich does not describe nor provide any correlations between samples taken within 12 hours of the brain injury and correlation with patient prognosis, as presently recited in claim 105, as amended. In contrast, Wunderlich notes only a correlation from 72 hours after stroke onset (see Wunderlich, page 561, first column, first full paragraph).

In view of at least the above remarks, it is believed that the rejection of independent claim 105 as being unpatentable in view of Quanterix and Wunderlich has been overcome, and that the patentability of claim 105 has been established. Accordingly, withdrawal of the claim objection is respectfully requested.

Claim 105 was rejected under pre-AIA 35 U.S.C § 103(a) as being unpatentable over Quanternix 2010a in view of Mortberg (previously cited).

As noted above in connection with the § 103(a) rejection over Quanterix in view of Wunderlich, and without acceding to the merits of the rejection, independent claim 105 has been amended to recite that at least one of the plurality of samples is obtained within 12 hours of the brain injury. As previously mentioned, in the Office Action, the Examiner indicated that claims requiring that samples taken at 12 hours or less be included in the data analysis are allowable over the prior art. Furthermore, nowhere does Quanterix teach or suggest this feature, and nowhere does Wunderlich cure the deficiencies of Quanterix. A person of ordinary skill in the art, when reading

Mortberg, would not be able to determine whether tau is present in any level, let alone a detectable level, within 12 hours of the brain injury. Accordingly, Mortberg does not teach or suggest the claimed methods.

In view of at least the above remarks, it is believed that the rejection of independent claim 105 as being unpatentable in view of Quanterix and Mortberg has been overcome, and that the patentability of claim 105 has been established. Accordingly, withdrawal of the claim objection is respectfully requested.

Double Patenting

Claims 1-15 and 20-24 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-11, 15 and 23-30 of copending Application No. 14/111,331.

It is respectfully requested that these rejections be deferred until the claims of this application are otherwise in condition for allowance.

New Claims

New independent claims 111 and 114 have been added. For at least the reasons described above in connection with claim 12, these new claims meet all of the requirements of 35 U.S.C. §§ 101 and 103.

Moreover, independent claim 111 meets all of the requirements of 35 U.S.C. § 101 at least for the additional reason that they are directed to methods of treating patients following a brain injury, and not directed to a judicial exception. Such claims directed to methods of treatment are patent-eligible under 35 U.S.C. § 101 (See *Vanda Pharmaceuticals Inc. v. West-Ward Pharmaceuticals*, 887 F.3d 1117 (Fed. Cir. 2018)). As explained in the USPTO Memorandum of June 7, 2018 with the subject: "Recent Subject Matter Eligibility Decision: *Vanda Pharmaceutical Inc. v. West-Ward Pharmaceuticals*," "'method of treatment' claims that practically apply natural relationships should be considered **patent eligible** under Step 2A of the USPTO's subject matter eligibility guidance".

Additionally independent claim 114 meets all of the requirements of 35 U.S.C. § 101 at least for the reason that it is directed to determining a measure of tau protein in blood or blood-derived samples, and not to a judicial exception (e.g., a natural phenomenon). Claim 114 recites the performance of an assay on a plurality of blood or blood-derived samples obtained from a patient, with at least one of the samples having been obtained within 12 hours of the patient's brain injury, and determining an area under a curve based on the tau protein molecule concentrations detected from each of the plurality of samples. Claim 114 follows the pattern of *Julitis* claim 1 from Example 29 of the USPTO's May 2016 "Subject Matter Eligibility Examples: Life Sciences"), which recites steps of obtaining samples and performing an assay to detect a JUL-1 from the samples, but does not recite or involve any correlation/diagnosis steps. *Julitis* claim 1 is held to be patent-eligible subject matter because the steps of the method do not recite or describe any recognized exception (e.g., natural laws, natural correlations), and as such, the claim is not directed to an exception (Step 2A: No), and is eligible. Analogously, claim 114 does not recite or describe a recognized exception. It should be understood that Applicant does not suggest that the inclusion of diagnosis/correlation steps necessarily directs a claim to a judicial exception, but, as the commentary for *Julitis* claim 1 makes clear, the lack of such steps definitively precludes a claim from being directed to such a judicial exception.

Claim 114 meets the requirements of 35 U.S.C. § 103 at least because at least one of the plurality of blood or blood derived samples from which a tau protein molecule concentration is determined is collected within 12 hours of the patient's brain injury, and then used in the step of determining an area under a curve of tau protein molecule concentrations. As pointed out above, the Examiner has previously indicated that such a feature is non-obvious over the prior art of record.

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee

occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US02.

Dated: November 14, 2018

Respectfully submitted,

By /Michael J. Pomianek/

Michael J. Pomianek
Registration No.: 46,190
WOLF, GREENFIELD & SACKS, P.C.
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617.646.8000

5954593.1

DOCKET NO.: Q0052.70018US02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	A. M. Weidner
Art Unit:	1649

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: November 14, 2018 Electronic Signature for: /Eileen M. MacKenzie/

MAIL STOP RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action after the filing of a Request for Continued Examination under 37 C.F.R. § 1.114.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending applications that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
*15/487,889	04-14-2017	Duffy et al.	Q0052.70011US05
*15/921,429	03-14-2018	Fournier et al.	Q0052.70014US02
*15/808,969	11-10-2017	Rissin et al.	Q0052.70015US02
*14/963,421	12-09-2015	Wilson et al.	Q0052.70016US02
*14/111,331	12-18-2013	Wilson et al.	Q0052.70017US01
*15/942,379	03-30-2018	Duffy et al.	Q0052.70019US02
*14/889,982	11-09-2015	Rissin et al.	Q0052.70020US01
*15/543,401	07-13-2017	Duffy et al.	Q0052.70021US01
*15/205,497	07-08-2016	Walt et al.	T0605.70005US06

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR .

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. Q0052.70018US02 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Michael J. Pomianek/

Michael J. Pomianek, Reg. No. 46,190
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: Q0052.70018US02
Date: November 14, 2018

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/269,142	ATTY. DOCKET NO.: Q0052.70018US02
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: September 19, 2016	CONFIRMATION NO.: 6662
				FIRST NAMED INVENTOR: David Wilson	
Sheet	1	of	1	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		9,482,622	B2	Sato et al.	11-01-2016
		9,809,838		Walt et al.	11-07-2017
		9,846,155	B2	Rissin et al.	12-19-2017
		9,932,626	B2	Duffy et al.	04-03-2018
		9,952,237	B2	Fournier et al.	04-24-2018
		2003-0096227	A1	Shinitzky et al.	05-22-2003
		2004-0018561	A1	Decrescenzo et al.	01-29-2004
		2005-0053996	A1	Tong	03-10-2005
		2018-0003703	A1	Duffy et al.	01-04-2018
		2018-0017552	A1	Duffy et al.	01-18-2018
		2018-0224451	A1	Rissin et al.	08-09-2018
		2018-0306830	A1	Fournier et al.	10-25-2018

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

6767621.1

PTO/AIA/22 (03-13)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): Q0052.70018US02

Application Number	15/269,142-Conf. #6662	Filed	September 19, 2016

For: METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Art Unit	1649	Examiner	A. M. Weidner

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☒ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1,400.00
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☒ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 46,190 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Michael J. Pomianek/ — Signature
November 14, 2018 — Date

Michael J. Pomianek — Typed or printed name
617.646.8000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: November 14, 2018 Electronic Signature for: /Eileen M. MacKenzie/

6769010.1

FEE TRANSMITTAL

Complete if known	
Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Examiner Name	A. M. Weidner
Art Unit	1649
Practitioner Docket No.	Q0052.70018US02

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)4,460.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below

☐ Charge fee(s) indicated below, **except for the filing fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
33	- 20 or HP =	13	x	$100.00	=	$1,300.00

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**		**Fee ($)**		**Fee Paid ($)**
4	- 3 or HP =	1	x	$460.00	=	$460.00

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims		
	Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**		**Fee ($)**		**Fee Paid ($)**
	- 100 =		/50 =		(round **up** to a whole number) x		=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1253 Extension for response within third month	1,400.00
1801 Request for continued examination (RCE) - 1st ...	1,300.00

SUBMITTED BY

Signature	/Michael J. Pomianek/	Registration No. (Attorney/Agent)	46,190	Telephone	617.646.8000
Name (Print/Type)	Michael J. Pomianek			Date	November 14, 2018

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: November 14, 2018 Electronic Signature for: /Eileen M. MacKenzie/

6769008.1

Electronic Patent Application Fee Transmittal

Application Number:	15269142
Filing Date:	19-Sep-2016
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Michael Pomianek/Eileen MacKenzie
Attorney Docket Number:	Q0052.70018US02

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	13	100	1300
INDEPENDENT CLAIMS IN EXCESS OF 3	1201	1	460	460
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1300	1300
		Total in USD ($)		**4460**

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/269,142	Filing Date 09/19/2016	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	11/14/2018	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 33	Minus	** 20	= 13		x $100 =	1300
	Independent (37 CFR 1.16(h))	* 4	Minus	*** 3	= 1		x $460 =	460
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	1760

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/MOLIKI I MAY/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	34314630
Application Number:	15269142
International Application Number:	
Confirmation Number:	6662
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Michael Pomianek/Eileen MacKenzie
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US02
Receipt Date:	14-NOV-2018
Filing Date:	19-SEP-2016
Time Stamp:	19:59:46
Application Type:	Utility under 35 USC 111(a)

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RAM confirmation Number	111518INTEFSW20003800
Deposit Account	232825
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.17 (Patent application and reexamination processing fees)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	Q005270018US02-RCE-MJP.pdf	28259 59cff8f2a6173105b6f319209454969a8f179eb0	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2	Fee Worksheet (SB06)	Q005270018US02-FEE-MJP.pdf	32952 ace29802d758c417063f1c83238f962e6b37ffa8	no	1
Warnings:					
Information:					
3	Extension of Time	Q005270018US02-EXT-MJP.pdf	24896 1d4f0f1d47c30389ffc5250f3895629611d3f767	no	1
Warnings:					
Information:					
4		Q005270018US02-AMN-MJP.pdf	94372 685c87a84e7586424d6e2eadaf5f5953a421117a	yes	21

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Arguments/Remarks Made in an Amendment	10	21
Claims	2	9
Amendment Submitted/Entered with Filing of CPA/RCE	1	1

Warnings:

Information:

5		Q005270018US02-IDS-MJP.pdf	54262 a994268bf3d8dcd5b061802cf7334e4669cc0851	yes	4
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Information Disclosure Statement (IDS) Form (SB08)		4	4	
	Transmittal Letter		1	3	
Warnings:					
Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	36221 4563e94cb41100fe26c0f15cdeab3c54734c4778	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	270962		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/269,142	09/19/2016	David Wilson	Q0052.70018US02	6662

23628 7590 12/31/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
WEIDNER, ADAM M	

ART UNIT	PAPER NUMBER
1649	

NOTIFICATION DATE	DELIVERY MODE
12/31/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	**Application No.** 15/269,142	**Applicant(s)** Wilson et al.	
	Examiner ADAM M WEIDNER	**Art Unit** 1649	**AIA Status** No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 14 November 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 12-13,21-24,92-95,97-103 and 105-120 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 12-13,21-24,92-95,97-103 and 105-120 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 11/14/18 has been entered.

Claims 12-13, 21-24, 92-95, 97-103, and 105-120 are pending and under examination.

Notice

Nowhere in the Remarks filed 11/14/18 does Applicant provide any substantive argument against the double patenting rejection, instead asking that the rejection be deferred until the Application is otherwise allowable. Note that nonstatutory double patenting rejections, including provisional ODP rejections, are not held in abeyance (MPEP §804(I)(B)(1)). Resolution of this rejection is necessary and future correspondence must provide a complete response to the double patenting rejections.

Withdrawn Rejections

The rejection under §103 is withdrawn as all of the independent claims require measuring tau within 12 hours, which was non-obvious as previously discussed.

The rejection under §101 over claim 12 and dependent claims thereof is withdrawn. This is specifically because of Applicant's argument on page 14 of the Remarks filed 11/14/18, noting that the decision in Berkheimer clearly requires the Office to first present evidence of the routine nature of the

assay. The use of a highly sensitive assay, including to specifically measure tau and to measure tau in blood/serum, has already been made of record. Art has also been made of record noting that calculating the area under a curve (AOC) was also well known. However, no art of record combines such an assay with the AOC manipulations of the claim. The specification makes no statement that this combination of elements was routine or conventional, no Court decision explicitly discusses this technology coupled to the data manipulations required, no publications indicate that the combination of elements was prevalent (though the individual elements were), and the Examiner cannot fairly take official notice of the conventional nature of this combination. Further, claims must be examined for eligibility as an ordered set of steps and should consider the purpose of the judicial exception, which is to mitigate the risk that it will 'tie-up' the excepted subject matter and pre-empt others from using [the judicial exception]" (federal register, p.74622, C1).

Taking the claimed elements as instantly amended and weighing all the factors and evidence of record, the combination of using an assay which requires a limit of detection less than about 0.2 pg/ml, requiring steps i-iv, which are included in the single molecule digital ELISA, and requiring that all the gathered data including the measurements pre-12 hours be included in an area under a curve analysis is deemed sufficient to render the claims patent eligible. This rationale also applies to claim 111 and dependent claims thereof. While the treatment step is no more than generic instructions to "treat it", i.e., use any conceivable therapeutic, the claim still contains the combination of elements discussed above. The method of claim 114 is a method of detection and is eligible for the same reasons.

Current Rejections

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 105-110 are rejected under 35 U.S.C. § 101 because the claims as a whole are not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole,

these claims do not recite something significantly more than a judicial exception. The rationale for this determination is explained below:

Guidance with respect to patent eligibility under 35 U.S.C. § 101 has been provided, effective 16 December 2014 and published in the Federal Register V 79 No 241 (please visit http://www.uspto.gov/patents/law/exam/interim_guidance_subject_matter_eligibility.jsp for the published guidance). This guidance has been provided in light of recent court decisions such as *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. __, 133 S. Ct. 2107, 2116, 106, USPQ2d 1972 (2013), *Mayo Collaborative Services v. Prometheus Laboratories, Inc.*, 566 U.S. __, 132 S. Ct. 1289, 101 USPQ2d 1961 (2012), and *Alice Corporation PTY. LTD v CLS Bank International*.

The first step under this guidance is determining if the claim is directed to one of the four statutory categories (process, machine, manufacture, or composition of matter). In this case, the claims are methods (a process). The second step is determining if the claims recite or involve judicial exception(s), such as laws of nature, natural phenomena, natural products, or an abstract idea. In this case, the claims are drawn to judicial exceptions: "determining a prognosis…based on the measurement of tau".

Thus, it must be determined if the claim as a whole recites something significantly more than the judicial exceptions.

Where a claim describes a judicial exception, such a claim "requires closer scrutiny for eligibility because of the risk that it will 'tie-up' the excepted subject matter and pre-empt others from using [the judicial exception]" (federal register, p.74622, C1). While all inventions to some degree involve natural laws, products, and other judicial exceptions, the new guidance regarding patent eligibility makes clear that a practical application of these exceptions is necessary, offering "significantly more" than the exception itself. Limitations that were found not to be enough to qualify as "significantly more" include:

- Mere instructions to implement an abstract idea on a computer
- Adding generic instructions that the judicial exception should be used ("apply it")
- Simply appending well-understood, routine and conventional activities previously known to the industry, specified at a high level of generality
- Adding insignificant extrasolution activity to the exception ("mere data gathering")

- Generally linking the use of the exception to a particular technological environment or field of use

Independent claim 105 introduces the judicial exception of calling out the natural correlation between tau levels in blood observed within 12 hours of a brain injury and its relevance to prognosis. Such limitations follow the pattern of Julitis claim 2, which is ineligible. This is also similar to the fact pattern of the claims at issue in *Mayo*, where a drug was administered, the natural metabolites were measured, and a conclusion was made about treatment efficacy; this was deemed ineligible by the Supreme Court.

In Mayo, a level of the metabolite "less than about 230 pmol per $8x10^8$ red blood cells [indicated] a need to increase the amount of said drug". Such claim limitations were found to be a description of the correlation itself and not "significantly more". It was also found that steps prior to this constituted routine data gathering, instructing the artisan to engage in conventional, well-known practices and then "warning" the user of the implications of the data; this was not eligible in Mayo.

"Determining a prognosis" is the same as "warning" the user of the implications of the tau levels in blood shortly after brain injury, i.e., it is correlated to the prognosis without more ("apply it").

As above, "mere data gathering" is both necessary to utilize the judicial exception as well as insignificant extrasolution activity. Limitations such as obtaining the samples within 12/48 hours or that the relevant tau concentrations require a sensitivity of less than about 0.2 pg/mL are merely a description of the natural correlation itself, informing the artisan of the ranges in which the correlation is observable/relevant. In another light, it is generic instructions to use assays of high sensitivity; using sensitive assays to detect proteins represents no more than conventional wisdom in the art and such descriptions do not offer significantly more. As discussed previously, such techniques were known and routinely used in this manner. See for example Rissin ("single molecule enzyme linked immunosorbent assay detects serum proteins as subfemtomolar concentrations"; previously cited). Here, Rissin describes antibodies attached to beads, which then bind a target antigen, followed by binding the antigen with a biotin-labeled antibody, where only some of the beads bind the biotin-antibody, dividing these beads into wells, and imaging them to determine the number of locations containing the biotin-antibody by exposure to a streptavidin-ß-gal (streptavidin-enzyme) conjugate in order to determine the concentration of the

antigen (e.g., figure 1). While Rissin does not explicitly use the technique to measure tau, Rissin teaches the technology is broadly applicable to detecting single protein molecules in blood for sensitive diagnostic assays (abstract). The instant claims do not require any particular, novel antibody or other unique reagent. Thus, once informed of the relevant correlation, the "digital ELISA" technique would have been a well-known choice using conventional, commercially available monoclonal antibodies in order to measure tau concentration at the required sensitivity (such as by single molecule detection). Thus, such an assay does not provide significantly more than instructions to a relevant audience to use known techniques to observe a natural phenomenon and to "apply it". This is not patent eligible.

See also (previously cited):

- Harma 2000 *Clinical Chemistry*
- Gorris 2010 "analytical chemistry on the femtoliter scale"
- Ekins 2010 "*Clinical Chemistry*
- Duffy 2011 "immunoassays with broad dynamic ranges based on combining digital and digitally enhanced analog detection of enzyme labels"
- *Chen 2009 *AIChE*
- Xie 2009 *JACS*
- Burton 2010 *Lab Chip*
- Agrawal 2007 *J Agric Food Chem1*
- Quaternix 2010 *IQT Technology Focus Day*
- 2011 "Quanterix discovers link between heart attack-induced hypoxia suspected Alzheimer's disease pathway"
- US 20110245097
- US 20100075407
- US 20110212537
- US 20110212848

This list is not exhaustive, but illustrates that at the time of or before the instant invention, an assay meeting the criteria of the instantly claimed assay was already publicly known and had been disclosed as

measuring numerous different proteins from cancer antigens to Aß to tau. For example, the Quaternix document above ("single molecule arrays for digital detection in complex samples" *IQT technology focus day* presented 25 March 2010, more than one year before the instant priority date of 12 April 2011) describes using antibodies bound to beads to capture a "target protein", followed by a biotin-labeled detection antibody, performed in a plurality of wells (p.13), suggests its use relative to neurological diseases (p.19), and explicitly states that the method was used to measure tau in blood of AD patients and age-matched controls (p.22). The document also teaches measuring tau in relation to TBI (p.29).

Thus, taken as a whole, instant claim 105 does not provide significantly more than the recitation of the judicial exception and is thus not patent eligible.

Claims 106-110 provide further steps of the detection method, which were known steps as discussed in previous actions.

Therefore, claims 105-110 are not patent eligible.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 111-113 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.