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(54) **Title:** IN VITRO POTENCY ASSAY FOR PROTEIN-BASED MENINGOCOCCAL VACCINES

(57) **Abstract:** The invention uses ELISA or similar assays for analysing a meningococcal vaccine. The assay uses antibodies which bind to meningococcal proteins within the vaccine, and in particular monoclonal antibodies which are bactericidal for meningococcus and/or which recognise conformational epitopes within the meningococcal proteins. By performing the assay on a series of dilutions of a test vaccine, and by comparing the results with those obtained using a reference vaccine of known potency, it is possible to determine the relative potency of the test vaccine. This value can be used as a parameter for determining whether a manufactured batch of a vaccine is suitable for release to the public, or whether it has experienced a production failure and so should not be used.



IN VITRO POTENCY ASSAY FOR PROTEIN-BASED MENINGOCOCCAL VACCINES

This application claims the benefit of US provisional application 61/608,293 (filed March 8th, 2012), the complete contents of which are hereby incorporated herein by reference for all purposes.

TECHNICAL FIELD

- 5 This invention is in the field of *in vitro* assays for assessing the potency of protein-containing vaccines for protecting against *Neisseria meningitidis* (meningococcus).

BACKGROUND ART

- Unlike live vaccines that are quantified by *in vitro* titration, the potency of inactivated or subunit vaccines normally requires an *in vivo* test for each batch prior to its release for public use [1],
10 although a number of exceptions exist *e.g.* the SRID (single radial immunodiffusion) potency test for the influenza vaccine and the use of ELISA for hepatitis B vaccines.

- Typical *in vivo* tests involve an immunisation-challenge test using small rodents (mice or rats) as the experimental model. Depending on the type of vaccine, different endpoints are used, such as death/survival ratios (whole cell pertussis, diphtheria toxoid and tetanus toxoid, rabies vaccine),
15 clinical signs (diphtheria, tetanus) or colonisation (whole cell and acellular pertussis). By establishing a dose-response curve in parallel to a standard preparation with known potency, the potency of the vaccine can be expressed relative to that preparation *e.g.* in standard units.

- A challenge model is not always available. In those cases potency testing is usually limited to serological responses, with antibody responses being measured after immunisation of test animals. At
20 least part of the functionality of these antibodies can be determined by their ability to neutralise the pathogen *in vitro* or to their ability to kill bacteria in the presence of complement (such as the serum bactericidal antibody assay, or SBA, for meningococcus).

The SBA assay is useful but cumbersome, and involves the sacrifice of many mice. As explained in reference 1 it is thus desirable to provide *in vitro* alternatives for assessing vaccine potency.

- 25 One *in vitro* assay for analysing MenB vaccines is the “MATS” ELISA test disclosed in references 2 and 3. The relative potency measured by MATS was shown to correlate with the ability of MenB strains to be killed in SBA.

- The MATS test is used to evaluate the strain coverage of a MenB vaccine, rather than to analyse the vaccine’s immunogenicity. There remains a need for further and improved *in vitro* assays for
30 assessing the immunogenicity of meningococcal vaccines. Such *in vitro* assays could be used to confirm that a particular vaccine will have an expected *in vivo* activity in human recipients.

DISCLOSURE OF THE INVENTION

- The invention uses binding assays, such as ELISA, for analysing a meningococcal vaccine. The assay uses antibodies which bind to meningococcal proteins within the vaccine, and in particular
35 monoclonal antibodies which are bactericidal for meningococcus and/or which recognise

conformational epitopes within the meningococcal proteins. By performing the assay on a series of dilutions of a test vaccine, and by comparing the results with those obtained using a standard or reference vaccine of known potency, it is possible to determine the relative potency of the test vaccine. This value can be used as a parameter for determining whether a manufactured batch of a vaccine is suitable for release to the public, or whether it has experienced a production failure and so should not be used. Assays of the invention are particularly useful for analysing vaccines which contain multiple different antigens and/or which contain adsorbed antigen(s).

Thus the invention provides a binding assay for *in vitro* analysis of a meningococcal vaccine sample, comprising steps of: (i) permitting a meningococcal protein immunogen within the sample to interact with a monoclonal antibody which either (a) is bactericidal for meningococcus or (b) recognises a conformational epitope in the meningococcal antigen; then (ii) measuring the interaction between the immunogen and antibody from step (i).

The invention also provides an assay for *in vitro* analysis of a meningococcal test vaccine sample, comprising steps of: (i) performing the above binding assay on the test sample and, optionally, on at least one dilution of the test sample; (ii) performing the above binding assay on a standard vaccine sample and, optionally, on at least one dilution of the standard vaccine sample; and (iii) comparing the results from steps (i) and (ii) to determine the potency of immunogen(s) in the test vaccine relative to the potency of immunogen(s) in the standard vaccine.

The invention also provides a process for analysing a bulk vaccine, comprising steps of: (i) assaying the relative potency of immunogen(s) in the bulk as described above; and, if the results of step (i) indicate an acceptable relative potency, (ii) preparing unit doses of vaccine from the bulk.

The invention also provides a process for analysing a batch of vaccine, comprising steps of: (i) assaying the relative potency of immunogen(s) in at least one vaccine from the batch as described above; and, if the results of step (i) indicate an acceptable relative potency, (ii) releasing further vaccines from the batch for *in vivo* use.

The invention also provides a competitive ELISA assay for *in vitro* analysis of a meningococcal vaccine sample, wherein the assay uses (i) a solution-phase anti-vaccine monoclonal antibody (ii) an immobilised antigen which is recognised by the anti-vaccine antibody, and (iii) a labelled antibody which binds to the anti-vaccine antibody, wherein the antibody either (a) is bactericidal for meningococcus or (b) recognises a conformational epitope in the meningococcal antigen.

The invention also provides a binding assay for *in vitro* analysis of a meningococcal vaccine sample, wherein the assay uses immunogens in a vaccine to inhibit the binding of a monoclonal antibody to a control antigen, wherein the monoclonal antibody binds to both an immunogen in the vaccine and the control antigen.

The invention also provides a vaccine which has been released following use of an assay as described herein.

The invention also provides a kit for performing the assay of the invention. This kit may include *e.g.* a microwell plate, a microwell plate including well-immobilised immunogens, a dilution buffer, and/or an anti-immunogen antibody.

Binding assays and ELISA formats

The invention uses a binding immunoassay. Typically this will be an enzyme-linked immunosorbent assay (ELISA) as is well known in the art. The invention can use any ELISA format, including those conventionally known as direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA.

Step (i) of the ELISA assay of the invention involves permitting a meningococcal protein immunogen within the sample to interact with a monoclonal antibody. The characteristics of this interaction (*e.g.* homogeneous or heterogeneous) will vary according to the chosen ELISA format. The interaction between the monoclonal antibody and the immunogen is then detected in step (ii). As typical for ELISA, the interaction can be measured quantitatively, such that step (ii) provides a result which indicates the concentration of the monoclonal antibody's target epitope within the vaccine sample. By using a monoclonal antibody which binds to a bactericidal or conformational epitope, the result in step (ii) indicates the concentration of the corresponding functional epitope in the vaccine sample, and can distinguish between immunogens which retain the relevant epitope (and function) and those which have lost the epitope (*e.g.* due to denaturation, aggregation or breakdown during storage or by mishandling). By comparison with values obtained with a standard vaccine of known potency, results from step (ii) can be used to calculate relative potency of a test vaccine.

The preferred ELISA format for use with the invention is the competitive ELISA (Figure 5). In this format the vaccine sample is incubated with the monoclonal antibody (primary antibody) so that complexes can form between the antibody and immunogens in the sample. These complexes are then added to a container in which competitor antigens are immobilised. Antibody which is not complexed with immunogens from the vaccine sample is able to bind to these immobilised competitor antigens; if the sample contains a lot of target for the antibody then there will be less uncomplexed antibody to bind to the immobilised competitor antigens, whereas less target in the sample (whether due to lower amounts of immunogen, for example after dilution, or to loss of the antibody's epitope, for example after denaturation of immunogens) leads to more uncomplexed antibody. The antibody which is bound to the immobilised competitor antigens (after usual washing steps, *etc.*) can then be detected by adding a labelled secondary antibody which binds to the monoclonal anti-vaccine (*i.e.* primary) antibody. The label is used to quantify the amount of immobilised primary antibody in the normal ways. The use of competitive ELISA avoids the need to have two different anti-immunogen antibodies which recognise different epitopes on the same immunogen, and also can give better results in vaccines which include multiple different immunogen

components. It also permits the test vaccine to be analysed directly, without requiring any manipulation prior to testing (although such manipulations can be performed if desired).

Suitable competitor antigens for immobilisation include the meningococcal proteins which are present in the vaccine, or proteins comprising these vaccine proteins (*e.g.* fusion proteins), or proteins comprising fragments of the vaccine proteins (*e.g.* truncated forms). The immobilised competitor antigen must retain the epitope recognised by the relevant monoclonal antibody, so that it can compete with the vaccine's immunogens for binding to the antibody. Typically this can be achieved by immobilising antigen from fresh batches of bulk vaccine or, preferably, from fresh batches of bulk purified immunogen prior to preparation of bulk vaccine.

Labelling of antibodies in an ELISA can take various forms. In the preferred competitive format the secondary antibody is labelled. In an ELISA the antibody is labelled with an enzyme, which is then used to catalyse a reaction whose product is readily detectable. The linked enzyme can cause a detectable change in an enzyme substrate which is added to the labelled antibody after it becomes immobilised *e.g.* to modify a substrate in a manner which causes a colour change. For example the enzyme may be a peroxidase (*e.g.* horseradish peroxidase, HRP), or a phosphatase (*e.g.* alkaline phosphatase, AP). Other enzymes can also be used *e.g.* laccase, β -galactosidase, *etc.*

The choice of substrate will depend on the choice of linked enzyme. Moreover, substrates differ in terms of cost, ease-of-use, sensitivity (*i.e.* lower limit of detection) and compatibility with available imaging equipment. These parameters are familiar to those skilled in ELISA. Preferred substrates undergo a colorimetric change, a chemiluminescent change, or a chemifluorescent change when contacted with the linked enzyme. Colorimetric substrates (and their enzymatic partners) include, but are not limited to: PNPP or p-Nitrophenyl Phosphate (AP); ABTS or 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (HRP); OPD or o-phenylenediamine dihydrochloride (HRP); and TMB or 3,3',5,5'-tetramethylbenzidine (HRP). Chemiluminescent substrates include luminol or 5-amino-2,3-dihydro-1,4-phthalazinedione (HRP), particularly in the presence of modified phenols such as p-iodophenol. Chemifluorescent substrates include p-hydroxyhydrocinnamic acid. Various proprietary substrates are also available and these can be used with the invention if desired *e.g.* QuantaBlu, QuantaRed, SuperSignal, Turbo TMB, *etc.*

Where an ELISA reagent is immobilised on a solid surface, this surface take various forms. Usually the reagent is immobilised on a plastic surface, such as a surface made from polystyrene, polypropylene, polycarbonate, or cyclo-olefin. The plastic will usually be transparent and colourless, particularly when using chromogenic enzyme substrates. White or black plastics may be preferred used when using luminescent or fluorescent substrates, as known in the art. The plastic will generally be used in the form of a microwell plate (microtitre plate) as known in the art for ELISA (a flat plate having multiple individual and reaction wells). Such plates include those with 6, 24, 96, or 384 sample wells, usually arranged in a 2:3 rectangular matrix. Microwell plates facilitate the preparation

of dilution series and also the transfer of materials from one plate to another while maintaining spatial relationships *e.g.* in the step of transferring a mixture of antibody and vaccine into a different microwell plate for measuring the interaction between the antibody and vaccine.

5 During an ELISA it may be desirable to add a blocking reagent and/or detergent *e.g.* to reduce non-specific binding interactions which might distort the assay's results. Blocking procedures are familiar to people working in the ELISA field.

10 In addition to the ELISA formats discussed above, the invention can use any suitable variants of ELISA, such as M&P ELISA or ELISA Reverse [4], the rapid ELISA of reference 5, *etc.*, and can also be extended to use alternatives to ELISA, such as flow injection immunoaffinity analysis (FIIAA), AlphaLISA or AlphaScreen [6], dissociation-enhanced lanthanide fluorescent immunoassay (DELFA), ELAST, the BIO-PLEX Suspension Array System, MSD, *etc.* Any of these binding assays can be used.

15 As an alternative to using a conjugated enzyme as the label, other labelling is possible. For instance, other indirect labels (*i.e.* alternative to enzymes) can be used, but it is also possible to label the antibody by conjugation to a direct label such as a coloured particle, an electrochemically active reagent, a redox reagent, a radioactive isotope, a fluorescent label or a luminescent label.

As a further alternative, the primary antibody can be conjugated to a high affinity tag such as biotin, avidin or streptavidin. An enzyme conjugated to a ligand for the tag, such as avidin, streptavidin or biotin can then be used to detect immobilised primary antibody.

20 Any of these variations can be used within the scope and spirit of the overall invention.

25 In some ELISA formats, rather than labelling a secondary antibody, the anti-vaccine monoclonal antibody (whether a bactericidal antibody or one which recognises a conformational epitope) will be labelled. Thus the invention provides a monoclonal antibody which immunospecifically binds to a meningococcal protein (such as NHBA, *etc.*, as disclosed herein) and which is conjugated to an enzyme (such as AP or HRP). Immunospecific binding can be contrasted with non-specific binding, and antibodies of the invention will thus have a higher affinity (*e.g.* at least 100-fold higher affinity) for the meningococcal target protein than for an irrelevant control protein, such as bovine serum albumin.

The vaccine sample

30 Assays of the invention are used to analyse vaccines. The assay is performed on at least one sample of the vaccine, and this analysis reveals information about the sampled vaccine. The assay can be performed on a sample(s) taken from a bulk vaccine, in which case the assay's results can be used to determine the fate of that bulk *e.g.* whether it is suitable for further manufacturing use (*e.g.* for preparing packaged doses of the vaccine), or whether it should instead be modified or discarded. The
35 assay can also be performed on a sample(s) taken from a batch of vaccines, in which case the assay's

results can be used to determine the fate of that batch *e.g.* whether the batch is suitable for release for use by healthcare professionals. Usually, enough samples will be taken from bulks/batches to ensure compliance with statistical practices which are normal for vaccine release assays. Testing of batches of final vaccine (formulated and packaged) in the form in which they would be released to the public is most useful.

The vaccine sample can be analysed at full strength *i.e.* in the form in which it is taken from the bulk or batch. In some cases, however, it is useful to analyse the vaccine at a fraction of full strength *e.g.* after dilution. The most useful assays analyse a series of strengths, the strongest of which may be a full strength sample or may be at fractional strength. Dilutions will typically be achieved using buffer rather than with plain water. Such buffers can sometimes include surfactants such as polysorbate 20 or polysorbate 80.

It is useful to analyse a series of dilutions of the vaccine. For instance, serial 1:2, 1:5 or 1:10 (by volume) dilutions can be used. The dilution series will include at least 2 members, but usually will include more *e.g.* 5, 10, or more members. For instance, 9 serial dilutions at 1:2 gives 10 samples at 1:2⁰, 1:2¹, 1:2², ..., 1:2⁹, and 1:2¹⁰-fold strengths relative to the strongest sample. The dilution series can be tested using the assays of the invention to provide a series of measurements which can be plotted (literally or notionally) against dilution. This series of measurements can be used to assess the vaccine's relative potency, as described below. The vaccine includes at least one meningococcal protein immunogen *i.e.* a protein which, when administered to human beings, elicits a bactericidal immune response. Various such proteins are known in the art, including but not limited to NHBA, fHbp and NadA as found in the BEXSERO™ product [7,8]. Further protein immunogens which can be analysed are HmbR, NspA, NhhA, App, Omp85, TbpA, TbpB, and Cu,Zn-superoxide dismutase. A vaccine may include one or more of these various antigens *e.g.* it can include each of NHBA, fHbp and NadA. It can also include variant forms of a single antigen *e.g.* it can include more than one variant of meningococcal fHbp (*i.e.* two fHbp proteins with different sequences [9]), using different monoclonal anti-fHbp antibodies to recognise each different variant separately.

The vaccine can include meningococcal vesicles *i.e.* any proteoliposomic vesicle obtained by disruption of or blebbing from a meningococcal outer membrane to form vesicles therefrom that retain antigens from the outer membrane. Thus this term includes, for instance, OMVs (sometimes referred to as 'blebs'), microvesicles (MVs) and 'native OMVs' ('NOMVs'). Various such vesicles are known in the art (*e.g.* see references 10 to 24) and any of these can be included within a vaccine to be analysed by the invention. In some embodiments, however, the vaccine is vesicle-free. Where a vaccine does include vesicles it is preferred to use a competitive ELISA format as this tends to give better results in samples which contain multiple components.

An analysed vaccine can preferably elicit an immune response in human beings which is protective against serogroup B meningococcus. For instance, the vaccine may elicit an immune response which

is protective at least against a prototype serogroup B strain such as MC58, which is widely available (e.g. ATCC BAA-335) and was the strain sequenced in reference 25. Other strains can also be tested for vaccine efficacy [2] but a response against MC58 is easily tested.

A preferred vaccine which can be analysed according to the invention is BEXSERO™ [7]. This vaccine includes three different recombinant proteins, consisting of amino acid sequences SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6. It also contains NZ98/254 outer membrane vesicles.

In addition to meningococcal protein immunogens, a vaccine can include other immunogens. These can be non-protein immunogens from meningococcus and/or immunogens from other bacteria and/or immunogens from non-bacterial pathogens, such as viruses. Thus, for instance, an analysed vaccine might include: (a) one or more capsular saccharides from meningococci e.g. from serogroups A, C, W135 and/or Y, as in the MENVEO, MENACTRA, and NIMENRIX products which all include conjugated capsular saccharides; (b) an antigen from *Streptococcus pneumoniae*, such as a saccharide (typically conjugated), as in the PREVNAR and SYNFLORIX products; (c) an antigen from hepatitis B virus, such as the surface antigen HBsAg; (d) an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3; (e) a diphtheria antigen, such as a diphtheria toxoid; (f) a tetanus antigen, such as a tetanus toxoid; (g) a saccharide antigen from *Haemophilus influenzae* B (Hib), typically conjugated; and/or (h) inactivated poliovirus antigens.

The vaccine is a pharmaceutical composition and so, in addition to its immunogens, typically includes a pharmaceutically acceptable carrier, and a thorough discussion of such carriers is available in reference 26.

The pH of an analysed vaccine is usually between 6 and 8, and more preferably between 6.5 and 7.5 (e.g. about 7). Stable pH in an analysed vaccine may be maintained by the use of a buffer e.g. a Tris buffer, a citrate buffer, phosphate buffer, or a histidine buffer. Thus an analysed vaccine will generally include a buffer.

An analysed vaccine may be sterile and/or pyrogen-free. Compositions of the invention may be isotonic with respect to humans.

An analysed vaccine comprises an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. The antigen content of

compositions of the invention will generally be expressed in terms of the mass of protein per dose. A dose of 10-500µg (*e.g.* 50µg) per immunogen can be useful.

Analysed vaccines may include an immunological adjuvant. Thus, for example, they may include an aluminium salt adjuvant or an oil-in-water emulsion (*e.g.* a squalene-in-water emulsion). Suitable aluminium salts include hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates), (*e.g.* see chapters 8 & 9 of ref. 27), or mixtures thereof. The salts can take any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*), with adsorption of antigen to the salt being preferred. The concentration of Al⁺⁺⁺ in a composition for administration to a patient is preferably less than 5mg/ml *e.g.* ≤4 mg/ml, ≤3 mg/ml, ≤2 mg/ml, ≤1 mg/ml, *etc.* A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred. Aluminium hydroxide adjuvants are particularly suitable for use with meningococcal vaccines. The invention has been shown to give useful results despite the adsorption of protein immunogens within the vaccine, and analysis is possible without requiring a desorption step (*i.e.* analysis can be performed without a desorption pre-treatment of the vaccine). Where a vaccine includes adsorbed immunogen it is preferred to use a competitive ELISA format as this tends to give better results.

Analysed vaccines may include an antimicrobial, particularly when packaged in multiple dose format. Antimicrobials such as thiomersal and 2-phenoxyethanol are commonly found in vaccines, but it is preferred to use either a mercury-free preservative or no preservative at all.

Analysed vaccines may comprise detergent *e.g.* a Tween (polysorbate), such as Tween 80. Detergents are generally present at low levels *e.g.* <0.01%. Analysed vaccines may include residual detergent (*e.g.* deoxycholate) from OMV preparation. The amount of residual detergent is preferably less than 0.4µg (more preferably less than 0.2µg) for every µg of MenB protein.

If an analysed vaccine includes LOS, the amount of LOS is preferably less than 0.12µg (more preferably less than 0.05µg) for every µg of protein.

Analysed vaccines may include sodium salts (*e.g.* sodium chloride) to give tonicity. A concentration of 10±2 mg/ml NaCl is typical *e.g.* about 9 mg/ml.

The standard vaccine

The assay of the invention can provide quantitative information about the amount of functional epitopes in a vaccine. If this amount is compared to the amount in a vaccine of known potency then it is possible to calculate the relative potency of a test vaccine. Thus in some embodiments the analysed vaccine is a standard vaccine which has known potency in an *in vivo* assay *e.g.* it has a known SBA titre. In other embodiments the analysed vaccine is a test vaccine which does not have a known potency in an *in vivo* assay. In further embodiments the assay is used to analyse both a standard vaccine and a test vaccine, and the results of the analysis of the test vaccine are compared to the analysis of the standard vaccine, and this comparison is used to express the test vaccine's potency relative to the known potency of the standard vaccine.

For instance, after manufacture of a new bulk preparation of BEXSERO™, or after storage of a batch or bulk of manufactured vaccine, a test sample from the batch/bulk can be tested using the assay of the invention, and the results can be compared to those obtained with BEXSERO™ having known *in vivo* potency. This comparison will reveal whether the new/stored batch/bulk (the test sample) is as potent as it should be. If so, the batch/bulk can be released for further use; if not, it can be investigated and/or discarded. For instance, unit doses can be prepared from the bulk, or the batch can be released for public distribution and use.

For assessing relative potency it is useful to analyse the test vaccine and the standard vaccine at a variety of strengths. As discussed above, a series of dilutions of the vaccines can be analysed. The dilution series can be tested using the assays of the invention to provide a curve (literally or notionally) of binding assay results against dilution. This curve can be compared to a standard curve (*i.e.* the same curve, but obtained with the standard vaccine) to determine relative potency. For instance, by plotting the logarithm of the binding titer against the logarithm of dilution for the test and reference vaccines, the horizontal distance between the two parallel regression lines indicates relative potency (no horizontal separation indicating a relative potency of 100% or 1.0).

To simplify comparisons, the dilutions used for the test vaccine should be the same as those used for the reference vaccine (*e.g.* a series of 1:2, 1:5, or 1:10 dilutions for both vaccines).

A test for relative potency can be carried out multiple times in order to determine variance of the assay *e.g.* multiple times (duplicate, triplicate, *etc.*) on a single sample, and/or performed on multiple samples from the same bulk/batch. The invention can involve determining the variation in such multiple assays (*e.g.* the coefficient of variation) as a useful parameter, and in some embodiments the results of the assay are considered as useful only where variation falls within acceptable limits *e.g.* <15%. Sometimes a wider variation is permitted *e.g.* <20%, depending whether tests are performed within (intra-assay) or in different (inter-assay) experimental sessions.

Where a vaccine includes multiple different immunogen, the potency of each of these is ideally tested separately. These results can then be combined for an analysis of the vaccine sample as a whole, but it is useful to identify the specific cause of any loss of overall potency.

The antibody

Assays of the invention use monoclonal antibodies which recognise protein immunogens which are present within the analysed vaccines. The invention can use antibodies which are bactericidal for meningococcus and/or which recognise conformational epitopes in the protein immunogens. In both cases the antibodies can thus distinguish between functional immunogen and denatured or non-functional immunogen. The use of bactericidal antibodies is preferred.

Determining whether an antibody is bactericidal against meningococcus is routine in the art, and can be assessed by SBA [28-31]. Reference 32 reports good inter-laboratory reproducibility of this assay when using harmonised procedures. SBA can be run against strain H44/76 (reference strain 237 from

the PubMLST database; strain designation B: P1.7,16: F3-3: ST-32 (cc32); also known as 44/76-3 or Z3842). For present purposes, however, an antibody can be regarded as bactericidal if it kills strain MC58 using human complement.

Determining whether an antibody recognises a conformational epitope is also straightforward. For instance, the antibody can be tested against a panel of linear peptide fragments from the target antigen (*e.g.* using the Pepscan technique) and the binding can be compared to the antibody's binding against the complete antigen. As an alternative, binding can be compared before and after denaturation of the target antigen.

Assays of the invention can use a single monoclonal antibody or a mixture of monoclonal antibodies.

Typically a vaccine will include multiple different immunogens and each of these will require a different monoclonal antibody for its analysis. Thus an assay can use: a single monoclonal antibody which recognises a single immunogen; a plurality of different monoclonal antibodies which recognise a single immunogen (typically different epitopes on the immunogen); a plurality of different monoclonal antibodies which recognise a plurality of different immunogens, in which there is one or more antibody/s per immunogen (typically recognising different epitopes if they target the same immunogen). Rather than perform a single assay to recognise multiple immunogens simultaneously, it is preferred to perform multiple assays with a single monoclonal antibody per assay. These results can then be combined for an overall analysis of the vaccine sample. By using multiple assays, each immunogen within a multi-immunogen vaccine can be assessed separately *e.g.* to isolate the cause of any loss of potency relative to a standard vaccine.

An antibody can be tested to ensure that it does not cross-react with other antigens which might be present in a vaccine. This test is straightforward, and such cross-reacting antibodies can either be used with caution and proper controls, or can be rejected in favour of antibodies which do not have the cross-reacting activity.

To facilitate determination of relative potency, the monoclonal antibody should show a linear binding response when a target antigen diluted *i.e.* dilution of the target antigen should bring about a corresponding reduction in binding by the antibody. Linearity can be assessed by linear regression *e.g.* to have $R^2 \geq 0.95$.

The monoclonal antibodies can be obtained from any suitable species *e.g.* murine, rabbit, sheep, goat, or human monoclonal antibodies. Advantageously, the chosen species can be selected such that secondary antibodies are readily available *e.g.* labelled goat anti-mouse secondary antibodies are easy to obtain, so mouse monoclonal antibodies are easily usable in ELISA.

The monoclonal antibodies can have any heavy chain type *e.g.* it can have α , δ , ϵ , γ or μ heavy chain, giving rise respectively to antibodies of IgA, IgD, IgE, IgG, or IgM class. Classes may be further divided into subclasses or isotypes *e.g.* IgG1, IgG2, IgG3, IgG4, IgA, IgA2, *etc.* Antibodies may also be classified by allotype *e.g.* a γ heavy chain may have G1m allotype a, f, x or z, G2m allotype n, or

G3m allotype b0, b1, b3, b4, b5, c3, c5, g1, g5, s, t, u, or v; a κ light chain may have a Km(1), Km(2) or Km(3) allotype. IgG monoclonal antibodies are preferred. A native IgG antibody has two identical light chains (one constant domain C_L and one variable domain V_L) and two identical heavy chains (three constant domains C_{H1} , C_{H2} & C_{H3} and one variable domain V_H), held together by disulfide bridges.

The monoclonal antibodies can have any light chain type *e.g.* it can have either a kappa (κ) or a lambda (λ) light chain.

The term “antibody” is not limited to native antibodies, as naturally found in mammals. The term encompasses any similar molecule which can perform the same role in an immunoassay such as ELISA. Thus the antibody may be, for example, a fragment of a native antibody which retains antigen binding activity (*e.g.* a Fab fragment, a Fab' fragment, a F(ab')₂ fragment, a Fv fragment), a “single-chain Fv” comprising a V_H and V_L domain as a single polypeptide chain, a “diabody”, a “triabody”, a single variable domain or VHH antibody, a “domain antibody” (dAb), a chimeric antibody having constant domains from one organism but variable domains from a different organism, a CDR-grafted antibody, etc. The antibody may include a single antigen binding site (*e.g.* as in a Fab fragment or a scFv) or multiple antigen binding sites (*e.g.* as in a F(ab')₂ fragment or a diabody or a native antibody). Where an antibody has more than one antigen-binding site, however, it is preferably a mono-specific antibody *i.e.* all antigen-binding sites recognize the same antigen. The antibody may have a constant domain (*e.g.* including C_H or C_L domains), but this is not always required. Thus the term “antibody” as used herein encompasses a range of proteins having diverse structural features (usually including at least one immunoglobulin domain having an all- β protein fold with a 2-layer sandwich of anti-parallel β -strands arranged in two β -sheets), but all of the proteins possess the ability to bind to the target protein immunogens.

The term “monoclonal” as originally used in relation to antibodies referred to antibodies produced by a single clonal line of immune cells, as opposed to “polyclonal” antibodies that, while all recognizing the same target protein, were produced by different B cells and would be directed to different epitopes on that protein. As used herein, the word “monoclonal” does not imply any particular cellular origin, but refers to any population of antibodies that all have the same amino acid sequence and recognize the same epitope(s) in the same target protein(s). Thus a monoclonal antibody may be produced using any suitable protein synthesis system, including immune cells, non-immune cells, acellular systems, *etc.* This usage is usual in the field *e.g.* the product datasheets for the CDR grafted humanised antibody Synagis™ expressed in a murine myeloma NS0 cell line, the humanised antibody Herceptin™ expressed in a CHO cell line, and the phage-displayed antibody Humira™ expressed in a CHO cell line all refer the products as monoclonal antibodies. The term “monoclonal antibody” thus is not limited regarding the species or source of the antibody, nor by the manner in which it is made.

Known monoclonal antibodies can be used with the invention, or new monoclonal antibodies can be generated using known techniques (*e.g.* injection of a reference vaccine's immunogen into mice with Freund's complete adjuvant), followed by screening for those with suitable properties *e.g.* for bactericidal activity, *etc.* The invention does not require the use of particular known antibodies, but a number of antibodies useful for analysis of the immunogens in BEXSERO™ are described below:

- A suitable monoclonal antibody for assaying NHBA as found in the BEXSERO™ product is the 42A4A2 antibody (murine IgG1) which likely recognises a conformational epitope.
- Suitable monoclonal antibodies for assaying fHbp as found in the BEXSERO™ product include, but are not limited to, the MAb502 antibody [33,34], the 12C1/D7 antibody (see below) and the 11F10/G6 antibody (see below). These three antibodies are all bactericidal. MAb502 (murine IgG2a) does not give good linearity when diluted and so the other two antibodies (both murine IgG2b) are preferable. Two other useful anti-fHbp monoclonal antibodies are 30G11/H3 and 14B3/D4 (see below) The JAR3 and JAR5 antibodies (ref. 35; GenBank VL and VH genes are JF715927, F715926, JF715929 and JF715928) can also be used, as can other prior art JAR antibodies *e.g.* up to JAR35 [36]. The anti-fHbp monoclonal antibody can bind to a single variant of fHbp, or can bind to more than one variant (such as the JAR3 and JAR5 antibodies, as reported in reference 37).
- A suitable monoclonal antibody for assaying NadA as found in the BEXSERO™ product is the bactericidal 9F11/19 antibody (murine IgG2b).

Assaying a vesicle component in a vaccine can use any antigen in the vesicle, but it is convenient to use anti-PorA antibodies as these are readily available for serosubtype analysis (*e.g.* from NIBSC). Thus for assaying the OMV component as found in the BEXSERO™ product a suitable monoclonal antibody recognises serosubtype P1.4.

A secondary antibody used with the invention (*e.g.* in the assay's competitive format) can recognise the primary antibody when the primary antibody has become immobilised. The secondary antibody is typically polyclonal. For instance, if the primary antibody is murine then the secondary antibody can be an anti-murine antibody *e.g.* goat anti-mouse IgG. Suitable criteria for choosing secondary antibodies are well known in the ELISA field.

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, references 38-44, *etc.*

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value *x* is optional and means, for example, $x \pm 10\%$.

Where the invention concerns an “epitope”, this epitope may be a B-cell epitope and/or a T-cell epitope, but will usually be a B-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [45,46] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [47], matrix-based approaches [48], MAPITOPE [49], TEPITOPE [50,51], neural networks [52], OptiMer & EpiMer [53, 54], ADEPT [55], Tsites [56], hydrophilicity [57], antigenic index [58] or the methods disclosed in references 59-63, *etc.*). Epitopes are the parts of an antigen that are recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as “antigenic determinants”.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and % homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 64. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 65.

The word “substantially” does not exclude “completely” *e.g.* a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

Meningococcal protein immunogens

NHBA (Neisserial Heparin Binding Antigen)

NHBA [68] was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB2132 (GenBank accession number GI:7227388; SEQ ID NO: 9 herein). Sequences of NHBA from many strains have been published since then. For example, allelic forms of NHBA (referred to as protein ‘287’) can be seen in Figures 5 and 15 of reference 66, and in example 13 and figure 21 of reference 67 (SEQ IDs 3179 to 3184 therein). Various immunogenic fragments of NHBA have also been reported.

Preferred NHBA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 9, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 9.

The most useful NHBA antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 9. Advantageous NHBA antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

Over-expression of NHBA has previously been achieved in various ways *e.g.* introduction of a NHBA gene under the control of an IPTG-inducible promoter [68].

NadA (Neisserial adhesin A)

5 The NadA antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB1994 (GenBank accession number GI:7227256; SEQ ID NO: 10 herein). The sequences of NadA antigen from many strains have been published since then, and the protein's activity as a Neisserial adhesin has been well documented. Various immunogenic fragments of NadA have also been reported.

10 Preferred NadA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 10; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 10, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 10.

15 The most useful NadA antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 10. Advantageous NadA antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject. SEQ ID NO: 6 is one such fragment.

HmbR

20 The full-length HmbR sequence was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB1668 (SEQ ID NO: 7 herein). Reference 69 reports a HmbR sequence from a different strain (SEQ ID NO: 8 herein), and reference 70 reports a further sequence (SEQ ID NO: 19 herein). SEQ ID NOs: 7 and 8 differ in length by 1 amino acid and have 94.2% identity. SEQ ID NO: 19 is one amino acid shorter than SEQ ID NO: 7 and they have 99%
25 identity (one insertion, seven differences) by CLUSTALW. The invention can use any such HmbR polypeptide.

The invention can use a polypeptide that comprises a full-length HmbR sequence, but it will often use a polypeptide that comprises a partial HmbR sequence. Thus in some embodiments a HmbR sequence used according to the invention may comprise an amino acid sequence having at least *i*%
30 sequence identity to SEQ ID NO: 7, where the value of *i* is 50, 60, 70, 80, 90, 95, 99 or more. In other embodiments a HmbR sequence used according to the invention may comprise a fragment of at least *j* consecutive amino acids from SEQ ID NO: 7, where the value of *j* is 7, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more. In other embodiments a HmbR sequence used according to the invention may comprise an amino acid sequence (i) having at least
35 *i*% sequence identity to SEQ ID NO: 7 and/or (ii) comprising a fragment of at least *j* consecutive amino acids from SEQ ID NO: 7.

Preferred fragments of *j* amino acids comprise an epitope from SEQ ID NO: 7. Such epitopes will usually comprise amino acids that are located on the surface of HmbR. Useful epitopes include those with amino acids involved in HmbR's binding to haemoglobin, as antibodies that bind to these epitopes can block the ability of a bacterium to bind to host haemoglobin. The topology of HmbR, and its critical functional residues, were investigated in reference 71. Fragments that retain a transmembrane sequence are useful, because they can be displayed on the bacterial surface *e.g.* in vesicles. Examples of long fragments of HmbR correspond to SEQ ID NOs: 15 and 16. If soluble HmbR is used, however, sequences omitting the transmembrane sequence, but typically retaining epitope(s) from the extracellular portion, can be used.

The most useful HmbR antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 7. Advantageous HmbR antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

fHbp (factor H binding protein)

The fHbp antigen has been characterised in detail. It has also been known as protein '741' [SEQ IDs 2535 & 2536 in ref. 67], 'NMB1870', 'GNA1870' [72-74], 'P2086', 'LP2086' or 'ORF2086' [75-77]. It is naturally a lipoprotein and is expressed across all meningococcal serogroups. The structure of fHbp's C-terminal immunodominant domain ('fHbpC') has been determined by NMR [78]. This part of the protein forms an eight-stranded β -barrel, whose strands are connected by loops of variable lengths. The barrel is preceded by a short α -helix and by a flexible N-terminal tail.

The fHbp antigen falls into three distinct variants [79] and it has been found that serum raised against a given family is bactericidal within the same family, but is not active against strains which express one of the other two families *i.e.* there is intra-family cross-protection, but not inter-family cross-protection. The invention can use a single fHbp variant, but a vaccine will usefully include a fHbp from two or three of the variants. Thus it may use a combination of two or three different fHbps, selected from: (a) a first protein, comprising an amino acid sequence having at least *a*% sequence identity to SEQ ID NO: 1 and/or comprising an amino acid sequence consisting of a fragment of at least *x* contiguous amino acids from SEQ ID NO: 1; (b) a second protein, comprising an amino acid sequence having at least *b*% sequence identity to SEQ ID NO: 2 and/or comprising an amino acid sequence consisting of a fragment of at least *y* contiguous amino acids from SEQ ID NO: 2; and/or (c) a third protein, comprising an amino acid sequence having at least *c*% sequence identity to SEQ ID NO: 3 and/or comprising an amino acid sequence consisting of a fragment of at least *z* contiguous amino acids from SEQ ID NO: 3.

The value of *a* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more.

The value of *b* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more.

The value of *c* is at least 85 *e.g.* 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, or more.

The values of *a*, *b* and *c* are not intrinsically related to each other.

The value of x is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The value of y is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The value of z is at least 7 *e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 225, 250). The values of x , y and z are not intrinsically related to each other.

Where the invention uses a single fHbp variant, a composition may include a polypeptide comprising (a) an amino acid sequence having at least $a\%$ sequence identity to SEQ ID NO: 1 and/or comprising an amino acid sequence consisting of a fragment of at least x contiguous amino acids from SEQ ID NO: 1; or (b) an amino acid sequence having at least $b\%$ sequence identity to SEQ ID NO: 2 and/or comprising an amino acid sequence consisting of a fragment of at least y contiguous amino acids from SEQ ID NO: 2; or (c) an amino acid sequence having at least $c\%$ sequence identity to SEQ ID NO: 3 and/or comprising an amino acid sequence consisting of a fragment of at least z contiguous amino acids from SEQ ID NO: 3.

Where the invention uses a fHbp from two or three of the variants, a composition may include a combination of two or three different fHBps selected from: (a) a first polypeptide, comprising an amino acid sequence having at least $a\%$ sequence identity to SEQ ID NO: 1 and/or comprising an amino acid sequence consisting of a fragment of at least x contiguous amino acids from SEQ ID NO: 1; (b) a second polypeptide, comprising an amino acid sequence having at least $b\%$ sequence identity to SEQ ID NO: 2 and/or comprising an amino acid sequence consisting of a fragment of at least y contiguous amino acids from SEQ ID NO: 2; and/or (c) a third polypeptide, comprising an amino acid sequence having at least $c\%$ sequence identity to SEQ ID NO: 3 and/or comprising an amino acid sequence consisting of a fragment of at least z contiguous amino acids from SEQ ID NO: 3. The first, second and third polypeptides have different amino acid sequences.

Where the invention uses a fHbp from two of the variants, a composition can include both: (a) a first polypeptide, comprising an amino acid sequence having at least $a\%$ sequence identity to SEQ ID NO: 1 and/or comprising an amino acid sequence consisting of a fragment of at least x contiguous amino acids from SEQ ID NO: 1; and (b) a second polypeptide, comprising an amino acid sequence having at least $b\%$ sequence identity to SEQ ID NO: 2 and/or comprising an amino acid sequence consisting of a fragment of at least y contiguous amino acids from SEQ ID NO: 2. The first and second polypeptides have different amino acid sequences.

Where the invention uses a fHbp from two of the variants, a composition can include both: (a) a first polypeptide, comprising an amino acid sequence having at least $a\%$ sequence identity to SEQ ID NO: 1 and/or comprising an amino acid sequence consisting of a fragment of at least x contiguous amino acids from SEQ ID NO: 1; (b) a second polypeptide, comprising an amino acid sequence

having at least $c\%$ sequence identity to SEQ ID NO: 3 and/or comprising an amino acid sequence consisting of a fragment of at least z contiguous amino acids from SEQ ID NO: 3. The first and second polypeptides have different amino acid sequences.

Another useful fHbp which can be used according to the invention is one of the modified forms disclosed, for example, in reference 80 *e.g.* comprising SEQ ID NO: 20 or 23 therefrom. These modified forms can elicit antibody responses which are broadly bactericidal against meningococci. SEQ ID NO: 77 in reference 80 is another useful fHbp sequence which can be used.

fHbp protein(s) in a OMV will usually be lipidated *e.g.* at a N-terminus cysteine. In other embodiments they will not be lipidated.

One vaccine which can be analysed by the methods of the invention includes two different variants of fHbp. The first variant can have amino acid sequence SEQ ID NO: 29, and the second can have amino acid sequence SEQ ID NO: 30. These are preferably lipidated at their N-terminus cysteines. This vaccine can include an aluminium phosphate adjuvant, and can also include a histidine buffer and polysorbate 80. Ideally it includes equal masses of the two different fHbp polypeptides.

NspA (Neisserial surface protein A)

The NspA antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB0663 (GenBank accession number GI:7225888; SEQ ID NO: 11 herein). The antigen was previously known from references 81 & 82. The sequences of NspA antigen from many strains have been published since then. Various immunogenic fragments of NspA have also been reported.

Preferred NspA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 11; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 11, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 11.

The most useful NspA antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 11. Advantageous NspA antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

NhhA (Neisseria hia homologue)

The NhhA antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB0992 (GenBank accession number GI:7226232; SEQ ID NO: 12 herein). The sequences of NhhA antigen from many strains have been published since *e.g.* refs 66 & 83, and various immunogenic fragments of NhhA have been reported. It is also known as Hsf.

Preferred NhhA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 12; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 12, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 12.

The most useful NhhA antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 12. Advantageous NhhA antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

App (Adhesion and penetration protein)

The App antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB1985 (GenBank accession number GI:7227246; SEQ ID NO: 13 herein). The sequences of App antigen from many strains have been published since then. It has also been known as 'ORF1' and 'Hap'. Various immunogenic fragments of App have also been reported.

Preferred App antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 13; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 13, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 13.

The most useful App antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 13. Advantageous App antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

Omp85 (85kDa outer membrane protein)

The Omp85 antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB0182 (GenBank accession number GI:7225401; SEQ ID NO: 14 herein). The sequences of Omp85 antigen from many strains have been published since then. Further information on Omp85 can be found in references 84 and 85. Various immunogenic fragments of Omp85 have also been reported.

Preferred Omp85 antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 14; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 14, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16,

18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 14.

The most useful Omp85 antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 14.

5 Advantageous Omp85 antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

TbpA

The TbpA antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB0461 (GenBank accession number GI:7225687; SEQ ID NO: 17
10 herein). The sequences of TbpA from many strains have been published since then. Various immunogenic fragments of TbpA have also been reported.

Preferred TbpA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 17; and/or (b) comprising a fragment of at
15 least 'n' consecutive amino acids of SEQ ID NO: 17, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 17.

The most useful TbpA antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 17. Advantageous
20 TbpA antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

TbpB

The TbpB antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB1398 (GenBank accession number GI:7225686; SEQ ID NO: 18
25 herein). The sequences of TbpB from many strains have been published since then. Various immunogenic fragments of TbpB have also been reported.

Preferred TbpB antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 18; and/or (b) comprising a fragment of at
30 least 'n' consecutive amino acids of SEQ ID NO: 18, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 18.

The most useful TbpB antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 18. Advantageous

TbpB antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

Cu,Zn-superoxide dismutase

5 The Cu,Zn-superoxide dismutase antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 [25] as gene NMB1398 (GenBank accession number GI:7226637; SEQ ID NO: 20 herein). The sequences of Cu,Zn-superoxide dismutase from many strains have been published since then. Various immunogenic fragments of Cu,Zn-superoxide dismutase have also been reported.

10 Preferred Cu,Zn-superoxide dismutase antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 20; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 20, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 20.

15 The most useful Cu,Zn-superoxide dismutase antigens can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 20. Advantageous Cu,Zn-superoxide dismutase antigens for use with the invention can elicit bactericidal anti-meningococcal antibodies after administration to a subject.

Monoclonal antibodies

20 The invention also provides monoclonal antibodies which recognise meningococcal antigens. These can be used with the assays of the invention, or can be used more generally.

One antibody of the invention is "12C1/D7". Its V_L region has amino acid sequence SEQ ID NO: 21 and its V_H region has amino acid sequence SEQ ID NO: 22.

25 Another antibody of the invention is "11F10/G6". Its V_L region has amino acid sequence SEQ ID NO: 23 and its V_H region has amino acid sequence SEQ ID NO: 24.

Another antibody of the invention is "30G11/H3". Its V_L region has amino acid sequence SEQ ID NO: 25 and its V_H region has amino acid sequence SEQ ID NO: 26.

Another antibody of the invention is "14B3/D4". Its V_L region has amino acid sequence SEQ ID NO: 27 and its V_H region has amino acid sequence SEQ ID NO: 28.

30 The invention also provides monoclonal antibodies which bind to meningococcal antigens and which include the CDRs from the V_L and V_H regions of 12C1/D7, 11F10/G6, 30G11/H3, or 14B3/D4.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows relative potency plots for NHBA, fHbp, NadA and OMV immunogens in BEXSERO™ using monoclonal antibodies (A) 42A4A2 (B) MA502 (C) 12C1/D7 (D) 11F10/G6

(E) 9F11/19 (F) Anti-PorA. Each plot shows $\log(\text{OD}_{405-620\text{nm}})$ against $\log(\text{dilution})$. Circles show data for the standard vaccine; triangles for the test vaccine.

Figure 2 shows relative potency plots for two further batches of OMV in BEXSERO™.

Figure 3 shows RP values for vaccines heated overnight. The four groups of four bars are, from left to right: fHbp; NHBA; NadA; and OMVs. Within each group, the four bars are: 37°C; 50°C; 60°C; and 80°C.

Figure 4 shows RP plots for standard vaccine (circles) and for adjuvant (triangles) using monoclonal antibodies (A) MAb502 (B) 42A4A2 (C) 9F11/19 and (D) Anti-PorA.

Figure 5 illustrates an ELISA of the invention in competitive format. At the top, monoclonal antibody (A) for one of the vaccine immunogens is mixed with the vaccine sample (B) in ten wells having increasingly-diluted vaccine in each well. In (C) this mixture is transferred into the wells of a second plate, the wells of which are coated with immobilised vaccine immunogen. After incubation the plates are washed (D), then enzyme-conjugated anti-mAb serum is added in step (E), after which the enzyme is used to catalyse a detectable reaction for ELISA output.

15 MODES FOR CARRYING OUT THE INVENTION

The BEXSERO™ product is described in reference 7, and it includes 50 µg of each of NadA (subvariant 3.1; SEQ ID NO: 6), fHbp subvariant 1.1 (as a GNA2091-fHbp fusion protein; SEQ ID NO: 5), and NHBA subvariant 1.2 (as a NHBA-GNA1030 fusion protein; SEQ ID NO: 4), adsorbed onto 1.5 mg aluminium hydroxide, and with 25µg OMVs from *N.meningitidis* strain NZ98/254.

20 The following monoclonal antibodies are available:

(A) 42A4A2 (murine IgG1 against NHBA)

(B) MAb502 (murine IgG2a against fHbp)

(C) 12C1/D7 (murine IgG2b against fHbp)

(D) 11F10/G6 (murine IgG2b against fHbp)

25 (E) 9F11/19 antibody (murine IgG2b against NadA)

(F) Anti-PorA(P1.4), available from NIBSC.

These antibodies are bactericidal, except for 42A4A2 (which is non-bactericidal but seems to recognise a conformational epitope).

30 The BEXSERO™ product is serially diluted 9 times, either 1:2 or 1:5 each time. Six of these dilution series are present in rows (A) to (F) of a first microtitre plate (plate 1), from columns 1 (strongest) to 10 (most dilute). Each row receives one of the six monoclonal antibodies (A) to (F) described above, each used at the same strength in each column. After incubation the contents of these 60 wells are transferred into 60 wells in a second plate (plate 2). The wells in rows (A) to (F) in plate 2 are coated with the individual recombinant proteins (A) NHBA (B-D) fHbp (E) NadA and (F) PorA. In other

embodiments, all wells in a single ELISA plate are coated using the same antigen, and each antigen is tested separately by using a different ELISA microtiter plate.

The mixture is incubated for 2 hours at 37°C (for fHbp) or at room temperature (for NHBA, NadA and PorA), then washed. Monoclonal antibodies which were unbound to the vaccine antigens are retained on the plates. Anti-mouse IgG, conjugated to alkaline phosphatase, is then added to all 60 wells with pNPP and the amount of retained monoclonal antibody is assessed by OD_{405-620nm}. Thus the vaccine immunogen (serially diluted) inhibits the binding of the monoclonal antibodies to the immobilised antigens in plate 2. Higher levels of epitope in the vaccine sample will lead to more inhibition of this binding, and thus to less detectable signal after adding the pNPP.

- Figures 1A to 1F show the results from the six rows. The graphs also include data measured with a reference vaccine, and comparison of the two parallel lines reveals the following relative potencies:

	A	B	C	D	E	F
R.P.	0.915	2.344	0.859	0.895	1.037	1.033

The aberrant value in Figure 1B (*i.e.* using MAb502) arose because the curves were not linear and were not parallel to each other. In all other cases the curves were linear with good R^2 values. Thus the assay is suitable for assessing relative potency.

To check for inter-assay consistency the anti-PorA measurement was checked for two further BEXSERO™ batches (Figures 2A and 2B). The results in Figures 1F, 2A and 2B show no big differences, and RP was 1.033, 0.917 and 0.893 in the three different vaccine batches.

The ability of this assay to identify damaged vaccine was tested by artificially exposing a BEXSERO™ product to thermal stress. Relative potency values for each of the four immunogen components after 2 hours at 80°C were as follows:

	NHBA	fHbp	NadA	OMV
R.P.	0.25	0.08	0.01	0.55

Figure 3 shows relative potency values for each of the four immunogen components after overnight incubation at 37°C, 50°C, 60°C and 80°C. Thus the assay can detect losses in potency caused by thermal mistreatment.

To confirm that the aluminium hydroxide adjuvant did not interfere with the assay, antibodies (A), (B), (E) and (F) were tested with standard vaccine or with adjuvant. As shown in Figure 4, the adjuvant always showed its inability to compete and/or interfere with the binding of each monoclonal antibody to the respective immunogen(s).

Anti-fHbp monoclonal antibodies

Four bactericidal murine anti-fHbp IgG2b subclass monoclonal antibodies were obtained: 12C1/D7; 11F10/G6; 30G11/H3; and 14B3/D4. RNA was isolated from the murine hybridoma cells using an Oligotex Direct mRNA Mini Kit according to the manufacturer's instructions. cDNA was produced via reverse transcription using ~200 ng of the poly(A)+RNA template, an oligo-(dT) primer, and SuperScript II RT. cDNA was amplified by PCR using immunoglobulin heavy (H)- and light (L)-chain degenerate primers as described in reference 86. The purified products were inserted into the pSTBlue-1 Perfectly Blunt vector for sequencing.

12C1/D7's V_L region has amino acid sequence SEQ ID NO: 21:

10 DIVLTQSPSSIIYASLGERVTLTCKASQDIHNYLNWFQQKPGKSPKTLIYRANRLVDGVPSRFSGGGSGQDY
SLTISSELEFEDIGIYYCLQYDEFPPPTFGGGTRLEIKRADAAPTVS

and its V_H region has amino acid sequence SEQ ID NO: 22:

QVQLQESGPPELVKPGASVKISCKASGYSFSDYNMSWVKQSNQKSLEWIGIIDPKYGTINYNQKFKGKATLT
VDQASSTAYMQLNSLTSEDSAVYYCVRDYYGSSYFDYWGQGTTLTVS

15 11F10/G6's V_L region has amino acid sequence SEQ ID NO: 23:

DIVLTQTPSSIIYASLGERVTLTCKASQDIHNYLNWFQQKPGKSPKTLIYRANRLVDGVPSRFSGGGSGQDY
SLTISSELEFEDIGIYYCLQYDEFPPPTFGGGTRLEIKRADAAPTVS

and its V_H region has amino acid sequence SEQ ID NO: 24:

20 EFQLQQSGPELVKPGASVKISCKASGYSFSDYNMSWVKQSNQKSLEWIGIIDPKYGTINYNQKFKGKATLT
VDQASSTAYMQLNSLTSEDSAVYYCVRDYYGSSYFDYWGQGTTLTVS

30G11/H3's V_L region has amino acid sequence SEQ ID NO: 25:

DIVMTQSQKFMSTSVGDRVSITCKASQHVRTAVAWYQQKPGQSPKGLIYLASNRRRTGVPDRFTASGSGTDF
TLTITNVQSEDLADYFCLQHWNYPPFTFGSGTKLEIKRADAAPTVS

and its V_H region has amino acid sequence SEQ ID NO: 26:

25 EVQLQESGPPELVKPGASVKISCKASGYSFSDYNMSWVKQSNQKSLEWIGIIDPKYGTINYNQKFKGKATLT
VDQASSTAYMQLNSLTSEDSAVYYCVRDYYGSSYFDYWGQGTTLTVS

14B3/D4's V_L region has amino acid sequence SEQ ID NO: 27:

DIVLTQSPSSSLTVTAGEKVTMSCRSSQSLNLSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGVDPDRFTGS
GSGTDFTLTISVQAEDLAIYYCQNDYNYPLTFGAGTKLELKR

30 and its V_H region has amino acid sequence SEQ ID NO: 28:

QVQLQQPGAELVKPGASVKLSCKASGYSFTTYYWMNWVKQRPGQGLEWIGMIHPNSGSTNYNEKFKNKATLT
TVDKSSSTAYIQLSSLTSEDSAVFYCAAHYNYEGYFYAMDYWGQGTSTVTVSS

In a FACS assay the 11F10/G6 and 30G11/H3 were able to bind to meningococcal strains having each of the three different fHbp variants: MC58 (variant 1); 961-5945 (variant 2); and M1239 (variant 3). Moreover, these two FACS-positive antibodies also showed bactericidal activity against strains having each of the three variants.

14B3/D4 was FACS-positive and bactericidal against MC58 and 961-5945, but not against M1239. 12C1/D7 was FACS-positive and bactericidal against MC58, but not against 961-5945 or M1239.

12C1/D7 and 11F10/G6 competed with fH for binding to fHbp; the other two antibodies did not.

The epitope for 11F10/G6 seems to include residue Lys-268 in fHbp (var 1.1).

The epitope for 12C1/D7 seems to include residue Val-270 in fHbp (var 1.1).

The epitope for 14B3/D4 seems to include residues 60-90 in fHbp.

- 5 The epitope for 30H11/H3 seems to include residue Lys-257 in fHbp (var 1.1).

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

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CLAIMS

1. A binding assay for *in vitro* analysis of a meningococcal vaccine sample, comprising steps of:
(i) permitting a meningococcal protein immunogen within the sample to interact with a monoclonal antibody which either (a) is bactericidal for meningococcus or (b) recognises a conformational epitope in the meningococcal antigen; then (ii) measuring the interaction between the immunogen and antibody from step (i).
2. The assay of claim 1, wherein the measurement in step (ii) quantitatively indicates the concentration of the monoclonal antibody's target epitope within the vaccine sample
3. The assay of any preceding claim, wherein the binding assay is a competitive ELISA.
4. The assay of claim 3, in which: step (i) involves incubating the sample with the monoclonal antibody so that complexes can form between the antibody and immunogens in the sample; these complexes are added to a container in which competitor antigens are immobilised, wherein the competitor antigens can bind to the monoclonal antibody; and then determining in step (ii) the amount of monoclonal antibody which binds to the competitor antigens.
5. The assay of any preceding claim, in which step (ii) uses a secondary antibody labelled with an enzyme.
6. The assay of any preceding claim, which takes place in a microwell plate.
7. The assay of any preceding claim, wherein the vaccine includes meningococcal NHBA, fHbp and/or NadA antigens, and wherein the monoclonal antibody used in step (i) recognises meningococcal NHBA, fHbp or NadA antigen.
8. The assay of any preceding claim, wherein the vaccine includes more than one meningococcal immunogen.
9. The assay of any preceding claim, wherein the vaccine includes a meningococcal vesicle.
10. The assay of any preceding claim, wherein the vaccine includes an adsorbed meningococcal immunogen.
11. The assay of any preceding claim, which uses a single monoclonal antibody in step (i).
12. The assay of any preceding claim, which uses a murine monoclonal IgG antibody in step (i).
13. A method for *in vitro* analysis of a meningococcal test vaccine sample, comprising steps of:
(i) performing the assay of any one of claims 1 to 12 on the test sample and, optionally, on at least one dilution of the test sample; (ii) performing the assay of any one of claims 1 to 12 on a standard vaccine sample of known *in vivo* potency and, optionally, on at least one dilution of the standard vaccine sample; and (iii) comparing the results from steps (i) and (ii) to determine the potency of immunogen(s) in the test vaccine relative to the potency of immunogen(s) in the standard vaccine.
14. A process for analysing a batch of vaccine, comprising steps of: (i) assaying the relative potency of immunogen(s) in at least one vaccine from the batch by the method of claim 13; and, if the

results of step (i) indicate an acceptable relative potency, (ii) releasing further vaccines from the batch for *in vivo* use.

15. A competitive ELISA assay for *in vitro* analysis of a meningococcal vaccine sample, wherein the assay uses (i) a solution-phase anti-vaccine monoclonal antibody (ii) an immobilised antigen which is recognised by the anti-vaccine antibody, and (iii) a labelled antibody which binds to the anti-vaccine antibody, wherein the antibody either (a) is bactericidal for meningococcus or (b) recognises a conformational epitope in the meningococcal antigen.
16. A binding assay for *in vitro* analysis of a meningococcal vaccine sample, wherein the assay uses immunogens in a vaccine to inhibit the binding of a monoclonal antibody to a control antigen, wherein the monoclonal antibody binds to both an immunogen in the vaccine and the control antigen.
17. A vaccine which has been released following use of an assay of any preceding claim.
18. A monoclonal antibody which recognises meningococcal fHbp, selected from: (a) a monoclonal antibody whose variable region comprises SEQ ID NO: 21 and SEQ ID NO: 22; (b) a monoclonal antibody whose variable region comprises SEQ ID NO: 23 and SEQ ID NO: 24; (c) a monoclonal antibody whose variable region comprises SEQ ID NO: 25 and SEQ ID NO: 26; (d) a monoclonal antibody whose variable region comprises SEQ ID NO: 27 and SEQ ID NO: 28.

FIGURE 1

FIGURE 1A

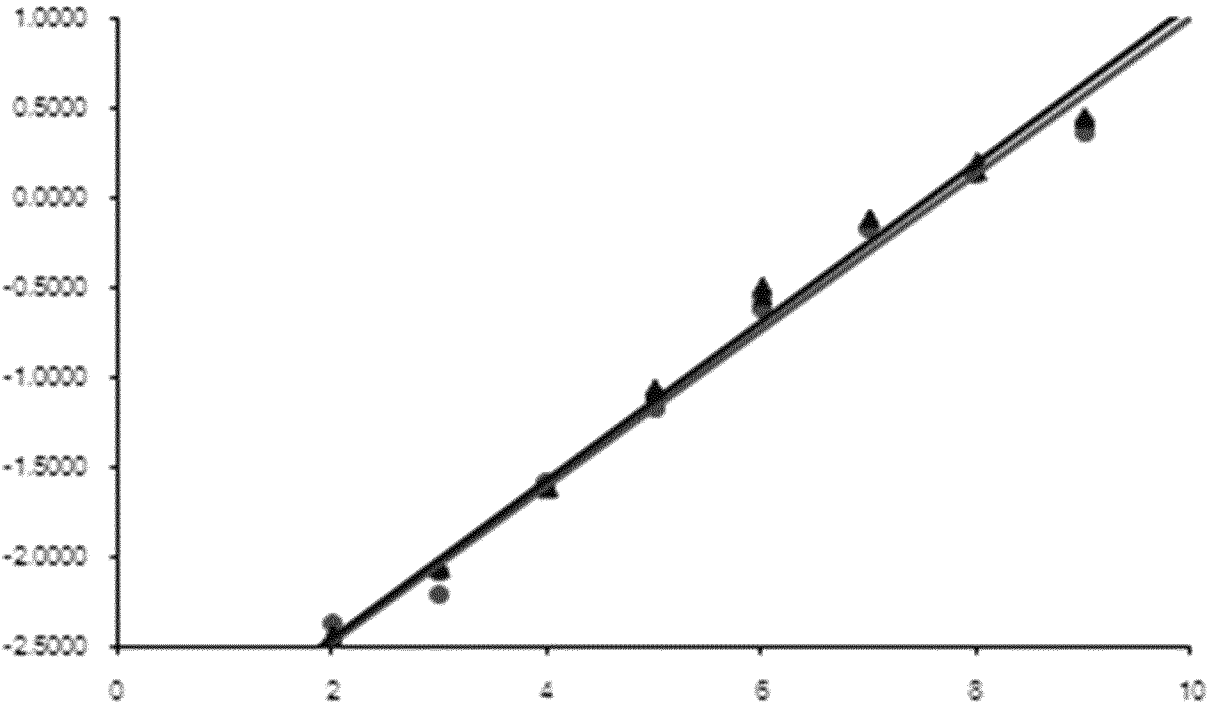
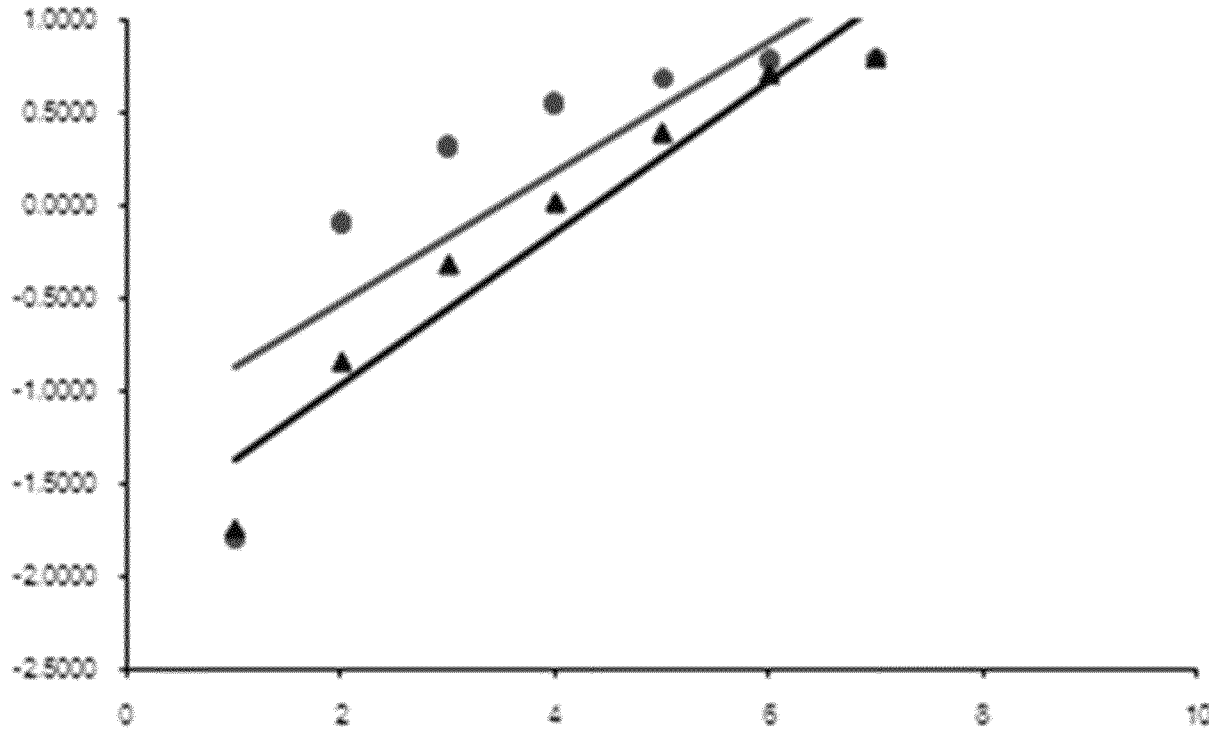
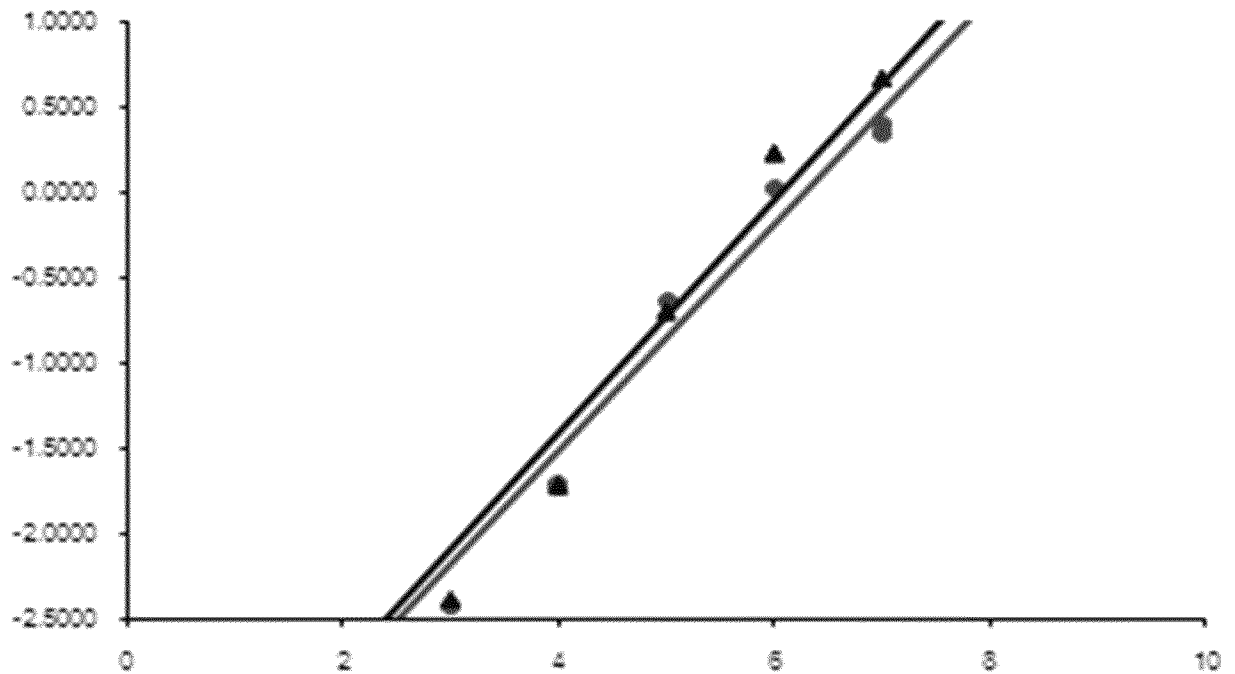
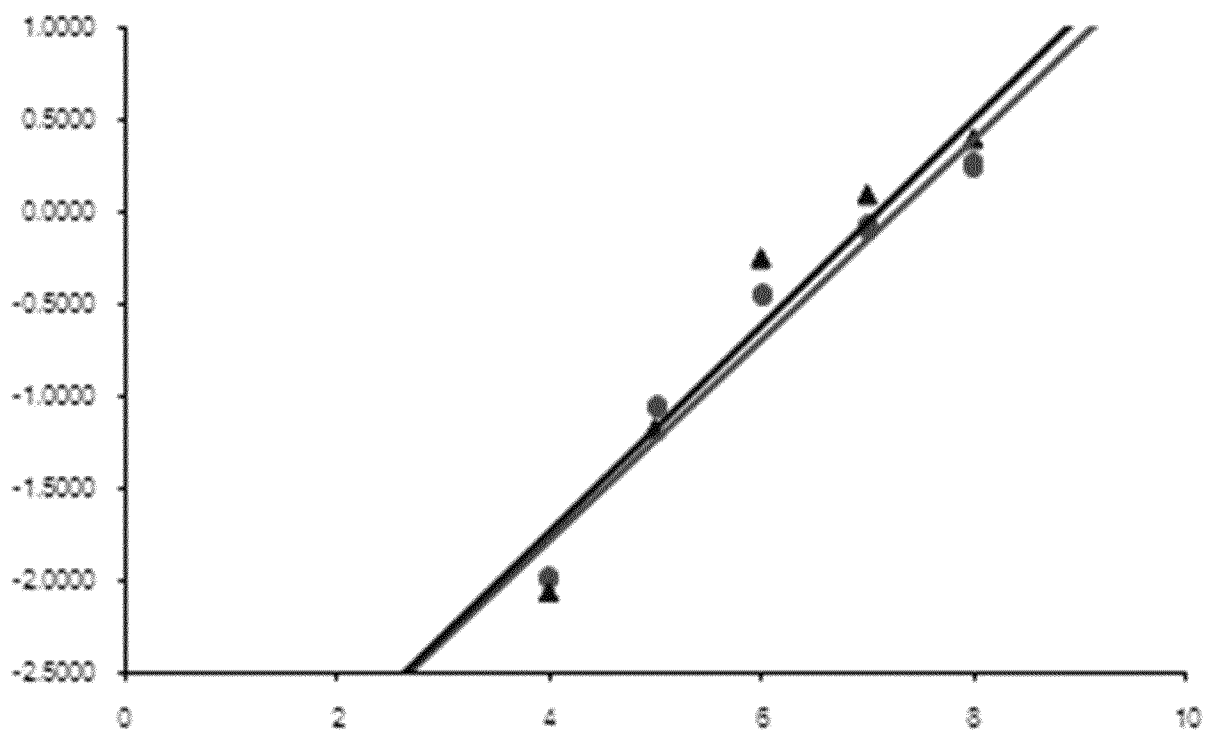


FIGURE 1B



2/8

FIGURE 1C**FIGURE 1D**

3/8

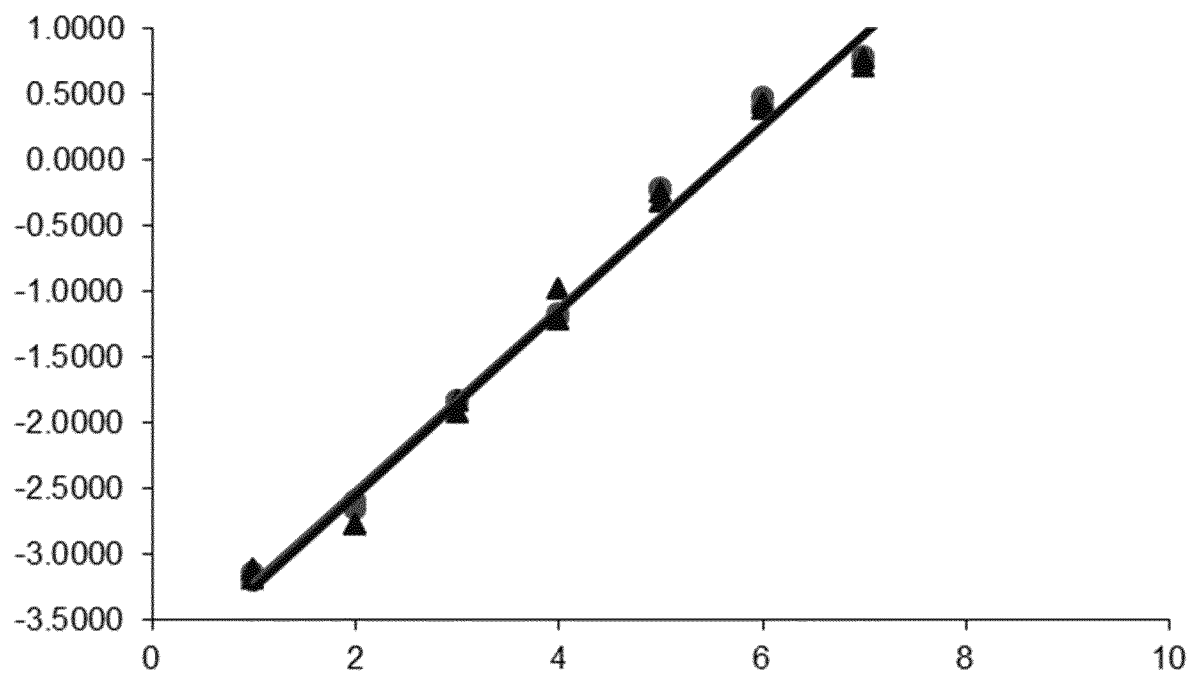
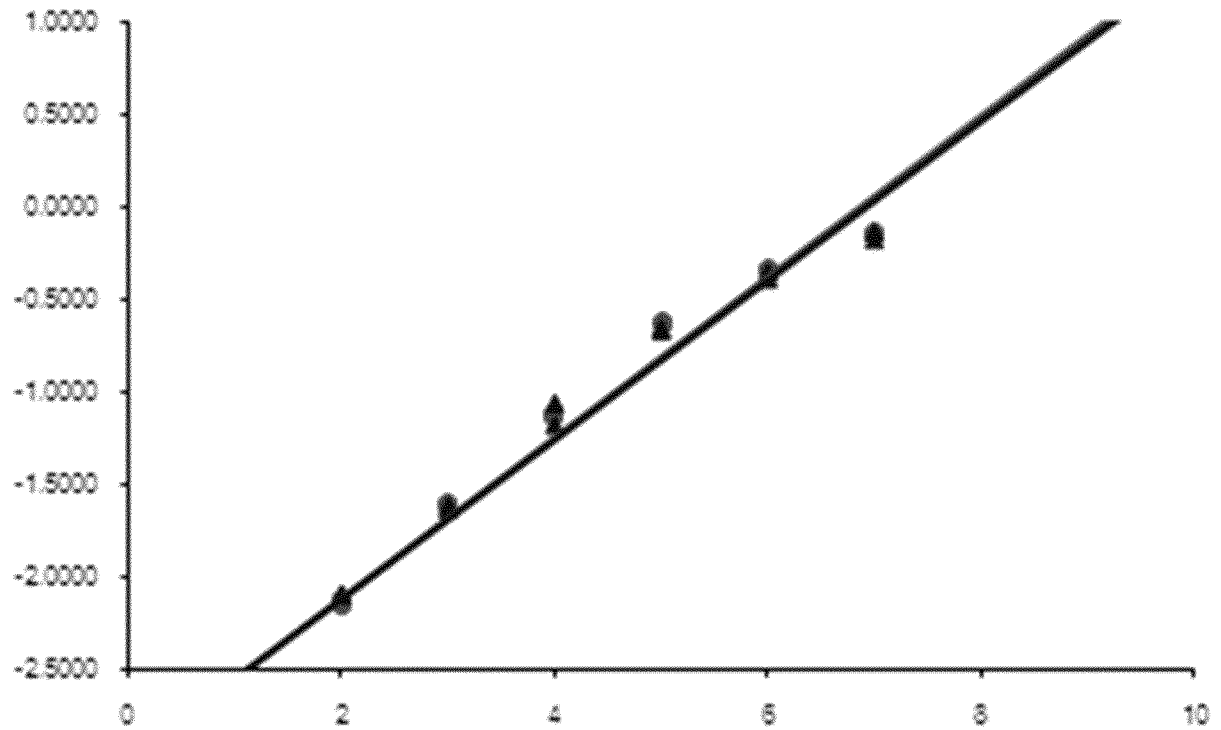
FIGURE 1E**FIGURE 1F**

FIGURE 1

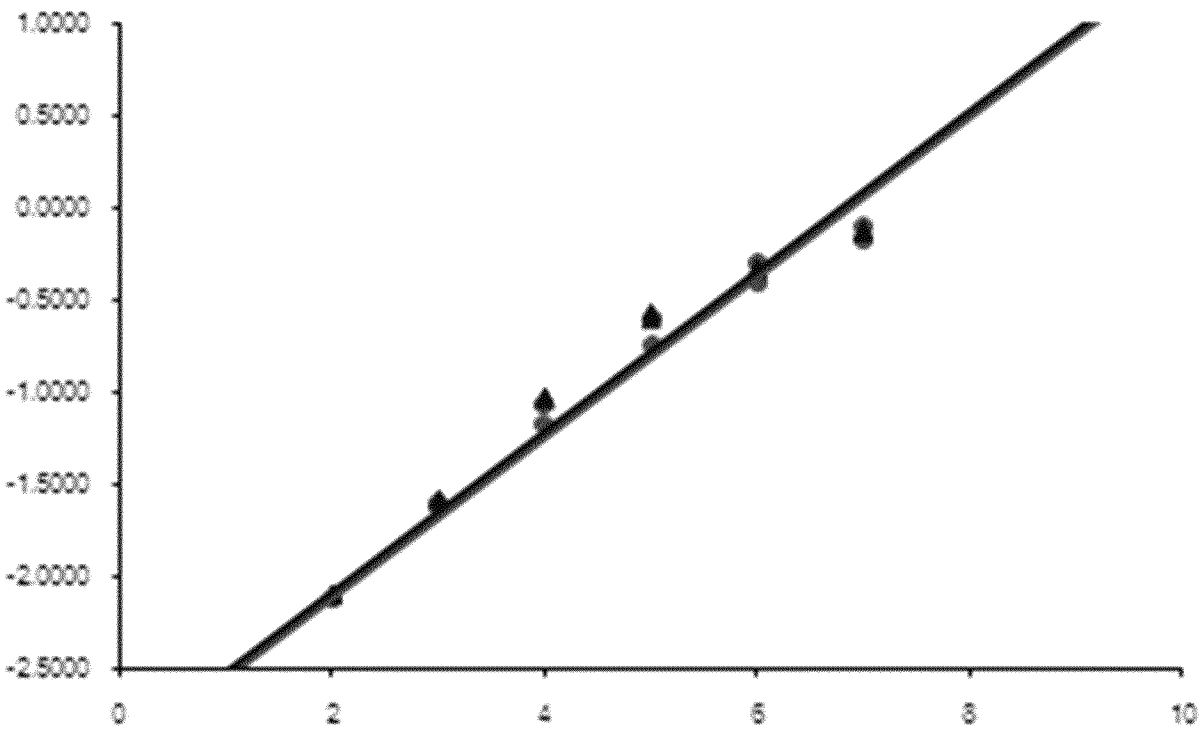


FIGURE 2B

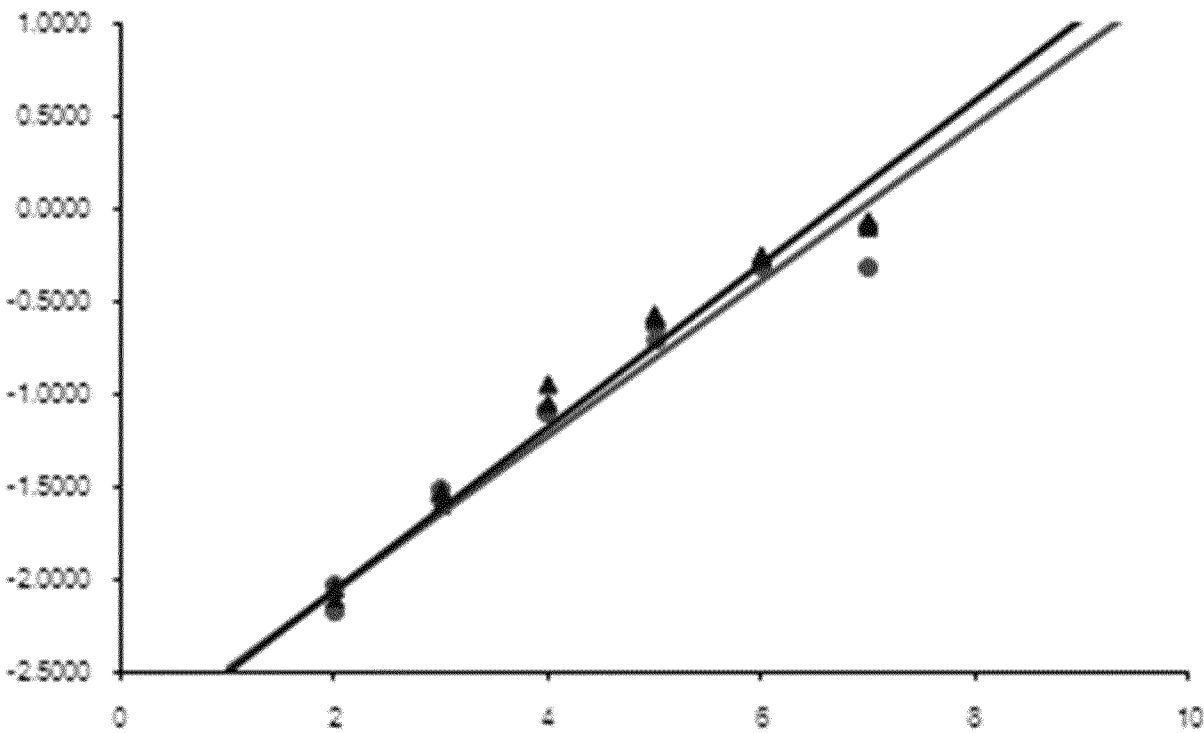


FIGURE 3

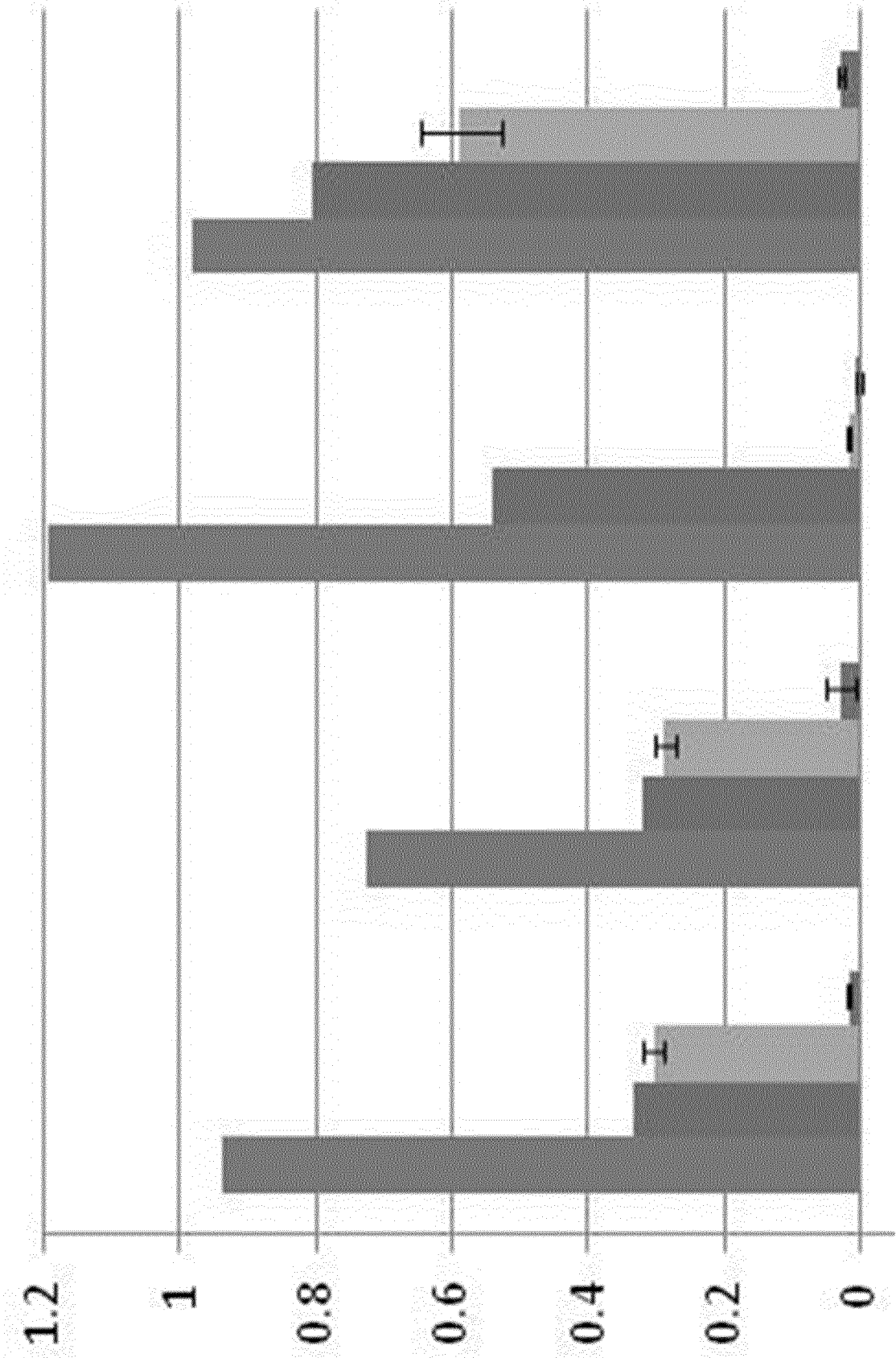
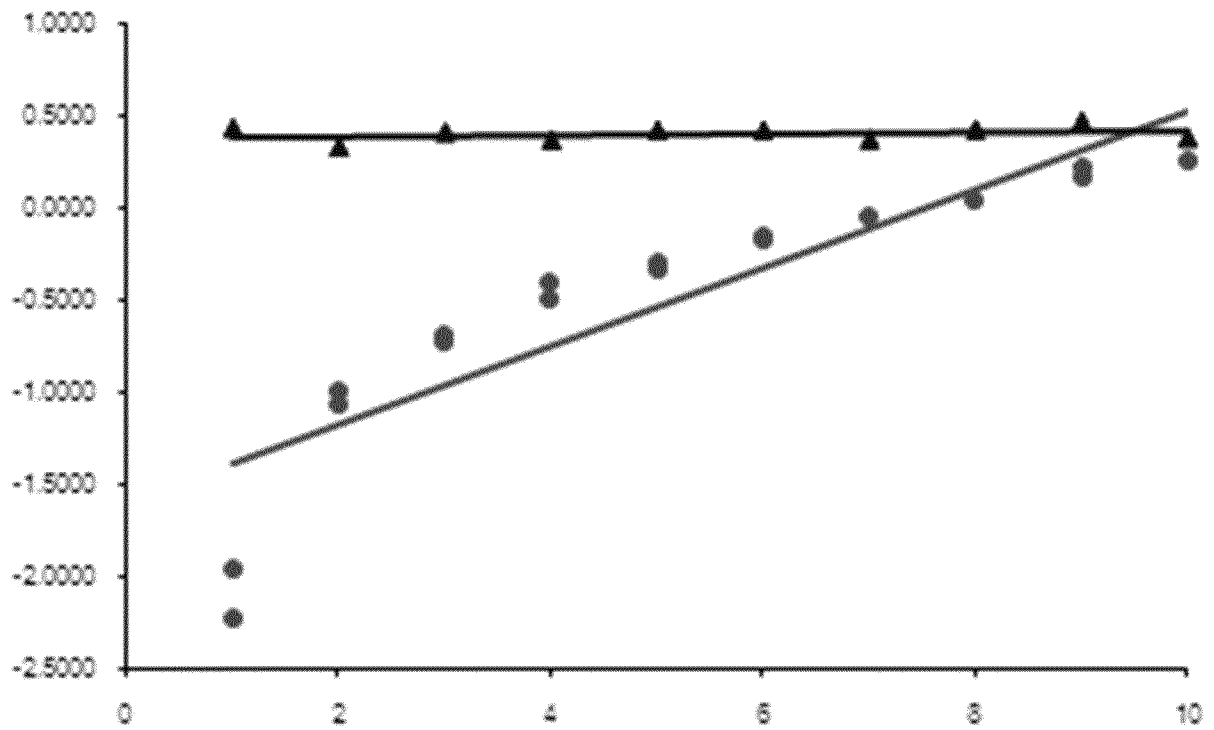
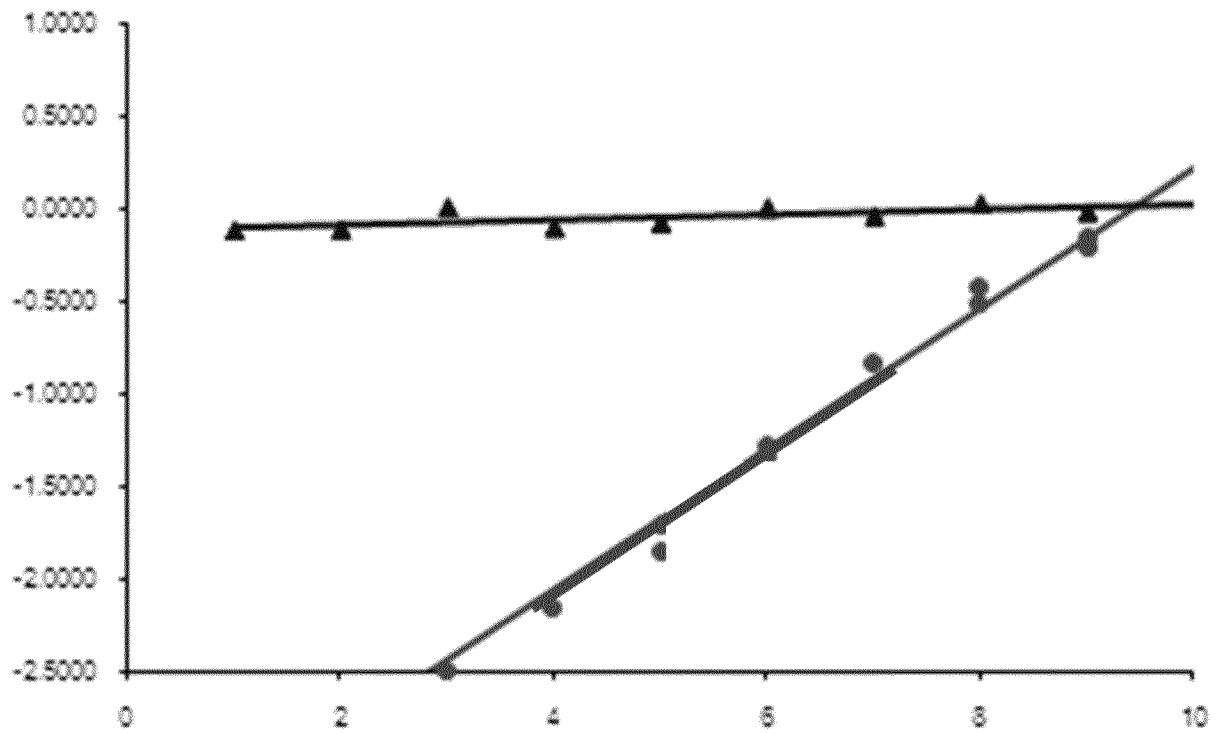
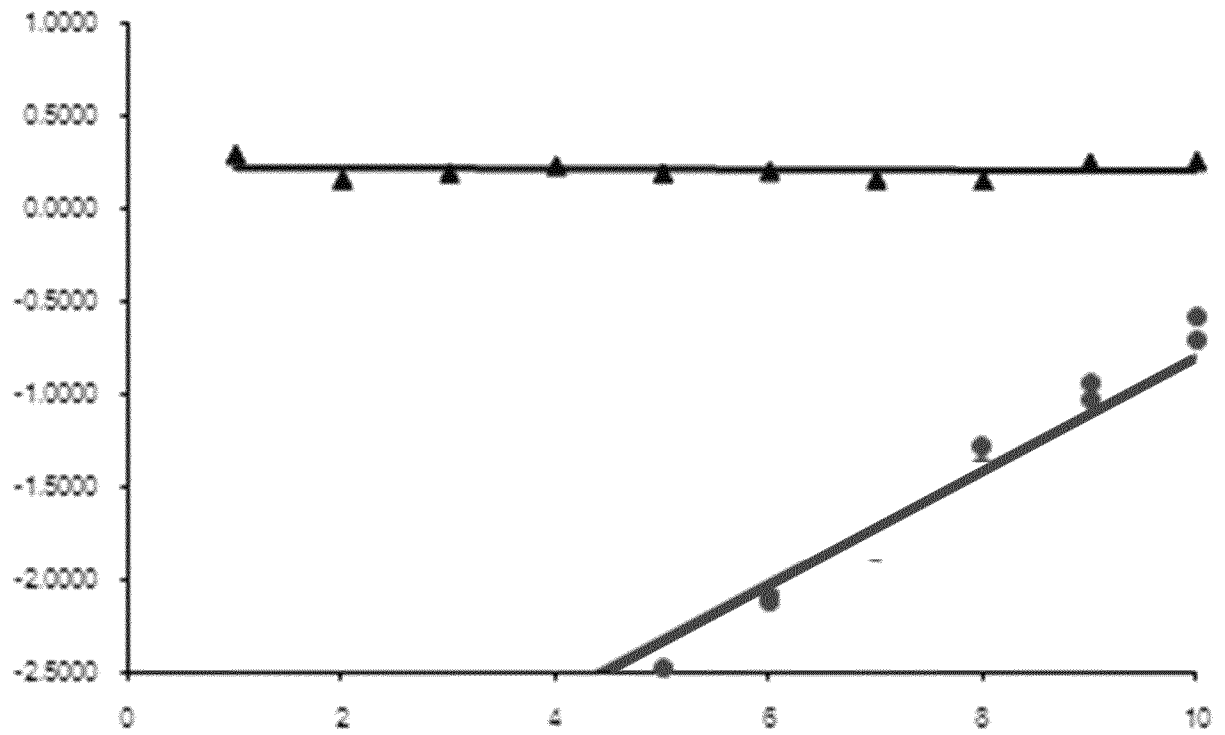
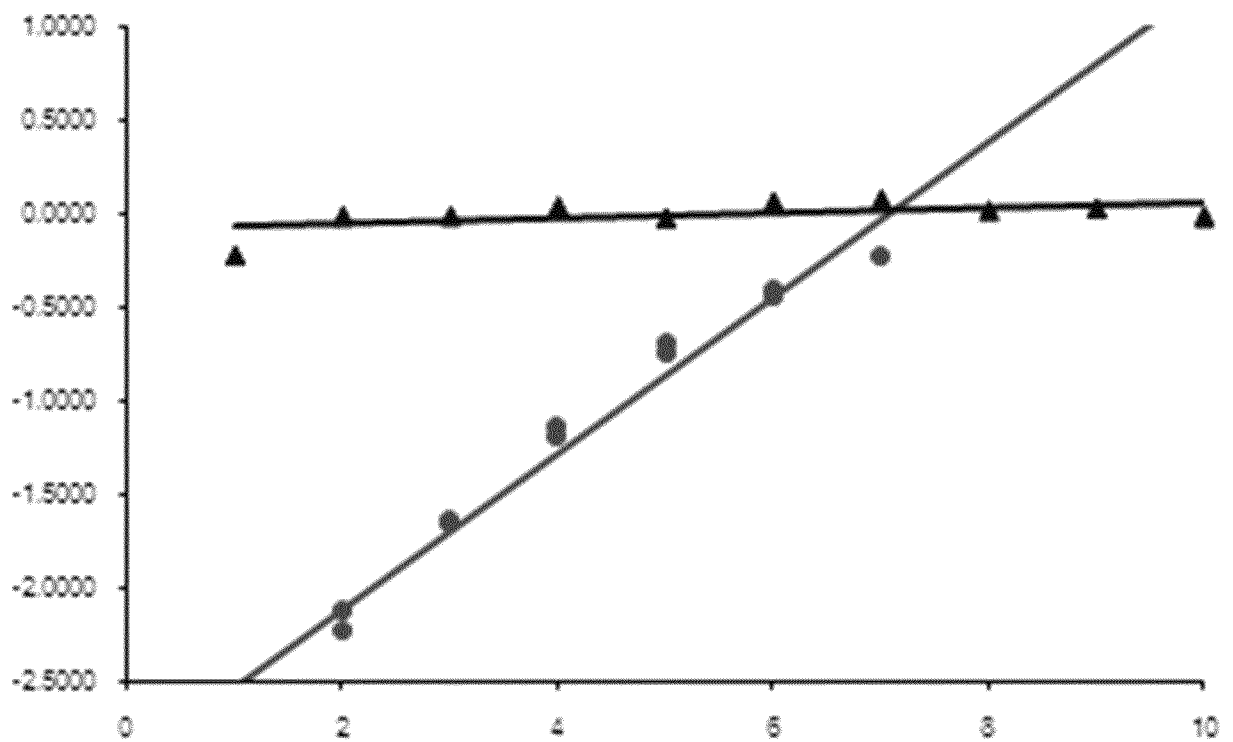


FIGURE 4**FIGURE 4A****FIGURE 4B**

7/8

FIGURE 4C**FIGURE 4D**

8/8

FIGURE 5