



(11) **EP 3 138 927 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
08.03.2017 Bulletin 2017/10

(51) Int Cl.:
C12Q 1/68 ^(2006.01) **G01N 33/53** ^(2006.01)
G01N 33/553 ^(2006.01)

(21) Application number: **16190239.0**

(22) Date of filing: **20.04.2011**

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

(30) Priority: **21.04.2010 US 326588 P**
12.08.2010 US 855147

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
11772608.3 / 2 561 099

(71) Applicant: **NanoMR, Inc.**
Albuquerque, NM 87106 (US)

(72) Inventors:
• **DRYGA, Sergey A**
Rio Rancho, NM 87144 (US)

- **ESCH, Victor C**
Albuquerque, NM 87111 (US)
- **SAUL, Richard G**
Gaithersburg, MD 20882 (US)
- **McDOWELL, Andrew F**
Albuquerque, NM 87106 (US)

(74) Representative: **Kirkham, Nicholas Andrew et al**
Graham Watt & Co. LLP
St. Botolph's House
7-9 St. Botolph's Road
Sevenoaks, Kent TN13 3AJ (GB)

Remarks:

This application was filed on 22.09.2016 as a
divisional application to the application mentioned
under INID code 62.

(54) **SEPARATING TARGET ANALYTES USING ALTERNATING MAGNETIC FIELDS**

(57) The invention generally relates to using magnetic particles and alternating magnet fields to separate a target analyte from a sample. In certain embodiments, methods of the invention involve contacting a sample with magnetic particles including first moieties specific for a target analyte, thereby forming target/particle complexes in the sample, flowing the sample through a channel including second moieties attached to at least one surface

of the channel, applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample, binding the target/particle complexes to the second moieties, and washing away unbound particles and unbound analytes of the sample.

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Description

Related Application

5 [0001] The present application claims the benefit of and priority to U.S. nonprovisional patent application serial number 12/855,147, filed August 12, 2010, which claims the benefit of and priority to U.S. provisional patent application serial number 61/326,588, filed April 21, 2010, the content of each of which is incorporated by reference herein in its entirety.

Field of the Invention

10 [0002] The invention generally relates to using magnetic particles and alternating magnet fields to separate a target analyte from a sample.

Background

15 [0003] Many laboratory and clinical procedures employ bio-specific affinity reactions. Such reactions are commonly utilized in diagnostic testing of biological samples, or for the separation of a wide range of target substances, especially biological entities such as cells, viruses, proteins, nucleic acids and the like. Various methods are available for analyzing or separating the above-mentioned target substances based upon complex formation between the substance of interest
20 and another substance to which the target specifically binds. Separation of complexes from unbound material may be accomplished gravitationally, e.g. by settling, or, alternatively, by centrifugation of finely divided particles or beads coupled to the target substance. If desired, such particles or beads may be made magnetic to facilitate the bound/free separation step. Magnetic particles are well known in the art, as is their use in immune and other bio-specific affinity reactions. See, for example, Whitehead et al. (U.S. 4,554,088) and Hunter et al. (Immunoassays for Clinical Chemistry, pp. 147-162,
25 eds., Churchill Livingstone, Edinborough, 1983). Generally, any material that facilitates magnetic or gravitational separation may be employed for this purpose. More recently, the superiority of magnetics for performing such separations has led to its use in many applications.

[0004] A problem with magnetic separation protocols is that magnetic beads must be added in excess to a sample to ensure a sufficient amount of binding of beads to a target analyte in the sample, thus producing a sample that contains
30 a very high percent of magnetic particles that are not bound to target analytes, as well as non-specific target entities. Non-specific target entities may for example be bound at a much lower efficiency, for example 1% of the surface area, while a target of interest might be loaded at 50% or nearly 100% of the available surface area or available antigenic sites. However, even 1% loading may be sufficient to impart force necessary for trapping in a magnetic gradient flow cell or sample chamber.

35 [0005] The presence of magnetic particles that are not bound to target analytes and non-specific target entities on the surface that includes the target/magnetic particle complexes interferes with the ability to successfully separate the target of interest of the remaining components of the mixture and unbound magnetic particles. The magnetic capture of the resulting mix, and close contact of magnetic particles with each other and labeled targets, result in the formation of aggregate that is hard to dispense and which might be resistant or inadequate for subsequent processing or analysis steps.

40 [0006] There is a need for methods for separating target analytes from a sample.

Summary

45 [0007] The present invention generally relates to using magnetic particles having a target-specific binding moiety and alternating magnet fields to separate a target analyte from a sample. Methods of the invention allow for rapid (less than 1 hr) and efficient capture of target analytes from a sample while eliminating non-specific binding and reducing background noise resulting from excess magnetic particles not bound to target analytes. Methods of the invention involve contacting a sample with magnetic particles including first moieties specific for a target analyte, thereby forming target/particle complexes in the sample, flowing the sample through a channel including second moieties attached to at least one
50 surface of the channel, applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample, binding the target/particle complexes to the second moieties, and washing away unbound particles and unbound analytes of the sample. A particular advantage of methods of the invention is for capture and isolation of bacteria and fungi directly from blood samples at low concentrations that are present in clinical samples (as low as 1 CFU/ml of
55 bacteria in a blood sample).

[0008] In certain embodiments, the free magnetic particles and the target/particle complexes both interact numerous times with the surface of the flow channel. The targets are bound due to the specific interaction between the target analyte and the second moiety on the surface of the flow channel, and the free magnetic particles are not bound, and

are thus continue flowing through the flow channel.

[0009] Methods of the invention may further involve eluting the bound target/particle complexes from the second moieties on the surface of the channel. Methods of the invention may further involve analyzing the eluted target/particle complexes. The target may be analyzed by a multitude of existing technologies, such as miniature NMR, Polymerase Chain Reaction (PCR), fluorescent labeling and visualization using microscopic observation, fluorescent *in situ* hybridization (FISH), growth-based antibiotic sensitivity tests, and variety of other methods that may be conducted with purified target without significant contamination from other sample components. In particular embodiments, analyzing involves flowing the target/particle complexes into an NMR instrument.

[0010] The target analyte refers to the target that will be captured and isolated by methods of the invention. The target may be a bacteria, a fungi, a protein, a nucleic acid, a receptor, a ligand, a cell, a virus, or any molecule known in the art. In a particular embodiment, the target is a bacteria.

[0011] Methods of the invention do not depend and are not limited by the type of sample. Any sample that includes a detectable target may be used with methods of the invention. The sample may be a biological sample (e.g., a human tissue or body fluid), an agricultural sample, or an environmental sample (e.g., a water or soil sample). In certain embodiments, the sample is a blood sample.

[0012] The first and second target-specific binding moieties will depend on the target to be captured. The moieties may be any capture moieties known in the art, such as an antibody, an aptamer, a phage, a nucleic acid, a protein, a receptor, or a ligand. In particular embodiments, the target-specific binding moieties are antibodies. In certain embodiments, the antibodies are specific for bacteria. In other embodiments, the antibodies are specific for fungi or viruses. In certain embodiments, the first and second moieties are the same. In alternative embodiments, the first and second moieties are different.

[0013] Generating alternating magnetic fields may be accomplished by any method known in the art. In certain embodiments, the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, in which the channel remains stationary and the first and second sets of magnets are moved to alternate proximity to the channel, thereby producing the alternating magnetic fields. In other embodiments, the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, in which first and second sets of magnets remain stationary and the channel is moved to alternate its proximity to the first and second sets of magnets, thereby producing the alternating magnetic fields.

[0014] Another aspect of the invention provides methods for detecting a target analyte in a sample including contacting a sample with magnetic particles including first moieties specific for a target analyte, thereby forming target/particle complexes in the sample, flowing the sample through a channel including second moieties attached to at least one surface of the channel, applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface of the channel to bind the second moieties and unbound particles remaining free in the sample, binding the target/particle complexes to the second moieties, washing away unbound particles and unbound analytes of the sample, eluting the target/particle complexes from the second moieties, and detecting the target/particle complexes.

Brief Description of the Drawings

[0015]

Figure 1 provides one exemplary configuration of a flow cell and first and second sets of magnets for generating alternating magnetic fields.

Figure 2 panel A provides an exemplary process chart for implementation of methods of the invention for separation of bacteria from blood. Panel B provides an magnified view of a target/magnetic particle complex.

Figure 3 is a graph showing recovery of bacteria from blood.

Detailed Description

[0016] The invention generally relates to using magnetic particles and alternating magnet fields to separate a target analyte from a sample. Methods of the invention involve contacting a sample with magnetic particles including first moieties specific for a target analyte, thereby forming target/particle complexes in the sample, flowing the sample through a channel including second moieties attached to at least one surface of the channel, applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample, binding the target/particle complexes to the second moieties, and washing away unbound particles and unbound analytes of the sample. Certain fundamental technologies and principles are associated with binding magnetic materials to target entities and subsequently separating by use of magnet fields and gradients. Such fundamental technologies and principles are known in the art and have been previously

described, such as those described in Janeway (Immunobiology, 6th edition, Garland Science Publishing), the content of which is incorporated by reference herein in its entirety.

[0017] Methods of the invention involve collecting a sample having a target analyte in a container, such as a blood collection tube (e.g., Vacutainer) in the case of blood. In certain embodiments, a solution is added that prevents or reduces aggregation of endogenous aggregating factors, such as heparin in the case of blood.

[0018] Methods of the invention do not depend and are not limited by the type of sample. Any sample that includes a detectable target may be used with methods of the invention. The sample may be a biological sample (e.g., a human tissue or body fluid), a food sample, an agricultural sample, or an environmental sample (e.g., a water or soil sample).

[0019] Exemplary biological samples include human tissue or body fluid. A tissue is a mass of connected cells and/or extracellular matrix material, e.g. skin tissue, nasal passage tissue, CNS tissue, neural tissue, eye tissue, liver tissue, kidney tissue, placental tissue, mammary gland tissue, placental tissue, gastrointestinal tissue, musculoskeletal tissue, genitourinary tissue, bone marrow, and the like, derived from, for example, a human or other mammal and includes the connecting material and the liquid material in association with the cells and/or tissues. A body fluid is a liquid material derived from, for example, a human or other mammal. Such body fluids include, but are not limited to, mucous, blood, plasma, serum, serum derivatives, bile, phlegm, saliva, sweat, amniotic fluid, mammary fluid, urine, sputum, and cerebrospinal fluid (CSF), such as lumbar or ventricular CSF. A sample may also be a fine needle aspirate. A sample also may be media containing cells or biological material. In particular embodiments, the sample is blood.

[0020] Exemplary agricultural samples include any plant material that is being interrogated by a method of the present invention. An agricultural sample includes, but is not limited to, seeds or plant tissue. Seeds include a single seed, a batch of seeds, a portion of a seed, or a seed scraping. Plant tissue includes, but is not limited to, any plant part such as leaf, flower, root, or petal. Plant tissue can also include a leaf punch.

[0021] Methods of the invention may be used to detect any target analyte. The target analyte refers to the substance in the sample that will be captured and isolated by methods of the invention. The target may be a bacteria, a fungi, a protein, a cell (such as a cancer cell, a white blood cell, a virally infected cell, or a fetal cell circulating in maternal circulation), a virus, a nucleic acid (e.g., DNA or RNA), a receptor, a ligand, a hormone, a drug, a chemical substance, or any molecule known in the art. In certain embodiments, the target is a pathogenic bacteria. In other embodiments, the target is a gram positive or gram negative bacteria. Exemplary bacterial species that may be captured and isolated by methods of the invention include *E. coli*, *Listeria*, *Clostridium*, *Mycobacterium*, *Shigella*, *Borrelia*, *Campylobacter*, *Bacillus*, *Salmonella*, *Staphylococcus*, *Enterococcus*, *Pneumococcus*, *Streptococcus*, and a combination thereof.

[0022] The sample is then mixed with magnetic particles including a target-specific binding moiety to generate a mixture that is allowed to incubate such that the particles bind to a target in the sample, such as a bacteria in a blood sample. The mixture is allowed to incubate for a sufficient time to allow for the particles to bind to the target analyte. The process of binding the magnetic particles to the target analytes associates a magnetic moment with the target analytes, and thus allows the target analytes to be manipulated through forces generated by magnetic fields upon the attached magnetic moment.

[0023] In general, incubation time will depend on the desired degree of binding between the target analyte and the magnetic beads (e.g., the amount of moment that would be desirably attached to the target), the amount of moment per target, the amount of time of mixing, the type of mixing, the reagents present to promote the binding and the binding chemistry system that is being employed. Incubation time can be anywhere from about 5 seconds to a few days. Exemplary incubation times range from about 10 seconds to about 2 hours. Binding occurs over a wide range of temperatures, generally between 15 °C and 40 °C.

[0024] Methods of the invention may be performed with any type of magnetic particle. Production of magnetic particles and particles for use with the invention are known in the art. See for example Giaever (U.S. 3,970,518), Senyi et al. (U.S. 4,230,685), Dodin et al. (U.S. 4,677,055), Whitehead et al. (U.S. 4,695,393), Benjamin et al. (U.S. 5,695,946), Giaever (U.S. 4,018,886), Rembaum (U.S. 4,267,234), Molday (U.S. 4,452,773), Whitehead et al. (U.S. 4,554,088), Forrest (U.S. 4,659,678), Liberti et al. (U.S. 5,186,827), Own et al. (U.S. 4,795,698), and Liberti et al. (WO 91/02811), the content of each of which is incorporated by reference herein in its entirety.

[0025] Magnetic particles generally fall into two broad categories. The first category includes particles that are permanently magnetizable, or ferromagnetic; and the second category includes particles that demonstrate bulk magnetic behavior only when subjected to a magnetic field. The latter are referred to as magnetically responsive particles. Materials displaying magnetically responsive behavior are sometimes described as superparamagnetic. However, materials exhibiting bulk ferromagnetic properties, e.g., magnetic iron oxide, may be characterized as superparamagnetic when provided in crystals of about 30 nm or less in diameter. Larger crystals of ferromagnetic materials, by contrast, retain permanent magnet characteristics after exposure to a magnetic field and tend to aggregate thereafter due to strong particle-particle interaction. In certain embodiments, the particles are superparamagnetic beads. In certain embodiments, the magnetic particle is an iron containing magnetic particle. In other embodiments, the magnetic particle includes iron oxide or iron platinum.

[0026] In certain embodiments, the magnetic particles include at least about 10% superparamagnetic beads by weight,

at least about 20% superparamagnetic beads by weight, at least about 30% superparamagnetic beads by weight, at least about 40% superparamagnetic beads by weight, at least about 50% superparamagnetic beads by weight, at least about 60% superparamagnetic beads by weight, at least about 70% superparamagnetic beads by weight, at least about 80% superparamagnetic beads by weight, at least about 90% superparamagnetic beads by weight, at least about 95% superparamagnetic beads by weight, or at least about 99% superparamagnetic beads by weight. In a particular embodiment, the magnetic particles include at least about 70% superparamagnetic beads by weight.

[0027] In certain embodiments, the superparamagnetic beads are less than 100 nm in diameter. In other embodiments, the superparamagnetic beads are about 150 nm in diameter, are about 200 nm in diameter, are about 250 nm in diameter, are about 300 nm in diameter, are about 350 nm in diameter, are about 400 nm in diameter, are about 500 nm in diameter, or are about 1000 nm in diameter. In a particular embodiment, the superparamagnetic beads are from about 100 nm to about 250 nm in diameter.

[0028] In certain embodiments, the particles are beads (e.g., nanoparticles) that incorporate magnetic materials, or magnetic materials that have been functionalized, or other configurations as are known in the art. In certain embodiments, nanoparticles may be used that include a polymer material that incorporates magnetic material(s), such as nanometal material(s). When those nanometal material(s) or crystal(s), such as Fe_3O_4 , are superparamagnetic, they may provide advantageous properties, such as being capable of being magnetized by an external magnetic field, and demagnetized when the external magnetic field has been removed. This may be advantageous for facilitating sample transport into and away from an area where the sample is being processed without undue bead aggregation.

[0029] One or more or many different nanometal(s) may be employed, such as Fe_3O_4 , FePt, or Fe, in a core-shell configuration to provide stability, and/or various others as may be known in the art. In many applications, it may be advantageous to have a nanometal having as high a saturated moment per volume as possible, as this may maximize gradient related forces, and/or may enhance a signal associated with the presence of the beads. It may also be advantageous to have the volumetric loading in a bead as high as possible, for the same or similar reason(s).

[0030] In order to maximize the moment provided by a magnetizable nanometal, a certain saturation field may be provided. For example, for Fe_3O_4 superparamagnetic particles, this field may be on the order of about 0.3T.

[0031] The size of the nanometal containing bead may be optimized for a particular application, for example, maximizing moment loaded upon a target, maximizing the number of beads on a target with an acceptable detectability, maximizing desired force-induced motion, and/or maximizing the difference in attached moment between the labeled target and non-specifically bound targets or bead aggregates or individual beads. While maximizing is referenced by example above, other optimizations or alterations are contemplated, such as minimizing or otherwise desirably affecting conditions.

[0032] In an exemplary embodiment, a polymer bead containing 80 wt% Fe_3O_4 superparamagnetic particles, or for example, 90 wt% or higher superparamagnetic particles, is produced by encapsulating superparamagnetic particles with a polymer coating to produce a bead having a diameter of about 250 nm.

[0033] Magnetic particles for use with methods of the invention have a target-specific binding moiety that allows for the particles to specifically bind the target of interest in the sample. The target-specific moiety may be any molecule known in the art and will depend on the target to be captured and isolated. Exemplary target-specific binding moieties include, nucleic acids, proteins, ligands, antibodies, aptamers, and receptors.

[0034] In particular embodiments, the target-specific binding moiety is an antibody, such as an antibody that binds a particular bacteria. General methodologies for antibody production, including criteria to be considered when choosing an animal for the production of antisera, are described in Harlow et al. (Antibodies, Cold Spring Harbor Laboratory, pp. 93-117, 1988). For example, an animal of suitable size such as goats, dogs, sheep, mice, or camels are immunized by administration of an amount of immunogen, such as target bacteria, effective to produce an immune response. An exemplary protocol is as follows. The animal is injected with 100 micrograms to 100 milligrams of antigen resuspended in adjuvant, for example Freund's complete adjuvant, dependent on the size of the animal, followed three weeks later with a subcutaneous injection of 100 micrograms to 100 milligrams of immunogen with adjuvant dependent on the size of the animal, for example Freund's incomplete adjuvant. Additional subcutaneous or intraperitoneal injections every two weeks with adjuvant, for example Freund's incomplete adjuvant, are administered until a suitable titer of antibody in the animal's blood is achieved. Exemplary titers include a titer of at least about 1:5000 or a titer of 1:100,000 or more, i.e., the dilution having a detectable activity. The antibodies are purified, for example, by affinity purification on columns containing protein G resin or target-specific affinity resin.

[0035] The technique of *in vitro* immunization of human lymphocytes is used to generate monoclonal antibodies. Techniques for *in vitro* immunization of human lymphocytes are well known to those skilled in the art. See, e.g., Inai, et al., Histochemistry, 99(5):335 362, May 1993; Mulder, et al., Hum. Immunol., 36(3):186 192, 1993; Harada, et al., J. Oral Pathol. Med., 22(4):145 152, 1993; Stauber, et al., J. Immunol. Methods, 161(2):157 168, 1993; and Venkateswaran, et al., Hybridoma, 11(6) 729 739, 1992. These techniques can be used to produce antigen-reactive monoclonal antibodies, including antigen-specific IgG, and IgM monoclonal antibodies.

[0036] Any antibody or fragment thereof having affinity and specific for the bacteria of interest is within the scope of the invention provided herein. Immunomagnetic beads against Salmonella are provided in Vermunt et al. (J. Appl. Bact.

72:112, 1992). Immunomagnetic beads against *Staphylococcus aureus* are provided in Johne et al. (*J. Clin. Microbiol.* 27:1631, 1989). Immunomagnetic beads against *Listeria* are provided in Skjerve et al. (*Appl. Env. Microbiol.* 56:3478, 1990). Immunomagnetic beads against *Escherichia coli* are provided in Lund et al. (*J. Clin. Microbiol.* 29:2259, 1991).

5 [0037] Methods for attaching the target-specific binding moiety to the magnetic particle are known in the art. Coating magnetic particles with antibodies is well known in the art, see for example Harlow et al. (*Antibodies*, Cold Spring Harbor Laboratory, 1988), Hunter et al. (*Immunoassays for Clinical Chemistry*, pp. 147-162, eds., Churchill Livingstone, Edinburgh, 1983), and Stanley (*Essentials in Immunology and Serology*, Delmar, pp. 152-153, 2002). Such methodology can easily be modified by one of skill in the art to bind other types of target-specific binding moieties to the magnetic particles. Certain types of magnetic particles coated with a functional moiety are commercially available from Sigma-
10 Aldrich (St. Louis, MO).

[0038] In certain embodiments, a buffer solution is added to the sample along with the magnetic beads. An exemplary buffer includes Tris(hydroxymethyl)-aminomethane hydrochloride at a concentration of about 75mM. It has been found that the buffer composition, mixing parameters (speed, type of mixing, such as rotation, shaking etc., and temperature) influence binding. It is important to maintain osmolality of the final solution (e.g., blood + buffer) to maintain high label
15 efficiency. In certain embodiments, buffers used in methods of the invention are designed to prevent lysis of blood cells, facilitate efficient binding of targets with magnetic beads and to reduce formation of bead aggregates. It has been found that the buffer solution containing 300 mM NaCl, 75 mM Tris-HCl pH 8.0 and 0.1% Tween 20 meets these design goals.

[0039] Without be limited by any particular theory or mechanism if action, it is believed that sodium chloride is mainly responsible for maintaining osmolality of the solution and for the reduction of non-specific binding of magnetic bead through ionic interaction. Tris(hydroxymethyl)-aminomethane hydrochloride is a well established buffer compound frequently used in biology to maintain pH of a solution. It has been found that 75 mM concentration is beneficial and sufficient for high binding efficiency. Likewise, Tween 20 is widely used as a mild detergent to decrease nonspecific attachment due to hydrophobic interactions. Various assays use Tween 20 at concentrations ranging from 0.01% to 1%. The 0.1% concentration appears to be optimal for the efficient labeling of bacteria, while maintaining blood cells intact.

25 [0040] An alternative approach to achieve high binding efficiency while reducing time required for the binding step is to use static mixer, or other mixing devices that provide efficient mixing of viscous samples at high flow rates, such as at or around 5 mL/min. In one embodiment, the sample is mixed with binding buffer in ratio of, or about, 1:1, using a mixing interface connector. The diluted sample then flows through a second mixing interface connector where it is mixed with target-specific nanoparticles. Additional mixing interface connectors providing mixing of sample and antigen-specific
30 nanoparticles can be attached downstream to improve binding efficiency. The combined flow rate of the labeled sample is selected such that it is compatible with downstream processing. At this point in the process, the mixture includes target/magnetic particle complexes, unbound magnetic particles, and the remaining components of the mixture. Prior art techniques for isolating target/magnetic particle complexes involve applying a magnetic field to the mixture to capture the complexes on a surface. Components of the mixture that are not bound to magnetic particles will not be affected by
35 the magnetic field and will remain free in the mixture.

[0041] The above described type of magnetic separation, produces efficient capture of a target analyte and the removal of a majority of the remaining components of a sample mixture. However, such a process produces a sample that contains a very high percent of magnetic particles that are not bound to target analytes because the magnetic particles are typically added in excess, as well as non-specific target entities. Non-specific target entities may be bound at a much
40 lower efficiency, for example 1% of the surface area, while a target of interest might be loaded at 50% or nearly 100% of the available surface area or available antigenic sites. However, even 1 % loading may be sufficient to impart force necessary for trapping in a magnetic gradient flow cell or sample chamber.

[0042] For example, in the case of immunomagnetic labeling of bacteria or fungi in a blood sample, the sample may include: labeled targets at a concentration of about 1/mL or a concentration less of about 10^6 /mL; background beads at a concentration of about 10^7 /ml to about 10^{10} /ml; and non-specific targets at a concentration of about 10/ml to about
45 10^5 /ml.

[0043] The presence of magnetic particles that are not bound to target analytes and non-specific target entities on the surface that includes the target/magnetic particle complexes interferes with the ability to successfully detect the target of interest. The magnetic capture of the resulting mix, and close contact of magnetic particles with each other and labeled
50 targets, result in the formation of aggregate that is hard to dispense and which might be resistant or inadequate for subsequent processing or analysis steps. Further, with addition of excess magnetic particles to the sample, a large number of particles may accumulate in the areas of high gradients, and thus a magnetically bound target analyte may likely be in the body of the accumulation of particles as opposed to the desired location adjacent the functionalized surface where specific binding may occur. Ignoring intra-bead forces (those forces associated with the magnetic field distribution of the individual beads and the forces these fields and associated gradients have on other beads), the beads
55 may accumulate into large amorphous piles. Such intra-label forces do occur, and thus the aggregates of beads tend to exist in chains and long linear aggregates that are aligned with the 'field lines' of the magnetic trap pieces.

[0044] Methods of the invention address this problem by applying alternating magnetic fields to the sample as it flows

through the channel. The frequency of the alternating magnetic field is selected such that the free magnetic nanoparticles cannot transverse the whole distance between top and bottom of the flow cell before the direction of the magnetic field is changed, causing nanoparticles to move in the opposite direction. Therefore, a majority of free nanoparticles will not come into close contact with active surfaces of the flow cell and will be washed away by liquid flow. Labeled target, due to higher magnetic moment, have higher velocity in the magnetic field and will reach a surface of the flow cell before change of the magnetic field, thus coming into close contact with the surface. This, in turn, will result in a specific binding event and result in a specific capture of the target analyte in the sample (such as a bacterium or other rare cell) to the surface coated with a second moiety. Components of the mixture that are not bound to magnetic particles will not be affected by the magnetic field and will remain free in the mixture.

[0045] The second target-specific moiety may be the same or different from the first target-specific moiety. The second moiety may be attached to the surface of the flow channel by methods described above relating to attaching first target-specific moieties to magnetic particles.

[0046] Fig. 1 provides one exemplary configuration of a flow cell and first and second sets of magnets for generating alternating magnetic fields. This figures shows that the flow cell is positioned between the first and second sets of magnetics. Either movement of the flow cell or movement of the magnets brings the flow cell closer to one set of magnets and further from the other set of magnets. Subsequent movement brings the flow cell within proximity of the other set of magnets. Such movements generate alternating magnetic fields within the channels of the flow cell that are felt by the unbound magnetic particles and the target/magnetic particle complexes.

[0047] In one embodiment, a flow cell may be about 15 mm wide and about 15 mm long, with a lead-in region and an lead-out section, and a height of about 0.5mm (Fig. 1). A flow rate for such a cell may be about 100 μ l/min, about 1 ml/min, about 10 ml/min, or from about 100 μ l/min to about 10 ml/min or other ranges therein. A magnetic configuration may be an array of magnets, for example, an array of 7 bar magnets, or 5 bar magnets, or 3 bar magnets (Fig. 1). Magnets may be configured with alternating magnet poles facing one another, n-n, s-s, etc., with the pole face being normal to the array's rectangular face in this embodiment.

[0048] In a flowing system, successive encounters of unbound magnetic particles with a surface of a flow channel, without a resulting binding event, will allow the unbound magnetic particles to travel through the system and subsequently out of the cell. The cycling of the magnetic bar trap assemblies may be optimized based on the flow characteristics of the target(s) of interest. The expression of force on a magnetic moment and of terminal velocity for such target(s) is the following:

$$m \cdot (\nabla B) = F \quad \text{Equation 1}$$

$$v_t = -F / (6 \cdot \eta \cdot n \cdot r) \quad \text{Equation 2}$$

where η is the viscosity, r is the bead diameter, F is the vector force, B is the vector field, and m is the vector moment of the bead.

[0049] A characteristic transit time across the height of the cell may be established. An efficient frequency of the alternating magnetic attractors, such that many surface interactions may be established prior to the exit from the flow cell, may be established. In certain embodiments, the transit time can be substantially different for the target of interest versus the unbound magnetic particles, or non-specific bound non-target. In such an embodiment, the target can be ensured to interact with surface a maximal amount of times, while the unbound magnetic particles or non-target can interact a minimal number of times, or not at all.

[0050] Because of the magnetizing characteristics of the particles, the unbound magnetic particles may form aggregates, which may be in the form of linear chains or clumps. This may be the case at high concentrations of beads. At all concentrations, the unbound magnetic particles may exhibit spatial poisson statistics, and there is some probability that there will be a neighboring bead close enough to be captured by the forces associated with the magnetic field of the beads themselves. By using alternating magnetic fields, methods of the invention break up these linear aggregates, particularly when the spatial gradient field from the trap magnets is shifted faster than the unbound magnetic particles can move mutually to reorient to the new distribution of trap gradient. Particles organized in chains, with N-S axis co-aligned, may quickly be subjected to an external field that produces particle moments with the N-S poles shifted by 90°, and may produce very strong intra-particle repulsive forces. Transverse motion of the trap magnets serves this purpose in concert with, or as a discrete step in addition to, the alternation of the trapping magnets from one surface to the other.

[0051] In the optimization of the cycling timing of the trap magnets, the flow characteristics of the cell may be considered along with the spatial distribution of the gradient of the trap magnets. Flow characteristics may dictate the transport of the magnetic materials from entrance to exit of the cell, so that parabolic flow, plug flow, or any particular flow characteristic

may be considered to facilitate obtaining desired deposition patterns and desired interactions with the surfaces of interest.

[0052] In certain embodiments, it may be desirable in various applications to maximize the encounters of the target/magnetic particle complexes with the functionalized surface of the channel, to minimize interference with the unbound magnetic particles, and/or to minimize adhesion of the unbound magnetic particles and non-specific materials to the surface. It may be advantageous to produce an array of pipes, or tubes, through which the flow of the sample materials may flow. By way of example, a 125 mm x 15 mm x 0.5 mm cell volume may be filled with tubes, longitudinally aligned with the cell flow direction, such that there is a great increase in the functionalized surface area and a limitation on the number of unbound magnetic particles that may interact and impede in the encounter of the target with the surface. Planar structures may be used for this purpose, in which the cell volume is constructed with multiple layers of smaller flow channels such that the surface area is increased and the number of unbound magnetic particles available to impede the target on its way to the surface is decreased. In this embodiment, the general approach of cycling the trap magnets is similar to that described above, but variables such as time constants, amplitudes and gradient field distributions, for example, are optimized for the particular situation. Similarly, in the case of transverse trap motion for the breaking-up of aggregates, the general approach is similar to that described above.

[0053] It may be desirable to shield the portion of the sample flow outside the trap cell from fringing magnetic field so that magnetic material does not have the opportunity to self-aggregate prior to entering the strong field and gradient zone of the trap. The magnetic materials and labeled target may also be trapped in flow tubes and other fluidic structures through magnetic forces in undesired areas. Shielding can be accomplished by the appropriate design of the trap magnets, for example, by managing the 'return path' of the field, and/or by using high permeability materials to capture and channel the field to minimize fringing field exposure.

[0054] It is possible to purify samples and fixate targets of interest and to then examine the target *in situ* on the surface, in any suitable manner known in the art, or the target can be removed from the surface by eluting off the surface through the use of an appropriate bonding chemistry that can be released such that the target can be analyzed in some subsequent process, for example, by use of a flowing system similar to a cytometer, or by collection of the individual targets for other analysis, such a NAT, PCR, etc, or some other post-processing and analysis as is enabled by the supply of target entity. In an exemplary case, the sample is analyzed using a flowing NMR detector.

[0055] Detection of bacteria of interest can be performed by use of nucleic acid probes following procedures which are known in the art. Suitable procedures for detection of bacteria using nucleic acid probes are described, for example, in Stackebrandt et al. (U.S. 5,089,386), King et al. (WO 90/08841), Foster et al. (WO 92/15883), and Cossart et al. (WO 89/06699), each of which is hereby incorporated by reference.

[0056] A suitable nucleic acid probe assay generally includes sample treatment and lysis, hybridization with selected probe(s), hybrid capture, and detection. Lysis of the bacteria is necessary to release the nucleic acid for the probes. The nucleic acid target molecules are released by treatment with any of a number of lysis agents, including alkali (such as NaOH), guanidine salts (such as guanidine isothiocyanate), enzymes (such as lysozyme, mutanolysin and proteinase K), and detergents. Lysis of the bacteria, therefore, releases both DNA and RNA, particularly ribosomal RNA and chromosomal DNA both of which can be utilized as the target molecules with appropriate selection of a suitable probe. Use of rRNA as the target molecule(s), may be advantageous because rRNAs constitute a significant component of cellular mass, thereby providing an abundance of target molecules. The use of rRNA probes also enhances specificity for the bacteria of interest, that is, positive detection without undesirable cross-reactivity which can lead to false positives or false detection.

[0057] Hybridization includes addition of the specific nucleic acid probes. In general, hybridization is the procedure by which two partially or completely complementary nucleic acids are combined, under defined reaction conditions, in an anti-parallel fashion to form specific and stable hydrogen bonds. The selection or stringency of the hybridization/reaction conditions is defined by the length and base composition of the probe/target duplex, as well as by the level and geometry of mis-pairing between the two nucleic acid strands. Stringency is also governed by such reaction parameters as temperature, types and concentrations of denaturing agents present and the type and concentration of ionic species present in the hybridization solution.

[0058] The hybridization phase of the nucleic acid probe assay is performed with a single selected probe or with a combination of two, three or more probes. Probes are selected having sequences which are homologous to unique nucleic acid sequences of the target organism. In general, a first capture probe is utilized to capture formed hybrid molecules. The hybrid molecule is then detected by use of antibody reaction or by use of a second detector probe which may be labelled with a radioisotope (such as phosphorus-32) or a fluorescent label (such as fluorescein) or chemiluminescent label.

[0059] Detection of bacteria of interest can also be performed by use of PCR techniques. A suitable PCR technique is described, for example, in Verhoef et al. (WO 92/08805). Such protocols may be applied directly to the bacteria captured on the magnetic beads. The bacteria is combined with a lysis buffer and collected nucleic acid target molecules are then utilized as the template for the PCR reaction.

[0060] For detection of the selected bacteria by use of antibodies, isolated bacteria are contacted with antibodies

specific to the bacteria of interest. As noted above, either polyclonal or monoclonal antibodies can be utilized, but in either case have affinity for the particular bacteria to be detected. These antibodies, will adhere/bind to material from the specific target bacteria. With respect to labeling of the antibodies, these are labeled either directly or indirectly with labels used in other known immunoassays. Direct labels may include fluorescent, chemiluminescent, bioluminescent, radioactive, metallic, biotin or enzymatic molecules. Methods of combining these labels to antibodies or other macromolecules are well known to those in the art. Examples include the methods of Hijmans, W. et al. (1969), Clin. Exp. Immunol. 4, 457-, for fluorescein isothiocyanate, the method of Goding, J. W. (1976), J. Immunol. Meth. 13, 215-, for tetramethylrhodamine isothiocyanate, and the method of Ingrassia, E. (1980), Meth. in Enzymol. 70, 419-439 for enzymes.

[0061] These detector antibodies may also be labeled indirectly. In this case the actual detection molecule is attached to a secondary antibody or other molecule with binding affinity for the anti-bacteria cell surface antibody. If a secondary antibody is used it is preferably a general antibody to a class of antibody (IgG and IgM) from the animal species used to raise the anti-bacteria cell surface antibodies. For example, the second antibody may be conjugated to an enzyme, either alkaline phosphatase or to peroxidase. To detect the label, after the bacteria of interest is contacted with the second antibody and washed, the isolated component of the sample is immersed in a solution containing a chromogenic substrate for either alkaline phosphatase or peroxidase. A chromogenic substrate is a compound that can be cleaved by an enzyme to result in the production of some type of detectable signal which only appears when the substrate is cleaved from the base molecule. The chromogenic substrate is colorless, until it reacts with the enzyme, at which time an intensely colored product is made. Thus, material from the bacteria colonies adhered to the membrane sheet will become an intense blue/purple/black color, or brown/red while material from other colonies will remain colorless. Examples of detection molecules include fluorescent substances, such as 4-methylumbelliferyl phosphate, and chromogenic substances, such as 4-nitrophenylphosphate, 3,3',5,5'-tetramethylbenzidine and 2,2'-azino-di-[3-ethylbenz-thiazoliane sulfonate (6)]. In addition to alkaline phosphatase and peroxidase, other useful enzymes include β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, galactose oxidase, glucose oxidase and hexokinase.

[0062] Detection of bacteria of interest using NMR may be accomplished as follows. In the use of NMR as a detection methodology, in which a sample is delivered to a detector coil centered in a magnet, the target of interest, such as a magnetically labeled bacterium, may be delivered by a fluid medium, such as a fluid substantially composed of water. In such a case, the magnetically labeled target may go from a region of very low magnetic field to a region of high magnetic field, for example, a field produced by an about 1 to about 2 Tesla magnet. In this manner, the sample may traverse a magnetic gradient, on the way into the magnet and on the way out of the magnet. As may be seen via equations 1 and 2 above, the target may experience a force pulling into the magnet in the direction of sample flow on the way into the magnet, and a force into the magnet in the opposite direction of flow on the way out of the magnet. The target may experience a retaining force trapping the target in the magnet if flow is not sufficient to overcome the gradient force.

[0063] Magnetic fields on a path into a magnet may be non-uniform in the transverse direction with respect to the flow into the magnet. As such, there may be a transverse force that pulls targets to the side of a container or a conduit that provides the sample flow into the magnet. Generally, the time it takes a target to reach the wall of a conduit is associated with the terminal velocity and is lower with increasing viscosity. The terminal velocity is associated with the drag force, which may be indicative of creep flow in certain cases. In general, it may be advantageous to have a high viscosity to provide a higher drag force such that a target will tend to be carried with the fluid flow through the magnet without being trapped in the magnet or against the conduit walls.

[0064] Newtonian fluids have a flow characteristic in a conduit, such as a round pipe, for example, that is parabolic, such that the flow velocity is zero at the wall, and maximal at the center, and having a parabolic characteristic with radius. The velocity decreases in a direction toward the walls, and it is easier to magnetically trap targets near the walls, either with transverse gradients force on the target toward the conduit wall, or in longitudinal gradients sufficient to prevent target flow in the pipe at any position. In order to provide favorable fluid drag force to keep the samples from being trapped in the conduit, it may be advantageous to have a plug flow condition, wherein the fluid velocity is substantially uniform as a function of radial position in the conduit.

[0065] When NMR detection is employed in connection with a flowing sample, the detection may be based on a perturbation of the NMR water signal caused by a magnetically labeled target (Sillerud et al., JMR (Journal of Magnetic Resonance), vol. 181, 2006). In such a case, the sample may be excited at time 0, and after some delay, such as about 50ms or about 100ms, an acceptable measurement (based on a detected NMR signal) may be produced. Alternatively, such a measurement may be produced immediately after excitation, with the detection continuing for some duration, such as about 50ms or about 100ms. It may be advantageous to detect the NMR signal for substantially longer time durations after the excitation.

[0066] By way of example, the detection of the NMR signal may continue for a period of about 2 seconds in order to record spectral information at high-resolution. In the case of parabolic or Newtonian flow, the perturbation excited at time 0 is typically smeared because the water around the perturbation source travels at different velocity, depending on radial position in the conduit. In addition, spectral information may be lost due to the smearing or mixing effects of the differential motion of the sample fluid during signal detection. When carrying out an NMR detection application involving

a flowing fluid sample, it may be advantageous to provide plug-like sample flow to facilitate desirable NMR contrast and/or desirable NMR signal detection.

5 [0067] Differential motion within a flowing Newtonian fluid may have deleterious effects in certain situations, such as a situation in which spatially localized NMR detection is desired, as in magnetic resonance imaging. In one example, a magnetic object, such as a magnetically labeled bacterium, is flowed through the NMR detector and its presence and location are detected using MRI techniques. The detection may be possible due to the magnetic field of the magnetic object, since this field perturbs the magnetic field of the fluid in the vicinity of the magnetic object. The detection of the magnetic object is improved if the fluid near the object remains near the object. Under these conditions, the magnetic perturbation may be allowed to act longer on any given volume element of the fluid, and the volume elements of the fluid so affected will remain in close spatial proximity. Such a stronger, more localized magnetic perturbation will be more readily detected using NMR or MRI techniques.

10 [0068] If a Newtonian fluid is used to carry the magnetic objects through the detector, the velocity of the fluid volume elements will depend on radial position in the fluid conduit. In such a case, the fluid near a magnetic object will not remain near the magnetic object as the object flows through the detector. The effect of the magnetic perturbation of the object on the surrounding fluid may be smeared out in space, and the strength of the perturbation on any one fluid volume element may be reduced because that element does not stay within range of the perturbation. The weaker, less-well-localized perturbation in the sample fluid may be undetectable using NMR or MRI techniques.

15 [0069] Certain liquids, or mixtures of liquids, exhibit non-parabolic flow profiles in circular conduits. Such fluids may exhibit non-Newtonian flow profiles in other conduit shapes. The use of such a fluid may prove advantageous as the detection fluid in an application employing an NMR-based detection device. Any such advantageous effect may be attributable to high viscosity of the fluid, a plug-like flow profile associated with the fluid, and/or other characteristic(s) attributed to the fluid that facilitate detection. As an example, a shear-thinning fluid of high viscosity may exhibit a flow velocity profile that is substantially uniform across the central regions of the conduit cross-section. The velocity profile of such a fluid may transition to a zero or very low value near or at the walls of the conduit, and this transition region may be confined to a very thin layer near the wall.

20 [0070] Not all fluids, or all fluid mixtures, are compatible with the NMR detection methodology. In one example, a mixture of glycerol and water can provide high viscosity, but the NMR measurement is degraded because separate NMR signals are detected from the water and glycerol molecules making up the mixture. This can undermine the sensitivity of the NMR detector. In another example, the non-water component of the fluid mixture can be chosen to have no NMR signal, which may be achieved by using a perdeuterated fluid component, for example, or using a perfluorinated fluid component. This approach may suffer from the loss of signal intensity since a portion of the fluid in the detection coil does not produce a signal.

25 [0071] Another approach may be to use a secondary fluid component that constitutes only a small fraction of the total fluid mixture. Such a low-concentration secondary fluid component can produce an NMR signal that is of negligible intensity when compared to the signal from the main component of the fluid, which may be water. It may be advantageous to use a low-concentration secondary fluid component that does not produce an NMR signal in the detector. For example, a perfluorinated or perdeuterated secondary fluid component may be used.

30 The fluid mixture used in the NMR detector may include one, two, or more than two secondary components in addition to the main fluid component. The fluid components employed may act in concert to produce the desired fluid flow characteristics, such as high-viscosity and/or plug flow. The fluid components may be useful for providing fluid characteristics that are advantageous for the performance of the NMR detector, for example by providing NMR relaxation times that allow faster operation or higher signal intensities.

35 [0072] A non-Newtonian fluid may provide additional advantages for the detection of objects by NMR or MRI techniques. As one example, the objects being detected may all have substantially the same velocity as they go through the detection coil. This characteristic velocity may allow simpler or more robust algorithms for the analysis of the detection data. As another example, the objects being detected may have fixed, known, and uniform velocity. This may prove advantageous in devices where the position of the detected object at later times is needed, such as in a device that has a sequestration chamber or secondary detection chamber down-stream from the NMR or MRI detection coil, for example.

40 [0073] In an exemplary embodiment, sample delivery into and out of a 1.7T cylindrical magnet using a fluid delivery medium containing 0.1% to 0.5 % Xanthan gum in water was successfully achieved. Such delivery is suitable to provide substantially plug-like flow, high viscosity, such as from about 10cP to about 3000cP, and good NMR contrast in relation to water. Xanthan gum acts as a non-Newtonian fluid, having characteristics of a non-Newtonian fluid that are well known in the art, and does not compromise NMR signal characteristics desirable for good detection in a desirable mode of operation.

45 [0074] Figure 2 panel A provides an exemplary process chart for implementation of methods of the invention for separation of bacteria from blood. Sample is collected in sodium heparin tube by venipuncture, acceptable sample volume is 1 - 10 mL. Superparamagnetic particles having target-specific binding moieties are added to the sample, followed by incubation on a shaking incubator at 37 °C for 30 -120 min.

5 [0075] Capture of the labeled targets allows for the removal of blood components and reduction of sample volume from 30 mL to 5 mL. The capture is performed by injecting the mixture of sample, unbound particles, and target/particle complexes into a channel that has target-specific antibodies coated on the surface. During the injection step, alternating magnetic fields are applied by a mechanical system that moves NdFeB permanent magnets (3 bars arranged in opposing orientation) on either side of the channel such that a magnet is present in close proximity only on one side of the channel. The frequency of the alternating magnetic field (1 Hz) was selected so that labeled targets would have sufficient time to reach one or both surfaces of the channel multiple times, while the majority of free beads would be prevented from reaching either surface of the channel.

10 [0076] After separation, target/particle complexes were eluted from the second moiety on the surface of the channel and flowed into an NMR machine for analysis. The detection method is based on a miniature NMR detector tuned to the magnetic resonance of water. When the sample is magnetically homogenous (no labeled targets), the NMR signal from water is clearly detectable and strong. The presence of magnetic material in the detector coil disturbs the magnetic field, resulting in reduction in water signal. One of the primary benefits of this detection method is that there is no magnetic background in biological samples which significantly reduces the requirements for stringency of sample processing. In addition, since the detected signal is generated by water, there is a built-in signal amplification which allows for the detection of a single labeled bacterium.

15 [0077] Using methods described herein, the following has been accomplished: binding of magnetic particles having bacteria-specific moieties to about 10,000 bacteria in about 5ml of blood using about 5×10^9 /ml of about 250 nm-diameter immunomagnetic label beads in an about 20 ml solution optimized for binding; separating the target from other components of the sample to produce a sample of about 5ml targets and unbound magnetic particles; and removing virtually all unbound magnetic particles to enrich the sample into about 0.5ml with about 61% of the starting bacteria.

20 [0078] Methods of the invention may also be combined with other separation and isolation protocols known in the art. Particularly, methods of the invention may be combined with methods shown in co-pending and co-owned U.S. patent application serial number 12/850,203, filed August 4, 2010, entitled Isolating A Target Analyte From A Body Fluid, the content of which is incorporated by reference herein in its entirety.

Incorporation by Reference

25 [0079] References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

30 [0080] Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

EXAMPLES

Example 1: Sample

35 [0081] Blood samples from healthy volunteers were spiked with clinically relevant concentrations of bacteria (1-10 CFU/mL) including both laboratory strains and clinical isolates of the bacterial species most frequently found in blood-stream infections.

Example 2: Antibody preparation

40 [0082] In order to generate polyclonal, pan-Gram-positive bacteria-specific IgG, a goat was immunized by first administering bacterial antigens suspended in complete Freund's adjuvant intra lymph node, followed by subcutaneous injection of bacterial antigens in incomplete Freund's adjuvant in 2 week intervals. The antigens were prepared for antibody production by growing bacteria to exponential phase ($OD_{600} = 0.4-0.8$). Following harvest of the bacteria by centrifugation, the bacteria was inactivated using formalin fixation in 4% formaldehyde for 4 hr at 37 °C. After 3 washes of bacteria with PBS (15 min wash, centrifugation for 20 min at 4000 rpm) the antigen concentration was measured using BCA assay and the antigen was used at 1 mg/mL for immunization. In order to generate Gram-positive bacteria-specific IgG, several bacterial species were used for inoculation: Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecium

and *Enterococcus faecalis*.

[0083] The immune serum was purified using affinity chromatography on a protein G sepharose column (GE Healthcare), and reactivity was determined using ELISA. Antibodies cross-reacting with Gram-negative bacteria and fungi were removed by absorption of purified IgG with formalin-fixed Gram-negative bacteria and fungi. The formalin-fixed organisms were prepared similar to as described above and mixed with IgG. After incubation for 1 hr at room temperature, the preparation was centrifuged to remove bacteria and absorption was repeated. Final antibody preparation was clarified by centrifugation and used for the preparation of antigen-specific magnetic beads.

Example 3: Preparation of antigen-specific magnetic beads

[0084] Superparamagnetic beads were synthesized by encapsulating iron oxide nanoparticles (5-15 nm diameter) in a latex core and labeling with goat IgG. Ferrofluid containing nanoparticles in organic solvent was precipitated with ethanol, nanoparticles were resuspended in aqueous solution of styrene and surfactant Hitenol BC-10, and emulsified using sonication. The mixture was allowed to equilibrate overnight with stirring and filtered through 1.2 and 0.45 μm filters to achieve uniform micelle size. Styrene, acrylic acid and divinylbenzene were added in carbonate buffer at pH 9.6. The polymerization was initiated in a mixture at 70 °C with the addition of $\text{K}_2\text{S}_2\text{O}_8$ and the reaction was allowed to complete overnight. The synthesized particles were washed 3 times with 0.1% SDS using magnetic capture, filtered through 1.2, 0.8, and 0.45 μm filters and used for antibody conjugation.

[0085] The production of beads resulted in a distribution of sizes that may be characterized by an average size and a standard deviation. In the case of labeling and extracting of bacteria from blood, the average size for optimal performance was found to be between 100 and 350 nm, for example between 200 nm to 250 nm.

[0086] The purified IgG were conjugated to prepared beads using standard chemistry. After conjugation, the beads were resuspended in 0.1% BSA which is used to block non-specific binding sites on the bead and to increase the stability of bead preparation.

Example 4: Attaching target-specific antibodies to surface of flow channel

[0087] Target-specific antibodies were attached to a surface of a flow channel using the following approach:

1. The surface was treated with 5M NaOH for 30 min at 37°C, followed by rinse with water and absorption of streptavidin, to coat the surface with streptavidin and provide means of attaching biotinylated antibodies;
2. Antibodies were biotinylated using des-thiobiotin, purified to remove excess biotin and attached to the surface through a specific binding to streptavidin.

The use of desthiobiotin allows for the elution of antibodies, and, therefore, bound target bacterium, using competitive elution with unmodified biotin. Other elution methods can be used, such as Ni^{2+} -chelating, GST-binding protein, etc.

Example 5: Separating target from remaining components of the sample

[0088] Target cells magnetically labeled using target-specific beads with the excess of free beads were injected into a channel that had target-specific antibodies coated on the surface. During the injection step, alternating magnetic field was applied by a mechanical system that moved NdFeB permanent magnets (3 bars arranged in opposing orientation) on either side of the channel such that a magnet was present in close proximity only on one side of the channel. The frequency of the alternating magnetic field (1 Hz) was selected so that labeled targets would have sufficient time to reach one or both surfaces of the channel multiple times, while the majority of free beads would be prevented from reaching either surface of the channel.

Example 6: Elution and analysis of target

[0089] The bound bacteria were eluted with 35 mM biotin solution in B29, followed by magnetic capture and re-suspension in the detection buffer containing Xanthan gum. The concentration of bacteria in samples were analyzed in flow-through NMR detector. Results indicate that the number of detected bacteria was directly proportional to the number of bacteria spiked into blood (Fig. 3).

[0090] Embodiments of the invention may include the features of the following enumerated paragraphs ("paras").

1. A method for separating a target analyte from a sample, the method comprising:

contacting a sample with magnetic particles comprising first moieties specific for a target analyte, thereby forming

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target/particle complexes in the sample;

flowing the sample through a channel comprising second moieties attached to at least one surface of the channel;

5 applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample;

binding the target/particle complexes to the second moieties; and

10 washing away unbound particles and unbound analytes of the sample.

2. The method according to para 1, further comprising eluting the bound target/particle complexes from the second moieties on the surface of the channel.

15 3. The method according to para 1, further comprising analyzing the eluted target/particle complexes.

4. The method according to para 3, wherein analyzing comprises flowing the target/particle complexes into an NMR instrument.

20 5. The method according to para 1, wherein the target analyte is selected from the group consisting of a bacteria, a virus, a fungus, a cell, a protein, and a nucleic acid.

6. The method according to para 5, wherein the target analyte is a bacteria.

25 7. The method according to para 1, wherein the sample is human tissue or body fluid.

8. The method according to para 8, wherein the body fluid is blood.

9. The method according to para 1, wherein the first and second moieties are the same.

30 10. The method according to para 1, wherein the first and second moieties are different.

11. The method according to para 1, wherein the first and second moieties are selected from the group consisting of antibodies, receptors, aptamers, proteins, and ligands.

35 12. The method according to para 1, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the channel remains stationary and the first and second sets of magnets are moved to alternate proximity to the channel, thereby producing the alternating magnetic fields.

40 13. The method according to para 1, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein first and second sets of magnets remain stationary and the channel is moved to alternate its proximity to the first and second sets of magnets, thereby producing the alternating magnetic fields.

45 14. A method for detecting a target analyte in a sample, the method comprising:

contacting a sample with magnetic particles comprising first moieties specific for a target analyte, thereby forming target/particle complexes in the sample;

50 flowing the sample through a channel comprising second moieties attached to at least one surface of the channel;

applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface of the channel to bind the second moieties and unbound particles remaining free in the sample;

55 binding the target/particle complexes to the second moieties;

washing away unbound particles and unbound analytes of the sample;

eluting the target/particle complexes from the second moieties; and

detecting the target/particle complexes.

5 15. The method according to para 14, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the channel remains stationary and the first and second sets of magnets are moved to alternate proximity of the first and second sets of magnets to the channel, thereby producing the alternating magnetic fields.

10 16. The method according to para 14, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the first and second sets of magnets remain stationary and the channel is moved to alternate its proximity to the first and second sets of magnets, thereby producing the alternating magnetic fields.

15 17. The method according to para 14, wherein detecting comprises flowing the target/particle complexes into an NMR instrument.

18. The method according to para 14, wherein the target analyte is a bacteria.

20 19. The method according to para 14, wherein the sample is human tissue or body fluid.

20. The method according to para 14, wherein the body fluid is blood.

25 21. The method according to para 14, wherein the first and second moieties are the same.

22. The method according to para 14, wherein the first and second moieties are different.

23. The method according to para 14, wherein the first and second moieties are selected from the group consisting of antibodies, receptors, aptamers, proteins, and ligands.

30 24. A method for separating a target analyte from a sample, the method comprising:
contacting a sample with magnetic particles comprising first moieties specific for a target analyte, thereby forming target/particle complexes in the sample;
35 applying a magnetic field to capture target/particle complexes on a surface;
washing the target/particle complexes;
40 removing the magnetic field, thereby releasing target/particle complexes from the surface;
flowing the sample through a channel comprising second moieties attached to at least one surface of the channel;
applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought
45 into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample;
binding the target/particle complexes to the second moieties; and
washing away unbound particles and unbound analytes of the sample.

Claims

55 1. A method for separating and analyzing a target analyte in a sample, the method comprising:
contacting a sample with magnetic particles comprising binding moieties specific for a target analyte;
flowing the sample through a channel while applying alternating magnetic fields to capture magnetic particles that are bound to said target analyte;

rinsing said channel to remove unbound components of said sample;
releasing captured magnetic particles from the surface of the channel; and
analyzing the released target analyte attached to said released magnetic particles,

- 5 wherein the target/particle complexes are not examined in situ on the surface.
2. The method according to claim 1, wherein analyzing comprises the use of one or more processes selected from the group consisting of lysis, hybridization, cytometry, NAT, PCR, and NMR.
- 10 3. The method according to claim 1, wherein the target analyte is selected from the group consisting of a bacteria, a virus, a fungus, a cell, a protein, and a nucleic acid.
4. The method according to claim 1, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the channel remains stationary and the first and second sets of magnets are moved to alternate proximity to the channel, thereby producing the alternating magnetic fields.
- 15 5. The method according to claim 1, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein first and second sets of magnets remain stationary and the channel is moved to alternate its proximity to the first and second sets of magnets, thereby producing the alternating magnetic fields.
- 20 6. A method for detecting a target analyte in a sample, the method comprising:
- contacting a sample with magnetic particles comprising binding moieties specific for a target analyte;
25 flowing the sample through a channel while applying alternating magnetic fields to capture magnetic particles that are bound to said target analyte;
rinsing said channel to remove unbound components of said sample;
releasing captured magnetic particles from the surface of the channel; and
detecting the released target analyte attached to said released magnetic particles,
- 30 wherein the target/particle complexes are not examined in situ on the surface.
7. The method according to claim 6, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the channel remains stationary and the first and second sets of magnets are moved to alternate proximity of the first and second sets of magnets to the channel, thereby producing the alternating magnetic fields.
- 35 8. The method according to claim 6, wherein the alternating magnetic fields result from the channel being positioned between first and second sets of magnets, wherein the first and second sets of magnets remain stationary and the channel is moved to alternate its proximity to the first and second sets of magnets, thereby producing the alternating magnetic fields.
- 40 9. The method according to claim 6, wherein detecting comprises the use of one or more processes selected from the group consisting of lysis, hybridization, cytometry, NAT, PCR, and NMR.
- 45 10. The method according to claim 1 or claim 6, wherein the target analyte is a bacterium.
11. The method according to claim 1 or claim 6, wherein the sample is human tissue or body fluid.
- 50 12. The method according to claim 11, wherein the body fluid is blood.
13. The method according to claim 1 or claim 6, wherein the binding moieties are selected from the group consisting of antibodies, receptors, aptamers, proteins, and ligands.
- 55 14. The method according to claim 13, wherein the binding moieties are antibodies.
15. A method for detecting a target analyte in a sample, the method comprising:

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contacting a sample with magnetic particles comprising binding moieties specific for a target analyte;
flowing the sample through a channel while applying a magnetic field to capture magnetic particles that are
bound to said target analyte;
5 rinsing said channel to remove unbound components of said sample;
releasing captured magnetic particles from the surface of the channel; and
detecting the released target analyte attached to said released magnetic particles, wherein the target/particle
complexes are not examined in situ on the surface.

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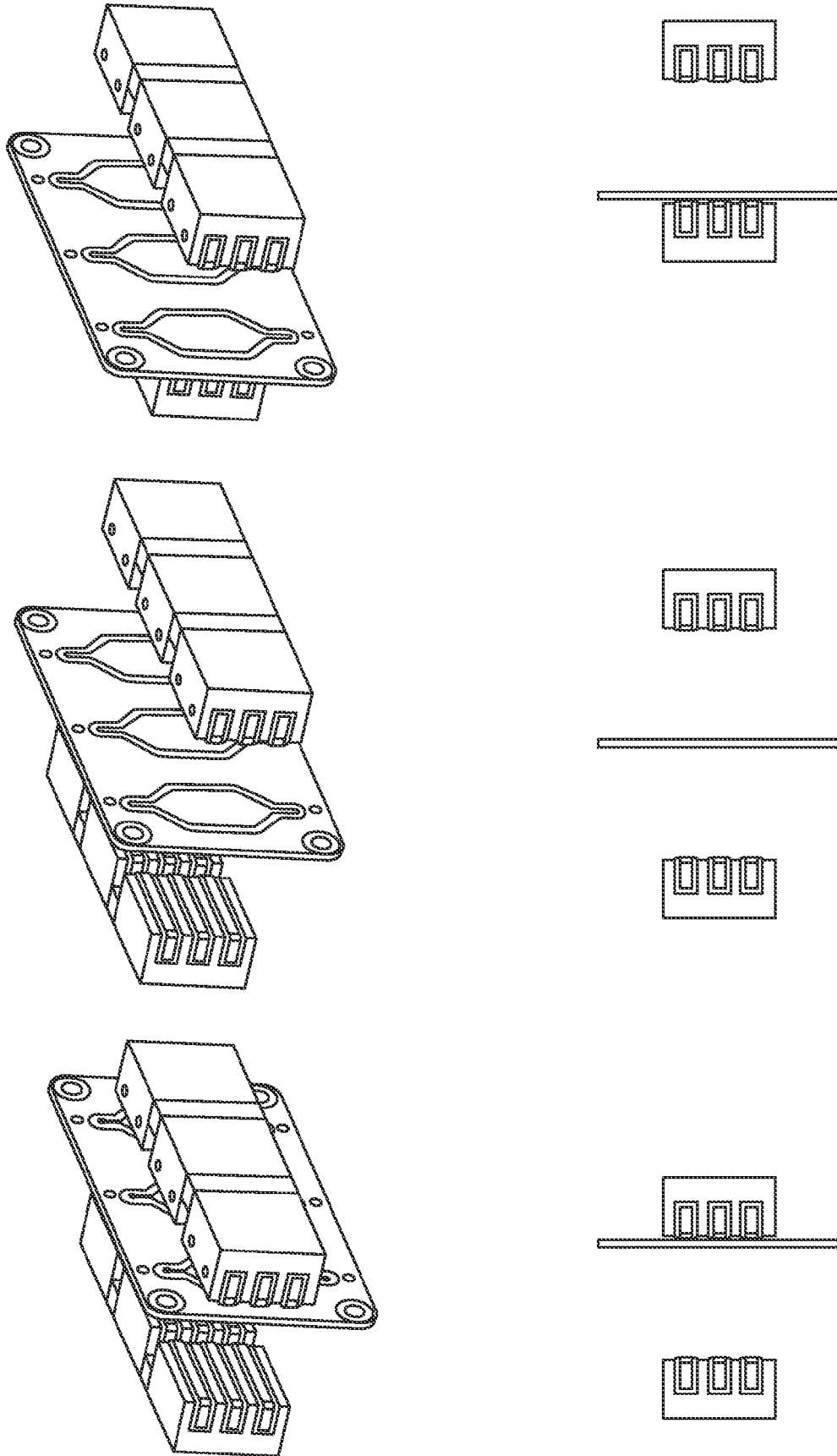


FIG. 1

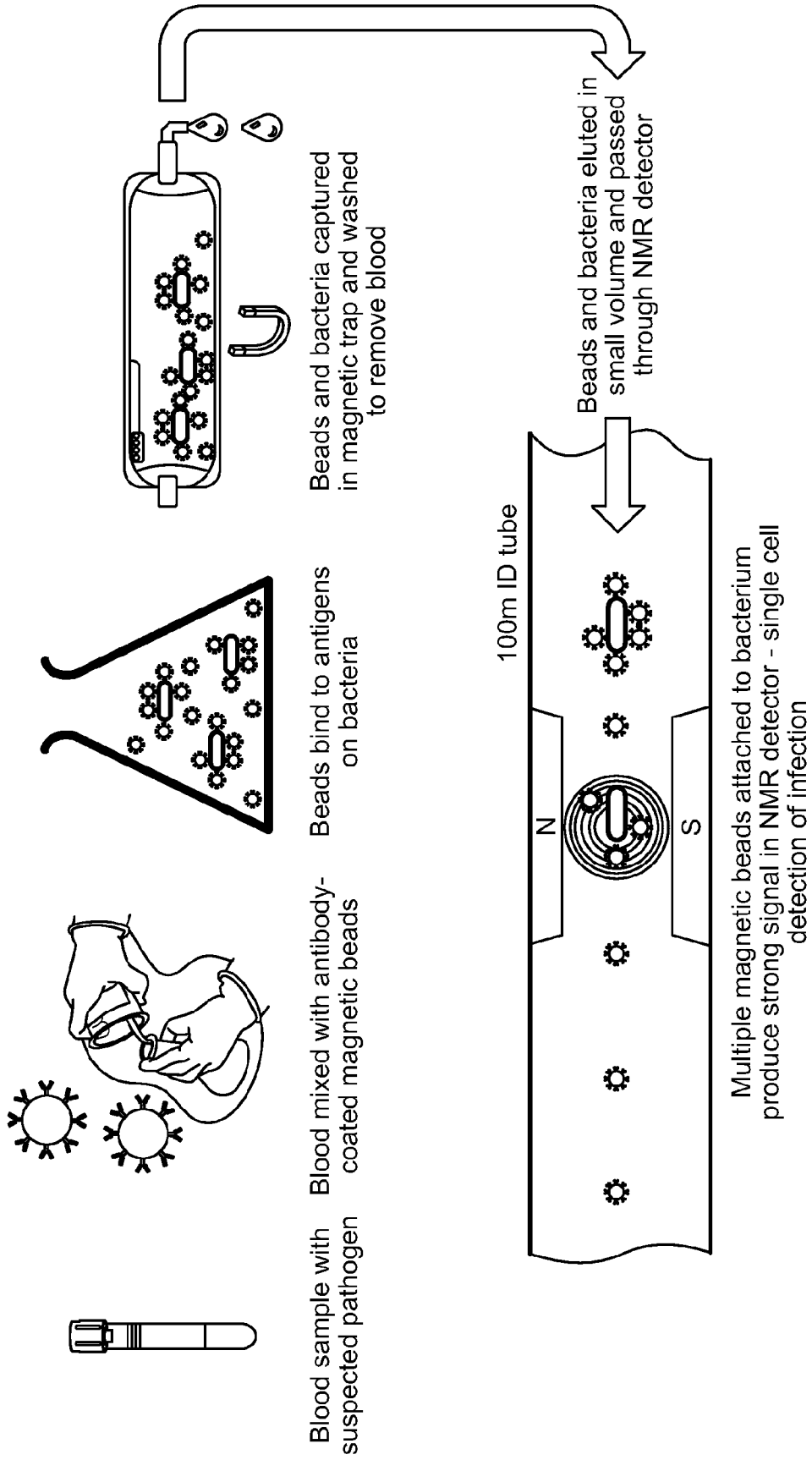


FIG. 2 Panel A

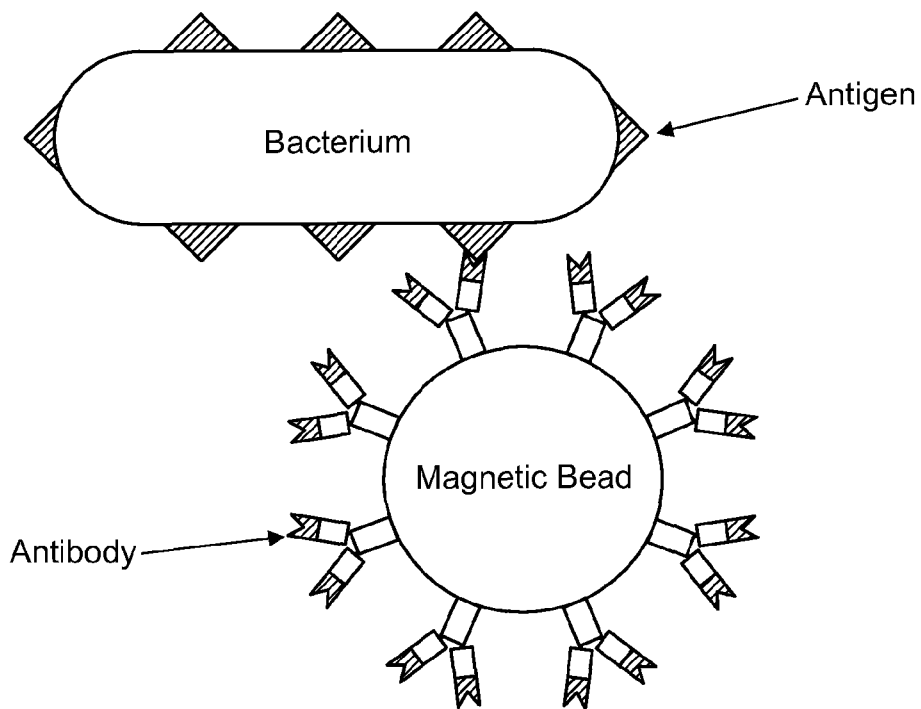


FIG. 2 Panel B

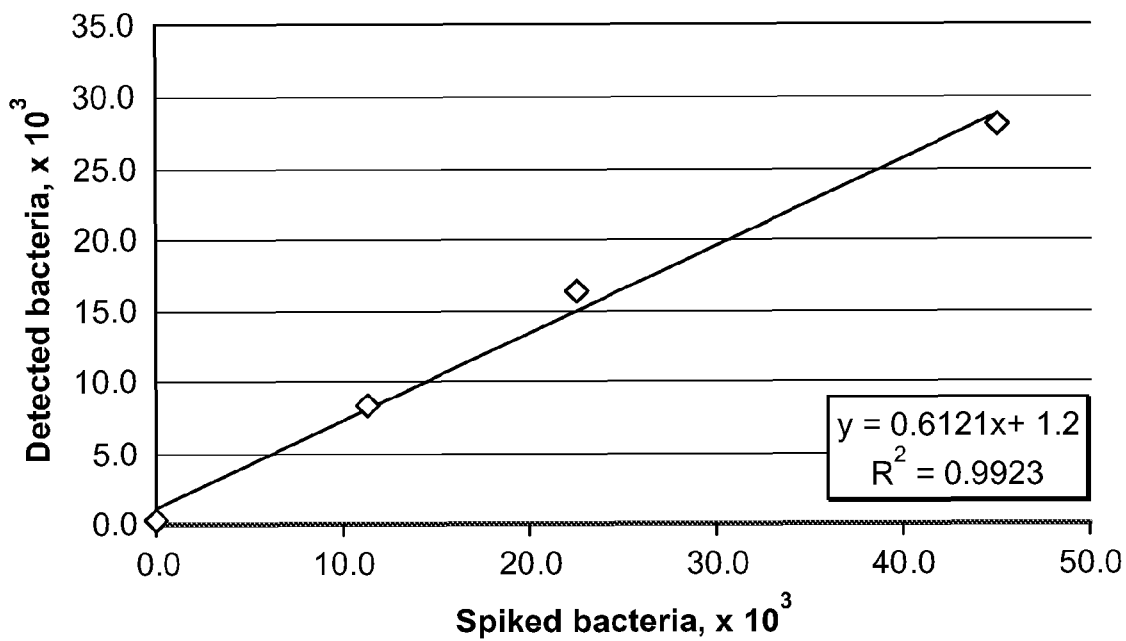


FIG. 3



EUROPEAN SEARCH REPORT

Application Number
EP 16 19 0239

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	VASILE I FURDUI ET AL: "Microfabricated electrolysis pump system for isolating rare cells in blood; Micro-electrolysis pumps for blood", JOURNAL OF MICROMECHANICS & MICROENGINEERING, INSTITUTE OF PHYSICS PUBLISHING, BRISTOL, GB, vol. 13, no. 4, 1 July 2003 (2003-07-01), pages S164-S170, XP020068953, ISSN: 0960-1317, DOI: 10.1088/0960-1317/13/4/324 * the whole document *	1-15	INV. C12Q1/68 G01N33/53 G01N33/553
X	FURDUI VASILE I ET AL: "Immunomagnetic T cell capture from blood for PCR analysis using microfluidic systems", 2004, LAB ON A CHIP, VOL. 4, NR. 6, PAGE(S) 614-618, XP002765758, ISSN: 1473-0197 * figures 1,2 *	1-15	
X	CHANDLER DARRELL P ET AL: "Automated immunomagnetic separation and microarray detection of E. coli O157:H7 from poultry carcass rinse", INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, ELSEVIER BV, NL, vol. 70, no. 1-2, 22 October 2001 (2001-10-22), pages 143-154, XP002261190, ISSN: 0168-1605, DOI: 10.1016/S0168-1605(01)00536-0 * the whole document *	15	TECHNICAL FIELDS SEARCHED (IPC) G01N C12Q
Y	* the whole document *	1-14	
Y	US 2009/227044 A1 (DOSEV DOSI [US] ET AL) 10 September 2009 (2009-09-10) * paragraph [0066]; figure 2; examples 4-12 *	1-14	
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 11 January 2017	Examiner Seroz, Thierry
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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EUROPEAN SEARCH REPORT

Application Number
EP 16 19 0239

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50

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	EP 2 138 835 A1 (HONG CHIN-YIH REX [TW]; HORNG HERNG-ER [TW]; YANG HONG-CHANG [TW]; YAN) 30 December 2009 (2009-12-30) * the whole document *	1-14	
Y	US 2006/257945 A1 (MASTERS BRETT P [US] ET AL) 16 November 2006 (2006-11-16) * the whole document *	1-14	
A	US 2009/146658 A1 (MCDOWELL ANDREW F [US] ET AL) 11 June 2009 (2009-06-11) * the whole document *	1-15	
A	WO 2005/106480 A1 (CANON KK [JP]; IKEDA TAKASHI [JP]; NISHIUMA SATORU [JP]; YAMAMICHI JUN) 10 November 2005 (2005-11-10) * page 2, lines 13-16 * * page 5, lines 10-13; figures 8, 9b * * page 7, lines 10-11 * * page 10, line 4 - page 11, line 9 * * page 16, lines 1-2 * * page 16, line 25 - page 17, line 7 * * page 17, lines 25,26; claim 2 * -----	1-15	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (IPC)
Place of search		Date of completion of the search	Examiner
Munich		11 January 2017	Seroz, Thierry
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03/02 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.

EP 16 19 0239

5 This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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11-01-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009227044 A1	10-09-2009	US 2009227044 A1 WO 2007089564 A2	10-09-2009 09-08-2007
EP 2138835 A1	30-12-2009	CN 101614796 A EP 2138835 A1 JP 5254136 B2 JP 2010051951 A TW 201000900 A US 2009325317 A1	30-12-2009 30-12-2009 07-08-2013 11-03-2010 01-01-2010 31-12-2009
US 2006257945 A1	16-11-2006	EP 1886142 A2 EP 1891441 A2 JP 5197357 B2 JP 2008541052 A JP 2008541053 A JP 2013015533 A KR 20080016595 A KR 20080027239 A US 2006257945 A1 US 2006286685 A1 US 2007037142 A1 US 2007037231 A1 US 2007042441 A1 US 2007281371 A1 WO 2006119308 A2 WO 2007030155 A2	13-02-2008 27-02-2008 15-05-2013 20-11-2008 20-11-2008 24-01-2013 21-02-2008 26-03-2008 16-11-2006 21-12-2006 15-02-2007 15-02-2007 22-02-2007 06-12-2007 09-11-2006 15-03-2007
US 2009146658 A1	11-06-2009	CA 2703687 A1 EP 2208081 A1 JP 5539889 B2 JP 2011501196 A US 2009146658 A1 US 2012112744 A1 WO 2009055587 A1	30-04-2009 21-07-2010 02-07-2014 06-01-2011 11-06-2009 10-05-2012 30-04-2009
WO 2005106480 A1	10-11-2005	CN 1947016 A JP 2005315677 A US 2006226832 A1 WO 2005106480 A1	11-04-2007 10-11-2005 12-10-2006 10-11-2005

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 85514710 A [0001]
- US 61326588 A [0001]
- US 4554088 A [0003] [0024]
- US 3970518 A, Giaever [0024]
- US 4230685 A, Senyi [0024]
- US 4677055 A, Dodin [0024]
- US 4695393 A, Whitehead [0024]
- US 5695946 A, Benjamin [0024]
- US 4018886 A, Giaever [0024]
- US 4267234 A, Rembaum [0024]
- US 4452773 A [0024]
- US 4659678 A, Forrest [0024]
- US 5186827 A, Liberti [0024]
- US 4795698 A, Own [0024]
- WO 9102811 A, Liberti [0024]
- US 5089386 A, Stackebrandt [0055]
- WO 9008841 A, King [0055]
- WO 9215883 A, Foster [0055]
- WO 8906699 A, Cossart [0055]
- WO 9208805 A, Verhoef [0059]
- US 85020310 A [0078]

Non-patent literature cited in the description

- HUNTER et al. Immunoassays for Clinical Chemistry. Churchill Livingstone, 1983, 147-162 [0003]
- HARLOW et al. Antibodies. Cold Spring Harbor Laboratory, 1988, 93-117 [0034]
- INAI et al. *Histochemistry*, May 1993, vol. 99 (5), 335-362 [0035]
- MULDER et al. *Hum. Immunol.*, 1993, vol. 36 (3), 186-192 [0035]
- HARADA et al. *J. Oral Pathol. Med.*, 1993, vol. 22 (4), 145-152 [0035]
- STAUBER et al. *J. Immunol. Methods*, 1993, vol. 161 (2), 157-168 [0035]
- VENKATESWARAN et al. *Hybridoma*, 1992, vol. 11 (6), 729-739 [0035]
- VERMUNT et al. *J. Appl. Bact.*, 1992, vol. 72, 112 [0036]
- JOHNE et al. *J. Clin. Microbiol.*, 1989, vol. 27, 1631 [0036]
- SKJERVE et al. *Appl. Env. Microbiol.*, 1990, vol. 56, 3478 [0036]
- LUND et al. *J. Clin. Microbiol.*, 1991, vol. 29, 2259 [0036]
- HARLOW et al. Antibodies. Cold Spring Harbor Laboratory, 1988 [0037]
- HUNTER et al. Immunoassays for Clinical Chemistry. 1983, 147-162 [0037]
- STANLEY. *Essentials in Immunology and Serology*, 2002, 152-153 [0037]
- HIJMANS, W. et al. *Clin. Exp. Immunol.*, 1969, vol. 4, 457 [0060]
- GODING, J. W. *J. Immunol. Meth.*, 1976, vol. 13, 215 [0060]
- INGRALL, E. *Meth. in Enzymol.*, 1980, vol. 70, 419-439 [0060]
- SILLERUD et al. *Journal of Magnetic Resonance*, 2006, vol. 181 [0065]

专利名称(译)	使用交变磁场分离目标分析物		
公开(公告)号	EP3138927A1	公开(公告)日	2017-03-08
申请号	EP2016190239	申请日	2011-04-20
[标]申请(专利权)人(译)	NANOMR		
申请(专利权)人(译)	NANOMR INC.		
当前申请(专利权)人(译)	NANOMR INC.		
[标]发明人	DRYGA SERGEY A ESCH VICTOR C SAUL RICHARD G MCDOWELL ANDREW F		
发明人	DRYGA, SERGEY A ESCH, VICTOR C SAUL, RICHARD G MCDOWELL, ANDREW F		
IPC分类号	C12Q1/68 G01N33/53 G01N33/553		
CPC分类号	C07K1/22 C07K16/1267 C12N13/00 C12Q1/6806 G01N33/54333 Y10T428/2982 C12Q2565/629 C12Q2563/143 A61B5/055 B01L3/502761 B01L2200/0647 B01L2400/043 C12N15/1013 C12Q1/689 C12Q2565/633 G01N24/08 G01N33/54326 G01N33/56911 G01N2333/195 G01N2446/20 G01R33/465		
优先权	61/326588 2010-04-21 US 12/855147 2010-08-12 US PCT/US2011/033186 2011-04-20 WO		
其他公开文献	EP3138927B1		
外部链接	Espacenet		

摘要(译)

本发明一般涉及使用磁性颗粒和交变磁场将目标分析物与样品分离。在某些实施方案中，本发明的方法包括使样品与磁性颗粒接触，所述磁性颗粒包括对靶分析物特异的第一部分，从而在样品中形成靶/颗粒复合物，使样品流过包括附着于至少一个表面的第二部分的通道。通道，将交变磁场施加到流动的样品上，使得靶/颗粒复合物接近表面以结合第二部分和未结合的颗粒在样品中保持游离，将靶/颗粒复合物结合到第二部分，并洗去未结合的颗粒和未结合的样品分析物。

(10) (11) EP 3 138 927 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication: 08.03.2017. Bulletin 2017/10 (51) Int. Cl.: C12Q 1/68 (2016.01) G01N 33/53 (2016.01)

(21) Application number: 16190239.0 (22) Date of filing: 20.04.2011

(84) Designated Contracting States: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HH, HU, IE, IT, LI, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR

(30) Priority: 21.04.2010 US 20098 P 12.08.2010 US 20098 P

(62) Document number(s) of the earlier application(s) in accordance with Art. 76(2) EPC: 11720616.3 / 2 561 059

(71) Applicant: NANOMR, Inc. Albuquerque, NM 87106 (US)

(72) Inventors: DRYGA, Sergey A Rio Rancho, NM 87144 (US)

(74) Representatives: Kirkham, Nicholas Andrew et al. Charles Watt & Co. LLP St. Botolph's House 7a St. Botolph's Street Sevenoaks, Kent TN13 3AJ (GB)

(54) SEPARATING TARGET ANALYTES USING ALTERNATING MAGNETIC FIELDS

(57) The invention generally relates to using magnetic particles and alternating magnetic fields to separate a target analyte from a sample. In certain embodiments, methods of the invention involve contacting a sample with magnetic particles including first moieties specific for a target analyte, thereby forming target/particle complexes in the sample. Flowing the sample through a channel including second moieties attached to at least one surface of the channel, applying alternating magnetic fields to the flowing sample to result in target/particle complexes being brought into proximity of the surface to bind the second moieties and unbound particles remaining free in the sample, binding the target/particle complexes to the second moieties, and washing away unbound particles and unbound analytes of the sample.