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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 February 2015 (05.02.2015)

(10) International Publication Number
WO 2015/014922 A2

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

(21) International Application Number: PCT/EP2014/066445

(22) International Filing Date: 30 July 2014 (30.07.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 13179014.9 1 August 2013 (01.08.2013) EP

(71) Applicant: **NOVARTIS AG** [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH).

(72) Inventors: **FINCO, Oretta**; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina, 1, I-53100 Siena (IT). **BUDRONI, Sonia**; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina, 1, I-53100 Siena (IT). **BURICCHI, Francesca**; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina, 1, I-53100 Siena (IT). **MEDINI, Duccio**; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina 1, I-53100 Siena (IT). **VOLPINI, Gianfranco**; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina, 1, I-53100 Siena (IT).

(74) Agents: **COURGEON, Antoine** et al.; Novartis Vaccines and Diagnostics S.r.l., Via Fiorentina 1, I-53100 Siena (IT).

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

[Continued on next page]

(54) **Title**: METHOD FOR MEASURING BINDING REACTIONS

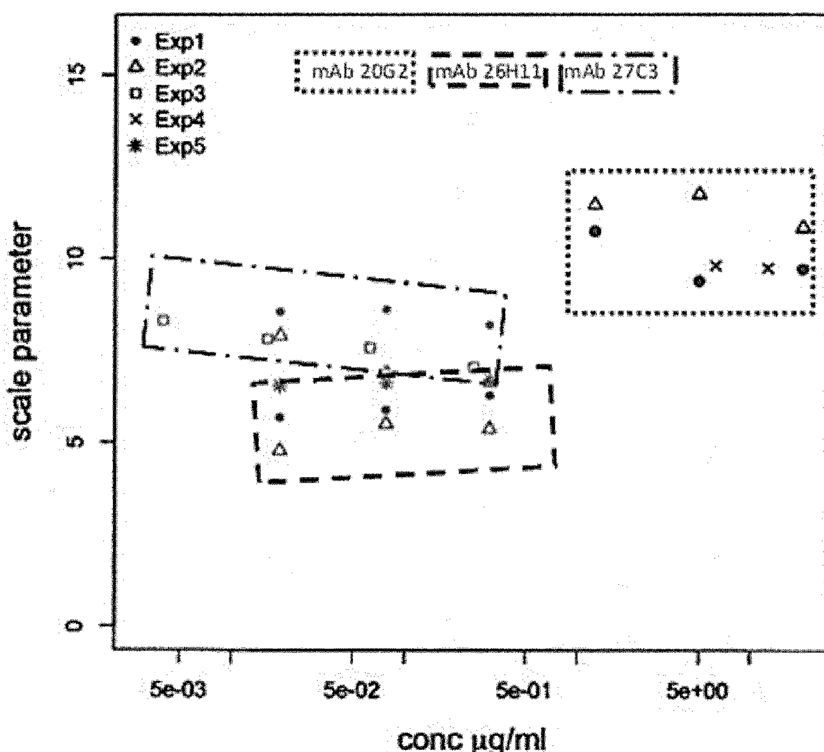

Fig. 6

(57) **Abstract**: The inventors have identified a method for measuring affinity of receptor for ligand that does not require a pre-incubation step. The method of the invention is particularly suitable for high-throughput affinity analyses of receptors and/or ligands.

WO 2015/014922 A2

TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

METHOD FOR MEASURING BINDING REACTIONS

This application claims the benefit of European patent application no. 13179014.9, filed on 2nd August 2013, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

5 This invention is in the field of measuring binding reactions between receptors and ligands, particularly antibodies and antigens.

BACKGROUND TO THE INVENTION

The ability to measure the affinity of a receptor for a ligand is particularly important in areas of biotechnology and medicine, particularly where the receptor is an antibody and the ligand is an 10 antigen *e.g.* in the development of vaccines and diagnostics. For example, during the immune response following immunisation, antibody affinity progressively increases over time. This results in the generation of higher-affinity antibodies (*i.e.* affinity maturation). Evaluation of antibody affinity maturation is a critical aspect of the antibody response, and can be useful when developing vaccines. Understanding antibody affinity enables determination of whether a vaccine is likely to be able to 15 induce long-term protection against an antigen.

A number of methods for determining antibody binding affinity and kinetic rate constants are available, but limitations are associated with each. Known methods include, for example, surface plasmon resonance (SPR), quartz crystal microbalance and solution equilibrium titration. These methods are time consuming and not well suited to high-throughput techniques. Typically, these 20 methods are unsuitable for analysing and characterising antibodies with particularly high affinities, and for analysing polyclonal antibodies. Moreover, assay techniques that require use of labelled or surface-immobilised analytes have been reported to produce incorrect affinity data, as compared to solution-based methods, which is thought to result from modification of the analytes (ref.1).

Methods in the prior art also require multiple washing and dilution steps to identify optimal antibody 25 concentrations before kinetic assays may be performed. This leads to significant waste of sample and reagents. Even when optimal concentrations have been determined, the time required to establish equilibrium is often in the order of days, particularly when characterising high affinity complexes. In view of the above limitations, it is an object of the invention to provide further and improved methods for measuring receptor/ligand affinity. It is a particular object of the invention to provide 30 faster methods for measuring receptor/ligand affinity, particularly for antibodies and antigens.

SUMMARY OF THE INVENTION

To overcome the limitations of existing methods for measuring binding reactions between ligands and their receptors, the inventors have developed a new method that does not require pre-incubation of the ligand and receptor. The method of the invention is therefore faster than methods in the prior 35 art.

The method of the invention is suitable for use with any affinity column that, after ligand has passed along the column, allows point by point analysis of the level(s) of bound ligand. The invention is particularly suitable for use with the "Gyrolab" system. This system (www.gyros.com) uses a microfluidic platform which advantageously reduces waste of sample and reagents.

5 Gyrolab allows faster acquisition of kinetic data than *e.g.* the KinExA method (ref. 2), but its speed is currently limited by the conventional requirement to pre-incubate antibody before being able to perform affinity studies (ref 2). For example, reference 2 used the Gyrolab system to measure binding affinity of an antibody for its antigen, but this required a 48 hour pre-incubation step before performing affinity studies. With the invention, however, pre-incubation is not required, and so the 10 Gyrolab system's advantages can be enjoyed while providing a quicker measurement of affinity.

Moreover, conventional binding assays such as surface plasmon resonance or the Kinexa method, as practised by those of skill in the art, are limited in that they are not capable of measuring very high affinity binding (*e.g.* Kd $\leq 10^{-11}$ M), and higher affinity binding (*e.g.* Kd $\leq 10^{-12}$ M) is currently measurable only by using in-solution titration methods. Methods of the invention advantageously 15 have a large dynamic range and are capable of determining a broad range of binding affinities, from very low (*e.g.* Kd $\leq 10^{-8}$ M) to very high (*e.g.* Kd $\leq 10^{-12}$ M).

In one embodiment, the invention provides a method for measuring the affinity of a receptor for a ligand comprising analysing the distribution of ligand/receptor complexes along an affinity column comprising immobilised ligand for the receptor. As the receptor passes along the column it becomes 20 distributed, and the pattern of distribution is analysed to derive the affinity of the ligand/receptor interaction.

In one embodiment, the method of the invention comprises the steps of: A. applying a sample comprising a receptor to an affinity column comprising an immobilised ligand for the receptor; B. passing the sample along the affinity column; C. detecting level(s) of ligand/receptor complex along 25 the affinity column; D. analysing distributions of ligand/receptor complex along the column; and E. using data from step D to determine the binding affinity of the receptor for the ligand.

Binding affinity of antibodies can be derived from an affinity score W, which can be derived by regression of Eq. 1 to the level(s) of ligand/receptor complex along the affinity column. The regression is preferably least-square regression within the linear range of the detection. The linear 30 range of detection may be identified by A) performing a method of the invention on an antibody concentration interval; B) estimating normalisation factor (parameter A) from the results obtained from step A); C) performing 4PL linear regression on the relationship between parameter A and the antibody concentration; and D) estimating the middle point of the linear range (x_{mid}).

Typically, the ligand is an antigen and the receptor is an antibody for the antigen. In some 35 embodiments, the antibody is a monoclonal antibody. In other embodiments, the antibody is a polyclonal antibody.

As an alternative, the immobilised ligand is antibody and the receptor is antigen. Such embodiments are particularly suitable for determining and/or comparing the binding affinity of antigen(s) with pre-defined antibody, *e.g.* for determining cross-reactivity of antigen(s) with pre-defined antibody. In such embodiments, the antigen(s) are preferably obtained from natural bacterial strain(s).

5 In some embodiments, detection of ligand/receptor complex comprises detection of a detection agent bound to the ligand/receptor complex. The detection agent is preferably added to the affinity column after introduction of the sample to the affinity column.

The method of the invention does not require a step of pre-incubating the ligand and receptor, and so the method of the invention preferably does not include such a step.

10 In some embodiments, the sample is passed through the affinity column under inertia.

In some embodiments of the invention, capture agent is attached to the affinity column via a biotin/avidin interaction. The capture agent may be biotinylated. The avidin is preferably streptavidin.

In some embodiments, the detection agent is a fluorophore.

15 In some embodiments, the detection agent is excited using a laser.

In some embodiments, the sample comprises a mixture of receptors that have different affinities for bound ligand, *e.g.* polyclonal antibody sera. In such embodiments, the invention may be used to separate population(s) of receptors from within a sample based on their affinities for bound ligand. This makes possible the identification of populations of receptors that have different affinities for
20 bound ligand *e.g.* low affinity, medium affinity and/or high affinity receptor populations. Advantageously, this makes possible the identification of receptors that have high affinity for the bound ligand. Thus, the invention provides a method for separating population(s) of receptors from within a mixture of receptors that have different affinities for bound ligand, comprising the steps of: A) applying a sample comprising a receptor to an affinity column comprising immobilised ligand for
25 the receptor; and B) passing the sample through the affinity column. Typically, this embodiment of the invention is used to separate antibody population(s) within polyclonal sera according to their affinity for an antigen.

The invention also provides a method for isolating receptor population(s) from within a mixture of receptors that have different affinities for bound ligand (*e.g.* to isolate antibodies from polyclonal
30 sera). In this embodiment, the method comprises the steps of A) applying a sample comprising a receptor to an affinity column comprising immobilised ligand for the receptor; B) passing the sample through the affinity column; C) deriving spatial information on ligand/receptor complex populations along the column; and D) isolating receptor(s) from the column. The spatial information is typically derived by de-convoluting and performing mathematical regression of the ligand/receptor complex
35 distribution data obtained by steps A) and B.

Antibodies isolated according to the invention may optionally undergo further characterisation, *e.g.* amino acid sequencing.

The invention also provides antibody identified by or isolated by a method of the invention. In some embodiments, the antibody is for use as a standard *e.g.* in antibody functional assays (such as serum bactericidal assays), *in vitro* potency assays, *etc*. In some embodiments, the antibody is for use in therapy. In some embodiments, the antibody is for use in passive immunisation.

The invention also provides apparatus for use in a method of the invention. The apparatus is preferably an affinity column.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows dose-response curves of anti-NadA mouse monoclonal antibodies. Y-axis shows Fluorescence Intensity values (FI) obtained by the Gyrolab system. Antibodies were tested at different concentrations (μg/ml, X-axis).

Figure 2 A) shows 3D reaction profiles of each monoclonal antibody tested, at a concentration of 0.02μg/ml for 26H11/37 (i); 0.08μg/ml for 27C3/11 (ii); 5.0μg/ml for 20G2/A6 (iii) visualized with the Gyrolab evaluator software. The X-axis represents the compact disc (CD) radial coordinate (μm), the Y-axis represents the Fluorescence Intensity (FI) radial distribution of each monoclonal antibody.

Figure 2 B) shows overlaps of the 2D reaction profiles obtained with the 3 monoclonal antibodies tested at 0.02μg/ml for 26H11/37; 0.08 μg/ml for 27C3/11; 5.0μg/ml for 20G2/A6. The X-axis represents the CD radial coordinate, the Y-axis represents the sum of Fluorescence Intensities detected at each point.

Figure 3 shows radial distributions of the antigen-antibody capture profiles. Graphs compare Fluorescence Intensity responses FI (Y-axis) *vs.* the radium direction (X-axis) for different monoclonal antibodies. a) Five monoclonal antibodies were tested against *Neisseria meningitidis* NadA protein. b) four monoclonal antibodies were tested against polysaccharide of *Streptococcus agalactiae* type la. An asymmetric unimodal shape characterized each profile analyzed.

Figure 4 shows best fit of Eq. 1 to row data for monoclonal antibodies specific for antigens of different chemical nature. Monoclonal antibody 26H11 *vs. Neisseria meningitidis* NadA protein (a), monoclonal antibody 15B11 *vs. Neisseria meningitidis* fHbp (variant 1) protein (b) and monoclonal antibody 30G1 *vs.* Ps *Streptococcus agalactiae* type lb (c). In each capture profile, dots (•) are experimental Gyrolab data and the continuous line (—) is the predicted shape obtained by fitting data with the function in Eq.l. Curves represent monoclonal antibody concentrations tested.

Figure 5 shows relationship between A and concentration (μg/ml) of three monoclonal antibodies anti-NadA: 20G2 (a), 27C3 (b) and 26H11 (c). For each monoclonal antibody, three experiments were performed, represented by different symbols in each panel. Shaded rectangles enclose the

points for which there is a linear relation between the normalization factor and the monoclonal antibody concentration.

Figure 6 shows affinity score *W* *vs.* monoclonal antibody concentration. For the anti-NadA monoclonal antibodies under investigation – as shown in the figure legend – the inventors estimate *W* for all measurements in the *A* linear response range. *W* trend is almost constant when monoclonal antibody concentration varies.

Figure 7 shows the relationship between Log(KD) and the *W* affinity score for the three tested monoclonal antibodies anti-NadA. The regression line (—) was obtained by the least-squares method. A strong correlation between the two affinity measurements exists, as demonstrated by the Pearson's product-moment correlation coefficient: $p = 0.99$ (p- value < 0.05).

Figure 8 shows Log(KD) *vs.* *W* affinity score from monoclonal antibodies anti-fHbp. The regression line (—) was obtained by the least-squares method. Five monoclonal antibodies were tested against three fHbp variants: variant 1 (Var1), variant 2 (Var2) and variant 3 (Var3). The dotted band (---) is the regression confidence interval @95%. Pearson's product-moment correlation coefficient is $\boldsymbol{p} = 0.96$ whir a p-value « 0.001.

Figure 9 shows variability of the affinity score *W*. For each monoclonal antibody tested, the inventors evaluated the assay precision by analyzing the trend of the coefficient of variation CV (vertical axis) associate to *W* value (horizontal axis). The non-statistically significant p-value (0.78), obtained from the Pearson test, suggests an absence of dependency between CV and *W*.

Figure 10 shows binding profiles of the anti-fHbp variant 1 antibodies present in the serum of a fHbp immunized mouse (top) and of the anti-NHBA antibodies in a NHBA immunized rabbit serum (bottom). In each capture profile, dots (●) are experimental Gyrolab® data and the continuous line (—) passing through the dots is the predicted shape obtained by fitting data with a linear composition of Landau function. In each plot it is possible to identify two main antibody sub-populations at different affinity composing the pool of antigen specific antibodies of the serum. The Pearson's product-moment correlation coefficient between the raw data and their corresponding fitted data exceeds 0.99 for each polyclonal antibody with a p-value < 0.001.

Figure 11 shows binding profiles of anti-fHbp antibodies in the serum of a Balb/c mouse immunized subcutaneously with 20 ug of MenB antigen fHbp-231 (fusion of fHbp variant2, variant3 and variant1) plus MF59. Sera samples were taken before vaccination (top), 3 weeks after the first dose (centre), 3 weeks after the second dose (bottom) and analysed for the presence of antibodies binding to fHbp variant 1. For the de-convolution analysis the samples were run at a dilution in the range of linearity of the response, *i.e.* the preimmune serum was tested at 1:4 dilution, while the sera at 3 weeks after the first and the second vaccination were tested at 1:60 dilution. In each polyclonal profile, the black continuous line represents the best fit of a linear composition of Landau function to

FI data. It was obtained by the least-squares method as implemented in the *nls* package of R version 3.0.2 (http://www.r-project.org/)

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the finding that it is possible to measure the affinity between ligands and receptors using an affinity support, without requiring pre-incubation. The method of the invention is advantageously faster than methods in the prior art.

The method of the invention is suitable for use with any affinity column that allows point by point analysis of levels of ligand/receptor complexes along the affinity column (*i.e.* assays and/or affinity columns that provide spatial information on binding levels along the affinity column).

The inventors realised that progression of a receptor (*e.g.* antibody) through an affinity column coated with ligand (*e.g.* antigen) is related to its affinity for the ligand. The inventors also realised that receptors (*e.g.* antibodies) that have higher affinity to ligand (*e.g.* antigen) bind faster as they progress along the affinity column, as compared to receptors with lower affinity, thereby focussing the higher affinity receptors onto a narrower region of the affinity column. Thus, in a plot of the level of bound receptor against its position along the affinity column, the width of the curve is dependent on antibody affinity for the antigen coated on the affinity column. High affinity receptors lead to a narrower curve than lower affinity receptors. The inventors found that the width of the curve is tightly connected to the affinity of the receptor for the ligand. Thus, the inventors found that by obtaining spatial information on receptor levels along the affinity column, it is possible to determine the affinity of the ligand/receptor interaction.

An example of an affinity assay that is particularly suitable for use with the methods of the invention is the Gyrolab system. The Gyrolab system typically uses a fluorescent detection agent to help detect binding of a receptor to immobilised ligand on an affinity column, and provides spatial information on binding levels along the affinity column.

The inventors have developed an experimental protocol and a mathematical model for use with any affinity column that allows point-by-point analysis of receptor levels (in the form of ligand/receptor complexes) along the affinity column. The method of the invention is particularly suitable for measuring binding between antibodies and antigens. The method of the invention is suitable for use with high-throughput assays, such as the Gyrolab system. The invention is also particularly suitable for measuring affinity of antibodies specific for different types of antigens (*e.g.* proteins, carbohydrates, glycoconjugates *etc.*) or antigen portions (*e.g.* peptides, domains *etc*). The invention is suitable for use with any type of antibody (*e.g.* monoclonal or polyclonal antibodies).

In an attempt to find an improved method for measuring the binding affinity of receptors and ligands, in particular antibodies and antigens, the inventors explored the antigen-antibody reaction profiles that are obtainable using the Gyrolab system.

The inventors found that the method of the invention allows exploitation of the data generated using, for example, the Gyrolab system. In particular, the method of the invention derives an *"affinity score"* from raw data. The inventors have termed this affinity score "*W*". *W* precisely describes the affinity of monoclonal antibodies, and is determined by (*e.g.* computerized) regression of a
5 mathematical function to the signal intensity profiles along the column. The mathematical function accurately describes the antibody-antigen interaction profile, for a variety of different antigens, and for well-defined ranges of antibody affinity. The avidity of polyclonal antibody populations can be determined by combining *W* values for each single antibody sub-population.

Sub-populations of receptor(s) having different affinities for bound ligand form ligand/receptor
10 complexes at different positions along the column. Therefore, when the sample comprises a mixture of receptors that have different affinities for bound ligand, *e.g.* polyclonal antibody sera, the invention can identify sub-populations of receptor/ligand complexes based on their location on the column. Once identified, the receptor(s) may be isolated from the column, *e.g.* by excision.

The method of the invention is suitable for use with any affinity column that makes possible point by
15 point analysis of ligand/receptor complexes levels along the affinity column. To the inventors' knowledge, Gyrolab is currently the only commercially available method that makes this type of analysis possible, but the method of the invention is not limited to use with the Gyrolab system.

Affinity

Methods of the present invention are suitable for measuring the binding energy between receptors
20 and ligands. This binding energy is referred to herein as "affinity".

Methods of the invention are particularly suitable for measuring the binding energy between antibodies and antigens. Accordingly, the term "affinity" is used herein to describe in general the binding energy between antibodies and antigens.

Antibody/ antigen binding energy is conventionally defined as "affinity" or "avidity", depending on
25 the valency of the antibody. The term "affinity" is conventionally used to describe the binding energy between *e.g.* a monovalent antibody and a single antigen epitope, and generally best describes binding of antibody Fab fragment(s) with antigen(s). The term "avidity" is conventionally used to describe the more complex interaction between *e.g.* antibodies containing multiple binding sites and their antigens. Accordingly, when methods of the invention are used to measure the binding energy
30 between multivalent antibodies and antigens, the methods provide a measurement of avidity.

As discussed above, the term "affinity" is used herein to describe in general the binding energy between receptors and ligands (particularly antibodies and antigens), and so is not restricted to the conventional meaning of antibody affinity. Methods of the invention are particularly useful for identifying antibodies that have high affinity for an antigen. Methods of the invention are also useful
35 for identifying antigens that induce formation of high affinity antibodies.

A "high affinity" receptor (*e.g.* antibody) typically binds to a ligand (*e.g.* antigen) with a Kd equal to or less than about 10^{-10}M *e.g.* 10^{-10}M, 10^{-11}M, 10^{-12}M, 10^{-13}M or more.

Immunoassays

Principles of antibody affinity are well known in the art, and are widely used to study binding of antibodies to antigens, and the formation of antibody/antigen complexes. A number of different assay formats are available, and may be broadly classed as "competitive" or "non-competitive". The method of the invention is particularly suitable for use with *e.g.* sandwich assays, indirect antibody assays and bridging assays.

A discussion of the Gyrolab system, including preparation of its microfluidic affinity columns may be obtained from the www.gyros.com website. An exemplary affinity assay using the Gyrolab system may comprises the steps of: (1) immobilising ligand (*e.g.* antigen) onto an affinity column; (2) interfacing a solution of receptor (*e.g.* antibody) with the affinity column, allowing binding of receptor to the ligand immobilised on the affinity column; (3) interfacing a detection agent with the affinity column, allowing binding of the detection agent to the receptor and; (4) detecting the detection agent on the affinity column (intensity of detection is proportional to the amount of detection agent and thus receptor on the affinity column).

Conventionally, the solution of antibody and ligand added in step (2) has been pre-incubated, so that affinity may be determined. Advantageously, the method of the invention does not require this pre-incubation step.

The specific arrangement of ligand, receptor, and detection agent is not critical to the invention. The method of the invention may also be performed by *e.g.* immobilising receptor onto an affinity column and then interfacing a solution of ligand with the affinity column. Other arrangements which may be contemplated fall within the scope of the invention.

The invention is not limited to use with any particular type of affinity column, so long as the affinity column makes possible point by point analysis of ligand/receptor complexes levels along the affinity column.

The invention is particularly useful for evaluating the affinity of an antibody for an antigen.

The sample

The sample is passed through the affinity column. The sample comprises receptor. In a preferred embodiment, sample is substantially free of the ligand *e.g.* less than 0.1% of the sample, by weight.

In a preferred embodiment of the invention, the receptor and ligand first come into contact inside the affinity column. In some embodiments, however, some degree of pre-mixing will be required *e.g.* due to technical restraints. In such cases, pre-mixing of the receptor and ligand typically occurs less

than 30 minutes prior to introduction to the affinity column, *e.g.* less than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute(s) prior to introduction to the affinity column. Pre-mixing of the receptor and ligand preferably occurs less than 10 minutes prior to introduction to the affinity column, *e.g.* less than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute(s) prior to introduction to the affinity column. Preferably, pre-mixing of the receptor and ligand occurs less than one minute prior to introduction to the affinity column, *e.g.* less than 45, 30, 15, 10, 5, 4, 3, 2, or 1 second(s) prior to introduction to the affinity column.

The sample may be diluted prior to, or during introduction to the affinity column, using any suitable diluent. Suitable diluents are selected according to the particular characteristics of an assay *e.g.* application objective, type of analyte, biological origin and components of the sample and assay format. Preferred diluents for use in methods of the invention are selected from Rexxip range of buffers (www.Gyros.com), which are particularly suitable buffers for dilution of samples, when using the Gyrolab system.

The affinity column

Affinity columns for use in the invention include an immobilised ligand which can bind and retain receptor which passes over it.

Samples can pass through the affinity column using any suitable means, such as under gravity or under inertia. The Gyrolab system is an inertia-based system, whereby sample passes through the affinity column under inertia generated by rotation.

Immunoaffinity columns (such as the affinity columns used in the Gyrolab system) typically comprise beads coated with a capture agent, *i.e.* the affinity column is coated with capture agent.

Methods for attaching capture agent to affinity columns are known in the art. Preferably, the capture agent is attached to the affinity column via a biotin/avidin interaction. Methods of biotinylation and avidinylaiton are known in the art.

In one embodiment, the affinity column is coated with an avidin (*e.g.* streptavidin), and the capture agent comprises biotin. Introduction of the capture agent to the affinity column causes binding of biotin to avidin, thereby coating the affinity column with capture agent. In the Gyrolab system, capture agent comprising biotin are introduced to beads coated with streptavidin. Binding of biotin to streptavidin thereby coats the beads with capture agent.

In another embodiment, the affinity column is coated with biotin, and the capture agent comprises avidin (*e.g.* streptavidin). Introduction of the capture agent to the affinity column also causes binding of biotin to avidin, thereby coating the affinity column with capture agent.

In a preferred embodiment of the invention, the capture agent is biotinylated antigen, and the sample comprises antibody, preferably monoclonal or polyclonal antibody.

The present invention is particularly suitable for use with the Gyrolab system. Gyrolab is a microfluidics-based platform comprising an affinity column incorporated into a compact disc (CD). Flow rate through the CD is controlled by rotational frequency. The present invention may be used, for example, with Gyrolab Bioaffy or Gyrolab ADA CDs (*e.g.* Bioaffy 1000, Bioaffy 200 or Bioaffy 20 HC).

Detection agent

A detection agent is required when it is difficult or not technically possible to detect ligand/receptor complex directly. A detection agent may also be used to make possible the use of preferred detectors, such as fluorescence detectors.

A detection agent comprises a detectable moiety and is able to bind to the ligand/receptor complex.

The detectable moiety is more easily detectable than *e.g.* receptor only, ligand only, or the ligand/receptor complex itself. Thus, a detection agent is typically used to help improve detection of ligand/receptor complex (*i.e.* ligand/receptor complex bound to the affinity column).

Detection agent may be present (a) in the sample before introduction to the affinity column; (b) added to the affinity column simultaneously with the sample; and/or (c) added to the affinity column after introduction of the sample to the affinity column. The term "introducing a detection agent to the affinity column" refers generally to any of these options. Typically, detection agent is added to the affinity column after introduction of the sample to the affinity column. When a detection agent is used, detection of level(s) of ligand/receptor complex, is generally achieved indirectly, by detection of the detection agent itself.

Detection of ligand/receptor complex may be achieved by *e.g.* binding of detection agent to receptor in the ligand/receptor complex, followed by detection of the detection agent. Detection of ligand/receptor complex may also be achieved by *e.g.* binding of detection agent to ligand in the ligand/receptor complex, followed by detection of the detection agent. In some embodiments, detection agent binds ligand/receptor complex, but not receptor alone or ligand alone. In such cases, detection of ligand/receptor complex may be achieved by *e.g.* binding of detection agent to the ligand/receptor complex, followed by detection of the detection agent. The term detection agent "bound to the ligand/receptor complex" all of these binding possibilities.

A detection agent preferably comprises an antibody. The detection agent typically has low binding affinity for the capture agent, but high binding affinity for the receptor and/or ligand in the ligand/receptor complex.

In one embodiment, the detection agent has high affinity for ligand in the ligand/receptor complex. The detection agent typically binds to a different epitope in the antigen than the receptor.

In a preferred embodiment, the detection agent has high affinity for receptor in the ligand/receptor complex. In a most preferred embodiment, the detection agent comprises an antibody and the receptor in the ligand/receptor complex is an antibody.

Thus, in a preferred embodiment of the invention, the detection agent comprises and antibody and a fluorophore.

In some embodiments, the ligand/receptor complex is already detectable *e.g.* fluorescent. In such cases, the ligand/receptor complex can already function as the detection agent, and so addition of a different detection agent is not necessary to detect the ligand/receptor complex. Accordingly, introduction of a different detection agent to the column may be optional, depending on whether the ligand/receptor complex may be detected. Additional detection agent may be introduced to the column, even when the ligand/receptor complex may already be detected *e.g.* to help improve detection.

The detectable moiety

Detectable moieties for use in affinity assays are known in the art. Detectable moieties may involve use of *e.g.* stains or dyes, such as protein-specific antibodies, DNA-specific probes, Hoechst (bisbenzimide) dyes, Quant-iT PicoGreen reagent (Molecular Probes), YO-PRO-1 (Molecular Probes), fluorescein dyes *e.g.* fluorescein isothiocyanate (FITC), Tetramethylrhodamine isothiocyanate (TRITC), aminomethylcoumarin acetate (AMCA). Typically, such compound(s) include fluorescence label(s), and/or bioluminescence label(s), *etc*. The detectable moiety may be a quantum dot. The detectable moiety is preferably a fluorophore. Preferred fluorophores for use with the invention include *e.g.* Alexa Fluor® 405, 350, 488, 430, 514, 532, 555, 546, 568, 594, 610, 633, 635, 647, 660, 680, 700 or 750.

Detection of detectable moiety

Detection of detectable the detectable moiety requires use of a suitable detector. Suitable detectors are known in the art, and are selected according to the type of signal produced by the detectable moiety. Higher concentrations of detection agent (and thus higher concentrations of detectable moiety) have higher signal intensity.

For example, where the detectable moiety comprises a fluorescent label, the detector is preferably a fluorescence detector, such as a filter fluorometer or a spectrofluorometer. The fluorescence detector may be single-channelled or multi-channelled. Where the invention involves use of a fluorescent label, the invention also typically involves use of an excitation source *e.g.* laser, photodiode, lamp, xenon arc, mercury-vapor lamp *etc*. Wavelengths suitable for excitation of fluorophore(s) and concomitant detection of emission may be readily determined.

In a preferred embodiment of the invention, the detectable moiety is a fluorophore. Fluorophores are preferably excited using a suitable laser *e.g.* He-Cd, He-Ne, Ar^+ ion, diode *etc*. Laser-induced

fluorescence is employed by the Gyrolab system, which is particularly suitable for use with methods of the invention.

Data analysis

The method of the invention is suitable for use with any affinity column that allows point-by-point analysis of ligand/receptor complex levels along the affinity column. Such assays and/or affinity columns thus provide spatial information relating to receptor/ligand concentrations along the affinity column, after this point-by-point analysis has been performed.

The method of the invention allows identification of an "affinity score", termed herein as "*W*", based on analysis of distributions of antibody-antigen capture profiles (such as the capture profiles provided by the Gyrolab system). The distributions are typically radial or linear, depending on the nature of the affinity column. The Gyrolab system involves use of a rotating CD, and so the distribution is radial. A *W* score may likewise be calculated by analysing linear distributions of antibody-antigen capture profiles.

The method of the invention is particularly suitable for use with the output data generated using the Gyrolab system (*e.g.* the Gyrolab Evaluator software).

The avidity score *W* is obtained by least-square regression of Eq.1 (see below) to Gyrolab raw data within the linearity range of parameter *A*. *W* is a precise measure of antibody affinity which may be obtained by performing assays of antigen-antibody binding using the Gyrolab system.

In the *Landau distribution* reported in Eq. 1 (see below), in the context of a Gyrolab experiment, $f(z)$ is the fluorescence intensity, x is the radial coordinate, y_0, *A*, *W* and x_c are free parameters of the regression. *Landau distribution* parameters are referred to below as:

- y_0 (noise level): defines the level of noise of the experiment (background), representing the lowest value assumed by the fit of Eq. 1 to data.
- *A* (normalization factor): In the linear range of the assay, *A* is linear with the total FI measured in the experiment; the area under the Landau curve subtracted by the noise level y_0, and is also expected to be proportional to the concentration of antibody tested. By analyzing the A parameters obtained with the same antibody run at different concentrations it is possible to define the linear range of the assay.
- x_c (location parameter): describes the position on the x axis of the curve's maximum, *i.e.* the radial coordinate where maximum FI is measured.
- *W* (scale parameter): determines the width of the curve, *i.e.* the value of *W* increases with the width of the curve. Since the width of the curve depends on the antibody affinity for the antigen coated on the substrate (antibodies with high affinity will bind faster along the radial

coordinate than antibodies with low affinity, producing sharper FI profiles) the parameter W is tightly connected to the antibody affinity to the substrate.

Analysis of the A parameters obtained with the same antibody run at different concentrations makes it possible to define the linear range of the assay. This avoids the need to perform multiple runs to
5 work out optimum dilutions required for maximum precision. The method of the invention automatically determines the optimum dilutions, after one run, which saves time and reagents compared to methods in the prior art.

To provide a more reproducible and higher-throughput procedure for evaluating the affinity score W, the inventors developed an improved technique for identifying a range of linear responses for the
10 assay. This improved technique is based on the four-parameter logistic (4PL) non-linear regression model (shown below) which is commonly used for curve-fitting analyses in immunoassays and dose-response curves. x represents the monoclonal antibody concentration (µg/ml) used in a Gyrolab experiment or the independent value, and $f(x)$ represents the response value (integral of the Landau function) or dependent value:

$$f(x) = D + \frac{B - D}{1 + e^{\frac{(x_{mid} - x)}{scal}}}$$

15 The 4PL model equation comprises four parameters:

- D = maximum asymptote. This is analogous to a response value for infinite (or saturate) antibody concentration.
- B = minimum asymptote. This is analogous to the response at 0 µg/ml standard concentration.
20
- x_{mid} = inflection point. This represents the point on the curve where the curvature changes direction or signs. The inflection point is where the curve changes *e.g.* from being concave upwards to concave downwards.
- $scal$ = slope factor, which refers to the steepness of the curve.

The inflection point x_{mid} represents the middle of the range in which the response $f(x)$ is linear
25 dependent by the dose (x).

To identify a range of linear response for the assay, the inventors performed the following steps:

1) perform Gyrolab experiment on a wide concentration interval;

2) from each experiment, estimate parameter A (as described in the Landau distribution section above);

30 3) perform 4PL linear regression on the relationship between A and the concentration of monoclonal antibody used.

4) estimate x_{mid}

Analyses are preferably performed in an interval between the two closest tested concentrations to x_{mid}.

Antigens

5 The antigen is typically an antigen against which antibody in the sample has been raised, or against which antibody in the sample has affinity. For example, when evaluating the binding affinity of an antibody with affinity to *Neisseria meningitis* NadA (*e.g.* antibodies obtained from an animal immunised with NadA), the immobilised ligand is preferably NadA antigen.

The invention can be used to determine antibody affinity for a wide range of antigens, typically 10 antigens that are implicated or involved in a wide range of diseases. The antigen (when used as an immunogen) may elicit an immune response that protects against a viral disease (*e.g.* due to an enveloped or non-enveloped virus), a bacterial disease (*e.g.* due to a Gram negative or a Gram positive bacterium), a fungal disease, a parasitic disease, an auto-immune disease, or any other disease.

15 In a preferred embodiment of the invention, the antigen is an antigen suitable for use in a vaccine or diagnostic composition.

The antigen may take various forms *e.g.* a polypeptide, a saccharide, a liposaccharide, a conjugate (*e.g.* of a carrier and a hapten, or of a carrier and a saccharide), *etc.* Where the antigen is a polypeptide, it will typically be a surface polypeptide *e.g.* an adhesin, a hemagglutinin, an envelope 20 glycoprotein, a spike glycoprotein, *etc.*

The antigen may be produced by expression in an organism that differs from the organism which causes the disease which the antigen provides an immune response against. However, a viral antigen is an antigen which elicits an immune response against a viral disease, even if the viral antigen is expressed in a bacterium. Similarly, an antigen that elicits an immune response against a bacterial 25 disease is a bacterial antigen, irrespective of how the antigen was produced. For example, an HIV antigen that is expressed in *E.coli* is a viral antigen, rather than a bacterial antigen.

Viral Antigens

Orthomyxovirus: Viral antigens include, but are not limited to, those derived from an Orthomyxovirus, such as Influenza A, B and C. In certain embodiments, 30 orthomyxovirus antigens are selected from one or more of the viral proteins, including hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M1), membrane protein (M2), one or more of the transcriptase components (PB1, PB2 and PA). In certain embodiments the viral antigen include HA and NA. In certain embodiments, the influenza antigens are derived from interpandemic (annual) flu strains, 35 while in other embodiments, the influenza antigens are derived from strains with the

potential to cause pandemic a pandemic outbreak.

Paramyxoviridae viruses: Viral antigens include, but are not limited to, those derived from Paramyxoviridae viruses, such as Pneumoviruses (RSV), Paramyxoviruses (PIV), Metapneumovirus and Morbilliviruses (Measles).

5 *Pneumovirus:* Viral antigens include, but are not limited to, those derived from a Pneumovirus, such as Respiratory syncytial virus (RSV), Bovine respiratory syncytial virus, Pneumonia virus of mice, and Turkey rhinotracheitis virus. Preferably, the Pneumovirus is RSV. In certain embodiments, pneumovirus antigens are selected from one or more of the following proteins, including surface proteins Fusion (F), 10 Glycoprotein (G) and Small Hydrophobic protein (SH), matrix proteins M and M2, nucleocapsid proteins N, P and L and nonstructural proteins NS1 and NS2. In other embodiments, pneumovirus antigens include F, G and M.

Paramyxovirus: Viral antigens include, but are not limited to, those derived from a Paramyxovirus, such as Parainfluenza virus types 1 – 4 (PIV), Mumps, Sendai viruses, 15 Simian virus 5, Bovine parainfluenza virus, Nipahvirus, Henipavirus and Newcastle disease virus. In certain embodiments, the Paramyxovirus is PIV or Mumps. In certain embodiments, paramyxovirus antigens are selected from one or more of the following proteins: Hemagglutinin –Neuraminidase (HN), Fusion proteins F1 and F2, Nucleoprotein (NP), Phosphoprotein (P), Large protein (L), and Matrix protein (M). In 20 other embodiments, paramyxovirus proteins include HN, F1 and F2. In other embodiments, the Paramyxovirus is Nipahvirus or Henipavirus and the antigens are selected from one or more of the following proteins: Fusion (F) protein, Glycoprotein (G) protein, Matrix (M) protein, Nucleocapsid (N) protein, Large (L) protein and Phosphoprotein (P).

25 *Poxviridae:* Viral antigens include, but are not limited to, those derived from *Orthopoxvirus* such as *Variola vera*, including but not limited to, *Variola major* and *Variola minor*.

Metapneumovirus: Viral antigens include, but are not limited to, Metapneumovirus, such as human metapneumovirus (hMPV) and avian metapneumoviruses (aMPV). In certain embodiments, metapneumovirus antigens are selected from one or more of the following 30 proteins, including surface proteins Fusion (F), Glycoprotein (G) and Small Hydrophobic protein (SH), matrix proteins M and M2, nucleocapsid proteins N, P and L. In other embodiments, metapneumovirus antigens include F, G and M. *Morbillivirus*: Viral antigens include, but are not limited to, those derived from a Morbillivirus, such as Measles. In certain embodiments, morbillivirus antigens are selected from one or more 35 of the following proteins: hemagglutinin (H), Glycoprotein (G), Fusion factor (F), Large protein (L), Nucleoprotein (NP), Polymerase phosphoprotein (P), and Matrix (M). *Picornavirus*: Viral antigens include, but are not limited to, those derived from Picornaviruses, such as Enteroviruses, Rhinoviruses, Heparnavirus, Cardioviruses and Aphthoviruses. In certain embodiments, the antigens are derived from Enteroviruses,

while in other embodiments the enterovirus is Poliovirus. In still other embodiments, the antigens are derived from Rhinoviruses. *Enterovirus*: Viral antigens include, but are not limited to, those derived from an Enterovirus, such as Poliovirus types 1, 2 or 3, Coxsackie A virus types 1 to 22 and 24, Coxsackie B virus types 1 to 6, Echovirus (ECHO) virus) types 1 to 9, 11 to 27 and 29 to 34 and Enterovirus 68 to 71. In certain embodiments, the antigens are derived from Enteroviruses, while in other embodiments the enterovirus is Poliovirus. In certain embodiments, the enterovirus antigens are selected from one or more of the following Capsid proteins VP0, VP1, VP2, VP3 and VP4. *Bunyavirus:* Viral antigens include, but are not limited to, those derived from an *Orthobunyavirus*, such as California encephalitis virus, a *Phlebovirus*, such as Rift Valley Fever virus, or a *Nairovirus*, such as *Crimean-Congo hemorrhagic fever* virus.

Rhinovirus: Viral antigens include, but are not limted to, those derived from rhinovirus. In certain embodiments, the rhinovirus antigens are selected from one or more of the following Capsid proteins: VP0, VP1, VP2, VP2 and VP4. *Heparnavirus*: Viral antigens include, but are not limited to, those derived from a Heparnavirus, such as, by way of example only, Hepatitis A virus (HAV). *Togavirus*: Viral antigens include, but are not limited to, those derived from a Togavirus, such as a Rubivirus, an Alphavirus, or an Arterivirus. In certain embodiments, the antigens are derived from Rubivirus, such as by way of example only, Rubella virus. In certain embodiments, the togavirus antigens are selected from E1, E2, E3, C, NSP-1, NSPO-2, NSP-3 or NSP-4. In certain embodiments, the togavirus antigens are selected from E1, E2 or E3. *Flavivirus*: Viral antigens include, but are not limited to, those derived from a Flavivirus, such as Tick-borne encephalitis (TBE) virus, Dengue (types 1, 2, 3 or 4) virus, Yellow Fever virus, Japanese encephalitis virus, Kyasanur Forest Virus, West Nile encephalitis virus, St. Louis encephalitis virus, Russian spring-summer encephalitis virus, Powassan encephalitis virus. In certain embodiments, the flavivirus antigens are selected from PrM, M, C, E, NS-1, NS-2a, NS2b, NS3, NS4a, NS4b, and NS5. In certain embodiments, the flavivirus antigens are selected from PrM, M and E. *Pestivirus*: Viral antigens include, but are not limited to, those derived from a Pestivirus, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).

Hepadnavirus: Viral antigens include, but are not limited to, those derived from a Hepadnavirus, such as Hepatitis B virus. In certain embodiments, the hepadnavirus antigens are selected from surface antigens (L, M and S), core antigens (HBc, HBe).

Hepatitis C virus: Viral antigens include, but are not limited to, those derived from a Hepatitis C virus (HCV). In certain embodiments, the HCV antigens are selected from one or more of E1, E2, E1/E2, NS345 polyprotein, NS 345-core polyprotein, core, and/or peptides from the nonstructural regions. In certain embodiments, the Hepatitis C virus antigens include one or more of the following: HCV E1 and or E2 proteins, E1/E2 heterodimer complexes, core proteins and non-structural proteins, or fragments of these

antigens, wherein the non-structural proteins can optionally be modified to remove enzymatic activity but retain antigenicity. *Rhabdovirus*: Viral antigens include, but are not limited to, those derived from a Rhabdovirus, such as a Lyssavirus (Rabies virus) and Vesiculovirus (VSV). Rhabdovirus antigens may be selected from glycoprotein (G), nucleoprotein (N), large protein (L), nonstructural proteins (NS). *Caliciviridae;* Viral antigens include, but are not limited to, those derived from Calciviridae, such as Norwalk virus, and Norwalk-like Viruses, such as Hawaii Virus and Snow Mountain Virus. *Coronavirus:* Viral antigens include, but are not limited to, those derived from a Coronavirus, SARS, Human respiratory coronavirus, Avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible gastroenteritis virus (TGEV). In certain embodiments, the coronavirus antigens are selected from spike (S), envelope (E), matrix (M), nucleocapsid (N), and Hemagglutinin-esterase glycoprotein (HE). In certain embodiments, the coronavirus antigen is derived from a SARS virus. *Retrovirus*: Viral antigens include, but are not limited to, those derived from a Retrovirus, such as an Oncovirus, a Lentivirus or a Spumavirus. In certain embodiments, the oncovirus antigens are derived from HTLV-1, HTLV-2 or HTLV-5. In certain embodiments, the lentivirus antigens are derived from HIV-1 or HIV-2. In certain embodiments, the antigens are derived from HIV-1 subtypes (or clades), including, but not limited to, HIV-1 subtypes (or clades) A, B, C, D, F, G, H, J. K, O. In other embodiments, the antigens are derived from HIV-1 circulating recombinant forms (CRFs), including, but not limited to, A/B, A/E, A/G, A/G/I, etc. In certain embodiments, the retrovirus antigens are selected from gag, pol, env, tax, tat, rex, rev, nef, vif, vpu, and vpr. In certain embodiments, the HIV antigens are selected from gag (p24gag and p55gag), env (gp160 and gp41), pol, tat, nef, rev vpu, miniproteins, (preferably p55 gag and gp140v delete). In certain embodiments, the HIV antigens are derived from one or more of the following strains: HIVIIIb, HIVSF2, HIVLAV, HIVLAI, HIVMN, HIV-1CM235, HIV-1US4, HIV-1SF162, HIV-1TV1, HIV-1MJ4,. In certain embodiments, the antigens are derived from endogenous human retroviruses, including, but not limited to, HERV-K ("old" HERV-K and "new" HERV-K).

Reovirus: Viral antigens include, but are not limited to, those derived from a Reovirus, such as an Orthoreovirus, a Rotavirus, an Orbivirus, or a Coltivirus. In certain embodiments, the reovirus antigens are selected from structural proteins λ1, λ2, λ3, μ1, μ2, σ1, σ2, or σ3, or nonstructural proteins σNS, μNS, or σ1s. In certain embodiments, the reovirus antigens are derived from a Rotavirus. In certain embodiments, the rotavirus antigens are selected from VP1, VP2, VP3, VP4 (or the cleaved product VP5 and VP8), NSP 1, VP6, NSP3, NSP2, VP7, NSP4, or NSP5. *Parvovirus:* Viral antigens include, but are not limited to, those derived from a Parvovirus, such as Parvovirus B19. In certain embodiments, the Parvovirus antigens are selected from VP-1, VP-2, VP-3, NS-1 and NS-2. In certain embodiments, the Parvovirus antigen is capsid protein VP1 or VP-2.

Delta hepatitis virus (HDV): Viral antigens include, but are not limited to, those derived from HDV, particularly δ -antigen from HDV.

Hepatitis E virus (HEV): Viral antigens include, but are not limited to, those derived from HEV.

5 *Hepatitis G virus (HGV)*: Viral antigens include, but are not limited to, those derived from HGV.

Human Herpesvirus: Viral antigens include, but are not limited to, those derived from a Human Herpesvirus, such as, by way of example only, Herpes Simplex Viruses (HSV), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), 10 Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8). In certain embodiments, the Human Herpesvirus antigens are selected from immediate early proteins (α), early proteins (β), and late proteins (γ). In certain embodiments, the HSV antigens are derived from HSV-1 or HSV-2 strains. In certain embodiments, the HSV antigens are selected from glycoproteins gB, gC, gD and gH, 15 fusion protein (gB), or immune escape proteins (gC, gE, or gI). In certain embodiments, the VZV antigens are selected from core, nucleocapsid, tegument, or envelope proteins. A live attenuated VZV vaccine is commercially available. Such antigens are suitable for use in methods of the invention. In certain embodiments, the EBV antigens are selected from early antigen (EA) proteins, viral capsid antigen (VCA), and glycoproteins of the 20 membrane antigen (MA). In certain embodiments, the CMV antigens are selected from capsid proteins, envelope glycoproteins (such as gB and gH), and tegument proteins. In other embodiments, CMV antigens may be selected from one or more of the following proteins: pp65, IE1, gB, gD, gH, gL, gM, gN, gO, UL128, UL129, gUL130, UL150, UL131, UL33, UL78, US27, US28, RL5A, RL6, RL10, RL11, RL12, RL13, UL1, UL2, 25 UL4, UL5, UL6, UL7, UL8, UL9, UL10, UL11, UL14, UL15A, UL16, UL17, UL18, UL22A, UL38, UL40, UL41A, UL42, UL116, UL119, UL120, UL121, UL124, UL132, UL147A, UL148, UL142, UL144, UL141, UL140, UL135, UL136, UL138, UL139, UL133, UL135, UL148A, UL148B, UL148C, UL148D, US2, US3, US6, US7, US8, US9, US10, US11, US12, US13, US14, US15, US16, US17, US18, US19, US20, US21, 30 US29, US30 and US34A. CMV antigens may also be fusions of one or more CMV proteins, such as, by way of example only, pp65/IE1 (Reap *et al.*, *Vaccine* (2007) 25:7441-7449). *Papovaviruses*: Antigens include, but are not limited to, those derived from Papovaviruses, such as Papillomaviruses and Polyomaviruses. In certain embodiments, the Papillomaviruses include HPV serotypes 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 35 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 and 65. In certain embodiments, the HPV antigens are selected from capsid proteins (L1) and (L2), or E1 – E7, or fusions thereof. In certain embodiments, the Polyomyavirus viruses include BK virus and JK virus. In certain embodiments, the Polyomavirus antigens are selected from VP1, VP2 or VP3.

Adenovirus: Antigens include those derived from Adenovirus. In certain embodiments, the

Adenovirus antigens are derived from Adenovirus serotype 36 (Ad-36).

Bacterial Antigens

Bacterial antigens suitable for use in methods of the invention include, but are not limited to, proteins, polysaccharides and lipopolysaccharides In certain embodiments, the bacterial antigens are produced by recombinant expression. .

Neisseria meningitidis: *N.meningitidis* antigens include, but are not limited to, proteins, saccharides (including a polysaccharide, or lipooligosaccharide), or outer-membrane vesicles purified or derived from *N. meningitidis* serogroup such as A, C, W135, Y, X or B. Useful *N.meningitidis* protein antigens include NHBA, a fHbp or NadA.

Streptococcus pneumoniae: *Streptococcus pneumoniae* antigens include, but are not limited to, a saccharide (including a polysaccharide or an oligosaccharide) and/or protein from *Streptococcus pneumoniae*. The saccharide may be a polysaccharide having the size that arises during purification of the saccharide from bacteria, or it may be an oligosaccharide achieved by fragmentation of such a polysaccharide. In certain embodiments saccharide antigens are selected from one or more of the following pneumococcal serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and/or 33F. In certain embodiments, protein antigens may be selected from a protein identified in WO98/18931, WO98/18930, US Patent 6,699,703, US Patent 6,800,744, WO97/43303, WO97/37026, WO 02/079241, WO 02/34773, WO 00/06737, WO 00/06738, WO 00/58475, WO 2003/082183, WO 00/37105, WO 02/22167, WO 02/22168, WO 2003/104272, WO 02/08426, WO 01/12219, WO 99/53940, WO 01/81380, WO 2004/092209, WO 00/76540, WO 2007/116322, LeMieux *et al.*, Infect. Imm. (2006) 74:2453-2456, Hoskins *et al.*, J. Bacteriol. (2001) 183:5709-5717, Adamou *et al.*, Infect. Immun. (2001) 69(2):949-958, Briles *et al.*, J. Infect. Dis. (2000) 182:1694-1701, Talkington *et al.*, Microb. Pathog. (1996) 21(1):17-22, Bethe *et al.*, FEMS Microbiol. Lett. (2001) 205(1):99-104, Brown *et al.*, Infect. Immun. (2001) 69:6702-6706, Whalen *et al.*, FEMS Immunol. Med. Microbiol. (2005) 43:73-80, Jomaa *et al.*, Vaccine (2006) 24(24):5133-5139. In other embodiments, *Streptococcus pneumoniae* proteins may be selected from the Poly Histidine Triad family (PhtX), the Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins, pneumolysin (Ply), PspA, PsaA, Spl28, SpIOl, Spl30, Spl25, Spl33, pneumococcal pilus subunits.

Streptococcus pyogenes (Group A Streptococcus): Group A Streptococcus antigens include, but are not limited to, a protein identified in WO 02/34771 or WO 2005/032582 (including GAS 40), fusions of fragments of GAS M proteins (including those described in WO 02/094851, and Dale, Vaccine (1999) 17:193-200, and Dale, Vaccine 14(10): 944-948), fibronectin binding protein (Sfb1), Streptococcal heme-associated protein (Shp), and Streptolysin S (SagA).

Moraxella catarrhalis: Moraxella antigens include, but are not limited to, antigens identified

in WO 02/18595 and WO 99/58562, outer membrane protein antigens (HMW-OMP), C-antigen, and/or LPS.

Bordetella pertussis: Pertussis antigens include, but are not limited to, pertussis toxoid (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*, optionally also combination with pertactin and/or agglutinogens 2 and 3.

Burkholderia: Burkholderia antigens include, but are not limited to Burkholderia mallei, Burkholderia pseudomallei and Burkholderia cepacia.

Staphylococcus aureus: Staph aureus antigens include, but are not limited to, a polysaccharide and/or protein from *S. aureus*. *S. aureus* polysaccharides include, but are not limited to, type 5 and type 8 capsular polysaccharides (CP5 and CP8) optionally conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A, such as StaphVAX™, type 336 polysaccharides (336PS), polysaccharide intercellular adhesions (PIA, also known as PNAG). *S. aureus* proteins include, but are not limited to, antigens derived from surface proteins, invasins (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), carotenoids, catalase production, Protein A, coagulase, clotting factor, and/or membrane-damaging toxins (optionally detoxified) that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin). In certain embodiments, *S. aureus* antigens may be selected from a protein identified in WO 02/094868, WO 2008/019162, WO 02/059148, WO 02/102829, WO 03/011899, WO 2005/079315, WO 02/077183, WO 99/27109, WO 01/70955, WO 00/12689, WO 00/12131, WO 2006/032475, WO 2006/032472, WO 2006/032500, WO 2007/113222, WO 2007/113223, WO 2007/113224. In other embodiments, *S. aureus* antigens may be selected from IsdA, IsdB, IsdC, SdrC, SdrD, SdrE, ClfA, ClfB, SasF, SasD, SasH (AdsA), Spa, EsaC, EsxA, EsxB, Emp, HlaH35L, CP5, CP8, PNAG, 336PS.

Staphylococcus epidermis: S. epidermidis antigens include, but are not limited to, slime-associated antigen (SAA).

Clostridium tetani (Tetanus): Tetanus antigens include, but are not limited to, tetanus toxoid (TT).

Clostridium perfringens: Antigens include, but are not limited to, Epsilon toxin from *Clostridium perfringen.*

Clostridium botulinums (Botulism): Botulism antigens include, but are not limited to, those derived from *C. botulinum*.

Cornynebacterium diphtheriae (Diphtheria): Diphtheria antigens include, but are not limited to, diphtheria toxin, preferably detoxified, such as CRM197.

Haemophilus influenzae B (Hib): Hib antigens include, but are not limited to, a Hib saccharide antigen. T

Pseudomonas aeruginosa: Pseudomonas antigens include, but are not limited to, endotoxin A, Wzz protein, *P. aeruginosa* LPS, LPS isolated from PAO1 (O5 serotype), and/or

Outer Membrane Proteins, including Outer Membrane Proteins F (OprF).

Brucella. Bacterial antigens derived from Brucella, including but not limited to, B. abortus, B. canis, B. melitensis, B. neotomae, B. ovis, B. suis and B. pinnipediae.

5 *Francisella.* Bacterial antigens derived from Francisella, including but not limited to, *F. novicida, F. philomiragia* and *F. tularensis.*

Streptococcus agalactiae (Group B Streptococcus): Group B Streptococcus antigens include, but are not limited to, a protein or saccharide antigen identified in WO 02/34771, WO 03/093306, WO 04/041157, or WO 2005/002619 (including proteins GBS 80, GBS 104, GBS 276 and GBS 322, and including saccharide antigens derived from serotypes Ia, Ib, 10 Ia/c, II, III, IV, V, VI, VII and VIII).

Neiserria gonorrhoeae: Gonorrhoeae antigens include,but are not limited to, Por (or porin) protein, such as PorB (see Zhu *et al*., *Vaccine* (2004) 22:660 - 669), a transferring binding protein, such as TbpA and TbpB (See Price *et al*., Infection and Immunity (2004) 71(1):277 - 283), a opacity protein (such as Opa), a reduction-modifiable protein 15 (Rmp), and outer membrane vesicle (OMV) preparations (see Plante *et al*, J Infectious Disease (2000) 182:848 - 855), also see, *e.g*., WO99/24578, WO99/36544, WO99/57280, WO02/079243).

Chlamydia trachomatis: Chlamydia trachomatis antigens include, but are not limited to, antigens derived from serotypes A, B, Ba and C (agents of trachoma, a cause of 20 blindness), serotypes L1, L2 & L3 (associated with Lymphogranuloma venereum), and serotypes, D-K. In certain embodiments, chlamydia trachomas antigens include, but are not limited to, an antigen identified in WO 00/37494, WO 03/049762, WO 03/068811, or WO 05/002619, including PepA (CT045), LcrE (CT089), ArtJ (CT381), DnaK (CT396), CT398, OmpH-like (CT242), L7/L12 (CT316), OmcA (CT444), AtosS 25 (CT467), CT547, Eno (CT587), HrtA (CT823), and MurG (CT761).

Treponema pallidum (Syphilis): Syphilis antigens include, but are not limited to, TmpA antigen.

Haemophilus ducreyi (causing chancroid): Ducreyi antigens include, but are not limited to, outer membrane protein (DsrA).

30 *Enterococcus faecalis or* Enterococcus faecium: Antigens include, but are not limited to, a trisaccharide repeat or other *Enterococcus* derived antigens.

Helicobacter pylori: H pylori antigens include, but are not limited to, CagA, VacA, NAP, HopX, HopY and/or urease antigen.

Staphylococcus saprophyticus: Antigens include, but are not limited to, the 160 kDa 35 hemagglutinin of *S. saprophyticus* antigen.

Yersinia enterocolitica Antigens include, but are not limited to, LPS.

E. coli: E. coli antigens may be derived from enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC),

enteropathogenic *E. coli* (EPEC), extraintestinal pathogenic *E. coli* (ExPEC) and/or enterohemorrhagic *E. coli* (EHEC). .

Bacillus anthracis (anthrax): *B. anthracis* antigens include, but are not limited to, A-components (lethal factor (LF) and edema factor (EF)).

5 *Yersinia pestis (plague)*: Plague antigens include, but are not limited to, F1 capsular antigen, LPS, Yersinia pestis V antigen.

Mycobacterium tuberculosis: Tuberculosis antigens include, but are not limited to, lipoproteins, LPS, BCG antigens, a fusion protein of antigen 85B (Ag85B), ESAT-6, Mycobacterium tuberculosis (Mtb) isocitrate dehydrogenase associated antigens, and 10 MPT51 antigens.

Rickettsia: Antigens include, but are not limited to, outer membrane proteins, including the outer membrane protein A and/or B (OmpB), LPS, and surface protein antigen (SPA).

Listeria monocytogenes: Bacterial antigens include, but are not limited to, those derived from Listeria monocytogenes.

15 *Chlamydia pneumoniae:* Antigens include, but are not limited to, those identified in WO 02/02606.

Vibrio cholerae: Antigens include, but are not limited to, proteinase antigens, LPS, particularly lipopolysaccharides of Vibrio cholerae II, O1 Inaba O-specific polysaccharides, V. cholera O139, antigens of IEM108 vaccine and Zonula occludens 20 toxin (Zot).

Salmonella typhi (typhoid fever): Antigens include, but are not limited to, capsular polysaccharides preferably conjugates (Vi, i.e. vax-TyVi).

Borrelia burgdorferi (Lyme disease): Antigens include, but are not limited to, lipoproteins (such as OspA, OspB, Osp C and Osp D), other surface proteins such as OspE-related 25 proteins (Erps), decorin-binding proteins (such as DbpA), and antigenically variable VI proteins, such as antigens associated with P39 and P13 (an integral membrane protein, VlsE Antigenic Variation Protein.

Porphyromonas gingivalis: Antigens include, but are not limited to, *P. gingivalis* outer membrane protein (OMP).

30 *Klebsiella:* Antigens include, but are not limited to, an OMP, including OMP A, or a polysaccharide optionally conjugated to tetanus toxoid.

In certain embodiments, any of the above bacterial-derived saccharides (polysaccharides, LPS, LOS or oligosaccharides) are conjugated to another agent or antigen, such as a carrier protein (for example CRM_{197}, tetanus toxoid or diphtheria toxoid).

35 *Fungal Antigens*

Fungal antigens suitable for use in methods of the invention include, but are not limited to, those derived from one or more of the fungi set forth below.

Fungal antigens are derived from Dermatophytes, including: Epidermophyton floccusum, Microsporum audouini, Microsporum canis, Microsporum distortum, Microsporum equinum, Microsporum gypsum, Microsporum nanum, Trichophyton concentricum, Trichophyton equinum, Trichophyton gallinae, Trichophyton gypseum, Trichophyton megnini, Trichophyton mentagrophytes, Trichophyton quinckeanum, Trichophyton rubrum, Trichophyton schoenleini, Trichophyton tonsurans, Trichophyton verrucosum, T. verrucosum var. album, var. discoides, var. ochraceum, Trichophyton violaceum, and/or Trichophyton faviforme; and

Fungal pathogens are derived from Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Aspergillus sydowi, Aspergillus flavatus, Aspergillus glaucus, Blastoschizomyces capitatus, Candida albicans, Candida enolase, Candida tropicalis, Candida glabrata, Candida krusei, Candida parapsilosis, Candida stellatoidea, Candida kusei, Candida parakwsei, Candida lusitaniae, Candida pseudotropicalis, Candida guilliermondi, Cladosporium carrionii, Coccidioides immitis, Blastomyces dermatidis, Cryptococcus neoformans, Geotrichum clavatum, Histoplasma capsulatum, Klebsiella pneumoniae, Microsporidia, Encephalitozoon spp., Septata intestinalis and Enterocytozoon bieneusi; the less common are Brachiola spp, Microsporidium spp., Nosema spp., Pleistophora spp., Trachipleistophora spp., Vittaforma spp Paracoccidioides brasiliensis, Pneumocystis carinii, Pythiumn insidiosum, Pityrosporum ovale, Sacharomyces cerevisae, Saccharomyces boulardii, Saccharomyces pombe, Scedosporium apiosperum, Sporothrix schenckii, Trichosporon beigelii, Toxoplasma gondii, Penicillium marneffei, Malassezia spp., Fonsecaea spp., Wangiella spp., Sporothrix spp., Basidiobolus spp., Conidiobolus spp., Rhizopus spp, Mucor spp, Absidia spp, Mortierella spp, Cunninghamella spp, Saksenaea spp., Alternaria spp, Curvularia spp, Helminthosporium spp, Fusarium spp, Aspergillus spp, Penicillium spp, Monolinia spp, Rhizoctonia spp, Paecilomyces spp, Pithomyces spp, and Cladosporium spp.

For example, the antigen may elicit an immune response against a Candida fungus such as *C.albicans*. For instance, the antigen may be a β-glucan, which may be conjugated to a carrier protein. The glucan may include β-1,3 and/or β-1,6 linkages. Suitable antigens include those disclosed in references 3 & 4.

Tumor Antigens

In certain embodiments, a tumor antigen or cancer antigen is used methods of the invention. In certain embodiments, the tumor antigen is a peptide-containing tumor antigens, such as a polypeptide tumor antigen or glycoprotein tumor antigens.

In certain embodiments, the tumor antigens include, but are not limited to, (a) cancer-testis antigens such as NY-ESO-1, SSX2, SCP1 as well as RAGE, BAGE, GAGE and MAGE family polypeptides, for example, GAGE-1, GAGE-2, MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, and

MAGE-12 (which can be used, for example, to address melanoma, lung, head and neck, NSCLC, breast, gastrointestinal, and bladder tumors), (b) mutated antigens, for example, p53 (associated with various solid tumors, e.g., colorectal, lung, head and neck cancer), p21/Ras (associated with, e.g., melanoma, pancreatic cancer and colorectal cancer), CDK4 (associated with, e.g., melanoma), MUM1 (associated with, e.g., melanoma), caspase-8 (associated with, e.g., head and neck cancer), CIA 0205 (associated with, e.g., bladder cancer), HLA-A2-R1701, beta catenin (associated with, e.g., melanoma), TCR (associated with, e.g., T-cell non-Hodgkins lymphoma), BCR-abl (associated with, e.g., chronic myelogenous leukemia), triosephosphate isomerase, KIA 0205, CDC-27, and LDLR-FUT, (c) over-expressed antigens, for example, Galectin 4 (associated with, e.g., colorectal cancer), Galectin 9 (associated with, e.g., Hodgkin's disease), proteinase 3 (associated with, e.g., chronic myelogenous leukemia), WT 1 (associated with, e.g., various leukemias), carbonic anhydrase (associated with, e.g., renal cancer), aldolase A (associated with, e.g., lung cancer), PRAME (associated with, e.g., melanoma), HER-2/neu (associated with, e.g., breast, colon, lung and ovarian cancer), alpha-fetoprotein (associated with, e.g., hepatoma), KSA (associated with, e.g., colorectal cancer), gastrin (associated with, e.g., pancreatic and gastric cancer), telomerase catalytic protein, MUC-1 (associated with, e.g., breast and ovarian cancer), G-250 (associated with, e.g., renal cell carcinoma), p53 (associated with, e.g., breast, colon cancer), and carcinoembryonic antigen (associated with, e.g., breast cancer, lung cancer, and cancers of the gastrointestinal tract such as colorectal cancer), (d) shared antigens, for example, melanoma-melanocyte differentiation antigens such as MART-1/Melan A, gp100, MC1R, melanocyte-stimulating hormone receptor, tyrosinase, tyrosinase related protein-1/TRP1 and tyrosinase related protein-2/TRP2 (associated with, e.g., melanoma), (e) prostate associated antigens such as PAP, PSA, PSMA, PSH-P1, PSM-P1, PSM-P2, associated with e.g., prostate cancer, (f) immunoglobulin idiotypes (associated with myeloma and B cell lymphomas, for example), and (g) other tumor antigens, such as polypeptide- and saccharide-containing antigens including (i) glycoproteins such as sialyl Tn and sialyl Le^x (associated with, e.g., breast and colorectal cancer) as well as various mucins; glycoproteins are coupled to a carrier protein (e.g., MUC-1 are coupled to KLH); (ii) lipopolypeptides (e.g., MUC-1 linked to a lipid moiety); (iii) polysaccharides (e.g., Globo H synthetic hexasaccharide), which are coupled to a carrier proteins (e.g., to KLH), (iv) gangliosides such as GM2, GM12, GD2, GD3 (associated with, e.g., brain, lung cancer, melanoma), which also are coupled to carrier proteins (e.g., KLH).

In certain embodiments, the tumor antigens include, but are not limited to, p15, Hom/Mel-40, H-Ras, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, EBNA, human papillomavirus (HPV) antigens, including E6 and E7, hepatitis B and C virus antigens, human T-cell lymphotropic virus antigens, TSP-180, p185erbB2, p180erbB-3, c-met, mn-23H1, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, p16, TAGE, PSCA, CT7, 43-9F, 5T4, 791 Tgp72, beta-HCG, BCA225, BTAA, CA 125, CA 15-3 (CA 27.29\BCAA), CA 195, CA 242, CA-50, CAM43, CD68\KP1, CO-029, FGF-5, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90 (Mac-2 binding protein\cyclophilin C-associated protein), TAAL6, TAG72, TLP, TPS, and the like.

Receptors

As discussed above, the "receptor" may be *e.g.* an antibody or an antigen. Antibodies bind to antigen to form an antibody/antigen complex, and so when the receptor is an antibody, the ligand is an antigen. Similarly, when the receptor is an antigen, the ligand is an antibody. The receptor is 5 typically an antibody.

Antibodies

The term "antibody" refers to a polypeptide or group of polypeptides that comprise at least one antigen-binding site. An "antigen binding site" is formed from the folding of the variable domains of an antibody molecule(s) to form three-dimensional binding sites with an internal surface shape and 10 charge distribution complementary to the features of an epitope of an antigen, which allows specific binding to form an antibody/antigen complex. An antigen-binding site may be formed from a heavy- and/or light-chain domain (VH and VL, respectively), which form hypervariable loops that contribute to antigen binding.

The term "antibody" as used herein includes antibodies obtained from both polyclonal and 15 monoclonal preparations, as well as, the following: (i) hybrid (chimeric) antibody molecules (*e.g* refs 5,6); (ii) F(ab')2 and F(ab) fragments; (iii) Fv molecules (noncovalent heterodimers (*e.g.* refs 7,8); (iv) single-chain Fv molecules (sFv) (*e.g.* ref 9); (v) dimeric and trimeric antibody fragment constructs; (vi) humanized antibody molecules (*e.g.* ref 10,11,12); (vii) Mini-antibodies or minibodies (i.e., sFv polypeptide chains that include oligomerization domains at their C-termini, 20 separated from the sFv by a hinge region; (*e.g.* refs 13,14); (vii) human antibodies; and, (viii) any functional fragments obtained from such molecules, wherein such fragments retain specific-binding properties of the parent antibody molecule.

Techniques for producing and processing polyclonal antisera are known in the art (*e.g.* ref. 15).

One skilled in the art can also readily produce monoclonal antibodies directed against an epitope. 25 The general methodology for making monoclonal antibodies by hybridomas is well known.

Thus, preferred samples for use in methods of the invention comprise a monoclonal antibody or a polyclonal antibody. Particularly preferred samples for use in methods of the invention include e.g. monoclonal antibody preparations or polyclonal antibody sera.

Ligand/receptor complex

30 Receptors bind ligand to form a ligand/receptor complex. The invention is based on the analysis of ligand/receptor complex formation, and so it does not matter which binding partner(s) in the ligand/receptor complex is/are termed "receptor" or "ligand". For example, the "receptor" may be an antibody or an antigen, and *vice versa* for the "ligand".

A ligand/receptor complex may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more receptors. In some embodiments, a ligand/receptor complex comprises 1 ligand and 1, 2, 3, 4, 5, or more receptors. In another embodiment, a ligand/receptor complex comprises 2 ligands and 1, 2, 3, 4, 5, or more receptors. In another embodiment, a ligand/receptor complex comprises 3 ligands and 1, 2, 3, 4, 5, or
5 more receptors.

A ligand/receptor complex may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more ligands. In some embodiments, a ligand/receptor complex comprises 1 receptor and 1, 2, 3, 4, 5, or more ligands. In another embodiment, a ligand/receptor complex comprises 2 receptors and 1, 2, 3, 4, 5, or more ligands. In another embodiment, a ligand/receptor complex comprises 3 receptors and 1, 2, 3, 4, 5, or
10 more ligands.

Therapy

Antibodies identified and/or isolated using methods of the invention can be used for passive immunisation or for immunotherapy. The invention thus provides an antibody identified by a method of the invention for use in therapy. The invention also provides the use of such antibodies in the
15 manufacture of a medicament. The invention also provides a method for treating a mammal comprising the step of administering an effective amount of antibody identified by a method of the invention. The invention also provides a composition comprising an antibody identified using a method of the invention. Compositions comprising antibodies identified using methods of the invention are useful for protecting a mammal against infection and/or disease.

20 ***General***

As used herein, the transitional term "comprising", which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. Finally, as used herein, the transitional
25 phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention as understood under United States patent law.

The term “about” in relation to a numerical value x means, for example, + two standard deviations of the value. In certain embodiments, “about” is understood as acceptable variation and tolerances
30 within the specific art. The term “about” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, particularly ±5%, even more particularly ±1%, and still more particularly ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The word “substantially” does not exclude “completely” e.g. a composition which is “substantially
35 free” from Y may be completely free from Y. For example, “substantially free” from Y can be

understood as a composition containing not more than 5% Y, not more than 4% Y, not more than 3% Y, not more than 2% Y, not more than 1% Y, or not more than 0.1% Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

As used herein, unless otherwise clear from context the term "or" is understood to be inclusive and can be used interchangeably with the term "and/or".

As used herein, "a" and "the" are understood to include both singular and plural unless otherwise clearly indicated by context.

Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, etc.

This invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLES

Gyrolab data were analyzed with the model of the invention and validated against Biacore. The inventors found that the affinity scores W are linearly correlated with the affinity constants (KD) determined by Biacore.

Analysis of the antigen-antibody reaction profiles obtained by Gyrolab system

The Gyrolab system is a technology that allows a user to perform miniaturized immunoassay in a high throughput manner. Using monoclonal antibodies specific for different antigens (bacterial proteins, viral proteins, polysaccharides) the inventors analyzed the profile of binding obtained by the Gyrolab system data. In a pilot study, to test the data output obtained by Gyrolab system in terms of profile of binding, the inventors used three monoclonal antibodies specific for *Neisseria meningitidis* NadA antigen with the following protocol:

Three mouse anti- *Neisseria meningitidis* NadA monoclonal antibodies were used:

- 26H11/37 (IgGl) concentrated 0.7 mg/ml raised against NadA var3 plus FCA.
- 27C3/11 (IgG2b) concentrated 1.1 mg/ml raised against NadA var3 plus FCA.
- 20G2/A6 (IgGl) concentrated 1.0 mg/ml raised against NadA var1 plus FCA.

26H11/37 and 27C3/11 were run with the Gyrolab system at 7 step 1:4 dilutions starting from 20μg/ml while 20G2/A6 starting from 400μg/ml. Dilutions were performed using Rexipp A™ buffer (Gyros). The runs were performed with the 200nl CD format using the Bioaffy200vl method.

As capture reagent biotinylated recombinant NadA was used at 100μg/ml (diluted in PBS/Tween20 0.01%). NadA was biotinylated using EZ-Link sulfo-NHS-LC-Biotin (Thermo Scientific) at a molar excess of 10 moles of biotin: 1 mole of protein.

As detection reagent anti-mouse IgG-Alexa 647 were used at 25 nM. Donkey anti-mouse IgG was labelled with Alexa 647 using the Alexa Fluor Monoclonal antibodies labelling kit from Molecular Probes. Dilutions were performed in Rexipp F™ buffer.

The fluorescence intensity (FI) response of each data-point was automatically provided by the instrument through the Gyrolab evaluator software. The obtained dose-response curves are reported in Figure 1.

The Gyrolab Viewer software allows the visualization of each single reaction profile with 3D and 2D images. The 3D reaction profiles of monoclonal antibodies are reported in Figure 2A. The overlapping of the 2D reaction profiles of the 3 analyzed monoclonal antibodies is reported in Figure 2B. The profile of monoclonal antibodies is shown both in Figure 2A and 2B at the concentration of 0.02 μg/ml for monoclonal antibody 26H11/37, 0.08 μg/ml for monoclonal antibody 27C3/11 and of 5 μg/ml for monoclonal antibody 20G2/A6. At these concentrations the 3 monoclonal antibodies displayed similar responses in terms of fluorescence intensity.

To confirm whether the experimental protocol allows us to describe the reaction profiles of binding to antigens of different chemical nature, the inventors tested a panel of monoclonal antibodies specific for different recombinant proteins (*Neisseria meningitis* NadA and fHbp) and polysaccharides (Psla of *Streptococcus agalactiae*). All of the monoclonal antibodies analyzed have a profile of interaction with the antigen that is represented by an asymmetric unimodal distribution (Figure 3).

Results

Definition of the best mathematical fit of the antibody-antigen reaction profile.

Trend of monoclonal antibody reaction profile is very similar with a mathematical function belonging to the General Extreme Value distribution class, known as *Landau distribution.*

The *Landau distribution* (16) was first introduced to describe the fluctuations of energy loss by ionization of a charged particle in a thin layer of matter and approximated by Behrens (17) as:

$$f(z) = y_0 + A\exp[-\frac{1}{2}(z + \exp(-z))] \quad \textbf{(Eq. 1)}$$

where $z = \frac{x - x_c}{w}$.

Fitting of the Landau distribution to data showed a very good agreement between the mathematical function and the experimental trends for all the monoclonal antibodies investigated, at all the concentrations providing signals above the detection limit of the instrument.

For each Gyrolab experiment described in the following, the best fit of Eq. 1 to FI (fluorescence intensity) data was obtained by the least-squares method as implemented in the *nls* package of R version 2.14 (ref. 18).

Figure 4 shows the best fit of Eq. 1 to raw Gyrolab data, for monoclonal antibodies specific for different antigens, and tested at different concentrations. The best fit of Eq. 1 describes accurately the Fluorescence Intensity (FI) profiles obtained experimentally. The Pearson's product-moment correlation coefficient r between the raw data and their corresponding fitted data exceeds 0.97 for
5 each monoclonal antibody tested with a *p-value* < 0.001.

Landau distribution parameters are defined above.

Determination of the "Affinity Score" (W) for monoclonal antibodies

To identify a range of linear response for the assay that allows to evaluate the affinity score W through mathematical regression of Eq. 1 to FI data, the inventors repeatedly tested, at different
10 concentrations, three monoclonal antibodies showing low, medium and high affinity for the *Neisseria meningitides* NadA protein (20G2, 27C3 and 26H11 respectively). From each experiment the inventors estimated the parameter, A according to the fitting procedure described above, and evaluated the relationship between A and the concentration of monoclonal antibody used. Results are shown in Figure 5.

15 Visual inspection of data reported in Figure 5 allowed the inventors to define for each monoclonal antibody a range of concentrations where the parameter A varies linearly with monoclonal antibody concentration, indicating that the fitting procedure of Eq.1 to data can capture accurately the monoclonal antibody -substrate interaction. The inventors confirmed these data by using the newly developed 4PL-based technique described above.

20 Results are shown in Table 1. As expected, monoclonal antibodies with lower affinity require higher concentrations than monoclonal antibodies with higher affinity to reach the linear range for the parameter A. The linear range of A is similar for the three monoclonal antibodies.

mAb	A linear range	Concentration range (µg/ml)
20G2	~ 0.1-5	1.250 - 20.00
27C3	~ 0.2-10	0.004-0.310
26H11	~ 0.05-5	0.004-0.310

Table 1

25 We estimated the affinity score W for each Gyrolab measurements in the linear range of parameter A. Results are shown in Figure 6. For each monoclonal antibody the value of W is substantially constant - within the experimental variability - for all the concentrations used. This indicates that W captures an intrinsic property of the monoclonal antibody, such as monoclonal antibody ' affinity to the substrate, not dependent on the concentration of the monoclonal antibody used in the experiment.

30 Table 2 reports the mean and the standard deviation (sd) of the affinity score W determined from the results shown in Figure 6. Each monoclonal has also been tested by Biacore through Surface

Plasmon Resonance (SPR) to assess the affinity-constant (KD value) to the NadA antigen. To perform the SPR assay the same biotynlated-NadA antigen, used also as capture reagent by Gyrolab, has been used and the KD values obtained by Biacore are shown in Table 2. The inventors observed that the affinity hierarchy obtained by Biacore corresponds to that obtained by Gyrolab.

5

mAb	Affinity score ***W*** mean ± sd	Number of Gyrolab measurements	KD (Biacore)
20G2	10.43 ±0.88	8	(1.14 ±0.04) x10^{-8}
27C3	7.73 ± 0.70	10	(1.73 ±0.02) x10^{-9}
26H11	5.92 ± 0.66	9	(6.00 ± 1.00) x10^{-10}

Table 2

The "Affinity Score" W is linearly correlated with Biacore's KD affinity constant

To validate the interpretation of *W* as a score indicating affinity of a antibody *vs.* a specific substrate, the inventors have performed a correlation analysis of *W vs.* the affinity-constant KD measured by

10 Biacore for the three monoclonal antibodies indicated in Table 2. The linear regression (log(KD) ~ W) is reported in Figure 7.

Even though only three monoclonal antibodies could be used in this first set of analysis, the inventors measured a significant linear correlation between the W and log(KD) as demonstrated by the Pearson's product-moment correlation coefficient $p = 0.99$ with p-value < 0.05.

15 To further confirm the approach and strengthen our data, the inventors have applied the same model to 5 different monoclonal antibodies binding to 3 different variants of *Neisseria meningitidis* fH binding protein (fHbp Varl, fHbp_Var2, fHbp_Var3).

W by Gyrolab and KD by Biacore were determined for all the five monoclonal antibodies (named 30G4, 30F11, 15B11, 11D1, 17C11) *vs.* three different variants of the FHbp protein (Table 3).

20

mAb	Affinity score ***W*** mean ± sd	Number of Gyrolab measurements	KD (Biacore)
30G4_Var3	7.48±0.51	9	5.3e-08
30G4_Var2	6.39±0.79	8	1.10e-08
30G4_Varl	4.29±0.31	9	6.10e-09
30Fll_Var3	8.32+1.14	9	2.8e-08
30Fll_Var2	6.54±0.96	9	9.10e-09
30Fll_Varl	4.16±0.45	9	5.50e-09
15Bll_Var3	7.85±0.99	9	2.10e-08
15Bll_Var2	6.13±0.54	8	8.50e-09
15Bll_Varl	4.09±0.24	9	5.90e-09
11Dl_Var3	9.97±0.90	9	2.20e-07
11Dl_Var2	8.77±0.69	9	3.60e-08
1lDl_Varl	6.74±0.41	9	1.7e-08
17Cll_Var3	10.31±0.70	12	3.00e-07
17Cll_Var2	8.67±0.43	12	2.90e-08
17Cll_Varl	6.66±0.50	11	1.7e-08

Table 3

Even though at the two extremes of the range investigated a slight departure from the linear trend was detected (Figure 8), a strong correlation between *Wand* KD was observed, with ***p*** = 0.96 (p-value « 0.001).

Variability of the affinity score *W*

The inventors have determined for each monoclonal antibody listed in Table 2 and Table 3 the coefficient of variation (CV, a standard measure of assay precision) associated with the value of *W* obtained in the experimental replicates.

Figure 9 shows that CVs of *W* ranged from 5% to 15%, with an average value of precision = 9%. No significant dependency of CV from the value of *W* was observed (p-value = 0.78), indicating that the variability of the parameter *W* determined with the methodology invented is homoscedastic.

Characterisation of polyclonal sera

The inventors have also used the method of the invention for determining qualitative changes in binding avidity of polyclonal antibodies. Accordingly, the method of the invention is also suitable for determining polyclonal antibody avidity.

When the sample comprises a mixture of polyclonal antibodies (*e.g.* polyclonal sera), the method is further improved by de-convolution of signal data. This helps provide affinity measurements for individual polyclonal antibodies within the mixture.

Binding profiles of polyclonal antibodies to the antigen are described by combinations of Landau-like distributions with different affinity scores

A polyclonal serum can be considered as a composition of n clones of mAbs of unknown affinity and concentration. On the basis of that assumption the inventors hypothesized that a polyclonal
5 population could be described as a composition of n "monoclonal-like" sub-populations fitted by a linear combination of Landau distributions. Populations of antibodies with different avidity (different *W* score) within the polyclonal sample were de-convoluted by the inventors. This kind of analysis is applicable to polyclonal samples of different origin. Figure 10 shows an example of the de-convolution in sub-populations of antigen specific antibodies present in mouse (top) and rabbit
10 (bottom) hyperimmune sera.

The inventors found that de-convolution could also be used to study affinity maturation of antigen-specific antibodies after repeated exposures to the antigen (see Figure 11).

Table 4 displays the *W* values obtained with the de-convolution of the polyclonal profiles by a two components fitting (W1= Higher affinity; W2= Lower affinity). Avidity maturation of the
15 antigen-specific antibodies is observed mainly in the high avidity population, where there is a progressive decrease of W1 value (increased affinity) after each single dose of vaccine.

	Preimmune	3 weeks after first dose	3 weeks after second dose
W1 (affinity score for the higher avidity component) ± sd	6.6±0.7	3.6± 0.6	2.9± 0.3
W2 (affinity score for the lower avidity component) ± sd	7.6± 0.7	6.7± 0.7	6.3± 0.6

Table 4

20 ***Conclusion***

The inventors found that that the parameter *W*, obtained by least-square regression of Eq.1 to raw antibody-antigen binding data (obtained using the Gyrolab system), within the linearity range of parameter *A*, is a precise measure of antibody affinity. The inventors have validated the method of the invention by direct comparison with SPR analysis (by Biacore), which is recognised as the
25 benchmark for measurement of KD affinity-constant. The inventors have applied the method of the invention to the binding profiles of polyclonal sera and to the study of affinity maturation of antigen-specific antibodies after repeated exposures to the antigen.

It will be understood that the invention has been described by way of example only and
30 modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES

[1] Darling, R.J. & Brault, P.A. *ASSAY and Drug Development Technologies*, Vol. 2(6). 2004, 647-657
[2] Salimi-Moosavi, *et al. Analytical Biochemistry*, Vol. 426. 2012, 134-141
[3] WO03/097091.
[4] Cassone & Torosantucci (2006) *Expert Rev Vaccines* 5:859-67.
[5] Winter *et al*. (1991) *Nature* 349:293-299
[6] U.S. Patent No. 4,816,567
[7] Inbar et al. (1972) Proc. Natl. Acad. Sci. USA 69:2659-2662
[8] Ehrlich et al. (1980) Biochem 19:4091-4096
[9] Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883
[10] Riechmann et al. (1988) Nature 332:323-327
[11] Verhoeyan et al. (1988) Science 239:1534-1536
[12] U.K. Patent Publication No. GB 2,276,169, published 21 September 1994
[13] Pack et al. (1992) Biochem 31:1579-1584
[14] Cumber et al. (1992) J. Immunology 149B:120-126
[15] Mayer and Walker, eds. (1987) IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London)
[16] L. Landau, On the Energy Loss of Fast Particles by Ionization, J. Phys. USSR 8 (1944) 201.
[17] S. Behrens, A.C. Melissinos, Univ. of Rochester Preprint UR-776 (1981)
[18] http://www.r-project.org/

CLAIMS

1. A method for measuring the binding affinity of receptor for ligand comprising analysing distributions of ligand/receptor complexes along an affinity column comprising immobilised ligand for the receptor.

5 2. The method of claim 1 comprising the steps of:

   A. applying a sample comprising a receptor to an affinity column comprising immobilised ligand for the receptor;

   B. passing the sample through the affinity column;

   C. detecting level(s) of ligand/receptor complex along the affinity column;

10 D. analysing distributions of ligand/receptor complex along the column; and

   E. using data from step D to determine the binding affinity of the receptor for the ligand.

3. The method of claim 1 or claim 2, wherein the binding affinity is derived from an affinity score *W*, which is derived by regression of Eq. 1 to the level(s) of ligand/receptor complex along the affinity column.

15 4. The method of claim 3, wherein the regression is least-square regression within the linear range of the detection.

5. The method of claim 4, wherein the linear range of detection is identified by:

   A) performing the method of any one of claims 1-3 on an antibody concentration interval;

   B) estimating normalisation factor (parameter *A*) from the results obtained from step A);

20 C) performing 4PL linear regression on the relationship between parameter *A* and the antibody concentration; and

   D) estimating the middle point of the linear range (x_{mid})

6. The method of any one of the preceding claims, wherein the receptor is an antibody.

7. The method of claim 6, wherein the antibody is a monoclonal antibody.

25 8. The method of claim 6, wherein the antibody is a polyclonal antibody.

9. The method of any one of the preceding claims wherein the ligand is an antigen.

10. The method of any one of claims 1-5 wherein the receptor is an antigen and the ligand is an antibody.

11. The method of claim 10 for determining cross-reactivity of antigen with antibody.

30 12. The method of claim 10 or 11 wherein the antigen is obtained from a natural bacterial strain.

13. The method of any one of claims 2-12, wherein detection of ligand/receptor complex comprises detection of a detection agent bound to the ligand/receptor complex.

14. The method of claim 13, wherein the detection agent is added to the affinity column after introduction of the sample to the affinity column.

15. The method of any one of the preceding claims, wherein the method does not comprise a step of pre-incubating receptor with ligand.

5 16. The method of any one of the preceding claims wherein the sample is passed through the affinity column under inertia.

17. The method of any one of the preceding claims, wherein ligand is immobilised on the affinity column via biotin/avidin interaction.

18. The method of claim 17, wherein the ligand is biotinylated.

10 19. The method of claim 17 or claim 18, wherein the avidin is streptavidin.

20. The method of any one of claims 13-19, wherein the detection agent is a fluorophore.

21. The method of claim 20, wherein the detection agent is excited using a laser.

22. A method for separating population(s) of receptors from within a mixture of receptors that have different affinities for bound ligand, comprising the steps of:

15 A) applying a sample comprising a receptor to an affinity column comprising immobilised ligand for the receptor; and

B) passing the sample through the affinity column.

23. A method for isolating population(s) of receptors from within a mixture of receptors that have different affinities for bound ligand, comprising the steps of:

20 A) performing steps A) and B) according to claim 22;

B) deriving spatial information on ligand/receptor complex populations along the column; and

C) isolating receptor(s) from the column.

24. The method of claim 23, wherein the spatial information is derived by de-convoluting and 25 performing mathematical regression of the ligand/receptor complex distribution data obtained by step A).

25. The method of any one of claims 22 to 24 wherein the sample is polyclonal sera.

26. An antibody identified by the method of claim 22 or an antibody isolated by the method of claim 23 or claim 24.

30 27. An antibody of claim 26 for use in therapy.

28. An antibody of claim 27 for use in passive immunisation.

29. Apparatus for use in the method of any one of the preceding claims.

30. Apparatus of claim 29, wherein the apparatus is an affinity column.

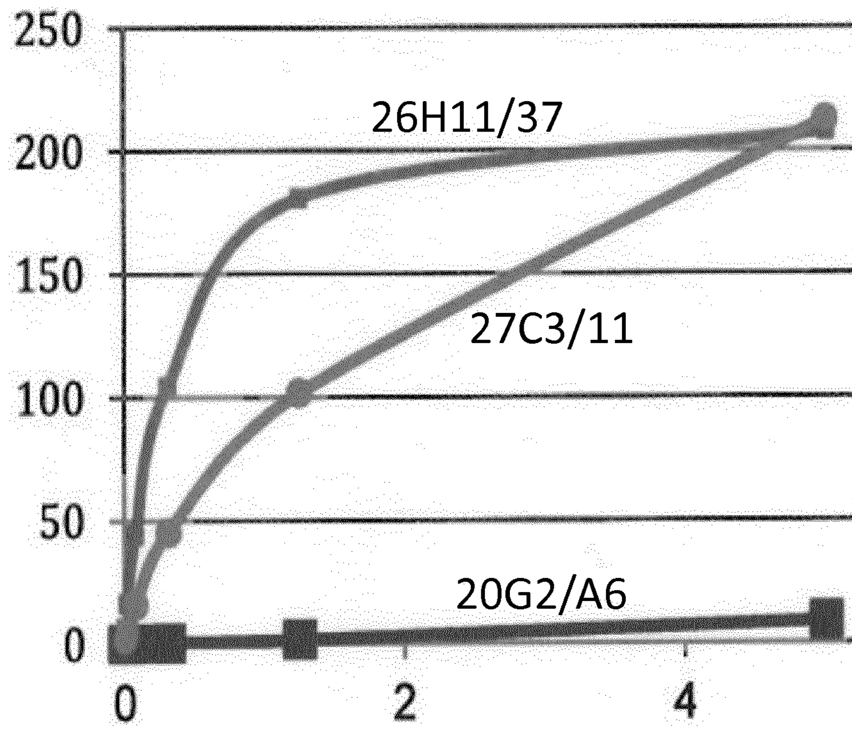

Fig. 1

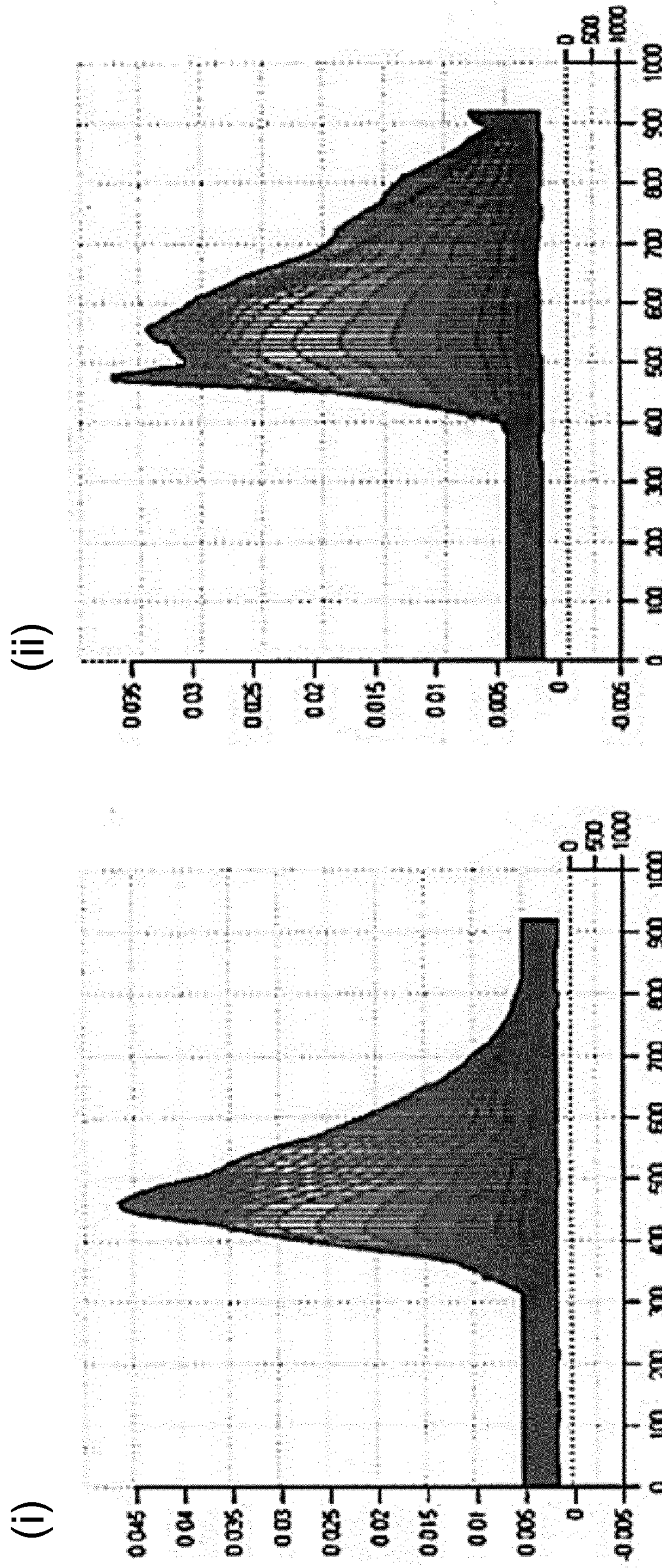

Fig. 2A

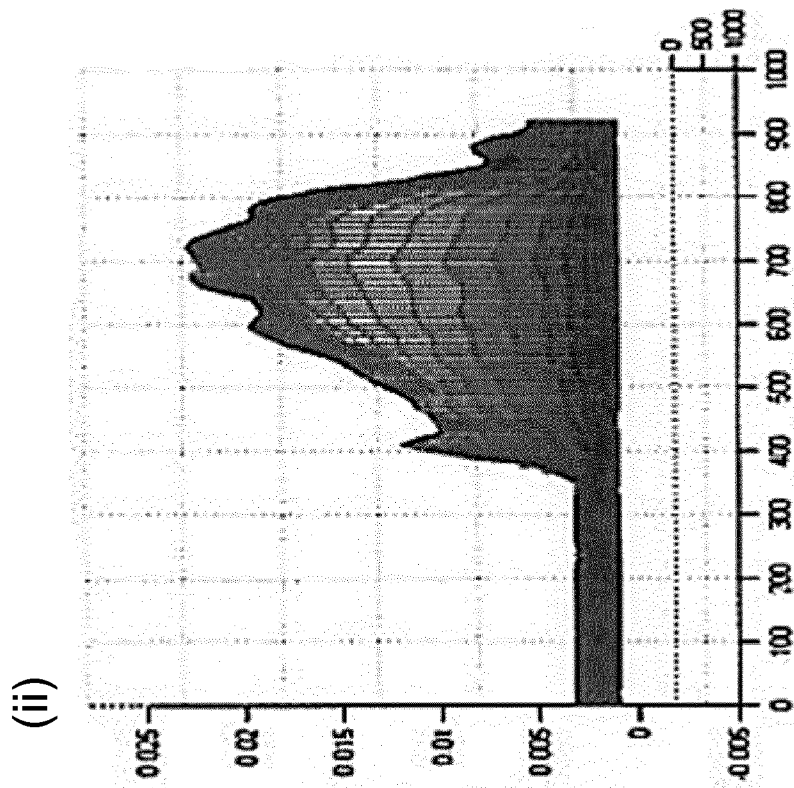

Fig. 2A(Cont.)

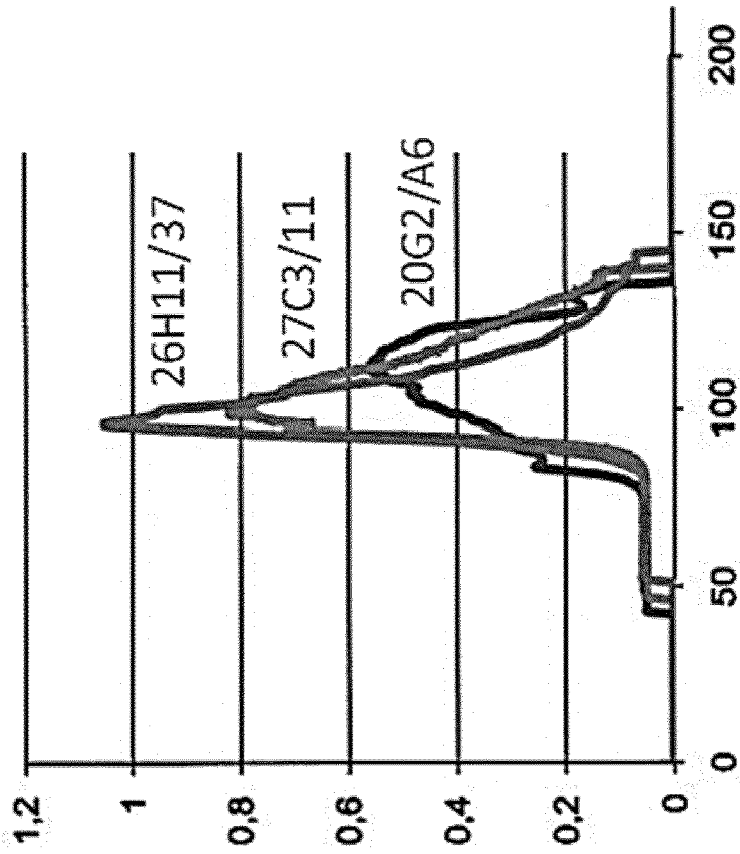

Fig. 2B

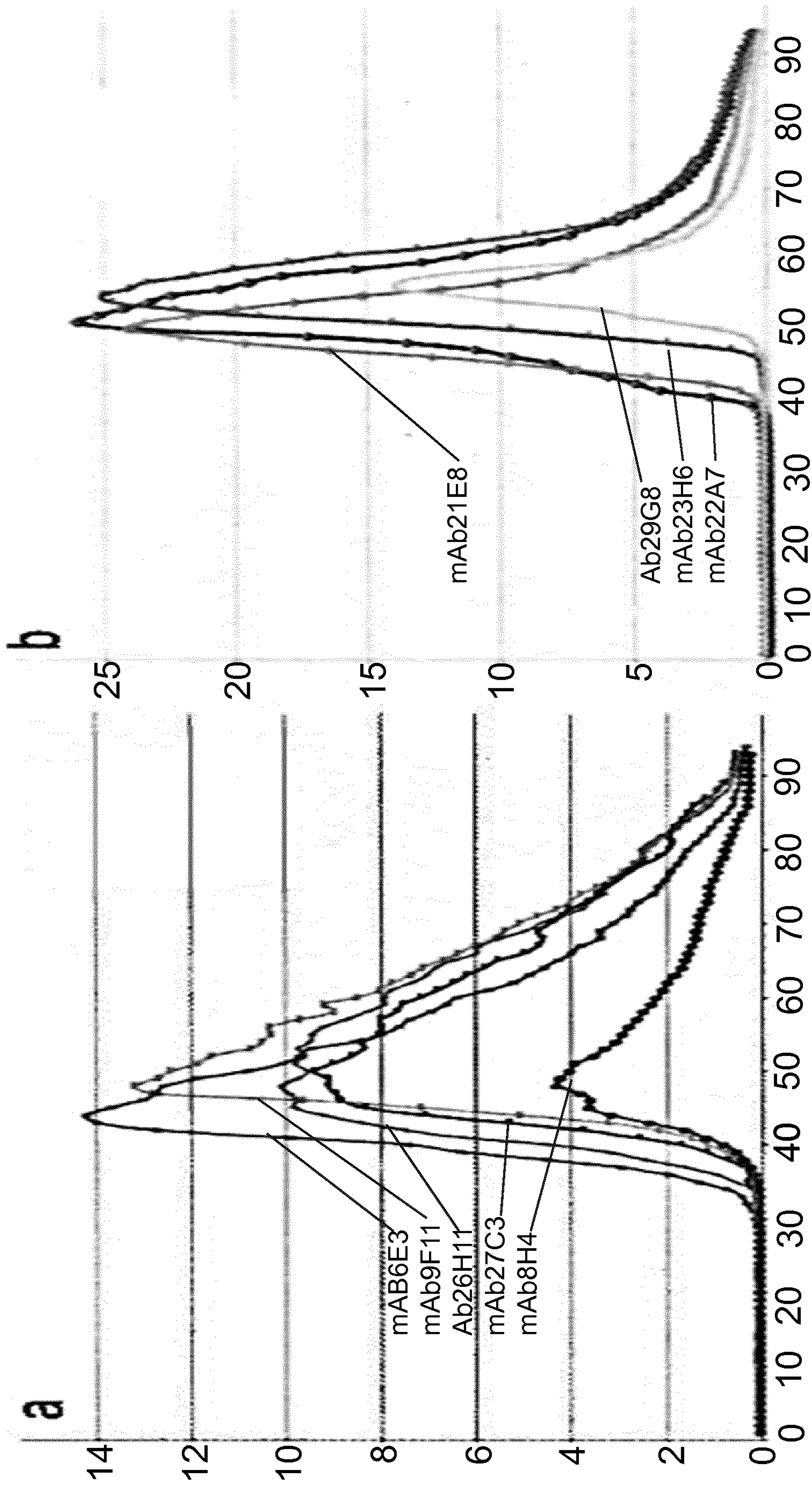

Fig. 3

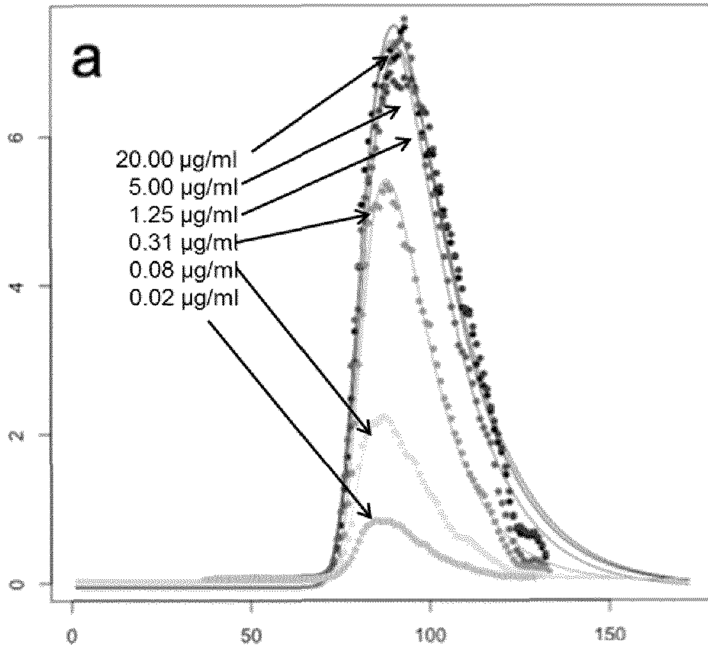

Fig. 4 (a)

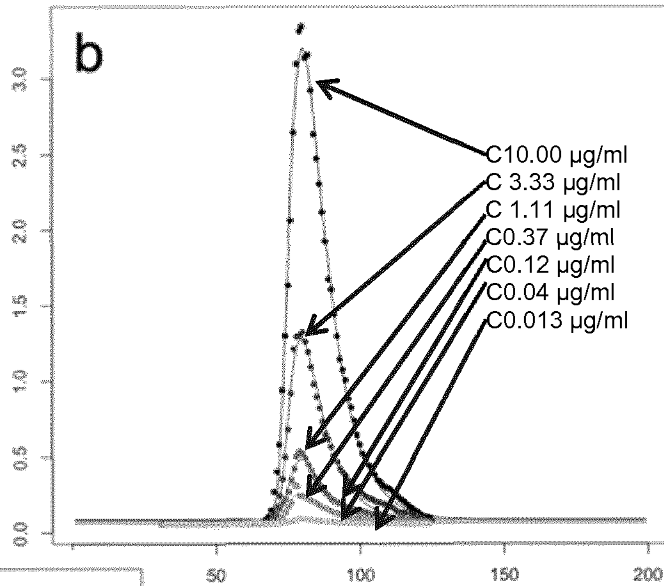

Fig. 4 (b)

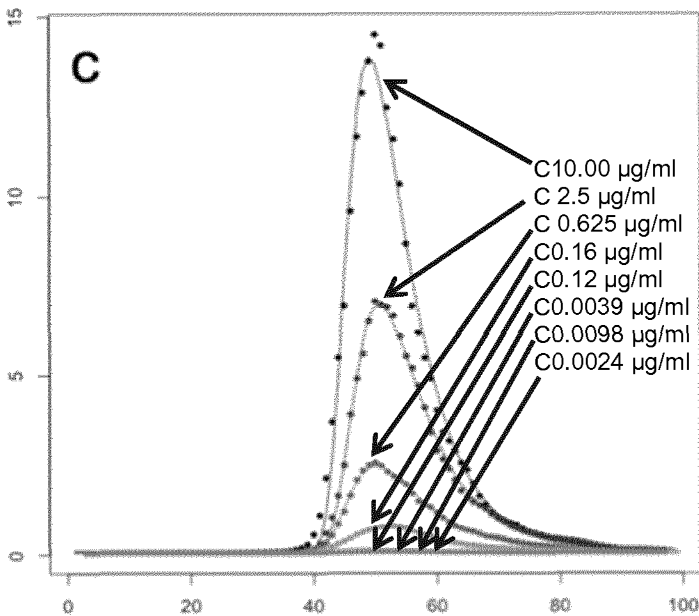

Fig. 4 (c)

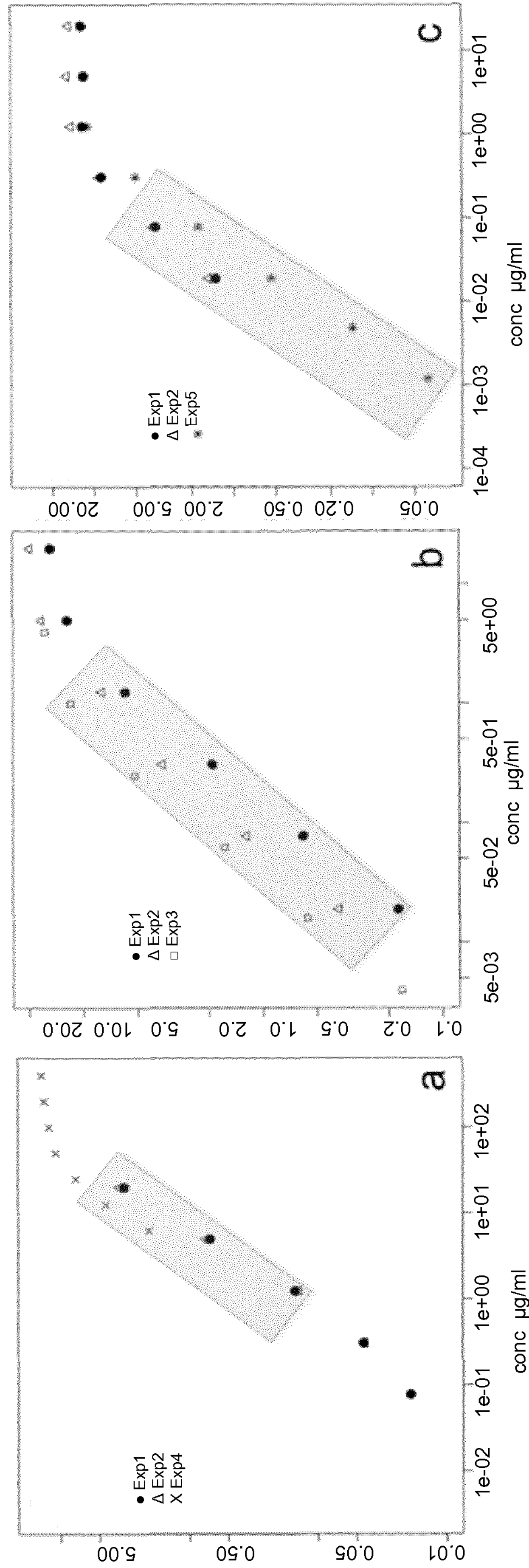

Fig. 5

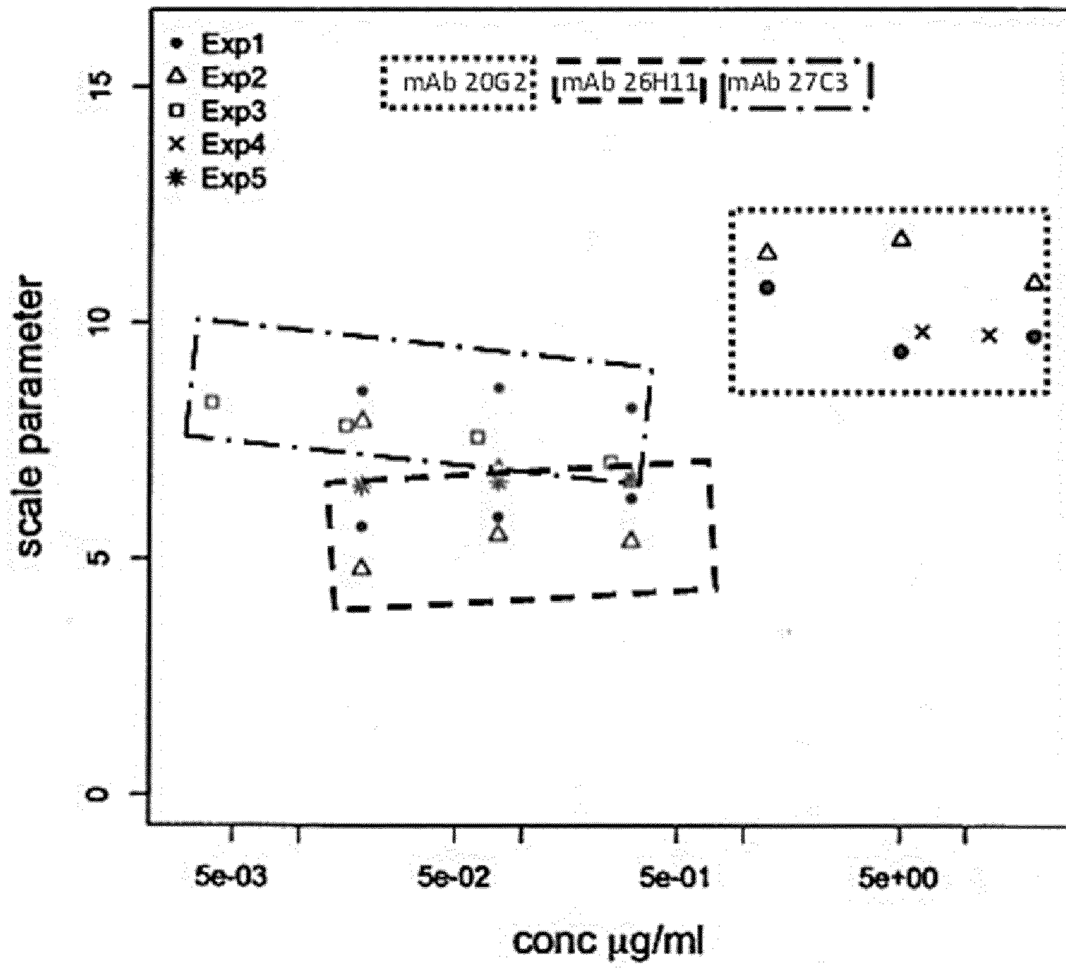

Fig. 6

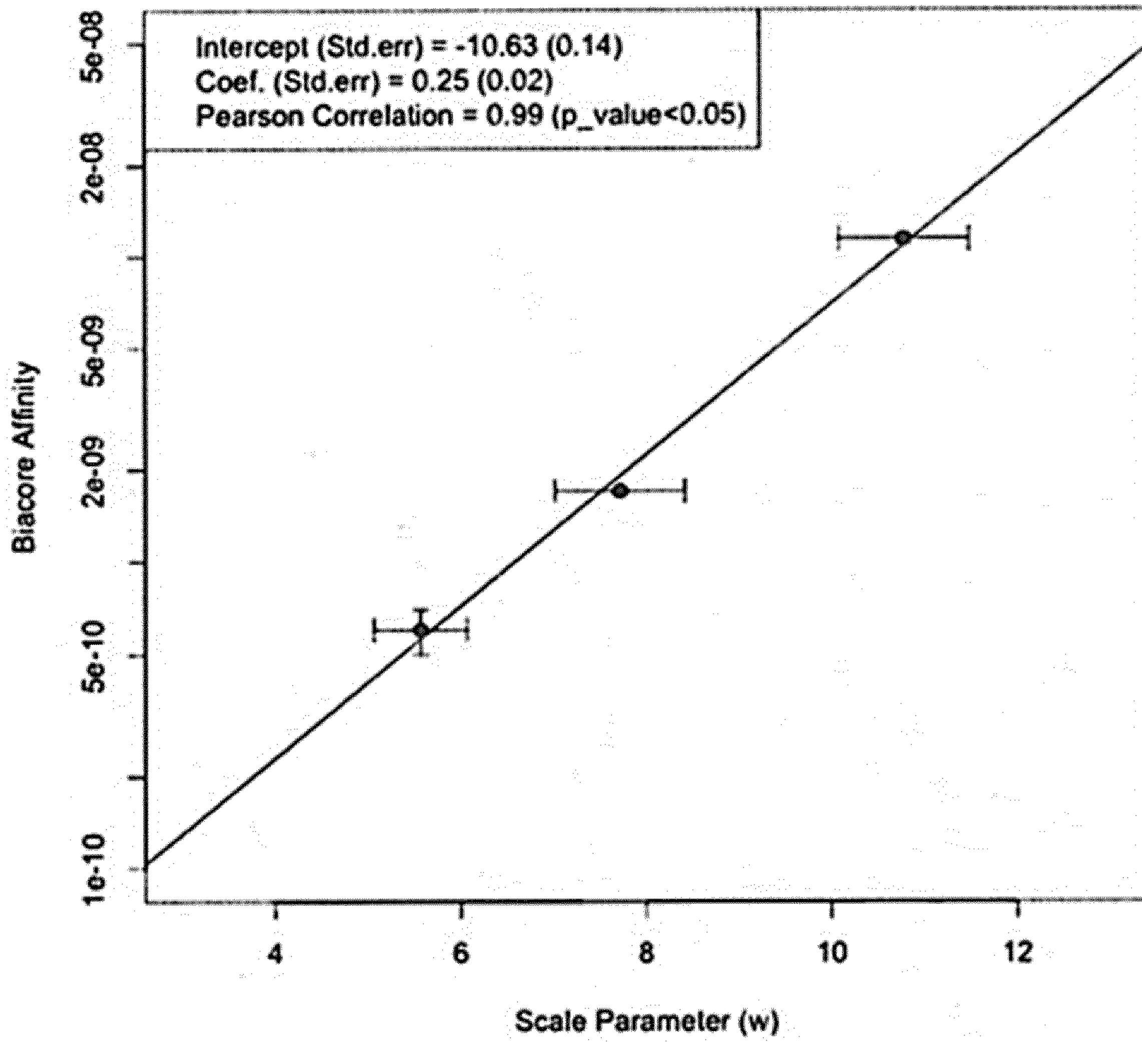

Fig. 7

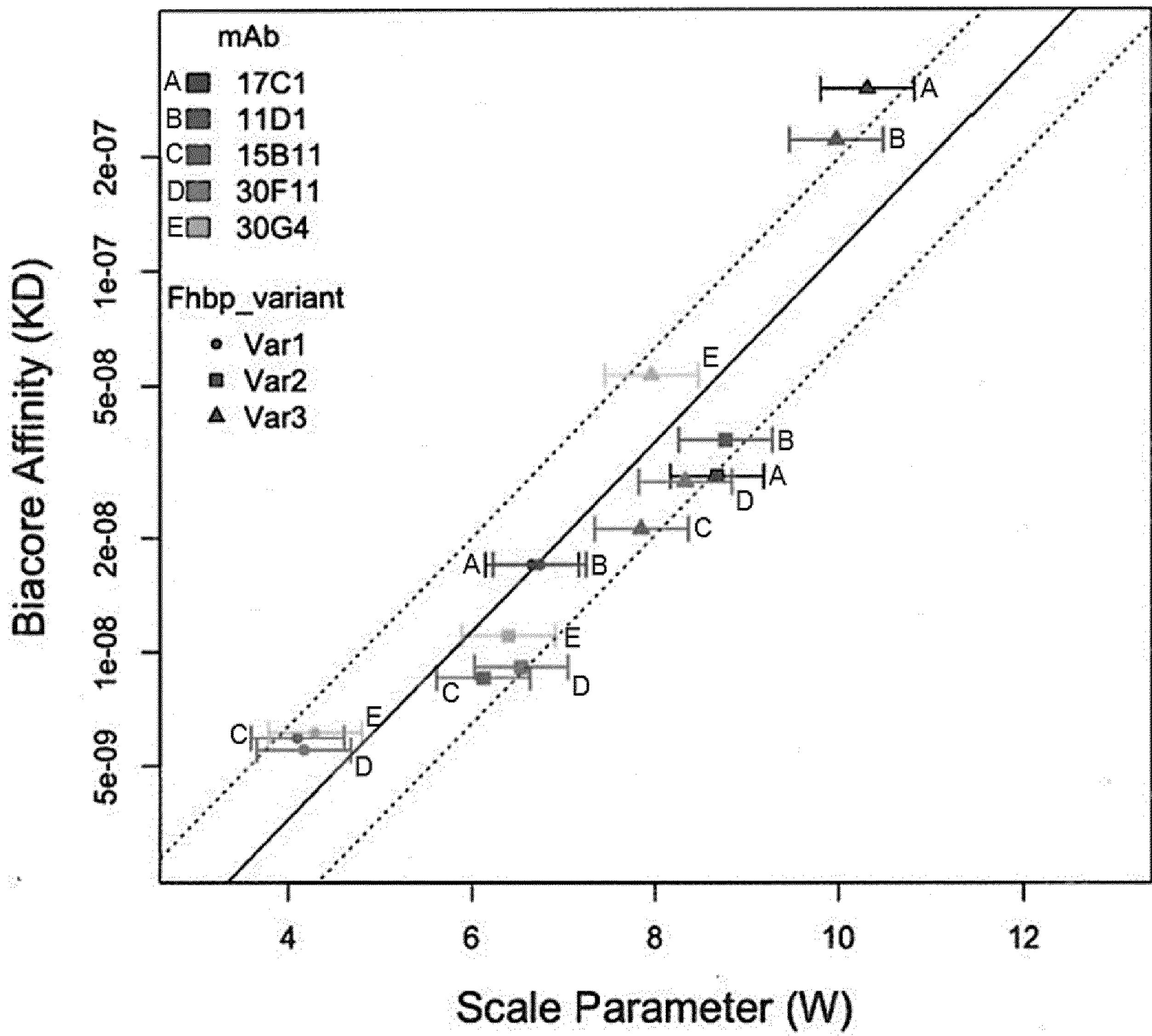

Fig. 8

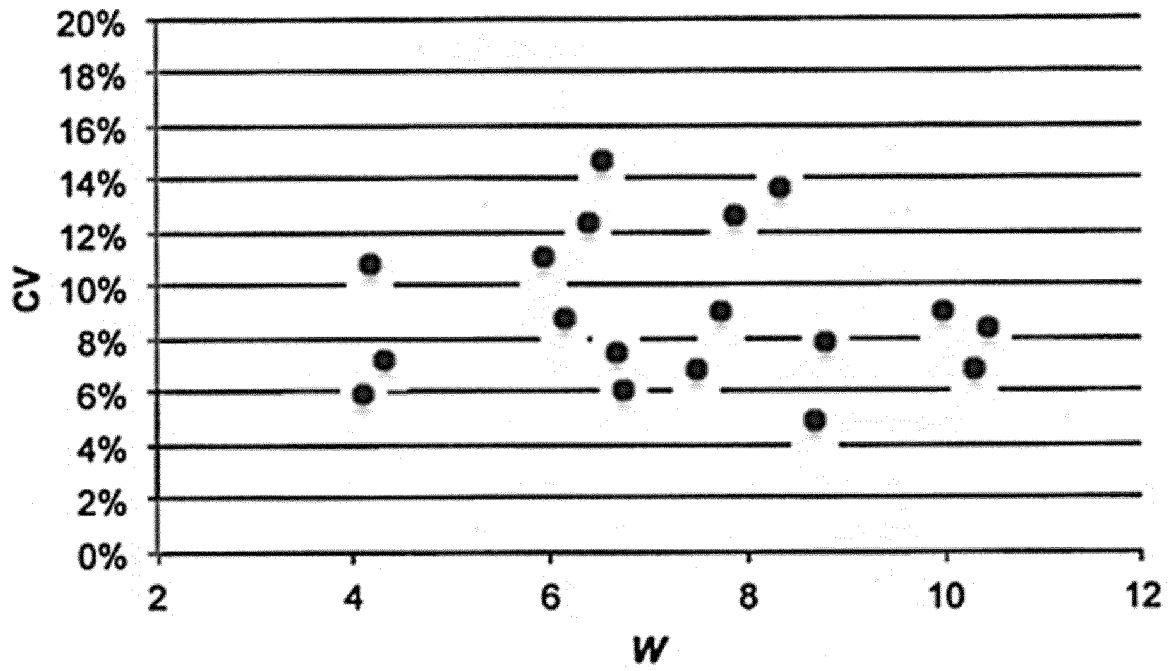

Fig. 9

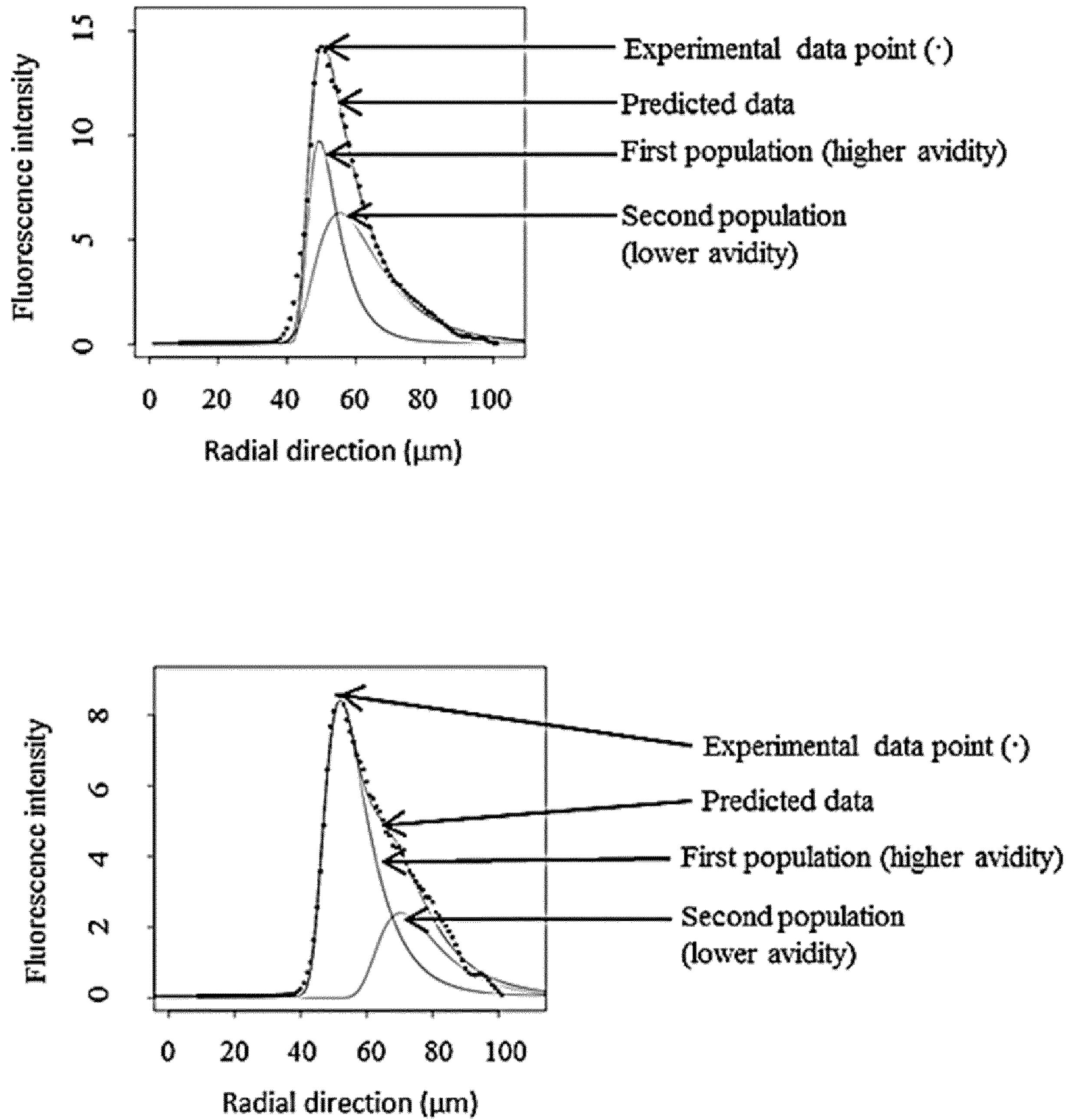

Fig. 10

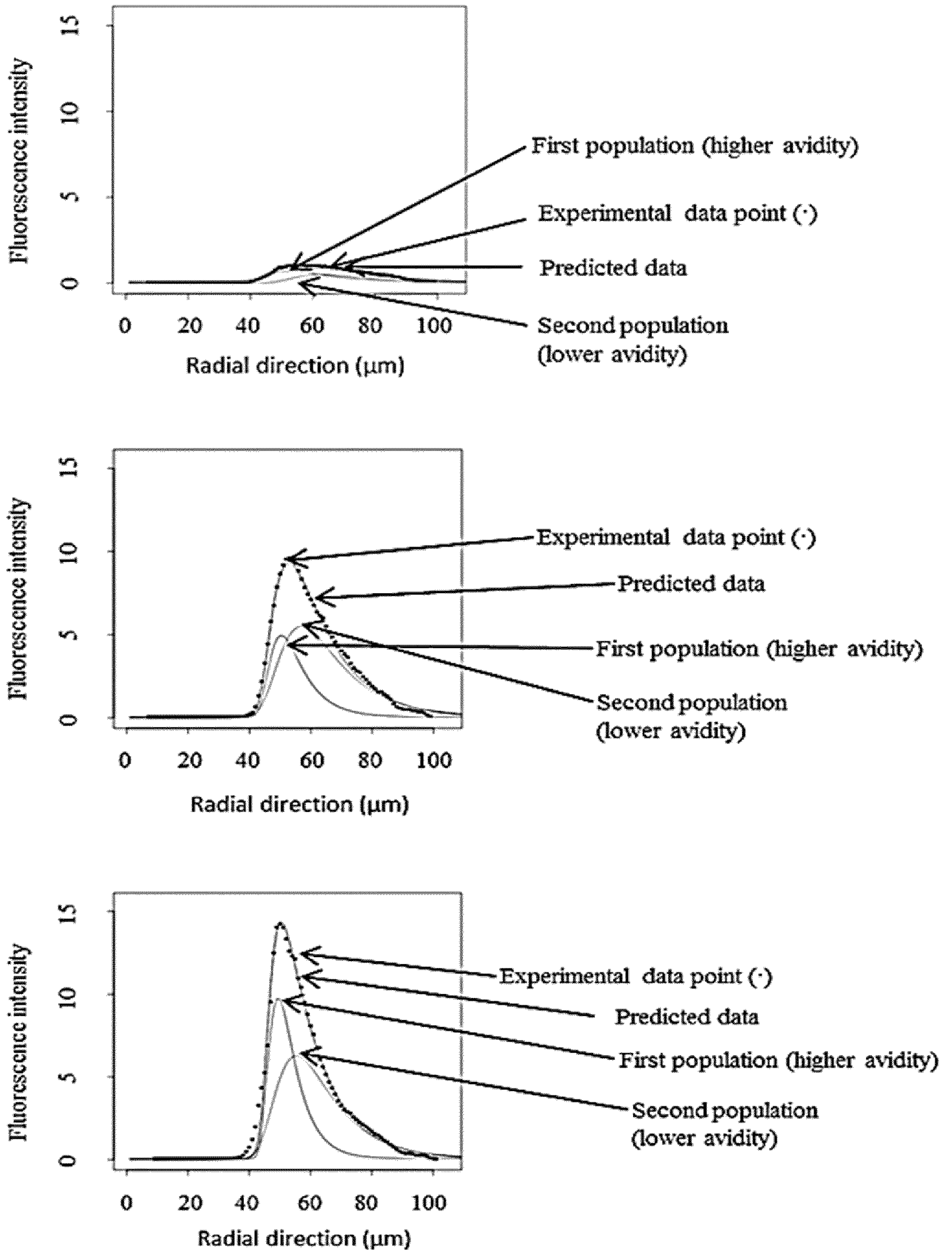

Fig. 11