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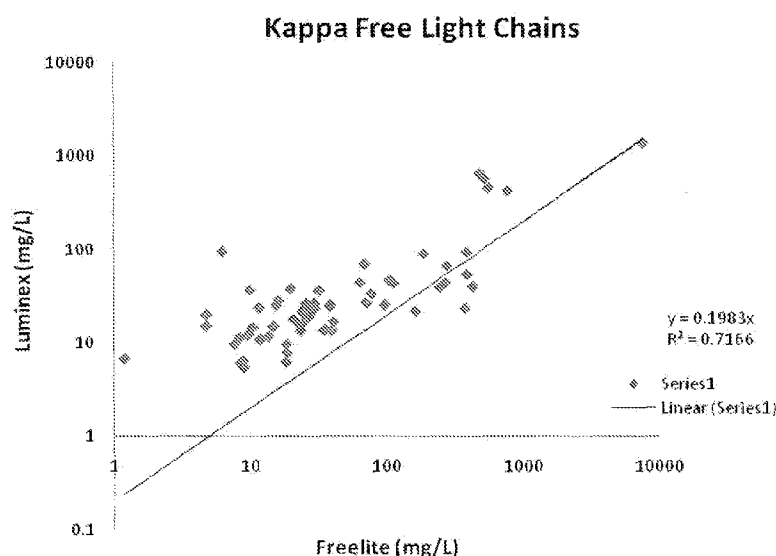
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(54) Title: BIOLOGICAL REAGENT

Figure 1a



(57) Abstract: A biological reagent is provided, the reagent comprising a biological fluid comprising a mixture of analytes present at substantially the same relative concentrations as when sampled, the mixture of analytes comprising at least one target analyte labelled with a detectable analyte-binding agent. Such a reagent finds use in diagnostic assays to determine the presence or concentration of at least one target analyte in a test sample. The biological reagent may be prepared by a method comprising contacting a sample of a biological fluid, comprising a mixture of analytes, with a detectable analyte-binding agent and effecting labelling of one or more of the analytes in the sample by the detectable analyte-binding agent.

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Biological reagent

The invention relates to a biological reagent and methods for the preparation and use thereof. In particular, the invention relates to a biological reagent for use in assays to
5 detect and/or determine the concentration of analytes present in a biological sample.

Background

Processes for detecting the presence of certain molecules in biological samples, and
10 for determining the concentration of such molecules, are used in many scientific fields. In particular, such methods are used in clinical laboratories for the diagnosis of a range of conditions and diseases. Assaying is a procedure commonly used for measuring the presence or concentration of a biochemical analyte in an organism or in a sample containing biological material. Medical and veterinary laboratories assay
15 a wide spectrum of biological fluid samples, which are used for the diagnosis of diseases and conditions. For example, in a clinical immunology laboratory, blood might be tested for antibodies to quantify acquired immunity against viruses, or allergens may be diagnosed by the detection of serum antibodies to particular common allergens.

20 In the evaluation of some conditions it is necessary to detect and/or quantify a number of different analytes. Multi-analyte assays can be formatted in different ways, the most common being micro-arrays or multi-plex bead or chip assays. The simultaneous assessment of a number of analytes at the same time can offer a more
25 accurate test because all analytes are exposed to the same physical assay treatment. This reduces the intra-assay error and eradicates inter-assay variation. Such methods are also economically beneficial as they dramatically reduce the 'hands-on' time of the assay, and reduce the need for additional personnel, training, instrumentation, and reagents.

30 Clinical assays capable of simultaneous multi-analyte measurement can be generally categorised into non-competitive and competitive formats. Non-competitive immunoassays are simple immunoassays whereby a first capture agent (such as a first antibody), specific to a target analyte of interest, is usually bound to a solid-
35 phase surface e.g., to a micro-array slide/multi-plex bead. When a test sample is added to the surface, the capture agent binds the target analyte in the sample and retains the analyte on the solid-phase structure. Next, a second capture agent (such

as a second antibody), also specific to the target analyte is added. This second antibody binds to the target analyte already bound to the first antibody on the solid-phase structure, thereby forming an analyte 'sandwich' between the two capture agents. The second capture agent is labelled so that the presence of the label can be used to generate a signal which is proportional to the amount of target analyte present in the test sample.

In competitive assays, the capture agents are usually bound to a solid-phase surface much in the same way as in non-competitive assays, although non-solid phase assays also exist. However, unlike non-competitive assays, the analyte in the test sample must compete with a corresponding labelled analyte to bind the capture agent. The amount of labelled analyte bound to the capture site is then measured. Unlike non-competitive assays where the signal intensity is directly proportional to the amount of unknown analyte, the signal intensity in competitive assays is inversely proportional to the quantity of unlabelled analyte in the test sample i.e. the lower the signal, the higher the quantity of unlabelled analyte.

Competitive immunoassays offer some important advantages over conventional sandwich / non-competitive immunoassay formats. Firstly, the issue of 'analyte excess', whereby the amount of analyte in the test sample saturates the first capture agent and the second capture agent to the extent that sandwich formation is inhibited causing a plateau or decrease in the projected maximal phase of signal output, is greatly reduced. Competitive immunoassays are also particularly useful when measuring small analytes or analytes with restricted epitope availability, because these assays require the binding of only one capture agent rather than two as in standard sandwich / non-competitive formats. Further, competitive assays offer greater assay detection ranges, and as a result these assays tend to have more sustained linearity. Competitive immunoassays also maintain the detection sensitivity of non-competitive assays, and therefore offer a more appealing assay format.

Despite the advantages offered by competitive assays, there are a number of reasons why they are not as popular as more conventional assay formats. Primarily, this is because it is difficult to obtain a solution to be inhibited that contains analytes of interest that are labelled to enable signal detection by an immunoassay reader and in their natural form.

A further limitation of competitive assays is that they can only be multiplexed for a specific type of analyte. For example, it is currently not possible to measure cytokines and antibodies in same assay. Current multiplex assays also cannot detect a variety of analytes outside a prescriptive concentration range, e.g. measurement of analytes at mg/L and g/L levels simultaneously is very difficult. Existing methods can also result in the detection of low affinity antibodies, rather than only the clinically significant high affinity antibodies, which reduces the accuracy of the diagnostic tests.

Some multiplex assays require the creation of a panel comprising each analyte of interest labelled with a marker. To create such a panel using existing approaches it is necessary to (i) separately isolate each analyte of interest in its pure form, (ii) label each analyte with a marker, and (iii) mix the labelled analytes together to create a panel containing all of the chosen analytes of interest.

Methods to obtain each analyte in a pure form vary depending on the chemical structure of the molecule of interest, and its intended application. For example, the manufacture of recombinant proteins may be desirable, particularly if a large quantity of analyte is required. However, such recombinant proteins bind with respective binding agents differently compared to their naturally produced counterparts in biological fluids, which often makes them unsuitable for use in assays. Further, the cost of developing and producing a large number of recombinant analytes is substantial, and requires intensive quality control and quality assurance steps. Alternatively, analytes may be extracted and purified from an existing source (e.g., plasma, serum) using high performance liquid chromatography, or similar separation techniques. These methods may expose the analytes to unnatural conditions in terms of pH, chemical exposure, temperature, tonicity, which can often result in the fractionation and disintegration of intact molecules. Such changes can affect the interaction of the target analytes with binding partners used in assays, and will require intensive quality control and quality assurance monitoring to ensure binding efficiency is maintained.

After separately isolating each molecule in its pure form and labelling it, each labelled molecule is then mixed together to create a panel containing all of the chosen analytes of interest. It can be difficult to recreate the normal relative and predefined concentrations of the analytes that would be found in the natural biological fluid from which each analyte originated.

The present invention therefore aims to address some of the problems identified above.

Summary of Invention

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Thus, according to a first aspect, there is provided a method for preparing a biological reagent for use in a diagnostic assay, the method comprising:

contacting a sample of a biological fluid, comprising a mixture of analytes, with a detectable analyte-binding agent; and

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effecting labelling of one or more of the analytes in the sample by the detectable analyte-binding agent.

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A “biological reagent”, as used herein, refers to an agent, preferably a fluid, which comprises biological material or molecules, and which can be used in a diagnostic assay to investigate, for instance by identifying or determining, the presence and/or concentration of one or more molecules of interest in a sample of biological fluid. A “diagnostic assay” is a type of experiment or procedure which is commonly used for measuring the presence or concentration of a biochemical analyte or a number of analytes in an organism or in a sample containing biological material. The presence or concentration of the analyte(s) may be indicative of a particular condition or disease of an organism.

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It will be understood that, according to the above aspect, the sample of biological fluid was previously obtained from a human or animal, such that the sampling itself does not form part of this aspect.

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In general, the biological fluid may have been sampled immediately prior to preparation of the biological reagent, or it may have been sampled a number of hours, weeks, months or even years prior to the preparation of the biological reagent.

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An “analyte”, as used herein, will be understood to mean a substance, chemical or molecule of interest which is present in the sample of biological fluid. The presence of an analyte, or the concentration of a particular analyte, may be indicative of a disease or condition of a human or animal subject. In all instances, it will be appreciated that reference to “analyte” includes a combination of analytes as well, unless otherwise apparent. “One or more” analytes mentioned herein of course can mean a single analyte or a combination of different analytes. Examples of analytes

that may be assayed include polypeptides and proteins (such as antibodies, cytokines), peptides (such as glucagon, secretin), glycoproteins (such as glycosylated antibodies), lipids (such as cholesterol), lipoproteins (such as low density lipoprotein, high density lipoprotein), glycolipids, phospholipids, carbohydrates, nucleic acids (such as RNA or DNA), steroids (such as glucocorticoids, oestrogens) antigens (such as bacteria, viruses, prions), soluble and cell-surface-bound cluster of differentiation (CD) antigens (e.g., CD62L, CD95), and autoantigens (such as anti-dsDNA antibodies, anti-nuclear antibodies).

10 It will be understood that the analytes are contacted with a detectable analyte-binding agent *in situ* in the biological fluid sample, preferably immediately after extraction from the patient or, if the sample is stored for instance by freezing, immediately after thawing, and most preferably without separation, isolation and/or purification of the analytes from or within the sample. In other words, a detectable analyte-binding agent is contacted directly with the sample of biological fluid and the analytes contained therein, to thereby label one or more of the analytes with the detectable analyte-binding agent. It will also be understood that "a" detectable analyte-binding agent may refer to one or more detectable analyte-binding agents.

20 Further agents or substances, such as suitable buffers may be added to the sample before or after the detectable analyte-binding agent has been contacted with the sample. Accordingly, dilution or aliquoting of the sample is envisaged. As such, it is preferred that the analytes are present in the sample of biological fluid at substantially the same relative concentrations as when sampled from the individual. However, it is particularly preferred that the detectable analyte-binding agent is contacted with the sample before the addition of any other agents or substances which may themselves label some of the analytes or interfere with labelling by the detectable analyte-binding agent.

30 In general, contacting the detectable analyte-binding agent with the sample is advantageous as it obviates the need to isolate, separate and/or purify the analytes, separately labelling each analyte and then optionally re-combining the labelled analytes. The labelling of analytes in a sample of biological fluid is also advantageous in that the analytes are present in their pre-existing i.e. native, *in-vivo* form. No isolation and purification steps are required which may degrade or alter the structure of the analytes, which may in turn affect the reliability of an assay using the resulting biological reagent. The labelling of a group of analytes while *in situ* in a

complex biological fluid is also quicker and more cost effective than the current procedure of isolating and labelling the analytes individually.

5 It will be understood that analytes in their “native” form, as referred to herein, are analytes which are substantially unchanged in structure, charge and/or conformation from the form in which they existed inside the individual’s body, immediately prior to sampling. The individual is preferably a mammal, most preferably a primate and especially a human.

10 It was generally previously perceived that the labelling of analytes *in situ* in a complex biological fluid was too difficult because of the presence of naturally occurring inhibitors/stabilisers found in biological fluids. These stabilisers maintain homeostasis and resist modification of endogenous molecules. It has been thought that these stabilisers would inhibit labelling, or would prevent analytes from being
15 labelled on an equi-molar basis. However, the present inventors have surprisingly found that contacting a detectable analyte-binding agent with a sample of biological fluid does in fact result in the efficient labelling of all analytes to which the detectable analyte-binding agent is capable of binding. The present invention thus provides a quicker, cheaper and more efficient method of preparing a biological reagent for use
20 in a diagnostic assay.

In some embodiments, some or all of the analytes in the sample of biological fluid and/or in the biological reagent are present at substantially the same relative concentrations as when sampled. By “substantially the same relative concentrations
25 as when sampled,” it will be understood that there is no significant difference between the relative concentration of the analytes in the biological fluid at or just prior to the moment of sampling, and in the resulting sample and/or biological reagent. For example, the relative concentrations of a given antibody and a given cytokine in the resulting biological agent may be approximately the same as the relative
30 concentrations of these analytes in the blood at a moment in time immediately prior to sampling.

The biological reagent of the present invention may, therefore, provide a snapshot of the relative concentrations of some or all of the analytes present in the body at the
35 moment of sampling. However, it will be understood that the absolute concentration of some or all of the analytes may change between sampling and the production of the final reagent, for example due to the addition of the detectable analyte binding

agent, which may be in solution, to the biological fluid. It will also be understood that the concentration of some analytes may be elevated (artificially or naturally) prior to sampling, for example by the administration of a vaccine to the individual from which the biological fluid is sampled, or by the exposure of that individual to a particular antigen.

In some embodiments, the method comprises a further step of adding a quantity of one or more analytes to the sample of biological fluid prior to or after the step of contacting the sample with the detectable analyte binding agent. This may be done to include an analyte in the biological reagent which is not, or is not thought to be, already present in the sample of biological fluid. Alternatively, or additionally, a quantity of an analyte may be added to the sample in order to increase the concentration of that analyte which is already present in the sample.

In some embodiments, a plurality of analytes is labelled by the detectable analyte-binding agent. In some embodiments, each of the plurality of analytes belongs to the same class of molecule, in that they are based on the same or related biochemical compounds or structure. For example, the analytes that are labelled by the detectable analyte binding agent may all be polypeptides and proteins (such as antibodies, cytokines), peptides (such as glucagon, secretin), glycoproteins (such as glycosylated antibodies), lipids (such as cholesterol), lipoproteins (such as low density lipoprotein, high density lipoprotein), glycolipids, phospholipids, carbohydrates, nucleic acids (such as RNA or DNA), steroids (such as glucocorticoids, oestrogens) antigens (such as bacteria, viruses, prions), soluble and cell-surface-bound cluster of differentiation (CD) antigens (e.g., CD62L, CD95), or autoantigens (such as anti-dsDNA antibodies, anti-nuclear antibodies). In another embodiment, the analytes that are labelled with the detectable analyte-binding agent belong to two or more different classes of molecule.

The type of detectable analyte-binding agent used to label the analyte or analytes will depend on the chemistry of the analyte or analytes of interest. For example, a detectable analyte-binding agent which reacts with or binds to a particular moiety or chemical group will be capable of labelling all analytes in a biological sample which contain that moiety or chemical group. For example, if (as is preferred) the analytes of interest are proteins or peptides, the detectable analyte binding agent may be one which reacts with chemical groups that are commonly present in proteins, such as primary amine groups which are present in lysine side chains and the N-terminus of

peptide chains. Analytes containing carbohydrate or lipid residues may also be labelled using free amines if available (e.g. in glycoproteins or lipoproteins). Therefore, a detectable analyte-binding agent may be selected which is capable of binding to all analytes present in the biological sample which contain a free primary amine group. By "free" primary amine group, it will be understood that the primary amine is conformationally available for binding, and is not obscured, for instance by folding within the analyte or by external sugar units in glycoproteins or PEG units (for instance) such that it is protected from reacting with the detectable analyte binding agent.

It will be appreciated that a suitable detectable analyte-binding agent will be one which is capable of binding to a chemical or biological group, substituent or recognition site on the analyte(s) of interest. For example, a detectable analyte-binding agent may be selected which is capable of binding to all analytes present in the biological sample which contain a free carboxyl group, a free sulfhydryl (–SH) group or a free hydroxyl (–OH) group.

In some embodiments, the method optionally comprises the further step of modifying the sample such that one or more of the analytes present therein are biologically, chemically or physically modified, prior to contacting the sample with the detectable analyte binding agent. For example, the step of modifying the sample may comprise treating the sample with a modifying agent, such as an oxidising or a reducing agent. In some embodiments it may be necessary to modify the sample to enable the detectable analyte binding agent to bind to the analyte(s) of interest. For example, some detectable analyte-binding agents that target carboxyl groups do not themselves have a carboxyl-reactive moiety, but instead rely on the introduction of carbodiimide crosslinker (e.g., 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)) into the reaction; this binds a primary amine on the label to the carboxyl group on the target analyte. The sample may be treated with a suitable oxidising agent in order to oxidise hydroxyl groups (e.g. of glycosylation groups) on the target analyte(s) so as to produce a reactive aldehyde, and/or the sample may be treated with a suitable reducing agent so as to reduce sulfhydryl groups.

Thus, a further advantage of the method is that it provides a reagent in which a large number of analytes may be labelled by a detectable analyte binding agent.

The current state of the art dictates that to create a reagent for a diagnostic assay containing, for example, five different labelled analytes, each analyte would need to be obtained in a purified form, separately labelled, and then all of the labelled analytes would have to be mixed together to create a panel. The resulting panel is then limited to use in assays which measure or detect only those five analytes. Conversely, the method of the present invention provides a reagent in which a plurality of analytes may be labelled, without prior separation of the analytes. This leaves the analytes in their native form. The resulting reagent comprising the labelled analytes can be used in a wide range of assays.

In a further embodiment, the method comprises contacting the sample of biological fluid with further detectable analyte-binding agents, e.g. two or more detectable analyte-binding agents. Each of the detectable analyte-binding agents may label a different class of analyte molecule by binding to a different moiety or chemical group. For example, all protein/peptide analytes may be labelled by one detectable analyte-binding agent while all lipid analytes may be labelled by a different detectable analyte-binding agent.

In an embodiment, the sample is contacted with an excess of the detectable analyte-binding agent(s). This ensures that all analytes which are capable of being labelled by the detectable analyte-binding agent(s) are so labelled, such that no unlabelled analytes remain. This is important since the presence of unlabelled analytes in the reagent could reduce the accuracy of assays performed using the biological reagent.

The detectable analyte-binding agent(s) may label the analyte or analytes by virtue of covalent or non-covalent interactions. In an embodiment, the detectable analyte-binding agent(s) binds to the analyte or analytes by covalent interactions.

The biological fluid may be blood plasma, blood serum, whole blood, urine, saliva, cell aspirate from bone marrow or other body sites, cerebrospinal fluid, oedema aspirate, mucous, aqueous humour or any other such biological fluid. The sample may have been obtained from a human or other mammal or animal body. If the diagnostic assay is for detecting the presence of, or determining the concentration of, an analyte in a human subject, it is preferred that the biological sample for producing the biological reagent is also obtained from a human. Similarly, if the diagnostic assay is for use in relation to an animal subject, it is preferred that the biological

sample for producing the biological reagent is obtained from an animal of the same species as the subject.

5 The sample may have been obtained from a single human or animal. Alternatively, the method may comprise mixing two or more samples of biological fluid, each having been obtained from a different individual, to provide a sample pool and adding the detectable analyte-binding agent directly to the sample pool. The pooling of samples is advantageous where a particular mixture of labelled analytes is desired but not all analytes are present in the biological fluid of a single human or animal. For
10 example, to create a panel containing human antibodies against common allergens, it would be necessary to pool samples from humans with different known allergies, to ensure that as many different antibodies against common allergens are encompassed in the biological reagent i.e., it is unlikely that one individual would have all common allergies, and hence it would be more beneficial to pool samples
15 from a range of individuals.

In some instances the sample of biological fluid may have been obtained from a human or an animal that is known to have high levels of a particular analyte or analytes of interest.

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The following paragraphs relating to the detectable analyte binding agent(s) apply to all aspects of the present invention, as appropriate.

25 The detectable analyte-binding agent is an agent which is capable of binding to one or more analytes in the sample of biological fluid. The detectable analyte-binding agent may itself comprise a detection moiety that produces a signal (e.g. a fluorescent signal) which can be detected. Alternatively, the detectable analyte-binding agent may itself not provide a detectable signal, but instead may be capable of binding to a marker that produces a detectable signal. Hence, the detectable
30 analyte-binding agent may be directly detectable, or it may be indirectly detectable.

The marker may comprise a single moiety which binds to the detectable analyte-binding agent and which also produces a detectable signal. Alternatively, the marker may comprise a binding moiety, for binding to the detectable analyte-binding agent,
35 linked or conjugated to a detection moiety or probe which produces a detectable signal.

The detection moiety (which may form part of the detectable analyte binding agent or part of the marker) may comprise a fluorescent moiety, a luminescent moiety, a bioluminescent moiety, a radioactive material, a colorimetric moiety, a nanoparticle having suitable detectable properties, or a chromogenic moiety. Suitable fluorescent moieties include fluorescent proteins (such as phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP) and allophycocyanin (APC)) fluorescent dyes (such as Fluorescein Isothiocyanate (FITC), rhodamines (Rs) and cyanines (Cys)), fluorescent tandem complexes (such as Allophycocyanin-Cyanine 7 (APC-Cy7), Peridinin-Chlorophyll-Protein complex-Cyanine 5 (PerCP-cy5) and Phycoerythrin-Texas Red (PE-TexasRed)), and nanocrystals (such as QDot 525, QDot 545 and QDot 625).

The detectable analyte-binding agent may comprise a prosthetic group. A "prosthetic group" will be understood by those skilled in the art as being a co-factor which is tightly bound to proteins or other macromolecules that enables indirect signal quantitation. For example, the prosthetic group may be biotin (also known as vitamin H or vitamin B₇) or iminobiotin (guanido analog of biotin) that can later be used to bind a marker comprising a streptavidin or avidin binding moiety conjugated to a detection moiety. In some embodiments, the detectable analyte-binding agent additionally comprises a co-factor, which is a chemical group that enable the prosthetic group to bind to the target analyte(s). The co-factor may be N-hydroxysuccinimide.

The detectable analyte-binding agent may comprise a photoactivatable moiety. Such a moiety enables binding of the detectable analyte-binding agent to one or more analytes in the sample of biological fluid upon activation with light. For example, the detectable analyte-binding agent may comprise photobiotin (e.g. photobiotin acetate), which is a derivative of biotin comprising biotin, a linker and a photoactivatable nitrophenyl azide group).

In some embodiments, the detectable analyte-binding agent comprises N-hydroxysuccinimide biotin (NHS-biotin), which labels (i.e. binds to) the free primary amine groups of the analytes present in the biological fluid to provide biotinylated analytes. In these embodiments the detectable analyte-binding agent does not comprise a detection moiety. Labelling analytes using biotin or NHS-biotin (also referred to as biotinylation) is particularly advantageous because it is a fast and specific process that is unlikely to affect the function of the analyte due to its small

size (~244 Da). The present inventors have surprisingly discovered that the addition of NHS-biotin to a biological fluid comprising a mixture of analytes results in the efficient labelling of all analytes containing free primary amine groups present in that fluid. This allows the detection of analytes in their native form, without having to
5 isolate and separately label all peptides and proteins of interest in the biological fluid sample. The use of an NHS-biotin to label analytes is particularly advantageous because it can easily be detected by its reaction with a marker comprising avidin or streptavidin (i.e. the binding moiety) conjugated to a detection moiety (e.g. a fluorescent probe) which can be detected by known techniques.

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In some embodiments, the detectable analyte-binding agent comprises N-hydroxysuccinimide fluorescein, which labels (i.e. binds to) the free primary amine groups of the analytes present in the biological fluid to provide fluorescein labelled analytes. Labelling analytes using a fluorescein-containing binding agent is
15 advantageous because it can easily be detected by known fluorescence techniques and it avoids the need for additional markers. Thus, in this embodiment the detectable analyte-binding agent itself comprises a detectable moiety.

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The detectable analyte-binding agent may be dissolved in a solvent prior to its addition to the sample of biological fluid. The solvent will depend on the chemical nature of the detectable analyte-binding agent. Suitable solvents may include dimethyl sulfoxide DMSO, dimethyl formamide (DMF), methylsulfonylmethane (MSM), dimethyl sulfite, dimethyl sulfide, or dimethyl sulfate.

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In some embodiments, the step of effecting labelling of one or more of the analytes in the sample by the detectable analyte-binding agent is carried out by simply mixing the sample of fluid with the agent. It is preferred, however, that the sample is incubated after the addition of the detectable analyte-binding agent. The mixture of the sample and the detectable analyte-binding agent may be incubated for a period
30 of time which is sufficient to allow the marker to bind to all of the analyte molecules which the detectable analyte-binding agent is capable of labelling. The period of time may be from 1 second to 4 hours, from 30 seconds minutes to 3 hours, from 1 minutes to 2 hours, from 10 minutes to 1.5 hours or from 30 minutes to 1 hour. In a particular embodiment, the mixture is incubated for a period of approximately 1 hour.

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The mixture of the sample and the detectable analyte-binding agent may be incubated at a temperature which facilitates the labelling of the analyte(s) by the

detectable analyte-binding agent. In some embodiments the mixture is incubated at a temperature of from 4 °C to 45 °C, from 10 °C to 40 °C or from 15 °C to 37 °C.

During incubation, the mixture of the sample and the detectable analyte-binding agent may be stirred, agitated or rotated (for example on a rotator wheel) to assist adequate mixing and efficient labelling of the analytes.

In some embodiments, the step of effecting labelling of one or more of the analytes in the sample by the detectable analyte-binding agent is carried out by activation of the detectable analyte-binding agent. In some embodiments wherein the detectable analyte-binding agent comprises a photoactivatable moiety, the detectable analyte-binding agent is activated by irradiation with light. The detectable analyte binding agent may be activated by UV light having a wavelength of from 40 to 400 nm, or from 250 to 350 nm. For example, the detectable analyte-binding agent may comprise photobiotin or photobiotin acetate, which is activated by irradiation with light having a wavelength of from 260 to 475 nm.

In an embodiment, the method comprises an additional step of adjusting the pH of the sample. This may be important to prevent degradation of the analytes and/or the detectable analyte-binding agent in the mixture. The pH of the sample may be adjusted to a pH of from 6 to 9, from 6.5 to 8.5 or from 7 to 8. The pH of the sample may be adjusted before or after the addition of the detectable analyte-binding agent, preferably before.

In an embodiment, the method comprises a further step of separating the analyte(s) which are labelled with the detectable analyte-binding agent from any unbound detectable analyte-binding agent. The separation may be achieved by passing the mixture of the sample and detectable analyte-binding agent through a buffer exchange column or by using size-exclusion chromatography. This process may also be used to separate labelled analytes from any unlabelled analytes, since the labelled analytes are heavier and thus elute from the column first. The unlabelled analytes are light and are not eluted, remaining in the column. Other methods of separating the labelled analytes from unbound detectable analyte-binding agent would be known to those skilled in the art.

According to a second aspect of the present invention, there is provided a biological reagent for use in a diagnostic assay to determine the presence or concentration of

at least one target analyte in a test sample, said reagent comprising a biological fluid comprising a mixture of analytes present at substantially the same relative concentrations as when sampled, wherein said mixture of analytes comprises said at least one target analyte labelled with a detectable analyte-binding agent.

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In an embodiment, the mixture of analytes comprises a plurality of target analytes labelled with the detectable analyte-binding agent. By virtue of labelling a plurality of analytes of potential interest in the biological fluid, the biological reagent of the invention can be used to measure or detect a panel of analytes in a test sample

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using a multiplexed competitive inhibition assay.

The analytes are advantageously present in the biological reagent at the relative concentrations at which they exist in the body at or immediately prior to the moment of sampling. The majority of analytes may be present at their natural physiological

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concentrations, which advantageously allows the measurement of different analytes with up to approximately 1000-fold concentration variation.

By the mixture of analytes being present in the biological sample at substantially the same relative concentrations as when sampled, it will be understood that there is no

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significant difference between the relative concentration of the analytes in the biological fluid at the moment of sampling and in the resulting reagent. However, it will be understood that the absolute concentration of the analytes may change as a result of the addition of the detectable analyte-binding agent (which may be in solution) to the biological fluid. It will also be understood that the concentration of

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some analytes may be elevated (artificially or naturally) prior to sampling, for example by the administration of a vaccine to the individual from which the biological fluid is sampled, or by the exposure of that individual to a particular antigen, or due to disease pathophysiology.

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The analytes that are labelled by the detectable analyte binding agent may belong to the class of proteins, peptides/polypeptides, glycoproteins, carbohydrates, lipids, lipoproteins, glycolipids, phospholipids, nucleic acids, antigens, autoantigens or steroids, or a combination thereof. Thus, all analytes that are labelled by the detectable analyte binding agent may belong to a single class of molecules, or they

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may belong to two or more different classes of molecules.

The mixture of analytes in the reagent may be the same as the mixture of analytes in the test sample. Alternatively, the mixture of analytes in the reagent may not be the same as the mixture of analytes in the test sample, for example if the reagent and the test sample may have been derived from different individuals.

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In an embodiment, each target analyte is labelled with the same detectable analyte-binding agent. In an alternative embodiment, the target analytes are labelled with two or more different detectable analyte-binding agents. For example, target analytes belonging to different classes of molecules may be labelled with different detectable analyte-binding agents.

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It will be appreciated that the biological reagent described herein can be used in a method for detecting the presence of and/or determining the concentration of one or more target analytes of a mixture of analytes in a sample of biological fluid obtained from a subject.

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Thus, according to a third aspect of the present invention, there is provided a method for detecting the presence of and/or determining the concentration of a target analyte in a test sample, the method comprising the use of a biological reagent in accordance with the second aspect of the present invention.

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The method may comprise a competitive binding assay.

Also provided is a method for determining the presence of a target analyte in a test sample may comprise the steps of:

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mixing a biological reagent as defined herein, said biological reagent comprising the target analyte labelled with a detectable analyte-binding agent, with the test sample,

separately, mixing said biological reagent with a control sample which does not comprise the target analyte;

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exposing each mixture to a capture agent which is capable of specifically binding the target analyte;

removing, from each mixture, any labelled target analyte which has not bound to the capture agent, for instance by washing; and

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comparing the level of binding of the labelled target analyte to the capture agent in the presence of the test sample to the level of binding of the

labelled target analyte to the capture agent in the presence of the control sample.

5 It will be understood that a "test sample" is a sample of biological fluid that has been obtained from a human or animal subject in which the presence and/or concentration of an analyte or number of analytes is to be determined. The analytes in the test sample are not labelled with a detectable analyte binding agent.

10 Further provided is a method of determining the concentration of a target analyte in a test sample may comprise the steps of:

exposing the biological reagent, comprising the target analyte labelled with a detectable analyte-binding agent, to a capture agent which is capable of specifically binding the target analyte, in the presence of the test sample;

15 removing any labelled target analyte which has not bound to the capture agent, for instance by washing;

measuring the level of binding of the labelled analyte to a capture agent; and

comparing the measured level of binding to a reference in order to determine the concentration of the target analyte.

20 The level of binding of the labelled analyte to the capture agent is measured by detecting the amount of labelled analyte bound to the capture agent, by virtue of the detectable analyte-binding agent. The reference enables the level of binding of the labelled analyte to the capture agent to be correlated against a concentration of the target analyte. The reference may be a data set, for example a table or a calibration
25 curve or line. This may be generated by mixing the biological reagent with a number of control samples or 'standards', each standard containing a different and known concentration of unlabelled target analyte, measuring the level of binding of the labelled analyte to a capture agent in the presence of the unlabelled target analyte in each of the standards, and plotting the level of binding of the labelled target analyte
30 versus the concentration of the unlabelled target analyte. The number of standards used to generate the calibration curve may be at least 2, at least 4 or at least 6. The competitive binding of the standards to the capture agent in the presence of the labelled analyte may be analysed in duplicate or in triplicate to provide a more accurate calibration curve or line.

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In some embodiments, the capture agent is bound to a solid-phase surface, such as a bead or an array.

In some embodiments, the method comprises simultaneously detecting the presence of and/or determining the concentration of a plurality of target analytes in a test sample. The analysis of a plurality of analytes may be carried out using a multiplex system, wherein a plurality of capture agents is arranged in a grid or array, each capture agent being specific for a single target analyte.

A variety of different multiplexing systems are available and will be known to those skilled in the art, including LuminexTM microbeads or aluminium based particles, such as the UltraPlexTM system from PronosticsTM. In such systems the monoclonal antibodies that bind to the analytes are attached to support materials that are distinguishable from each other. Other multiplexing systems protein arrays or microarrays, where different capturing agents may be spotted or fixed at certain positions of either a well, chip or slide.

The capture agent may be a monoclonal or a polyclonal antibody. Alternatively, the capture agent may include any natural or synthetic molecule that either replicates the paratope of the relevant capture antibody, or otherwise provides the necessary structure to enable binding to the target analyte. Examples of synthetic capture agents include 2D Molecular Imprinting Multimarker System.

If the target analyte(s) is an antibody (immunoglobulin), the capture agent may be an autoantigen or an antigen.

The antigen may be derived from a pathogen, e.g. from a bacterium, a virus, a prion, a parasite or a fungus. Detecting the level of antibodies in a test sample to certain antigens can be used to measure humoral immunity. An unlabelled target antibody in a test sample will only compete with a corresponding labelled analyte (in the biological reagent of the invention) for a capture agent in a competitive binding assay if the unlabelled antibody is of sufficiently high affinity to compete, and thus is of clinical significance. The method of the present invention therefore advantageously allows the detection of clinically significant antibodies only and would not detect insignificant low-affinity antibodies, in contrast to known assays such as ELISA. Thus, use of the reagent and methods of the present invention allows disease causing antibodies to be distinguished from low affinity antibodies which are more prevalent within patients without disease.

Examples of autoantigens include anti-doubled-stranded DNA antibodies (anti-dsDNAs), anti-nuclear antibodies (ANAs), anti-transglutaminase antibodies (ATAs), anti-neutrophil cytoplasmic antibodies (ANCAs), or extractable nuclear antigens (ENAs). The detection and quantification of antibodies to autoantigens has application in the diagnosis of autoimmune diseases.

In an embodiment, the capture agent is labelled with an element which generates a signal. The signal may be visual, audio, kinetic, radioactive or colorimetric. For example, the capture agent may be labelled with a fluorochrome.

The method may comprise the additional steps of adding a marker which is capable of binding to the detectable analyte-binding agent of the labelled analytes which have been bound by the capture agent, washing to remove any unbound molecules of the marker and detecting the presence of and/or determining the quantity of the marker.

A marker may be used when the detectable analyte binding agent itself does not provide a signal which can be detected. In an embodiment, the detectable analyte-binding agent comprises NHS-biotin. In this embodiment, the marker comprises a streptavidin or avidin binding moiety. The streptavidin or avidin moiety may be conjugated to any suitable detection moiety or probe that can be detected. Suitable detection moieties include horseradish peroxidase and fluorochromes (such as fluorescein phycoerythrin).

According to a fourth aspect of the present invention, there is provided a use of a biological reagent in accordance with the second aspect of the invention for detecting the presence of and/or determining the concentration of an analyte in a biological fluid.

Brief description of the drawings

Embodiments of the invention will now be described with reference to the following Figures in which:

Figure 1a shows the Kappa light chain concentration of 62 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding Site);

Figure 1b shows the Lambda light chain concentration of 62 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding Site);

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Figure 2a shows the IgG levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available IgG kit (COBAS, Roche);

10 Figure 2b shows the IgA levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available IgA kit (COBAS, Roche);

15 Figure 2c shows the IgM levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available IgM kit (COBRAS, Roche);

20 Figure 3a shows the Kappa light chain concentration of 20 human serum samples and 10 samples from multiple myeloma patients with elevated kappa light chains, measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding Site);

25 Figure 3b shows the Lambda light chain concentration of 20 human serum samples and 10 samples from multiple myeloma patients with elevated lambda light chains, measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding Site);

30 Figure 3c shows the kappa free light chain standard curve generated in accordance with an embodiment of the present invention; and

Figure 3d shows the lambda free light chain standard curve generated in accordance with an embodiment of the present invention.

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Detailed description

A biological reagent in accordance with an embodiment of the present invention is prepared as follows. Human blood is obtained by phlebotomy and stored in tubes with clot activators. The blood is centrifuged to separate the serum, which constitutes
5 a sample of a biological fluid. If necessary, the serum is stored at -20°C or -80°C until required.

A detectable analyte binding agent in the form of biotin-NHS (for indirect assay measurement) or fluorescein-NHS (for direct assay measurement) is provided. If
10 necessary, this is equilibrated to room temperature prior to use. The biotin-NHS or fluorescein-NHS is dissolved in dimethyl sulfoxide (DMSO) to provide a solution equating to 10 mg/ml. An excess of this solution is then added to an aliquot of human serum so that all proteins in the serum may be biotinylated. The mixture is incubated with continuous rotation to encourage binding of all available serum proteins
15 (analytes) to the biotin-NHS. The biotinylated serum is then passed down a sepharose bead based buffer exchange column (e.g. Sephadex G-25 DNA grade column) in order to separate the biotinylated proteins from free biotin-NHS. The biotinylated serum is eluted from the column using phosphate buffered saline to provide a biological reagent which is ready to use in a competitive inhibition assay to
20 detect and/or quantitate the level of one or more protein analytes of interest in one or more serum test samples. The reagent contains a complex mixture of labelled proteins at their normal relative and predefined serum concentrations.

To determine the concentration of a target analyte in a test serum sample obtained
25 from a subject, the reagent (which contains the labelled version of the target analyte) is mixed with the test sample (which contains the unlabelled analyte). A capture agent in the form of an antibody specific for the target analyte, and conjugated to micropsheres, is then added to the mixture. The unlabelled target analyte in the test sample will compete with the corresponding labelled (i.e. biotinylated or fluorescein
30 labelled) analyte in the reagent for the antibody binding site. The mixture is incubated and then wash steps are carried out to remove any labelled analytes which have not bound to the antibody molecules.

In the indirect assay, after washing, a marker in the form of streptavidin R-
35 phycoerythrin conjugate (SA-PE) is added to the mixture and incubated. The streptavidin binding moiety of the marker binds to the biotin label of any biotinylated target analytes which have been captured by the antibodies. The R-phycoerythrin

portion of the marker is the detectable moiety and fluoresces light in the red region of the spectrum. A further washing step is then carried out to remove any unbound SA-PE. The mixture is then analysed and the intensity of the red light produced by the R-phycoerythrin is measured. The intensity of the light is proportional to the level of biotinylated analytes bound to the antibodies. The greater the intensity of the light, the more labelled analytes have bound to the antibodies and the lower the concentration of the unlabelled analyte in the test sample. When the concentration of the unlabelled target analyte in the test sample is high, it out-competes the biotinylated analytes for the antibody binding sites. The SA-PE cannot bind to the unlabelled antibody-bound target analytes, and thus the intensity of the light is reduced.

Alternatively, in a direct assay, after the first series of washes, the mixture is then analysed and the intensity of the green light produced by the fluorescein is measured. The intensity of the light is proportional to the level of fluorescein labelled analytes bound to the antibodies. The greater the intensity of the light, the more labelled analytes have bound to the antibodies and the lower the concentration of the unlabelled analyte in the test sample. When the concentration of the unlabelled target analyte in the test sample is high, it out-competes the fluorescein labelled analytes for the antibody binding sites and thus the intensity of the light is reduced.

To quantify the level of target analyte in the test sample, the intensity of the light measured must be compared to a calibration curve. The calibration curve is generated by mixing the biological reagent (containing labelled target analyte) with a number of control samples, each containing a different known concentration of unlabelled target analyte. The light intensity produced using each control sample is detected as described above, and the measured light intensity is plotted against the analyte concentration to generate a calibration curve.

If quantification of the target analyte is not required, the presence of a target analyte in a test sample can be determined merely by detecting whether the light intensity generated by the reagent in the presence of the test sample is less than that generated by the reagent in the presence of a control sample which does not contain the target analyte. A reduction in light intensity produced by the test sample compared to the control sample indicates the presence of the target analyte in the test sample.

ExamplesExample 1A: Labelling of proteins in human serum with NHS-Biotin.

- 5 This method describes the reagents and procedure for labelling proteins in human serum with NHS-biotin to create a serum reagent in accordance with an embodiment of the present invention.

Reagents required:

- 10 • 1 M sodium bicarbonate buffer:
21 g sodium bicarbonate (sodium hydrogen carbonate NaHCO_3 ; Serva, electrophoresis research grade #30180) was dissolved in 200 ml distilled water with Teflon-coated magnetic stirrers. The pH of the solution was adjusted to pH 8.3 by addition of 1M sodium hydroxide.
- 15 • Dimethyl sulfoxide (DMSO), minimum 95% GC (Sigma. Product # D5879).
• Human serum:
Whole blood was obtained by phlebotomy and stored in tubes with clot activators. The blood was centrifuged for 5 minutes at 3,500 rpm, room temperature. The serum was removed using a sterile Pasteur pipette and stored at -20°C until required
- 20 for assaying.
- Biotin amidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (biotin NHS), minimum 95% TLC (Sigma. Product # B3295).

Procedure:

- 25 1. Biotin-NHS was removed from the freezer and equilibrated to room temperature.
2. 400 μl aliquots of human serum were equilibrated to room temperature.
3. A 10 mg/ml solution of biotin-NHS in DMSO was prepared.
4. 50 μl of the biotin-NHS-DMSO solution was added to each microtube containing a 400 μl aliquot of serum.
- 30 5. Each microtube was incubated for 1 hour at room temperature with continuous rotation on a rotator wheel to encourage mixing of all available serum proteins with Biotin-NHS.
6. After 1 hour, the tubes were removed from the rotator wheel and a buffer exchange was prepared using sepharose bead based buffer exchange columns;
- 35 GE Healthcare NAPTM 5 Columns (Sephadex G-25 DNA grade).

7. Prior to commencing, it was ensured that the NAP-5 columns had equilibrated to ambient temperature. The column was supported over a universal tube. The top and bottom caps were removed from the NAP-5 column and the excess liquid was allowed to flow through the column by gravity flow. The gel was equilibrated with approximately 10 ml of phosphate buffered saline (PBS) equilibration buffer. Note: this volume corresponds to 3 complete refills of the column. The equilibration buffer was allowed to completely enter the gel bed by gravity flow. Positive pressure was not applied.

8. The sample was added to the column (total volume to be added= 490 µl) and allowed to enter the gel bed completely.

9. An appropriate sized collection tube for sample collection was placed under the column. 1 ml of PBS was added to elute sample from the column and an approximate sample fraction size of 1 ml was collected.

10. This provided a reagent containing serum proteins, at their natural and normal relative concentrations, labelled with biotin (biotinylated serum). The biotinylated serum reagent was ready to be used in a competitive inhibition assay to quantitate unknown levels of protein in serum samples against a calibration curve with known protein concentration.

Example 1B: Utilisation of a reagent comprising an NHS-biotin detectable analyte binding agent in a two-step multi-plex assay to quantify free light chain levels (FLC) in human sera

This example describes the application of the reagent in a competitive inhibition multi-plex bead assay to measure levels of two different proteins in the sera of 62 human patients (test samples).

The biotinylated serum reagent was prepared as described above. Normal serum has naturally occurring low levels of kappa and lambda light chains (3-25mg/L), but serum was obtained from multiple myeloma patients who had high levels of IgA, IgG, IgM, free kappa and free lambda were mixed together and biotinylated using the methods as described above. The final concentration of the mixed serum prior to biotinylation was: free kappa: 384.6 mg/L; free lambda: 296.92 mg/L; IgA: 2.04 g/L; IgM: 3.07 g/L; IgG: 36.83 g/L.

Standard samples (containing a known concentration of unlabelled FLCs), control samples {containing different levels of FLC spanning the normal reference range for

serum FLC) and patient test samples were added to a 96-well filter plate. To this, the biotinylated serum reagent was added. This reagent contained labelled proteins including labelled FLC proteins that could compete against the unlabelled FLC proteins contained in the patient test samples, as well as the standards which contain
5 a known amount of free-kappa and free-lambda light chains. To each well, anti-kappa and anti-lambda FLC monoclonal antibodies (mAbs) bound to 5.5 μ m microspheres (Bio-Rad) were then added. The plate was incubated for 30 minutes, followed by a series of wash steps whereby 200 μ L of wash buffer was added to each well, incubated for 30 seconds and then aspirated using a vacuum manifold..

10 After washing, 100 μ L of wash buffer containing 0.2% streptavidin R-phycoerythrin conjugate (SA-PE, Molecular Probes) was added to each well and incubated in the dark on a plate shaker at room temperature for 30 minutes. Washing was repeated as described above. After washing, the microspheres were analysed on a Luminex machine. The machine measured the intensity of the red light refracted by each of
15 the microspheres (the intensity from any one microsphere is proportional to the amount of biotinylated FLC bound to the mAb on the microsphere). The machine computed the mean intensity of the light and related it to a code incorporated into the microspheres.

To validate the results from the above experiment, the 62 human patient test
20 samples were also analysed using a commercially available assay for measuring free-kappa and free-lambda light chains (Freelite, Binding Site) using a Roche Hitachi Modular Analyzer. The results obtained from both methods are depicted and correlated in Figures 1a and 1b.

25 Figure 1a shows the Kappa light chain concentration of 62 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding Site). A positive linear correlation is found between the results using the two separate methods. Kappa light chains within the normal reference range are
30 detected, as well as abnormally high malignant levels.

Figure 1b shows the Lambda light chain concentration of 62 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available assay (Freelite, Binding
35 Site). A positive linear correlation is found between the results using the two

separate methods. Lambda light chains within the normal reference range are detected, as well as abnormally high malignant levels.

5 Example 1C: Utilisation of a reagent comprising an NHS-biotin detectable analyte binding agent in a two-step multiplex assay to quantify total antibody levels in human sera

10 This example describes the application of the universal reagent in a competitive inhibition multiplex bead assay to measure levels of three different proteins in the sera of 54 human patients.

Levels of Immunoglobulin- α (IgA), Immunoglobulin- γ (IgG) and Immunoglobulin- μ (IgM) antibody molecules were quantified using a method similar to that described in Example 1b.

15 In this example, beads conjugated to anti-human IgG, anti-human IgA and anti-human IgM antibodies were used as the capture agent. All other reagents used were the same as in Example 1b. The universal labelled (NHS-Biotin) serum reagent was again used to determine the amount of inhibition caused by each of the test samples.

20 To validate the results from the above experiment, the 54 human patient samples were also analysed using a commercially available assay for measuring levels of human IgA, IgG and IgM (COBAS, Roche) using a Roche Hitachi Modular Analyzer. Results obtained from both methods are depicted and correlated in Figures 2a to 2c.

25 Figure 2a shows the IgG levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available IgG kit (COBAS, Roche). A positive linear correlation is found between the results using the two separate methods. IgG levels
30 within the normal reference range are detected, as well as abnormally high malignant levels.

Figure 2b shows the IgA levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained
35 using a commercially available IgA kit (COBAS, Roche). A positive linear correlation is found between the results using the two separate methods. IgA levels within the normal reference range are detected, as well as abnormally high malignant levels.

Figure 2c shows the IgM levels of 54 human serum samples measured in accordance with an embodiment of the present invention, compared with the results obtained using a commercially available IgM kit (COBAS, Roche). A positive linear correlation is found between the results using the two separate methods. IgM levels within the normal reference range are detected, as well as abnormally high malignant levels.

These results indicated that the biotinylation method accurately and reliably labelled proteins at a high concentration (~90 g/L) down to very low concentrations (<0.1 g/L). The results also indicated that the method showed similar specificity to that of the commercially available product.

Example 2A: Labelling of proteins in human serum with NHS-Fluorescein.

This method describes the reagents and procedure for labelling proteins in human serum with NHS-fluorescein to create a serum reagent in accordance with an embodiment of the present invention. As opposed to Example 1, where a two-step assay is used to quantify analyte(s) of interest, this approach utilises a one-step approach, offering improved speed whilst maintaining the accuracy of the test.

Reagents required:

- 1 M sodium bicarbonate buffer:
21 g sodium bicarbonate (sodium hydrogen carbonate NaHCO_3 ; Serva, electrophoresis research grade #30180) was dissolved in 200 ml distilled water with Teflon-coated magnetic stirrers. The pH of the solution was adjusted to pH 8.3 by addition of 1M sodium hydroxide.
- Dimethyl sulfoxide (DMSO), minimum 95% GC (Sigma. Product # D5879).
- Human serum:
30 Whole blood was obtained by phlebotomy and stored in tubes with clot activators. The blood was centrifuged for 5 minutes at 3,500 rpm, room temperature. The serum was removed using a sterile Pasteur pipette and stored at -20°C until required for assaying.
- 5/6-carboxyfluorescein succinimidyl ester
35 • (Fluorescein-NHS), minimum 90% purity by HPLC (Fisher Scientific #46410).

Procedure:

- Fluorescein-NHS was removed from the freezer and equilibrated to room temperature.
- 400 µl aliquots of human serum were equilibrated to room temperature.
- 5 • A 10 mg/ml solution of Fluorescein-NHS in DMSO was prepared.
- 60 µl of the fluorescein-NHS-DMSO solution was added to each microtube containing a 400 µl aliquot of serum.
- Each microtube was incubated for 1 hour at room temperature with continuous rotation on a rotator wheel to encourage mixing of all available serum proteins with Fluorescein-NHS.
- 10 • After 1 hour, the tubes were removed from the rotator wheel and a buffer exchange was prepared using sepharose bead based buffer exchange columns; GE Healthcare NAPTM 5 Columns (Sephadex G-25 DNA grade).
- Prior to commencing, it was ensured that the NAP-5 columns had equilibrated to ambient temperature. The column was supported over a universal tube. The top and bottom caps were removed from the NAP-5 column and the excess liquid was allowed to flow through the column by gravity flow. The gel was equilibrated with approximately 10 ml of phosphate buffered saline (PBS) equilibration buffer. Note: this volume corresponds to 3 complete refills of the column. The equilibration buffer was allowed to completely enter the gel bed by gravity flow.
- 15 • Positive pressure was not applied.
- The sample was added to the column (total volume to be added= 490 µl) and allowed to enter the gel bed completely.
- An appropriate sized collection tube for sample collection was placed under the column. 1 ml of PBS was added to elute sample from the column and an approximate sample fraction size of 1 ml was collected.
- 25 • This provided a reagent containing serum proteins, at their natural and normal relative concentrations, labelled with fluorescein (fluorescein labelled serum). The fluorescein labelled serum reagent was ready to be used in a competitive inhibition assay to quantitate unknown levels of protein in serum samples against a calibration curve with known protein concentration.
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Example 2B: Utilisation of a reagent comprising a NHS-fluorescein detectable analyte binding agent in a one-step multi-plex assay to quantify free light chain levels (FLC) in human sera

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This example describes the application of the reagent in a competitive inhibition multi-plex bead assay to measure levels of two different proteins in the sera of 20 normal human patients and 20 multiple myeloma patients (test samples).

- 5 The fluorescein labelled serum reagent was prepared as described above. Serum was obtained from a human patient with acute renal failure with elevated polyclonal immunoglobulins, including elevated polyclonal free kappa and free lambda light chains. The concentration of free kappa and lambda light chains in the patient serum prior to fluorescein labelling was: free kappa: 428 mg/L; free lambda: 351 mg/L
- 10 quantified using Freelite (The Binding Site, UK).

- Standard samples (containing a known concentration of unlabelled FLCs), control samples (containing different levels of FLC spanning the normal reference range for serum FLC) and patient test samples were added to a 96-well filter plate. To this, the fluorescein labelled serum reagent was added at a 1 in 200 dilution (40µL in 3960µL
- 15 PBS buffer containing 1% bovine serum albumin). This reagent contained labelled proteins including labelled FLC proteins that could compete against the unlabelled FLC proteins contained in the patient test samples, as well as the standards which contain a known amount of free-kappa and free-lambda light chains. To each well, anti-kappa and anti-lambda FLC monoclonal antibodies (mAbs) bound to 5.5 µm
- 20 microspheres (Bio-Rad) were then added. The plate was incubated for 30 minutes, followed by a series of wash steps whereby 200 µL of wash buffer was added to each well, incubated for 30 seconds and then aspirated using a vacuum manifold.. After washing, the microspheres were re-suspended in 120µL PBS and analysed on a BD FACS Canto II Flow Cytometer.
- 25 Beads were differentiated from extracellular material using a a dot-plot with axes dedicated to forward scatter versus side-scatter.

- Beads regions were addressed using a red laser (wavelength 633nm) with band-pass filters set at 780/60 (APC-Cy7) and 660/20 (APC). A minimum of 200 beads per bead region were counted. Fluorescein signal for each bead was detected using a
- 30 blue laser (wavelength 488nm) with a band-pass filter at 530/30. The light emission intensity at this wavelength, from any one microsphere, is directly proportional to the amount of fluorescein labelled FLC bound to the mAb on the microsphere. The flow cytometer (BD FACS Diva Software) then computes the median fluorescence intensity of the light emission. This data is transferred to curve-fitting software for

four parameter logistical regression analyses (ReaderFit, MiraiBio) where the median fluorescence intensities are converted to FLC concentration (mg/L) using the known standards.

To validate the results from the above experiment, the 20 human patient test samples were also analysed using a commercially available assay for measuring free-kappa and free-lambda light chains (Freelite, Binding Site) using a Roche Hitachi Modular Analyzer. The results obtained from both methods are depicted and correlated in Figures 3a, 3b, 3c and 3d.

Figure 3a shows the free kappa light chain concentration of 20 human serum samples with normal levels of free light chains and 10 samples from multiple myeloma patients with elevated levels of kappa light chains, compared with the results obtained using a commercially available assay (Freelite, Binding Site). A positive linear correlation is found between the results using the two separate methods. Kappa light chains within the normal reference range are detected, as well as abnormally high malignant levels.

Figure 3b shows the free lambda light chain concentration of 20 human serum samples with normal levels of free light chains and 10 samples from multiple myeloma patients with elevated levels of lambda light chains, compared with the results obtained using a commercially available assay (Freelite, Binding Site). A positive linear correlation is found between the results using the two separate methods. Lambda light chains within the normal reference range are detected, as well as abnormally high malignant levels.

Figure 3c shows the kappa free light chain standard curve generated.

Figure 3d shows the lambda free light chain standard curve generated.

Claims

1. A method for preparing a biological reagent for use in a diagnostic assay, the method comprising:
 - 5 contacting a sample of a biological fluid, comprising a mixture of analytes, with a detectable analyte-binding agent; and
 - effecting labelling of one or more of the analytes in the sample by the detectable analyte-binding agent.
- 10 2. The method of claim 1, wherein the analytes are present in the sample of biological fluid at substantially the same relative concentrations as when sampled.
3. The method of claim 1, further comprising adding a quantity of one or more
15 analytes to the sample of biological fluid prior to, or after, the addition of the detectable analyte-binding agent.
4. The method of any one of claims 1 to 3, wherein labelling of a plurality of analytes by the detectable analyte-binding agent is effected.
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5. The method of claim 4, wherein each of the plurality of analytes belongs to the same class of molecule.
6. The method of claim 4, wherein the analytes belong to two or more different
25 classes of molecule.
7. The method of any preceding claim, comprising contacting the sample with two or more detectable analyte-binding agents.
- 30 8. The method of claim 7, wherein each of the detectable analyte-binding agents labels a different class of analyte molecule.
9. The method of any preceding claim, wherein an excess of the detectable analyte-binding agent is added to the sample.
- 35 10. The method of any preceding claim, comprising the further step of modifying the sample such that one or more of the analytes present therein are biologically,

chemically or physically modified, prior to contacting the sample with the detectable analyte-binding agent.

- 5 11. The method of any preceding claim, wherein the biological fluid is blood plasma, blood serum, whole blood, urine, saliva, cell aspirate from bone marrow or other body sites, cerebrospinal fluid, oedema aspirate, mucous or aqueous humour.
- 10 12. The method of any preceding claim, wherein the sample has been obtained from a human or an animal that is known to have high levels of a particular analyte or analytes of interest.
13. The method of any preceding claim, wherein the sample is obtained from a single human or other mammal or animal.
- 15 14. The method of any one of claims 1 to 12, wherein the method comprises providing two or more samples, each having been obtained from a different individual, mixing said samples to provide a sample pool and adding the detectable analyte-binding agent directly to the sample pool.
- 20 15. The method of any preceding claim, wherein the detectable analyte-binding agent comprises a detection moiety which produces a detectable signal.
- 25 16. The method of any one of claims 1 to 14, wherein the detectable analyte-binding agent is capable of binding to a marker comprising a detection moiety which produces a detectable signal.
- 30 17. The method of claim 15 or claim 16, wherein the detection moiety is a fluorescent moiety, a luminescent moiety, a bioluminescent moiety, a radioactive material, a colorimetric moiety, a nanoparticle or a chromogenic moiety.
18. The method of claim 16, wherein the detectable analyte-binding agent comprises a prosthetic group.
- 35 19. The method of claim 18, wherein the prosthetic group is selected from the group consisting of streptavidin, avidin, biotin or iminobiotin.

20. The method of any preceding claim, wherein the step of effecting labelling of the one or more analytes by the detectable analyte-binding agent is carried out by incubating the sample for a period of time which is sufficient for the detectable analyte-binding agent to label all of the analyte molecules which the detectable analyte-binding agent is capable of binding.
21. The method of any one of claims 1 to 19, wherein the detectable analyte-binding agent comprises a photoactivatable moiety and the step of effecting labelling of the one or more analytes by the detectable analyte-binding agent is carried out by irradiating the sample with light.
22. A biological reagent for use in a diagnostic assay to determine the presence or concentration of at least one target analyte in a test sample, said reagent comprising a biological fluid, comprising a mixture of analytes present at substantially the same relative concentrations as when sampled, wherein said mixture of analytes comprises said at least one target analyte labelled with a detectable analyte-binding agent.
23. The biological reagent of claim 22, wherein the mixture of analytes comprises a plurality of target analytes, each target analyte labelled with a detectable analyte-binding agent.
24. The biological reagent of claim 22 or 23, wherein the analytes that are labelled by the detectable analyte-binding agent belong to the class of proteins, peptides/polypeptides, glycoproteins, carbohydrates, lipids, lipoproteins, glycolipids, phospholipids, nucleic acids, antigens, autoantigens or steroids, or a combination thereof.
25. The biological reagent of claim 23 or claim 24, wherein each of said plurality of target analytes is labelled with the same detectable analyte-binding agent.
26. The biological reagent of claim 23 or 24, wherein the target analytes are labelled with two or more different detectable analyte-binding agents.
27. A method for detecting the presence of and/or determining the concentration of a target analyte in a test sample, the method comprising the use of a biological reagent in accordance with any one of claims 22 to 26.

28. The method of claim 27, wherein the method comprises a competitive binding assay.

5 29. The method of claim 28 for determining the presence of a target analyte in a test sample, wherein the method comprises:

mixing the biological reagent with the test sample, said biological reagent comprising the target analyte labelled with a detectable analyte-binding agent ,

10 separately, mixing said biological reagent with a control sample which does not comprise the target analyte;

exposing each mixture to a capture agent which is capable of specifically binding the target analyte;

15 removing, from each mixture, any labelled target analyte which has not bound to the capture agent, for instance by washing; and

comparing the level of binding of the labelled target analyte to the capture agent in the presence of the test sample to the level of binding of the labelled target analyte to the capture agent in the presence of the control sample.

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30. The method of claim 28 for determining the concentration of a target analyte in a test sample, the method comprising the steps of:

25 exposing the biological reagent, comprising the target analyte labelled with a detectable analyte-binding agent, to a capture agent capable of specifically binding the target analyte, in the presence of the test sample, said biological reagent comprising the target analyte labelled with a detectable analyte-binding agent ;

removing any labelled target analyte which has not bound to the capture agent, for instance by washing;

measuring the level of binding of the labelled analyte to a capture agent; and

30 comparing the measured level of binding to a reference in order to determine the concentration of the target analyte.

31. The method of claim 29 or claim 30, wherein the capture agent is bound to a solid-phase surface.

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32. The method of any one of claims 27 to 31 when dependent on claim 23, comprising simultaneously detecting the presence of and/or determining the concentration of a plurality of target analytes in the test sample.

5 33. The method of claim 32, wherein a plurality of capture agents is arranged in a grid or an array, each capture agent being specific for a single target analyte.

34. The method of any one of claims 29 to 33, wherein the method further comprises adding a marker which is capable of binding to the detectable analyte-binding agent of the labelled analytes which have been bound by the capture agent,
10 washing to remove any unbound molecules of the marker and detecting the presence of and/or determining the quantity of the marker.

35. The method of claim 34, wherein the detectable analyte binding agent comprises
15 NHS-biotin and the marker comprises streptavidin or avidin conjugated to a detection moiety.

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Figure 1a

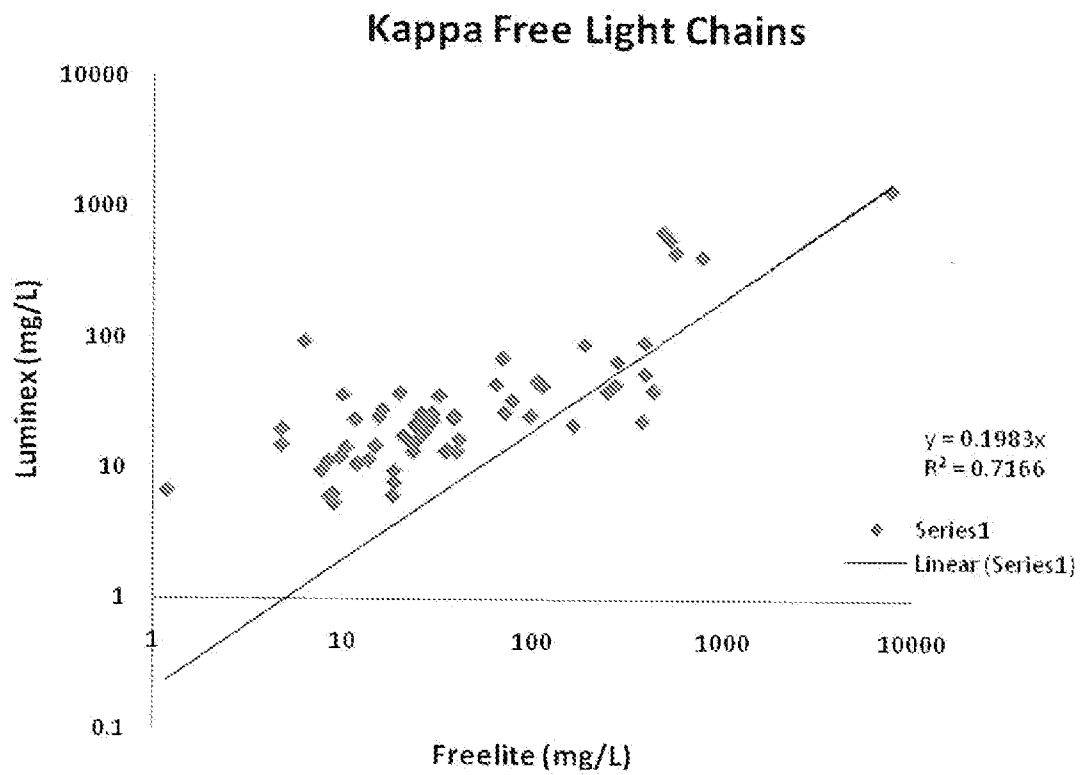


Figure 1b

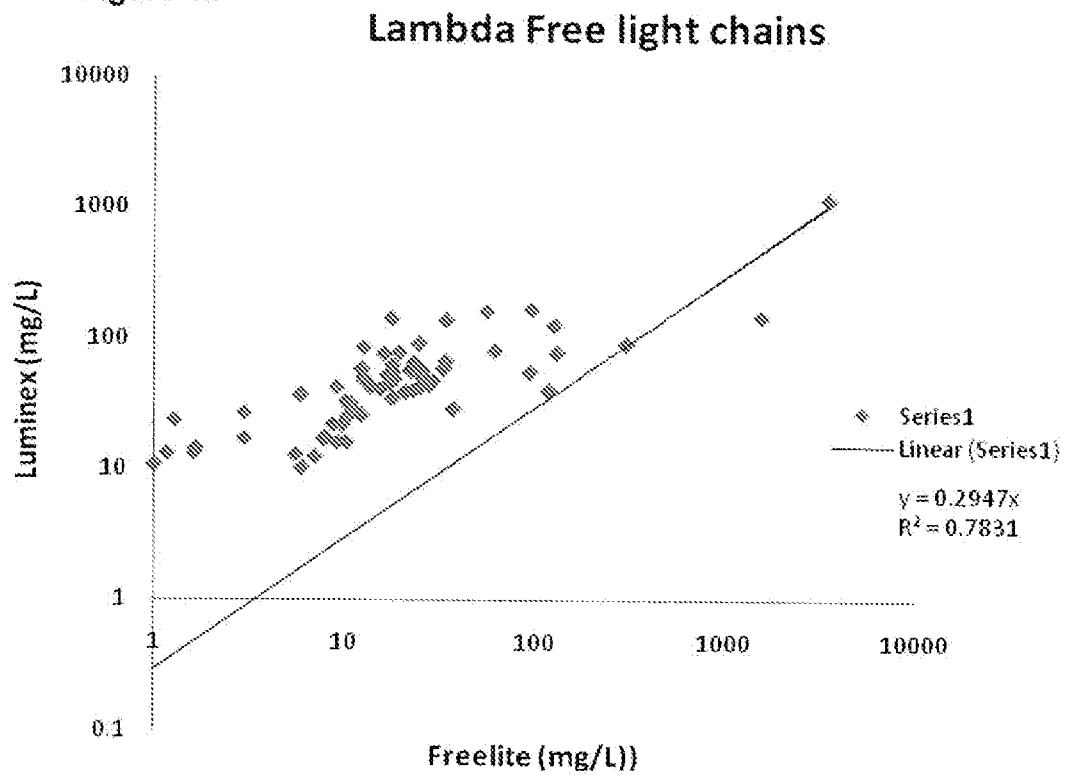


Figure 2a

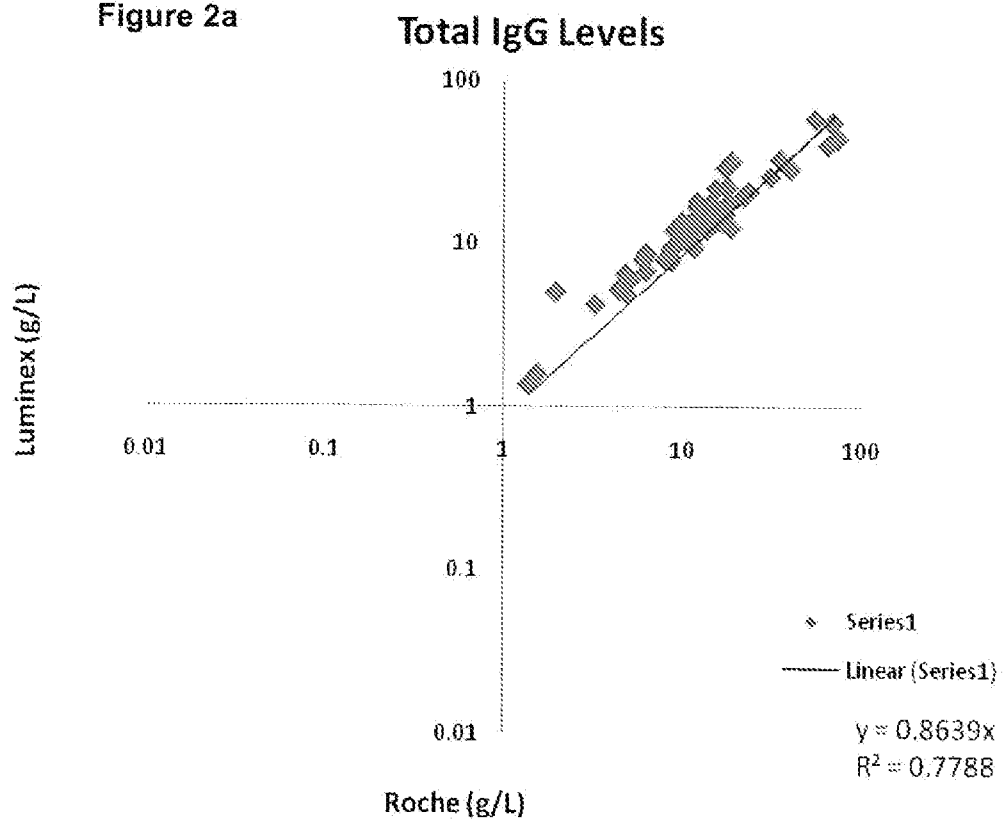
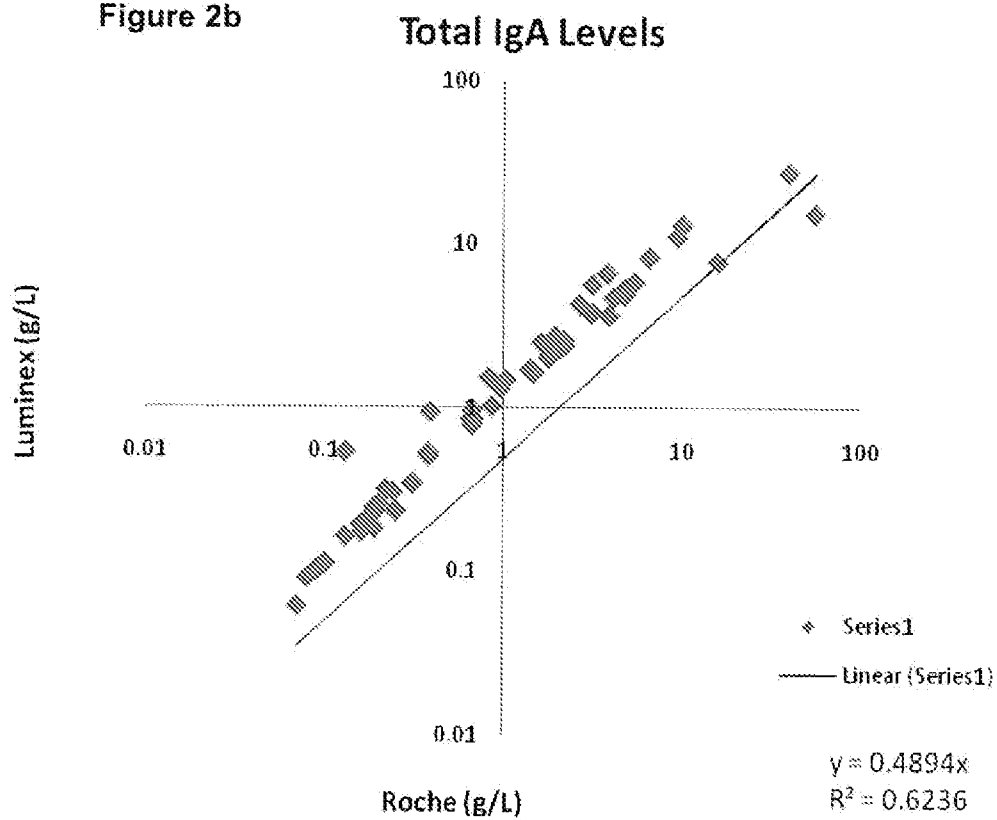


Figure 2b



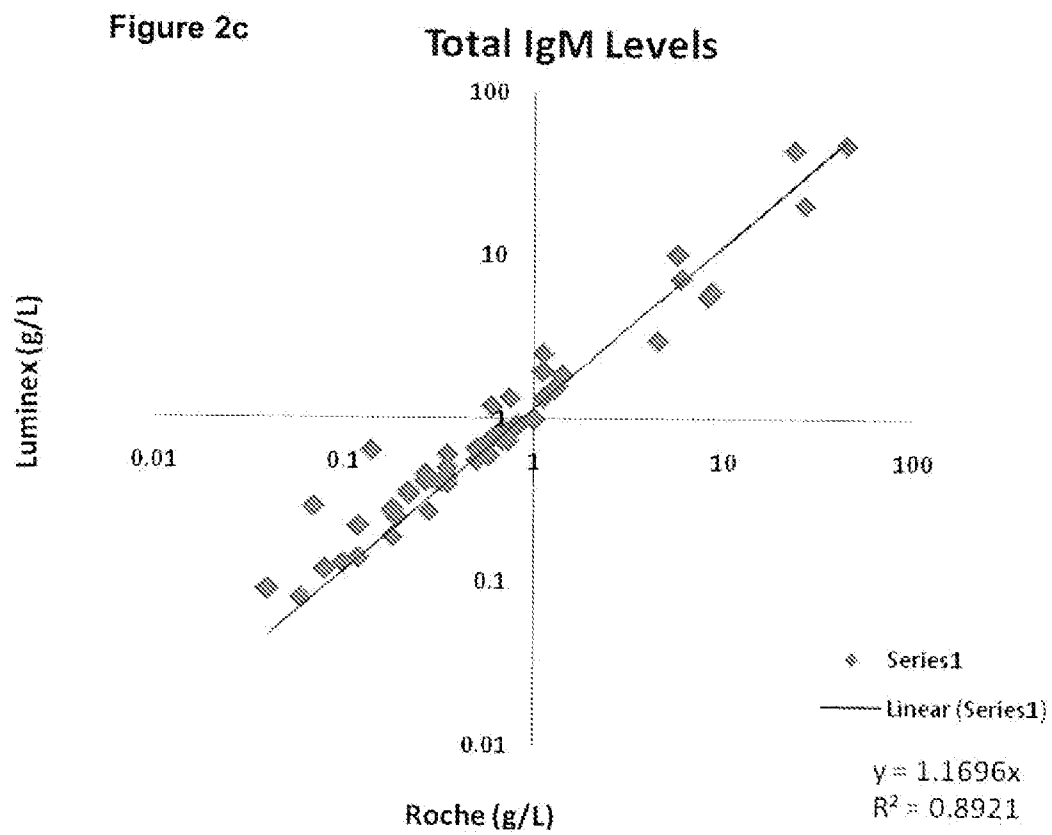


Figure 3a

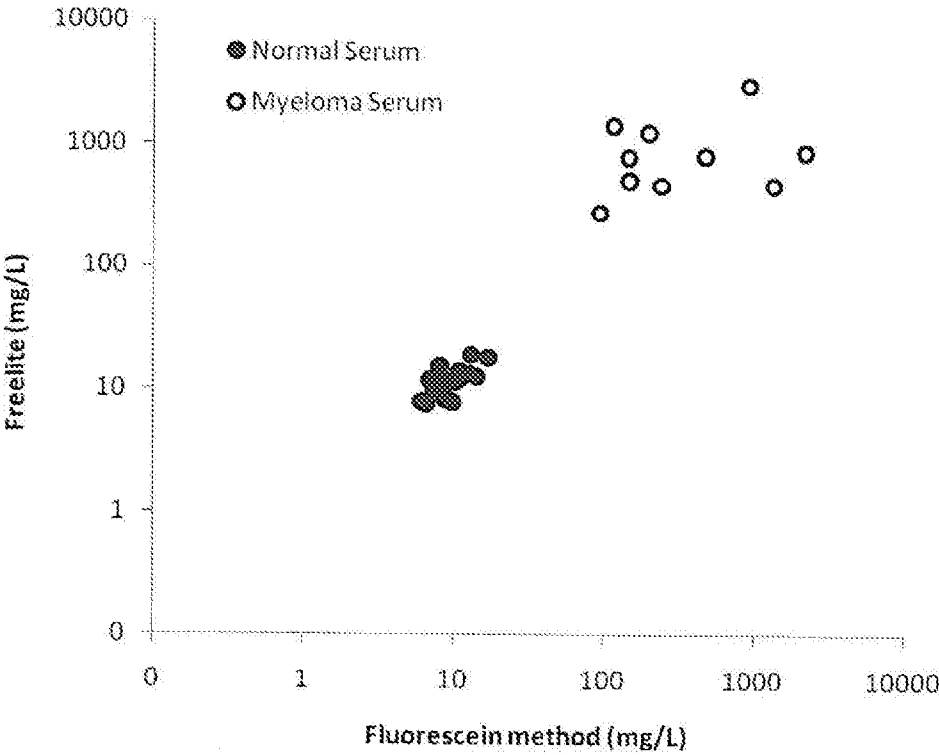


Figure 3b

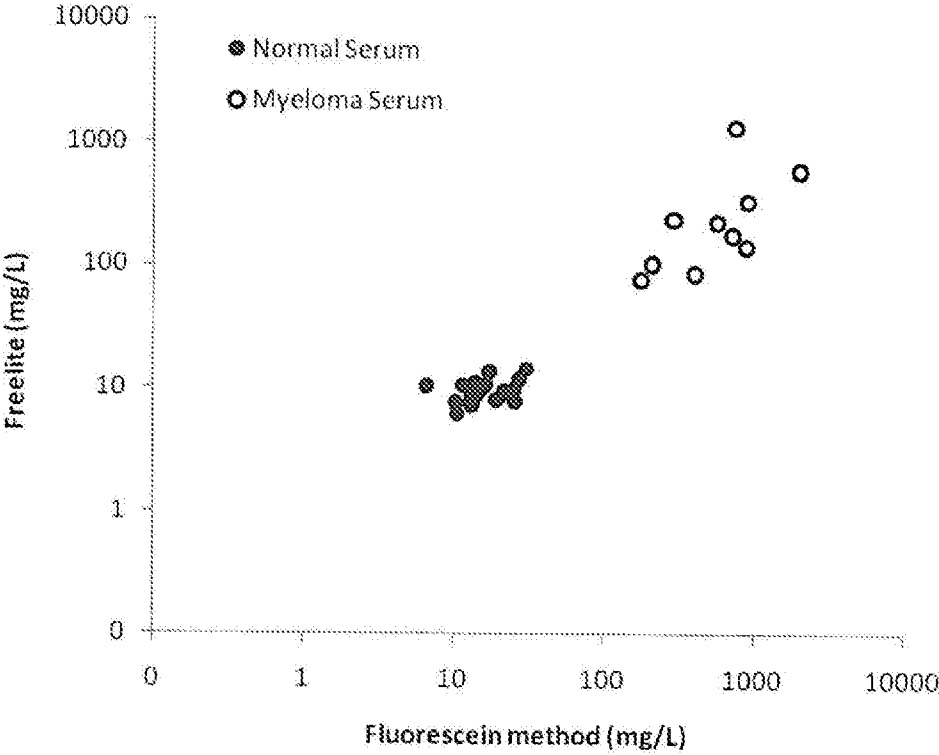


Figure 3c

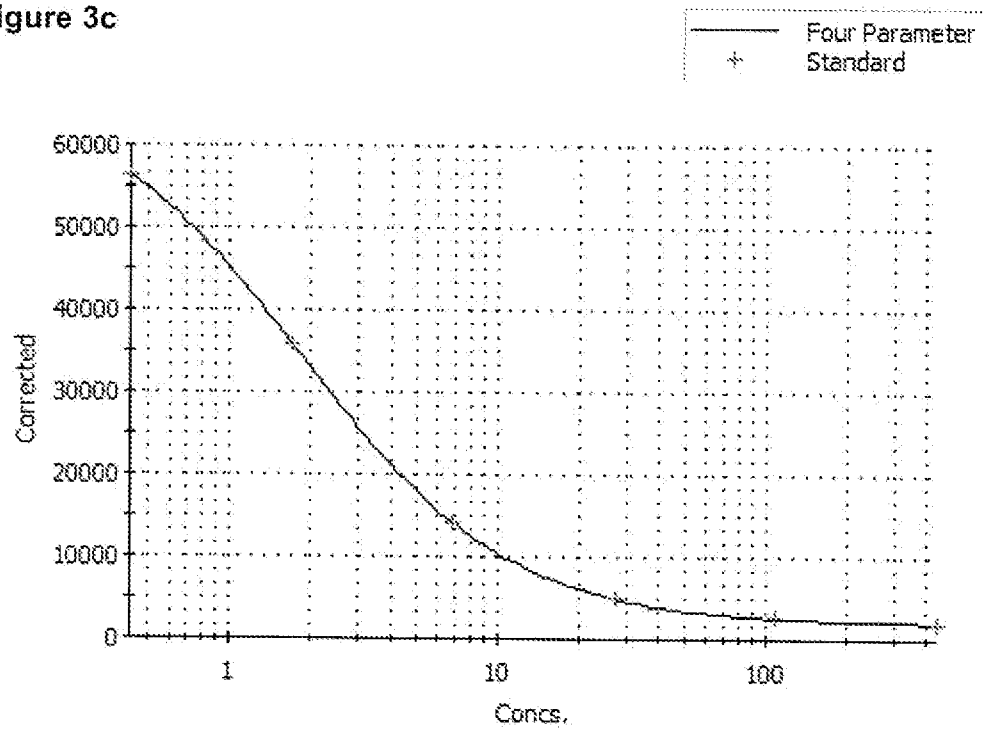
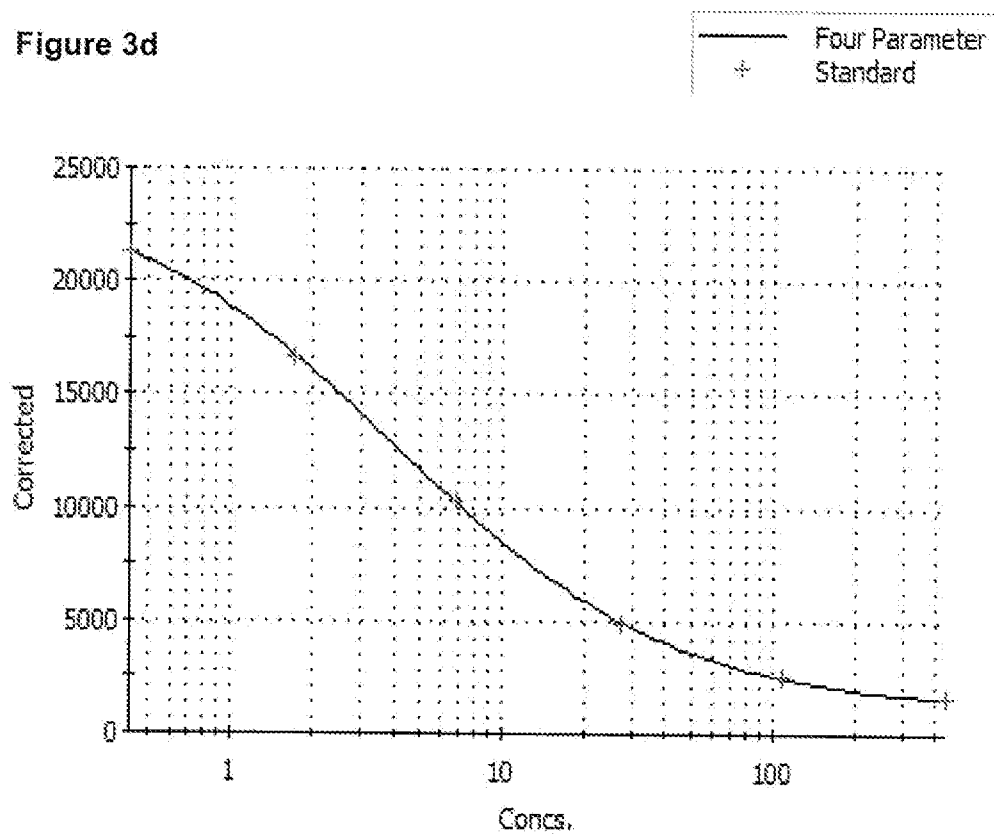


Figure 3d



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050793

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/53 G01N33/532 G01N33/96
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUO L Y ET AL: "Preliminary examination of time-resolved fluorometry for protein array applications.", LUMINESCENCE : THE JOURNAL OF BIOLOGICAL AND CHEMICAL LUMINESCENCE 2000 NOV-DEC, vol. 15, no. 6, November 2000 (2000-11), pages 409-413, XP002699064, ISSN: 1522-7235 abstract page 410, left-hand column, last paragraph -----	1-35
X	US 5 202 234 A (SHAH VIPIN D [US] ET AL) 13 April 1993 (1993-04-13) column 9, paragraph 1 -----	1-35
X	WO 2010/036470 A1 (US GOV HEALTH & HUMAN SERV [US]; BASILE ALISON JANE [US]) 1 April 2010 (2010-04-01) claims 1-5 -----	1-27



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

19 June 2013

Date of mailing of the international search report

08/07/2013

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2013/050793

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