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(54) **Title**: DIAGNOSTIC ASSAY

(57) **Abstract**: A method of detecting the presence of a target molecule in a biological sample from a subject, wherein the target molecule is capable of binding to a binding molecule, wherein the method comprises the steps of: a) subjecting the biological sample to a purification step to provide an enriched target molecule sample, wherein the purification step comprises: i) contacting binding molecules with the biological sample to enable binding of the target molecules to the binding molecules, and ii) removing target molecules from the binding molecules to provide the enriched target molecule sample; and b) subjecting the enriched target molecule sample to an assay to detect the presence of the target molecule.

Diagnostic Assay

Field of the Invention

The present invention relates to methods for detecting the presence of target molecules in a biological sample.

Background to the Invention

Several types of assays for detecting the presence of a target molecule are known. These include immunoassays such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunofluorescence assay (IFA), enzyme linked assay (EIA) and luminescence immunoassay (LIA). Immunoassays generally rely on highly labelled marker molecules (e.g. RIA) and/or on enhancing a signal (e.g. ELISA) in order to detect the presence of target molecules. However, the available assays are often not sufficiently sensitive to detect very low concentrations of target molecules.

Known assays generally require small amounts of test sera, often accompanied by sera dilution, in order to avoid excessive background noise. For example, ELISAs may require the sera to be diluted at least 50 times (e.g., by adding buffer to 2 microliters of serum such that a test sample volume of 100 microliters is obtained).

Radioimmunoprecipitation assays use only a few microliters (usually about 5 microliters) of test sera. This avoids the subsequent use of high volumes of anti-sera which would result in excessively large pellets which would in turn be associated with unacceptable levels of background noise (i.e. inappropriately high radioactivity values of the negative controls).

Although some specific ELISAs can use larger volumes (e.g. 50-100 microliters) of sera, they are of limited use and their specific characteristics mean they do not achieve higher sensitivity. For example, a commercial ELISA kit (by RSR Ltd) for the detection of antibodies to the nicotinic acetylcholine receptor (AChR) associated with Myasthenia Gravis uses a combination of 3 anti-AChR monoclonal antibodies in a sandwich assay using 100 microliters of serum. However, this does not increase the sensitivity of the assay over the regular RIA. Also, an ELISA kit (again by RSR Ltd) for detecting anti-aquaporin-4

antibodies associated with Neuromyelitis optica is based on the ability of antibodies to cross-link immobilized aquaporin-4 with soluble biotinilated aquaporin-4; this assay uses 50 microliters of serum but it probably detects only a small proportion of anti-aquaporin-4 antibodies which manage to cross-link immobilized with soluble aquaporin-4. Accordingly, its sensitivity is also limited.

Examples of disorders where current diagnosis techniques are inadequate are Myasthenia gravis (MG) and Neuromyelitis optica.

MG is a well characterized neurological autoimmune disease. It affects about one out of 5,000 people. In MG, the characteristic weakness and fatiguability of the voluntary muscles usually result from antibody-mediated loss of the nicotinic AChR at the neuromuscular junction. MG can either remain localized to a single muscle group (e.g. ocular MG) or can spread to several skeletal muscles (generalized MG). The pathogenic role of serum antibodies is clearly demonstrated by the dramatic clinical improvement that follows plasma exchange and by the passive transfer of experimental MG to animals by anti-AChR antibodies (reviewed in Drachman, N. Eng. J. Med. 330,1797-1810, 1994; Vincent, Lancet, 357, 2122-2128, 2001). The AChR is a transmembrane glycoprotein consisting of five homologous subunits with the stoichiometry of $\alpha 2\beta\gamma\delta$ or $\alpha 2\beta\varepsilon\delta$ (reviewed in Devillers-Thiery et al, J. Membr. Biol. 3, 133-191, 1993). Effector mechanisms responsible for antibody-dependent AChR loss are: (a) cross-linking of AChRs by bivalent antibodies; (b) activation of complement; and probably less importantly (c) direct interference with the ion channel (reviewed in Tzartos et al, Immunol. Rev. 163, 89-120, 1998). The mechanism that triggers the autoimmune response to the AChR is not yet clearly understood.

The predominant laboratory diagnostic test for the detection of anti-AChR antibodies in MG is an RIA based method for detection of antibodies against detergent-solubilised human AChR (Lindstrom, Clinical. Imm. Immunopath 7:36-43, 1977; Lindstrom patents US4033722, 1977, and US4789640, 1988). In this assay, a sample of detergent-solubilised human muscle or human cell lines expressing muscle-type AChR (usually 20-100 fmoles AChR) is preincubated with 125I-labelled alpha-bungarotoxin (usually a few times in molar excess over AChR with about 50,000-100,000 cpm); alpha-bungarotoxin is a small protein which is a very specific AChR ligand, resulting in indirect but specific labelling of the AChR. Then, the mixture is incubated with a small volume of the test patient's serum (usually up to 5

microliters; occasionally up to 10-20 microliters), followed by the addition of an anti-human immunoglobulin serum (usually about 10-50 microliters) to precipitate the patient's immunoglobulins together with any bound labeled AChR. The radioactivity of the pellet is a measure of the anti-AChR antibody titer which is calculated as nmoles/Liter (nM) of bound alpha-bungarotoxin binding sites (also referred to as anti-AChR antibody titer). By this assay positive titers are usually considered those above about 0.4-0.6 nM, titers of healthy individuals those below about 0.2 nM, whereas titers in between are considered ambiguous (depending on various publications, e.g. Lindstrom, Clinical. Imm. Immunopath 7:36-43, 1977; Vincent & Newsom-Davis, J Neurol Neurosurg Psychiatry. 48:1246-52, 1985). In addition, there are some exceptions where negative titers were considered only those below 0.1 nM (Hayashi et al., Brain & Dev, 17:38-41, 1995), 0.07 nM (Ohta et al., Clin. Chem. 36/6, 911-913, 1990) 0.04 nM (Oda & Ito, J. Neurol. 225:251-8, 1981) or even below 0.03 nM (Chan et al. Muscle & Nerve 36: 651–658, 2007). By this assay, only about 80-85% of the patients with generalised MG and only about 50% of the patients with ocular MG are found antibody-positive.

In addition to RIAs, several ELISAs have been developed (e.g. Gotti et al. Muscle & Nerve, 20: 800-808, 1997; Franciotta et al., Clin Chem. 45:400-5, 1999; Hewer et al., Clin Chim Acta. 364:159-66, 2006) with moderate success, but are generally less efficient than that of the RIA.

Leite et al. (Brain 131: 1940-52, 2008) developed a semi-quantitative fluorescence-based assay for the detection of low affinity antibodies that bind to AChR-rapsin clusters on the surface of human embryonic kidney cells co-transfected with cDNA encoding AChR subunits and rapsyn (a cytoskeletal protein which induces AChR clusters); by this assay they detected anti-AChR antibody binding in several previously seronegative MG sera.

Several anti-AChR positive MG patients, also have autoantibodies to striated muscle antigens, like titin and ryanodine receptors (Skeie GO, Romi F. Eur J Neurol. 15:1029-33, 2008). Such antibodies as well as antibodies to cytokines are very common in MG patients with thymoma and are used as a diagnostic marker for the presence of thymoma. These antibodies are usually detected by simple ELISAs (e.g. a commercial ELISA by DLD-Diagnostika, Hamburg) but more sensitive assays are desired.

Antibodies to extracellularly exposed antigenic AChR domain are disclosed in Oda K. & Shibasaki, H., Neurology 36:1374-1377, 1986.

In about one-third of the MG patients who do not have detectable anti-AChR antibodies, antibodies against muscle-specific kinase (MuSK) are detected (Hoch et al. Nature Medicine 7: 365-8, 2001; McConville et al. Annals of Neurology, 55: 580-4, 2004). MG patients with anti-MuSK antibodies constitute a specific subgroup of MG patients. These antibodies may play a role in the impairment of neuromuscular transmission, since MuSK is involved in postsynaptic differentiation and the clustering of AChRs. MuSK is a transmembrane protein which is selectively expressed in skeletal muscles. It consists of 4 extracellular immunoglobulin-like domains, one extracellular Cys-rich domain, and an intracellular kinase domain. MuSK seems to be part of a receptor for agrin, a protein secreted by nerves, which is involved in the formation of the neuromuscular junction. MuSK activation by agrin as a result of tyrosine phosphorylation leads to the phosphorylation of rapsyn, which, in turn, results in the clustering of AChRs and the phosphorylation of the AChR β-subunit. Despite the absence of anti-AChR antibodies, MuSK-MG is often characterized by moderate to severe MG symptoms. Patients with MuSK MG present predominantly with bulbar and ocular symptoms, dysphagia and dysarthria, and facial muscle symptoms and show less thymic pathology, more frequent respiratory crises, and a less satisfactory response to immunosuppressive treatment than MG patients with anti-AChR antibodies. Anti-MuSK antibodies in patients' sera are currently usually determined by an RIA which uses the whole extracellular domain of human MuSK directly labelled with 125I (Matthews et al. Clinica Chimica Acta 348: 95–99, 2004); a relevant commercial diagnostic kit is available (RSR Ltd). US patent no. 7,267,820 discloses a method for diagnosing neurotransmission or developmental disorders in a mammal comprising the step of detecting in a bodily fluid of said mammal autoantibodies to an epitope of MuSK.

The remaining ~ 5-10% of generalised and about 50% of ocular MG patients have yet unidentified antibodies; MG can be suspected from symptoms and other less specific diagnostic approaches but definite proof of the disease is lacking. This gap in MG diagnosis causes a major problem in most tested cases because a negative result (which is the most frequent result in the routine experimental diagnosis of MG) leaves an ambiguity as to whether there is MG or not. The remaining myasthenics may have amounts of circulating

anti-AChR or anti-MuSK antibodies that are too low to allow antibody detection with the currently available assays. Indeed, a very low anti-AChR or anti-MuSK titer could still be sufficient to explain the presence of MG, since there is no significant correlation between antibody titer and disease severity. In addition, patients with low anti-AChR or anti-MuSK antibody content in their blood could have most of their antibodies bound on their muscle AChRs or MuSK leaving only minimal amounts of free antibodies in the circulation. Therefore, a very sensitive assay is needed.

Neuromyelitis optica (NMO), or Devic's syndrome, is a chronic inflammatory demyelinating disease of the central nervous system (CNS), mainly affecting the optic nerves and the spinal cord. There is a debate as to whether it is a severe variant of multiple sclerosis (MS) or if it is a disease entity in itself. Like MS, NMO usually follows a relapsing–remitting course, but it often leads to more severe disability with impairment of functional vision and/or loss of ambulation. Its differentiation from MS and other similar CNS diseases is important in order to follow the appropriate treatment. However, such differentiation is usually very difficult due to overlap in clinical presentation and cerebrospinal fluid and magnetic resonance imaging findings. Autoantibodies to the aquaporin 4, the most abundant water channel protein in the CNS, which is highly concentrated in astrocytic foot processes, have been recently identified in patients with NMO (Lennon et al. Lancet 364: 2106–2112, 2004; Lennon et al. J Exp Med 202: 473–477, 2005). Indirect immunofluorescence, RIA and ELISAs are currently used for the detection of such autoantibodies with moderate success (Waters & Vincent, International MS Journal; 15: 99–105, 2008); an ELISA kit is commercially available (RSR Ltd). US patent no. 7,101,679 discloses methods and materials for diagnosing and treating NMO. Improved assays are clearly needed since no such antibodies may be detected in many patients that appear to have NMO.

Thus, there is an urgent need for higher sensitivity assays for detecting molecules associated with disease, since present methods are not always capable of detecting the presence of such molecules. Moreover, in many cases, the identified antibody titres are too close to the background values to be reliable and are considered ambiguous, being below the threshold for positive titre.

Summary

The detection method according to the present invention overcomes the problems associated with the prior art methods because it enables the use of large amounts of body fluids (e.g., serum or plasma), thereby facilitating the detection of very small concentrations of target molecules.

Put another way, the method of the present invention has far greater sensitivity than available immunoassays, since it allows the collection of the target molecules from much larger serum volumes. For example, serum volumes at least 100 times greater than available immunoassays may be used.

The present invention uses the principle of affinity purification in order to highly enrich body fluid target molecules prior to detecting the target molecules by regular assays. The present invention uses methods which involve a) the non-stringent purification of the target molecules from a biological sample (which may have too low a target molecule concentration to enable detection of the target molecule by conventional assays) and b) their subsequent detection by assays commonly known in the art.

The isolation of the target molecules in step a) does not have to be stringent - simply a high enrichment of target molecules is sufficient. This is because the specific target molecules are detected in step b). Indeed, it is advantageous that a stringent purification is not used as a stringent purification is generally time consuming and difficult to perform.

In step b) the semi-purified target molecules from the biological sample can then be easily determir by a regular assay. The assay may be an immunoassay (e.g. RIA, ELISA, immunofluoresence ass EIA or LIA), a ligand-binding assay, a surface plasmon resonance assay, or microchip-based assay.

The volume of body fluid used in the method of the present invention can be significantly greater than the volume of body fluid used in regular assays by virtue of the procedure of step a) prior to step b). Indeed, step a) may enable the amount of target molecule in the sample subjected to the assay to be, for example, at least 20, 40, 60, 80 or 100 times greater compared to a method that does not involve such a purification procedure.

According to a first aspect of the present invention, there is provided a method of detecting the presence of a target molecule in a biological sample, wherein the target molecule is capable of binding to a binding molecule, wherein the method comprises the steps of:

a) subjecting the biological sample to a purification step to provide an enriched target molecule sample, wherein the purification step comprises:

i) contacting the biological sample with binding molecules to enable binding of the target molecules to the binding molecules, and

ii) removing target molecules from the binding molecules to provide the enriched target molecule sample; and

b) subjecting the enriched target molecule sample to an assay to detect the presence of the target molecule.

The assay may be for example an immunoassay (e.g. RIA, ELISA, immunofluoresence assay, EIA LIA), a ligand-binding assay, a surface plasmon resonance assay, or a microchip-based assay.

Preferably the assay is an immunoassay.

After step (i), a portion of the biological sample that has not bound to the binding molecule may be removed using conventional techniques (e.g. washing), prior to step (ii).

The binding molecules are preferably immobilised. The immobilisation may be by covalent or non-covalent means.

In one embodiment, the binding molecules are specifically immobilised on surfaces carrying tags, specific ligands or monoclonal antibodies.

In another embodiment, the binding molecules are immobilised by non-specific adsorption on surfaces, such as plastic surfaces of ELISA plates.

In various embodiments, the binding molecules may be immobilised on a test-tube, a micro-well (which may be part of an ELISA plate), a membrane of a cell (which may naturally express the binding molecule or may be genetically engineered to express the binding

molecule), microchip, a support suitable for affinity chromatography, liposomes or beads (e.g. agarose beads, such as CNBr activated beads).

In step (a) target molecules may be removed from the binding molecules using, for example, a buffer such as a low or high pH buffer, or a specific antagonist.

The enriched sample comprising the released target molecules may then be brought to a desired pH by a small volume of high or low pH buffer as required. This liquid may then be subjected to step b).

When the target molecules are removed from the binding molecules, they may be released together with some non-specifically adsorbed proteins. However, this is acceptable. What is important is that the non-stringent purification enables an enriched target molecule sample to be obtained.

According to a second aspect of the present invention, there is provided a method of detecting the presence of a target molecule in a biological sample, wherein the method comprises the steps of:

a') subjecting the biological sample to a purification step to provide an enriched target molecule sample which comprises target molecules bound to a binding molecule, wherein the purification step comprises:

i) contacting the biological sample with binding molecules to allow the target molecule to bind to the binding molecule thereby forming target molecule-binding molecule complexes;

ii) removing the target molecule-binding molecule complexes from the biological sample to provide the enriched target molecule sample; and

b') subjecting the enriched target molecule sample to an assay to detect the presence of the target molecule.

The binding molecules used in step a')i) may be derived from a human.

The assay may be an assay as described above. Preferably the assay is an immunoassay.

The binding molecules may be immobilised as described above.

According to this aspect of the invention, the immobilised binding molecules may be on a membrane of a cell and the removal of target molecules bound to the binding molecules may comprise the step of solubilising the cell.

In one embodiment, the cell may be solubilised with a non-denaturing detergent.

In one embodiment, the target molecules bound to the binding molecules may be antigen-antibody complexes and when the cell is solubilised these whole antigen-antibody complexes may be released from the cell. In a preferred embodiment, the antigen-antibody complexes are immunoprecipitated with an anti-immunoglobulin material.

In the above methods of the present invention, the ratio of target molecule/total protein (wt/wt) in the enriched target molecule sample or the enriched target molecule-binding molecule sample is at least 20, at least 50, at least 100, at least 125, at least 150, at least 175 or at least 200 times greater than ratio of target molecule/total protein (wt/wt) of the biological sample prior to enrichment.

In the above methods of the present invention, at least 0.05ml, at least 0.1ml, at least 0.2ml, at least 0.3ml , at least 0.5ml, at least 0.6ml, at least 0.7ml, at least 0.8ml, at least 0.9ml, at least 1.0ml, at least 2.0ml, at least 3.0ml, at least 4.0ml, at least 5ml, at least 10ml, at least 20ml or at least 50 ml of biological sample may be subjected to the purification procedure of step a) or a').

In the above methods of the present invention, the target molecule or binding molecule may be an antibody or an antigen.

Preferably, the target molecule is an antibody and the binding molecule is an antigen immunospecific for said antibody.

Alternatively, the target molecule may be an antigen and the binding molecule may be an antibody, wherein the antigen is immunospecific for said antibody.

In the above methods of the present invention the target molecule or binding molecule may be, for example, a protein, a microbial or viral protein/constituent, a tumour marker, a cytokine, a chemokine, an amyloid peptide, a hormone, a lipid, a steroid, a polysaccharide, a drug, a drug metabolite, a vitamin, an alkaloid or other body fluid constituent, or a fragment or derivative thereof.

In some embodiments of the above methods of the present invention, the binding molecule is a receptor and the target molecule is a ligand for said receptor. The receptor may be a cytokine receptor and the ligand may be a cytokine. The receptor may be a chemokine receptor and the ligand may be chemokine.

In some embodiments of the above methods of the present invention, the target molecule is a receptor and the binding molecule is a ligand for said receptor. The receptor may be a cytokine receptor and the ligand may be a cytokine. The receptor may be a chemokine receptor and the ligand may be chemokine.

Preferably the target molecule is associated with a disease or disorder.

In the above methods of the present invention, detecting the presence of a target molecule may be indicative of, for example, an immune disorder, such as an autoimmune or inflammatory disorder, a neurological disorder or disease, an infectious disease such as a viral or bacterial infection or cancer. Thus, the target molecule may be associated with said disorders.

Preferably, the presence of the target molecule is indicative of myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), aquired neuromyotonia, peripheral neuropathy or a neurodegenerative disease.

Preferably the binding molecule is selected from the group consisting of an acetylcholine receptor (AChR) (e.g. muscle and neuronal type AChR), a muscle specific tyrosine kinase (MuSK), aquaporin-4, titin, a ryanodine receptor, a calcium channel, a potassium channel, an acetylcholinesterase, a myelin antigen, a ganglioside, a cytokine or a chemokine, or a

derivative or fragment thereof, and the target molecule is an antibody immunospecific for one of said group members.

The binding molecule or target molecule may be a cytokine or a chemokine or a fragment or derivative thereof.

In the above methods of the present invention, the assay may be for example an immunoassay (e RIA, ELISA, immunofluoresence assay, EIA or LIA), a ligand-binding assay, a surface plasm resonance assay, or a microchip-based assay.

Preferably the assay is an immunoassay. Preferably, the immunoassay comprises immunoprecipitation step.

The assay of step b) or b') may comprise adding an agent (e.g. a ligand) that binds to the target molecule. In this embodiment, the agent used in step b) may be the same as the binding molecule used in step a).

In embodiments of the above methods of the present invention where the target molecule is an antibody (e.g., an autoantibody) and the binding molecule is an antigen, step b) or b') may comprise the step of immunoprecipitating complexes of said antibody and said antigen.

In the above methods of the present invention, the assay of step b) or b') may comprise the step of detecting a label wherein said label is directly or indirectly bound to the binding molecule, target molecule or agent.

The binding molecule or agent may be a receptor and the label may be coupled to a ligand of said receptor. In one embodiment, the binding molecule or agent is an acetylcholine receptor and the ligand is α- bungarotoxin.

The binding molecule, target molecule or agent may be a biotinilated molecule and the label may be coupled to streptavidin or avidin.

In the above methods of the present invention, the label may be, for example, a heavy metal, a fluorescent or luminescent molecule, a radioactive (e.g., Iodine 125) or enzymatic tag (e.g. horseradish peroxidise).

In the above methods of the present invention, the biological sample may be, for example, blood, serum, plasma, spinal fluid including cerbrospinal fluid, lymph fluid and urine, bile, amniotic fluid, pleural fluid, ascites, tracheobronchial secretion, marrow fluid, milk, lacrimal fluid, nasal discharge, endocardial fluid, intra-articular fluid, bronchiole alveolus cleaning fluid, saliva, phlegm, semen, seminal fluids, joint fluid, infiltrating fluid, , sweat, tears, , faeces and the like; or extracts obtained from faeces, organs, tissues, cells, or the like or a sample derived therefrom.

Preferably, the biological sample is blood, serum, plasma or cerbrospinal fluid or a sample derived therefrom. The sample derived therefrom may be an immunoglobulin fraction.

The subject from which the biological sample is derived may be an animal. Preferably subject is a mammal. More preferably the subject is a human.

The binding molecule may be derived from a human and the subject from which the biological sample was obtained may also be human. However, the binding molecule used in the methods of the present invention is usually not derived from the same subject from which the biological sample was obtained.

In a particularly preferred embodiment of the present invention, there is provided a method for detecting the presence of an antibody in a biological sample, wherein the presence of said antibody is indicative of a disease, wherein the method comprises the steps of:

a) subjecting the biological sample to a purification step to provide an enriched antibody sample, wherein the purification step comprises:

i) contacting the biological sample with immobilised antigen immunospecific for said antibody to enable binding of the antibody to the antigen, thereby forming immobilised antibody-antigen complexes;

ii) washing said immobilised antibody-antigen complexes;

ii) removing said antibodies from the immobilised antigen to provide the enriched antibody sample; and

b) subjecting the enriched antibody sample to an immunoassay to detect the presence of the antibody. Said immunoassay preferably comprises contacting the enriched antibody sample with labelled antigen (preferably the same kind of antigen as used in step i). Preferably the immunoassay is ELISA or radioimmunoprecipitation.

Detailed description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Col d Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; and E. M. Shevach and W. Strober, 1992 and periodic supplements, *Current Protocols in Immunology*, John Wiley & Sons, New York, NY. Each of these general texts is herein incorporated by reference.

An "antibody" is understood within the scope of the invention to refer to an antibody that is an intact molecule as well as fragments or portions thereof, such as Fab, F(ab')2, Fv and scFv.

The term "antigen" is used to indicate any molecule or cell or particle that can be specifically recognised by the adaptive elements of the immune response, i.e. by B cells or T cells, or both.

The antigen may be an allergen. The term "allergen" is used to describe an antigen that elicits an unwanted immune hypersensitivity or allergic reaction.

A "biological sample" is understood within the scope of the invention to refer to a sample which is derived from a subject. The biological sample may be obtained directly from the subject or may be derived from cultured cells obtained from said subject. Preferred samples are those derived from blood which may comprise plasma, serum or fractions such as an immunoglobulin fraction. The biological sample may have been subjected to a treatment such as dilution in a carrier.

A "protein" is understood within the scope of the invention to include single-chain polypeptide molecules, as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulfide bonds. Portions of the protein may be referred to as a "subunit" or a "domain" or "fragment" as applicable. The term protein encompasses proteins that have been modified e.g. glycoproteins, lipoproteins etc. The term protein also encompasses homologues and derivatives of known proteins, and fragments thereof.

In addition to the specific target and binding molecules, antigens and proteins mentioned herein, the present invention also encompasses the use of variants, derivatives, analogues, homologues and fragments thereof.

In the context of the present invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be obtained by addition, deletion, substitution modification replacement and/or variation of at least one residue present in the naturally-occurring protein.

The term "derivative" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of and/or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide retains at least one of its endogenous functions.

The term "analogue" as used herein, in relation to polypeptides includes any mimetic, that is, a chemical compound that possesses at least one of the endogenous functions of the polypeptides which it mimics.

Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required activity or ability. Amino acid substitutions may include the use of non-naturally occurring analogues.

Proteins of use in the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the transport or modulation function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

"Fragments" are also variants and the term typically refers to a selected region of the polypeptide that is of interest either functionally or, for example, in an assay. "Fragment" thus refers to an amino acid sequence that is a portion of a full-length polypeptide.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

A "chemokine" is understood within the scope of the invention to refer to a group of cytokines which impart chemotaxis to any one of functionally mature blood cells. Depending on the conserved cysteine location at their N-terminus regions, chemokines are classified into four categories: CC, CXC, C, or CXXXC.

A "cytokine" is understood within the scope of the invention to refer to cytokines including those from the following known groups: "hematopoietic factors", “Interleukins”, "growth factors" "tumor necrosis factors" "interferons". Furthermore, any newly discovered members of any one of the aforementioned groups of cytokines, or any newly discovered cytokines

which do not belong to any one of the aforementioned groups of cytokines, may also be target molecules for the method according to the present invention. In particular, the method according to the present invention is applicable to cytokines which exist as soluble factors in blood circulation, have a biological activity in minuscule amounts, and are involved in various pathologies.

Examples of cytokines include inflammatory cytokines such as Tumor Necrosis factor (TNF), interleukin 1, interleukin 2, interleukin 6, interleukin 12, interleukin 17, interleukin 18, interleukin 21, Platelet Activating factor (PAF), G-CSF, GM-CSF, interferon. Further examples of cytokines include interleukin 5 and anti-inflammatory cytokines such as interleukin 4, interleukin 10, interleukin 13, TGF, examples of cytokine receptors include interleukin 1 receptor, soluble TNF receptor. Examples of "hematopoietic factors" include colony stimulating factors (CSFs) including granulocyte-macrophage-colony stimulating factors (GM-CSFs), stem cell factors, erythropoietin, thrombopoietin, and the like. Examples of "growth factors" include the TGF- beta family, the EGF family, the FGF family, the IGF family, the NGF family, blood platelet-derived growth factors (PDGFs), hepatic cell growth factors (HGFs), vascular endothelial cell growth factors (VEGFs), and the like. Examples of "tumor necrosis factors" include TNF- alpha , TNF- beta , and the like. Examples of "interferons" include INF- alpha , INF- beta , INF- gamma , and the like. Other known cytokines include endotheline, glial cell-derived neurotrophic factors (GDNFs), and the like.

An "assay" is understood within the scope of the invention to include an immunoassay, a ligand-binding assay, a surface plasmon resonance assay or a microchip-based assay or any other assay commonly known in the art. Details regarding such assays can be found in known literature and periodic supplements, such as *Current Protocols in Immunology*, Copyright © 2010 by John Wiley and Sons, Inc, Last Updated: February 08, 2010, Print ISSN: 1934-3671 (see e.g. chapters 2, 6 and 10), which is hereby incorporated by reference.

An "immunoassay" is understood within the scope of the invention to include an enzyme-link immunosorbant assay (ELISA), a radioimmunoassay (RIA), enzyme immunoassay (EIA), immunofluorescence assay or a luminescence assay (LIA) or any other immunoassay commoi known in the art.

An "autoimmune disease" is understood to include, but not limited to myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), peripheral neuropathy, Aquired Neuromyotonia, thyroiditis, Hashimoto's thyroiditis, insulitis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic), autoimmune hepatitis, thyroiditis, skleritis, uveitis, orchitis, idiopathic thrombocytopenic purpura, inflammatory bowel diseases (Crohn's disease, ulcerative colitis), Hashimoto's thyroiditis, Guillain-Barre syndrome, colitis, Grave's disease, pemphigus vulgaris, idiopathic myxedema, experimental autoimmune encephalomyelitis (EAE), autoimmune thrombocytopenia, sarcoidosis, experimental leishmaniasis, pernicious anemia, temporal artertis, dermatitis herpetiformis, vitiligo, primary biliary cirrhosis, autoimmune oophoritis and orchitis, autoimmune disease of the adrenal gland, dermatomyositis, dermatomyositis, spondyloarthropathies (such as ankylosing spondylitis), Goodpasture's syndrome, glomerulonephritis, psoriasis, pemphigoid, idiopathic leukopenia, rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, sjogren's syndrom, undifferentiated connective tissue syndrome, antiphospholipid syndrome, different forms of vasculitis (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essential (mixed) cryoglobulinemia, Psoriasis vulgaris and psoriatic arthritis, diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatoid granulomatosis, erythema nodosum, Reiter's syndrome and different forms of inflammatory dermatitis and the like.

An "infectious disease" is understood within the scope of the invention to include infectious diseases such as those caused by pathogens. The infectious diseases may be caused by bacteria (e.g. Haemophilus influenza type B, Pseudomonas and Mycobacteria), viruses (e.g. HIV, Cytomegalovirus and viruses associated with diseases such as measles and hepatitis e.g. hepatitis c). The infectious diseases may be caused by other organisms such as Toxoplasma, Echinococcus, Plasmodium species, Microfilariae, Helminths or Toxicara. The infectious diseases may be caused by prions.

A "neurological disorder or disease" is understood within the scope of the invention to include myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), Aquired Neuromyotonia, peripheral neuropathy, Down's syndrome, cerebrovascular disorder, cerebral stroke, spinal cord injury, Huntington's chorea, multiple sclerosis, amyotrophic lateral sclerosis, epilepsy, anxiety disorder, schizophrenia, depression and manic depressive psychosis, epilepsy, neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's chorea, dementia, cerebral apoplexy, autism and the like.

Figure legends

Fig. 1. Test for anti-AChR antibodies by the assay of Example 2

RIA of 5 positive anti-AChR sera [titers determined earlier by a commercial direct RIA, (RSR Ltd)], all diluted in normal human serum (NHS) to estimated titer 0.1 nM (i.e. below the cut-off of the commercial RIA for positive titers), and 8 sera from healthy individuals (NHS), by the 2-step assay of Example 2 (300 microliters serum, blank bars). The first two MG sera were also tested with the commercial direct RIA for comparison (5 microliters serum, filled bars). The cut-off for positive titers by the 2-step assay (horizontal bar at 350 Δcpm) was estimated to 0.02 nM. It is shown that the resulted (after dilution) sera are negative by the commercial RIA whereas were above the cut-off for positivity by the 2-step assay. With higher serum volumes the nM cut-off becomes almost proportionally lower (not shown). The cut-off value was estimated as: (average cpm of the normal control sera + 3.041 x SD) x 1.2.

Fig 2. Making a standard curve for titration of anti-AChR sera by the assay of Example 3

Titration of two MG sera of known titer (estimated earlier by the direct RIA) for anti-AChR antibodies. Specific amounts of anti-AChR antibodies (from 3 to 30 fmoles) were added to NHS to a total serum volume of 0.5 ml resulting in serum titers 0.01-0.1 nM. The assay was performed as described in the Example 3. The shown values are the averages of the values for the two sera. Fetal calf serum or 7% BSA in PBS were used as negative controls; these controls precipitated 40-70 cpm which were subtracted from the shown values.

Fig 3. Test of various groups of human sera for AChR binding by the assay of Example 3

0.3 ml from each serum were tested as described in the Example 3. Test sera included:

a. AChR-MG: Sera from 30 anti-AChR positive MG patients (as measured previously by the direct RIA) were diluted with NHS to calculated antibody titer 0.05 nM.

b. Ambiguous: Sera from 13 individuals with ambiguous (below the cut-off for positive) anti-AChR titer (0.2-0.45 nM), as measured previously by the classical RIA.

c. Normal: Sera from 32 healthy individuals.

d. Lupus: Sera from 10 patients with lupus erythematosus.

e. Anti-MuSK: Sera from 11 MG patients with anti-MuSK antibodies.

Background values obtained by the use of fetal calf serum or 7% BSA in PBS (40-70 cpm) were subtracted from the shown values of the test sera.

The dotted line marks the cut-off for positive sera. All sera identified as positive by the classical RIA, were found positive also with this assay (30/30) despite their dilution to titer of 0.05 nM (i.e. negative for the classical RIA). Variations in their bound Δcpm, despite the effort to make equal anti-AChR titers may be due to the original titration of the sera performed at different diagnostic experiments and to peculiarities of the different sera. Sera from non myasthenics (healthy individuals or patients with lupus erythematosus) were all found negative (titer <0.005 nM); several previously identified as ambiguous (below the cut-off for positive) were found here positive, whereas some previously identified as ambiguous are now shown to be negative. Anti-MuSK positive sera were found lacking anti-AChR antibodies. Therefore the present assay clarifies the situation for sera previously classified as ambiguous sera.

Fig 4. Titration of anti-MuSK antibodies by the assay of Example 4 compared with that of a commercial RIA

An MG serum of known titer for anti-MuSK antibodies, estimated earlier by a commercial RIA (RSR Ltd), was used at various dilutions in NHS to result in various calculated anti-MuSK antibody concentrations. For the commercial RIA, 5 microliters of the serum dilutions

were used (filled rhombuses) whereas for the 2-step RIA (performed as described in the Example 4) 500 microliters serum dilutions were used (empty squares).

Fig. 5. Test for anti-MuSK antibodies of 4 positive sera by the assay of Example 4

Four MG sera of known high titer for anti-MuSK antibodies, estimated earlier by the commercial RIA (RSR Ltd), were diluted 1000 times with NHS, to the indicated estimated titers, and tested with both the commercial RIA (filled bars; all below the cut-off for positive values) and the 2-step RIA as described in the Example 4 (blank bars, all well above the cut-off for positive values). For the commercial RIA 5 microliters serum were used, whereas for the 2-step RIA 100 microliters serum were used. The horizontal line marks the estimated cut-off for positive for both assays.

Fig 6. Test 'ambiguous' MG sera for anti-MuSK antibodies by the assay of Example 4

Six sera, which when tested with the commercial anti-MuSK RIA assay showed cpm values above the average values of the normal human sera but well below the cut-off for positive (classified as ambiguous) were tested with the 2-step assay of Example 4 (sera #1-6) and compared with seven normal human sera (sera #7-13). It is shown that 5 of the 6 'ambiguous' sera were definitely negative whereas serum no. 4 was found definitely positive (horizontal line marks the estimated cut-off for positive). 0.2 ml per serum was used.

Fig. 7. Test of selected sera for aquaporin-4 binding by the assay of Example 5

21 sera were tested for binding to 125I-streptavidin labelled biotinilated human aquaporin-4 as described in the Example 5. Serum #1 was found previously positive for anti-aquaporin-4 antibodies with a commercial ELISA kit (RSR Ltd) and was used as positive control; it was diluted 100 times with NHS. Sera #2-7 from putative NMO patients were found negative for anti-aquaporin-4 antibodies by the commercial ELISA whereas they are found here positive for anti-aquaporin-4 antibodies. Sera #8-21 were negative control sera from healthy persons. The cut-off for positive titer was estimated to 1500 cpm.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples.

Examples

Example 1 - A convenient method that allows the semi-purification of the antibodies from relatively large volumes of body fluids, before their measurement in a regular immunoassay

The assay consists of two steps:

Step 1. Semi-purification of the antigen-specific antibodies by an easy not necessarily stringent, affinity purification procedure

This step uses an immobilized/insoluble form of antigen to enable the semi-purification of the corresponding antibodies from body fluids (e.g., sera). The antigen can be recombinant or native, purified, semi-purified or in crude form. If it is a cell membrane molecule, the antigen-carrying cells or their isolated membranes can be used directly as the immobilized form of the antigen without the need for any further isolation and subsequent immobilization of the antigen. Alternatively, the antigen, or a fragment thereof, in soluble form, is immobilized on an insoluble matrix, like beads, ELISA plates etc, either covalently (for example by the use of CNBr-activated agarose beads) or non-covalently (for example through specific epitopes or other ligand binding sites carried by the expressed antigen or through non-specific attachment to an ELISA plate, etc).

We have tested the antigens AChR, MuSK and aquaporin-4, a. directly as cell membrane-embedded (AChR), b. as detergent-solubilised antigens immobilized on CNBr-activated beads (AChR, MuSK), c. as detergent-solubilised antigens immobilized on ELISA plates (all three), and d. as detergent-solubilised antigens immobilized on Nickel-carrying beads or Nickel-coated wells (MuSK).

In each test tube or well of a multi-well plate (e.g. a 96 deep-well plate), the insoluble matrix (e.g. cells, membranes, agarose beads etc) with the immobilized antigen (typically 10-200 fmoles per test tube) is added, or the ELISA plate wells with the immobilized antigen are directly used. Then, a large volume (typically 0.1-0.5 ml, but it can be several ml) of the test

body fluid is added. After incubation (overnight or shorter) preferably with shaking, the insoluble material may be washed a few times (e.g. by addition of about 0.3-1.0 ml buffer, centrifugation, and repeating the procedure another 1-2 times), thus almost all unbound body fluid proteins are discarded. (At this point of the procedure a direct detection of the bound antibodies would not be sensitive enough due to the unavoidable non-specific immunoglobulin binding to the matrix which occurs when using large volumes of serum). Then, a small volume (e.g., about 50 microliters) of a buffer capable of releasing the bound antibodies without harming their binding efficiency (e.g. glycine-HCl buffer, pH 2.0-3.0) is added to the washed pellet or well. Soon after, (e.g. 1-2 min), the insoluble material may be centrifuged, and the supernatant containing the released antibodies transferred to new test-tubes or ELISA wells and a small volume of a medium capable of neutralizing the antibody-releasing buffer may be added (e.g. concentrated Tris buffer). This step achieves a high enrichment of antibodies specific for the antigen in the sample while the total immunoglobulin content of the sample is very small (much less than that of the total immunoglobulin content of 1 microliter of human serum). Since the said isolation procedure (to be conveniently performed in a routine assay) may not be stringent, non-specific immunoglobulins may also be present.

Alternatively, in the case of non-covalently immobilized antigen (e.g. membrane-embedded antigen, tag-immobilized on beads antigen etc), the whole antigen-antibody complex can be solubilized, remaining as a complex, e.g. by non-denaturing detergent-solubilization of the cells/membranes or by releasing the His-tagged antigen from Ni+-beads with immidazolium buffer (see Example 3).

Step 2. Regular immunoassay for the determination of antigen-specific antibodies from the semi-purified antibody preparation

A regular immunoassay (e.g. ELISA, RIA or immunofluorescence) for the detection of the semi-purified and concentrated specific antibodies follows. If ELISA is followed the following protocol may be used: the above supernatant (after normalization of its pH) is added to an antigen-plated ELISA well, incubated for a period of time (e.g., from about 2 h to overnight), the well is washed, enzyme-conjugated anti-immunoglobulin or protein A/ protein G (e.g. horseradish peroxidise -conjugated goat anti-human immunoglobulin or protein A/G)

is added and incubated for about 1-2 hours, washed, the corresponding enzyme substrates are added and incubated for color development and measurement in an ELISA reader.

If a regular RIA is followed, the following protocol may be used: a small volume (e.g. 50 microliters) containing a directly or indirectly labeled antigen (e.g./preferably 125I-labelled) is added to the above semi-purified antibody-containing supernatant (after normalization of its pH) followed by incubation. Subsequently, the labeled antigen–specific antibody complexes are separated from the unbound labeled antigen either by incubation with soluble anti-human immunoglobulins (followed by centrifugation of the formed large complexes to result in a pellet) or by an insoluble matrix (e.g. agarose beads or ELISA plates) with immobilized material that binds human immunoglobulins (e.g. anti-human immunoglobulins, protein A or protein G). The material with the bound labeled-antigen - antibody complex is washed a few times and the signal -dependent on the labelling- (e.g. radioactivity) is measured as an indication of the amount of antigen-specific antibodies.

In the case that the whole antigen-antibody complex is solubilised, the antigen must be either already labeled, or be subsequently indirectly labeled (e.g. the AChR with the addition of the ligand 125I-alpha-bungarotoxin).

Using large body fluid volumes as above, plateau binding (i.e. when the target molecules bind all the available labeled antigen) during step 2 (regular immunoassay) may be achieved using relatively low titer sera. Thus, in order for the concentration (titer) to be determined for moderate to high serum titers (e.g. above about 0.1 nM anti-AChR titers) the assay may be repeated with lower serum volumes (e.g. 30-50 microliters), to determine titers up to about 1 nM. For measurement of higher titers much smaller serum volumes (e.g. 0.01-5 microliters) may be used directly for by the 2nd step. However, what is usually most important is the unequivocal determination of the presence or not of the specific antibodies (which usually determines the presence of the corresponding disease), rather than the actual titer.

In the case that in the second step the antigen is again immobilized (e.g. in ELISA), the method also favours the detection of low affinity antibodies; i.e. it combines the advantages of ELISAs which are known to detect low affinity antibodies, favoured by the immobilized and

concentrated antigen, (but with relatively low specificity) with the high sensitivity due to semi-purification and concentration of the specific antibodies in the first step.

Example 2 - Detection of very low titers of anti-AChR antibodies in MG patients' sera by the use of covalently immobilized AChR

A mixture of equal quantities of frozen human cells expressing embryonic and adult muscle-type AChR [cell line RD (ATCC CCL-136) for embryonic type AChR and cell line CN21 for adult type AChR] was detergent-solubilized by 2% Triton X-100 in phosphate buffered saline (PBS) plus a cocktail of protease inhibitors: 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 units/ml aprotinin, 5 micrograms/ml pepstatin (inhibitor cocktail) (PBS-Triton-cocktail). The AChR-containing extract was immobilized on CNBr-activated Sepharose 4B beads, according to the manufacturer's instructions (Amersham Pharmacia Biotech), so as about 100 fmoles AChR were immobilized on about 10 microliters beads.

To a series of test tubes, 20 microliters of 50% suspension of Sepharose-AChR in PBS-0.1% Triton was then added. To each test tube, 0.3 ml of test serum in presence of 0.2% Triton, were added and incubated overnight at 4 ^{0}C under constant rotation. Then the beads were washed with PBS-0.5% Triton and then with 10 mM phosphate buffer pH 7.0-0.5% Triton, followed each time by centrifugation for 1 min at 1000 rpm; then 75 microliters with freshly prepared 0.05 M glycine-HCl buffer, pH 2.3 was added to the pellets, vortexed and centrifuged immediately for 1 min at 1000 rpm. The antibody-containing supernatant was transferred into new test-tubes which already contained 4 microliters 1M Tris buffer pH 8.8 for neutralization.

To the above mixture, 50 microliters PBS-0.5% Triton containing 70 fmoles of detergent solubilized human AChR (a mixture of embryonic and adult AChR) labeled with about 50.000 cpm 125I-alpha-bungarotoxin and 5 microliters normal human serum (NHS) as carrier were added. After 2 h incubation at room temperature, 50 microliters goat anti-human immunoglobulin serum were added. After incubation for about 1 h, the formed pellet was washed twice with 1 ml PBS-0.5% Triton X-100. The radioactivity of the washed pellet was counted in a gamma counter. Control Sepharose-AChR beads were incubated with similar

volumes of NHS for the estimation of the non-specific background radioactivity (comments in the legend of Fig. 1).

Example 3 - Detection of very low titers of anti-AChR antibodies in MG patients' sera by the solubilization of non-covalently immobilized AChR-antibody complexes

Frozen cells (a mixture of equal volumes of RD and CN21 cells) were thawed and incubated with 125I-alpha-bungarotoxin (100.000 cpm per 40 fmoles cell-surface AChR) for 2 h at 4 ^{0}C and washed. The labelled cells were distributed in test-tubes (40 fmoles 125I-alpha-bungarotoxin labelled surface AChR in total 50 microliters PBS - 2% BSA – enzyme inhibitor cocktail per tube). To each test tube, 0.3-0.5 ml of test serum were added and incubated overnight at 4 ^{0}C under constant rotation. Then the cells were washed 2 times with 1ml PBS-0.2% BSA. 50 microliters 2% Triton were added to the pellets and incubated under rotation for 30 min at 4 ^{0}C. Subsequently, the tubes were centrifuged for 5 min at 6000 rpm and the antibody-AChR complex containing supernatants were transferred to new test tubes already containing 10 microliters PBS with 1 microliter NHS as carrier. To these, 10 microliters goat anti-human immunoglobulin serum were added. After incubation for 1 h, the formed pellets were washed two times with 1ml 0.5% Triton-PBS each. The radioactivity of the pellets was measured in a gamma counter.

Initially, a titration curve was constructed by testing various dilutions of two positive MG sera in 0.3ml NHS. The curve formed by the average values of two sera is shown in Fig. 2. Subsequently, various sera previously found (with the classical assay) as positive, ambiguous or negative were tested (presented in Fig. 3; comments in the legend).

Example 4 - Detection of very low titers of anti-MuSK antibodies in MG patients' sera

Purified recombinant human MuSK isoform #3 (its whole extracellular domain, i.e. amino acids 1-463, with myc and 6xHis tags) was immobilized on CNBr-activated Sepharose 4B beads, according to the manufacturer's instructions (Amersham Pharmacia Biotech), so as about 1 mg MuSK were immobilized on 1 ml beads.

To a series of test tubes, 10 microliters of 50% suspension of Sepharose-MuSK was then added. To each test tube, 0.1-0.5 ml of test serum (from putative MG patients) and 2-10 microliters 10% Triton, respectively, were added and incubated overnight at 4 ^{0}C under constant rotation. Then, the beads were washed 2 times with 1ml 0.1M glycine-HCl pH 6,

followed each time by centrifugation for 1 min at 1000 rpm; then 75 microliters with 0.1M glycine-HCl buffer, pH 2.4, was added to the pellets, vortexed and centrifuged immediately for 1 min at 1000 rpm. The antibody-containing supernatant was transferred to new test-tubes which already contained 4 microliters 1M Tris buffer, pH 8.8, so as the final pH becomes neutral.

To the above mixture, 50 microliters buffer containing about 70 fmoles 125I-labelled human MuSK (about 50.000 cpm) and 5 microliters NHS as carrier were added. After 2h incubation at room temperature, 50 microliters goat anti-human immunoglobulin serum were added. After incubation for 1 h, the formed pellet was washed twice with 1 ml PBS-0.5% Triton X-100. The radioactivity of the washed pellet was counted in a gamma counter. Control Sepharose-MuSK beads were incubated with similar volumes of NHS for the estimation of the non-specific background radioactivity, whereas known quantities of a positive MG serum (supplemented with NHS to a total volume 0.5 ml is used to make a titration curve (cpm versus titer), shown in Fig. 4. Fig. 5 shows that low titer MG sera (low titers were achieved by dilutions of very high titer sera) are determined as positive by the 2-step assay whereas are undetectable by the regular commercial RIA. In Fig. 6, a previous ambiguous serum is shown to be in fact positive (serum no 4) whereas other 'ambiguous' sera, are shown to be in fact negative.

Example 5 - Detection of very low titers of anti-aquaporin-4 antibodies in putative NMO patients' sera

Purified recombinant human aquaporin-4 (with 8His tag) was immobilized on ELISA plates: 50 microliters Na+-carbonate pH 9.0 buffer containing 0,2 micrograms aquaporin-4 were plated in each well. After overnight incubation at 4 ^{0}C, the plates were washed with PBS-0.05% Tween and blocked with 1% BSA in PBS for 1 h at room temperature, followed by 3 washings with PBS-0.05% Tween and one wash in PBS; finally the wells were emptied, shielded and stored at 4 ^{0}C.

0.15 ml of test serum (from putative NMO patients) was added to each well and incubated for 2h at room temperature with shaking, followed by overnight incubations at 4 ^{0}C under constant rotation. Then the wells were washed 2 times with ~0.3ml 0.05M glycine pH 6.0. To

the empty wells 50 microliters 0.1 M glycine-HCl pH 2.5 were added and shacked. In about 1 min, the 50 microliters liquid from each well, containing any released antibodies, were transferred to 1.5 ml test tubes already containing 7.5 microliters 0.5 M Tris buffer pH 8.8 and vortexed shortly.

To the above mixture, 20 microliters PBS-0.5% Triton containing about 20 fmoles biotinilated aquaporin-4 preincubated with 50.000 cpm 125I-streptavidin + 2 microliters NHS as carrier were added. The mix was incubated for 6 h at room temperature followed by the addition of 30 microliters goat anti-human immunoglobulin serum. After incubation for 2 h at room temperature, the formed pellet was washed twice with 1 ml PBS-0.5% Triton X-100. The radioactivity of the washed pellet was counted in a gamma counter. Control aquaporin-4 plated wells were incubated with similar volumes of NHS for the estimation of the non-specific background radioactivity. Fig. 7 shows that 6 sera which were found negative by the commercial ELISA were in fact found positive by the 2-step assay. Ten additional sera were found positive with the ELISA and gave high titers with the present RIA (from 0.10 to 1.81 nM), whereas 34 other sera provided for diagnostic purposes were found negative with both ELISA and the present RIA. Another 30 sera from healthy controls were tested and were also found negative (not shown).

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method for detecting the presence of a target molecule in a biological sample from a subject, wherein the target molecule is capable of binding to a binding molecule, wherein the method comprises the steps of:

a) subjecting the biological sample to a purification step to provide an enriched target molecule sample, wherein the purification step comprises:

i) contacting the biological sample with binding molecules to enable binding of the target molecules to the binding molecules, and

ii) removing target molecules from the binding molecules to provide the enriched target molecule sample; and

b) subjecting the enriched target molecule sample to an assay to detect the presence of the target molecule.

2. A method of claim 1, wherein the binding molecules are immobilised.

3. A method of claim 2, wherein the binding molecules are immobilised on a test-tube, micro-well, membrane of a cell, microchip or on a support suitable for affinity chromatography.

4. A method of claim 1, 2 or 3, where in step (a) target molecules are removed from the binding molecules using i) a low pH buffer or ii) a high pH buffer or iii) a specific antagonist.

5. A method for detecting the presence of a target molecule in a biological sample from a human subject, wherein the method comprises the steps of:

a') subjecting the biological sample to a purification step to provide an enriched target molecule-binding molecule sample which comprises target molecule bound to a binding molecule, wherein the purification step comprises:

i) contacting the biological sample with binding molecules to allow the target molecule to bind to the binding molecule thereby forming target molecule-binding molecule complexes, wherein the binding molecules are derived from a human, and

ii) removing the target molecule-binding molecule complexes from the biological sample to provide the enriched target molecule-binding molecule sample; and

b') subjecting the enriched target molecule-binding molecule sample to an assay to detect the presence of the target molecule.

6. A method of claim 5, wherein the binding molecules are immobilised.

7. A method of claim 6, wherein the binding molecules are immobilised on a test-tube, micro-well, membrane of a cell, microchip or on a support suitable for affinity chromatography.

8. A method of claim 7, wherein the immobilised binding molecules are on a membrane of a cell and wherein the removal of target molecules bound to the binding molecules in step a')ii) comprises the step of solubilising the cell.

9. A method of any preceding claim wherein the ratio of target molecule/total protein (wt/wt) in the enriched target molecule sample or the enriched target molecule-binding molecule sample is at least 50, at least 100, at least 125, at least 150, at least 175 or at least 200 times greater than the ratio of target molecule/total protein (wt/wt) of the biological sample prior to enrichment.

10. A method of any preceding claim, wherein the assay of step b) or b') comprises an immunoassa a ligand-binding assay, a surface plasmon resonance assay, or a microchip-based assay.

11. A method of claim 10, wherein the assay is an immunoassay.

12. A method of any preceding claim, wherein at least 0.05ml, at least 0.1ml, at least 0.2ml, at least 0.3ml , at least 0.5ml, at least 0.6ml, at least 0.7ml, at least 0.8ml, at least 0.9ml, at least 1.0ml, at least 2.0ml, at least 3.0ml, at least 4.0ml, at least 5ml or at least 10ml of biological sample is subjected to the purification procedure of step a) or a').

13. A method of any preceding claim, wherein the target molecule is an antibody or an antigen.

14. A method of any preceding claim, wherein the target molecule or binding molecule is a protein, a microbial or viral protein/constituent, a tumour marker, a cytokine, a chemokine, an amyloid peptide, a hormone, a lipid, a steroid, a polysaccharide, a drug, a drug metabolite, a vitamin, an alkaloid or other body fluid constituent, or a fragment or derivative thereof.

15. A method of any preceding claim, wherein detecting the presence of a target molecule is indicative of an autoimmune disease, a neurological disease or disorder, an infectious disease or an inflammatory disease.

16. A method of claim 15, wherein the presence of the target molecule is indicative of myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), neuromyotonia, peripheral neuropathy or a neurodegenerative disease.

17. A method of any preceding claim, wherein i) the target molecule is an antigen and the binding molecule is an antibody or ii) wherein the target molecule is an antibody and the binding molecule is an antigen.

18. A method of any preceding claim, wherein the target molecule is an antibody and the binding molecule is an antigen, wherein the antibody is associated with an autoimmune disease, a neurological disease or disorder, an infectious disease or an inflammatory disease and wherein the antibody is immunospecific for said antigen.

19. A method of claim 18, wherein the presence of the antibody is indicative of myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), neuromyotonia, peripheral neuropathy or a neurodegenerative disease.

20. A method of any preceding claim, wherein the target molecule or binding molecule is an acetylcholine receptor (AChR), a muscle specific tyrosine kinase (MuSK), aquaporin-4, titin, a ryanodine receptor, a calcium channel, a potassium channel, acetylcholinesterase, a myelin antigen, a ganglioside, a cytokine or a chemokine, or a derivative or fragment of said human acetylcholine receptor (AChR), muscle specific tyrosine kinase (MuSK), aquaporin-4, titin, ryanodine receptor, calcium channel, potassium channel, acetylcholinesterase, myelin antigen, ganglioside, cytokine or chemokine.

21. A method of any preceding claim, wherein the assay of step b) or b') comprises an immunoprecipitation step.

22. A method of any preceding claim, wherein the assay of step b) or b') comprises adding an agent that binds to the target molecule.

23. A method of claim 22, wherein the agent used in step b) is the same as the binding molecule used in step a).

24. A method of any preceding claim, wherein the target molecule is an autoantibody and the binding molecule is an antigen, wherein step b) or b') comprises the step of immunoprecipitating complexes of said autoantibody and said antigen.

25. A method of any preceding claim, wherein the assay of step b) or b') comprises the step of detecting a label on the binding molecule or the target molecule.

26. A method of any preceding claim, wherein the assay of step b) or b') comprises the step of detecting a label indirectly bound to the binding molecule or the target molecule.

27. A method according to claim 22 or 23, wherein the assay of step b) or b') comprises the step of detecting a label on the agent.

28. A method according to claim 27, wherein the assay of step b) or b') comprises the step of detecting a label indirectly bound to the agent.

29. A method of claim 25 to 28, wherein the binding molecule or agent used in the assay of step b) or b') is a receptor and the label is coupled to a ligand for said receptor.

30. A method of claim 29, wherein the binding molecule or agent is an acetylcholine receptor and the ligand is α- bungarotoxin.

31. A method of any one of claims 25 to 30, wherein the target molecule, binding molecule or agent is a biotinilated molecule and the label is coupled to streptavidin or avidin.

32. A method of any one of claims 25 to 31, wherein the label is a heavy metal, a fluorescent or luminescent molecule, a radioactive or enzymatic tag.

33. A method of claim 32, wherein the label is a radioactive tag that is Iodine 125.

34. A method of any preceding claim, wherein the biological sample is blood, serum, plasma or cerbrospinal fluid or a sample derived therefrom, wherein the sample derived therefrom is optionally an immunoglobulin fraction.

Fig. 1

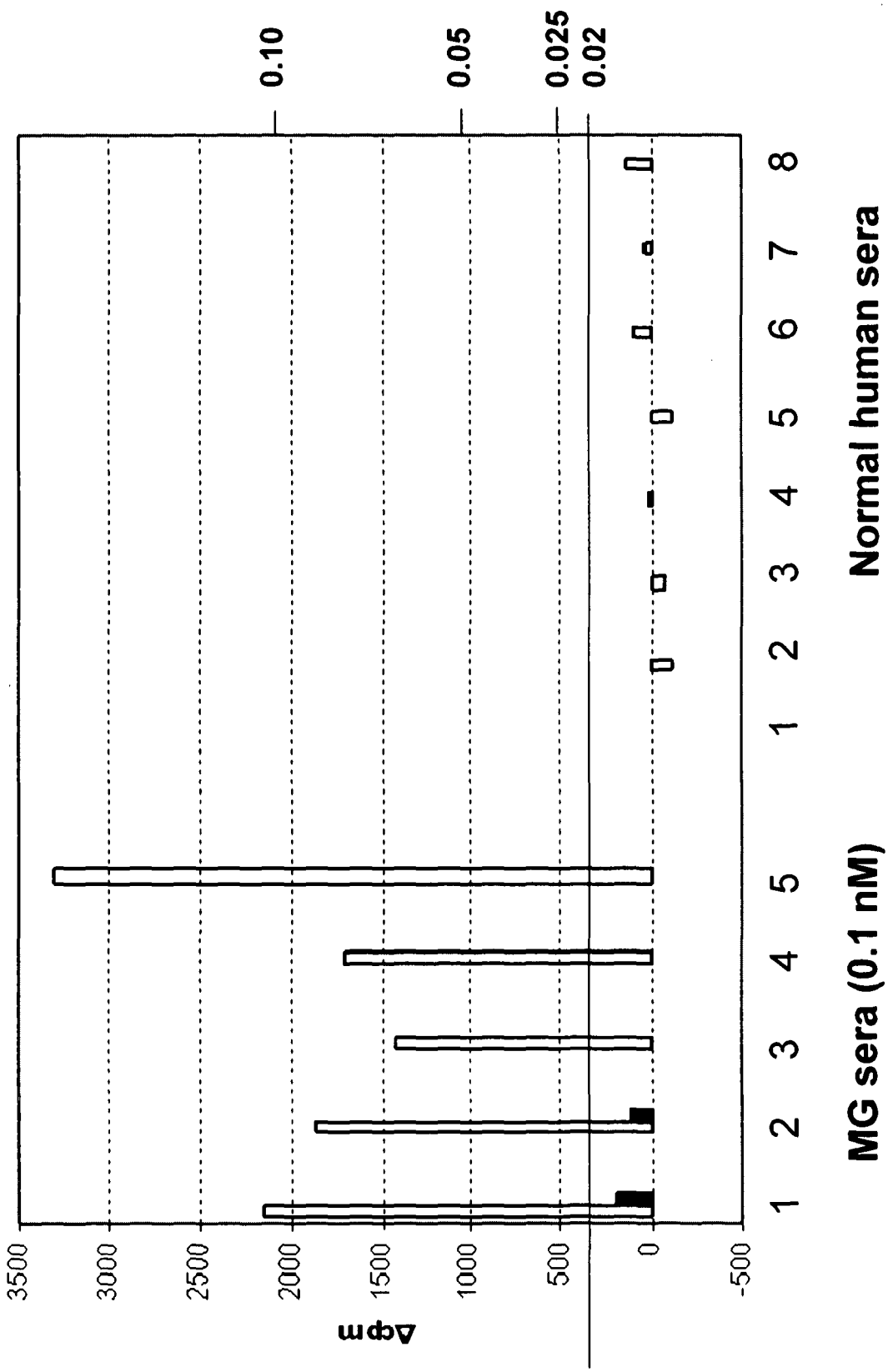

Titers for the 2-step assay (nM)
0.10
0.05
0.025
0.02
Δrpm
3500
3000
2500
2000
1500
1000
500
0
-500
1 2 3 4 5 1 2 3 4 5 6 7 8
MG sera (0.1 nM)
Normal human sera

Fig. 2

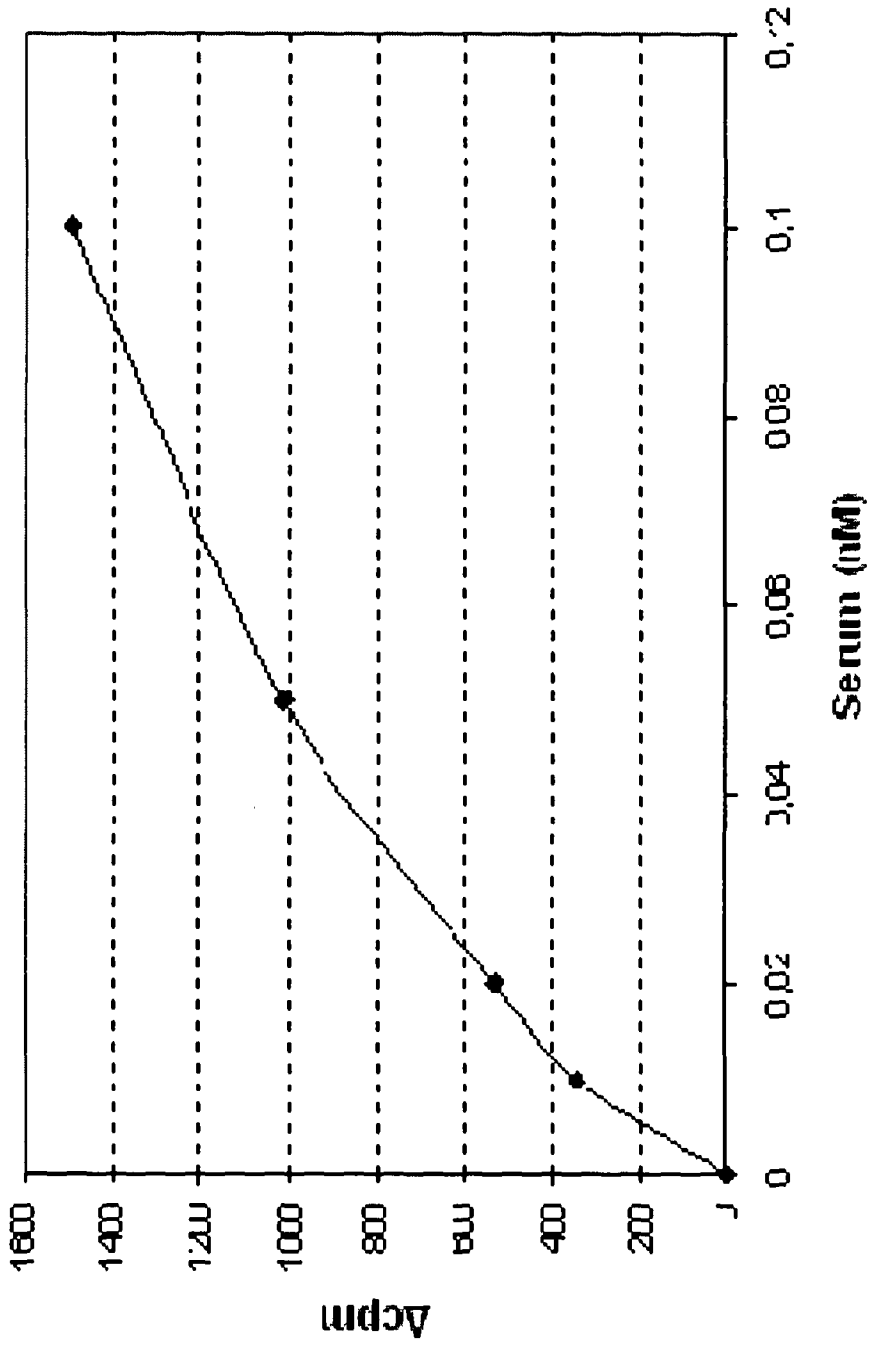

Serum (nM)
0
0,02
0,04
0,06
0,08
0,1
0,12
dcpm
0
200
400
600
800
1000
1200
1400
1600

Fig. 3

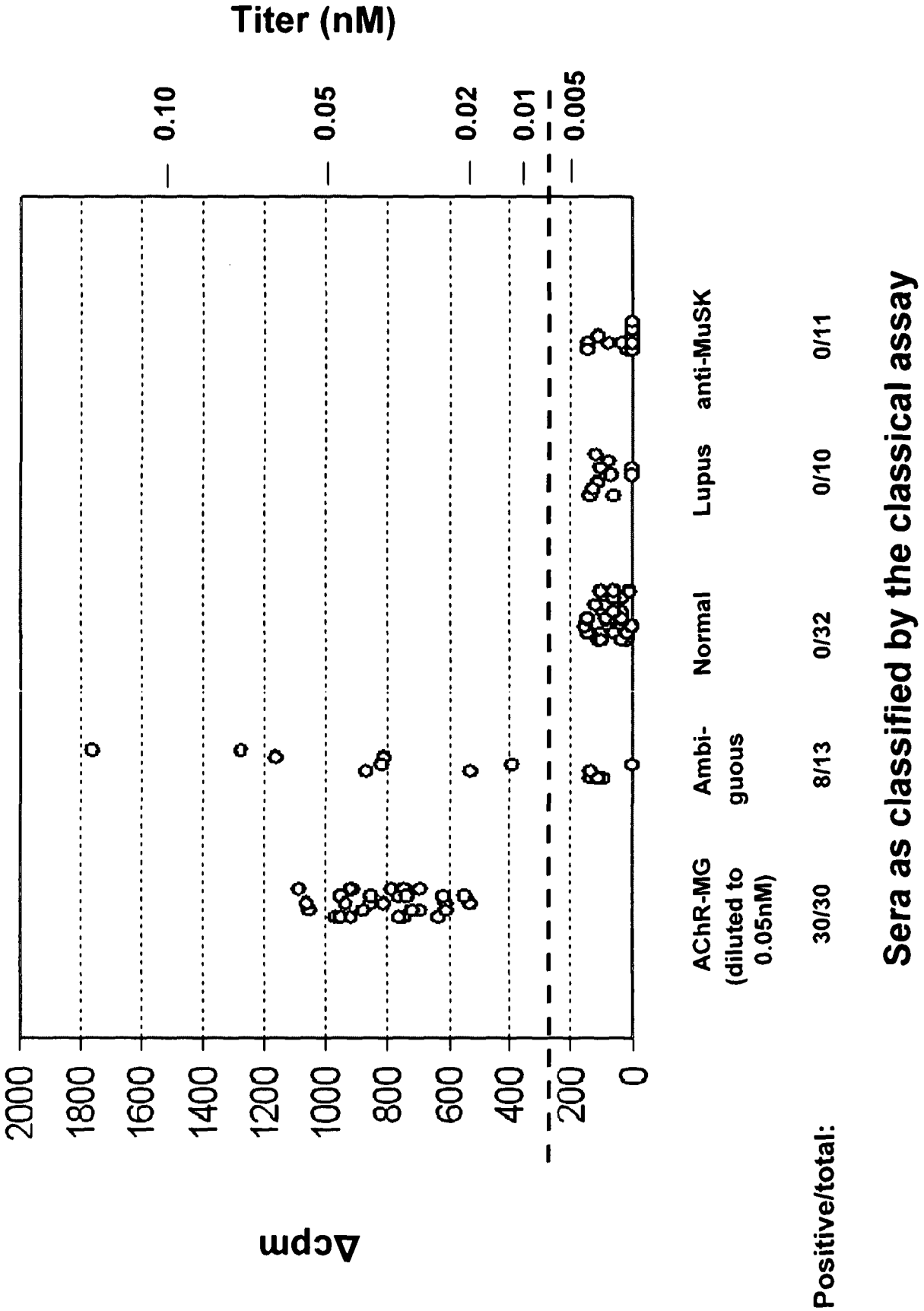

Titer (nM)
0.10
0.05
0.02
0.01
0.005
Δcpm
2000
1800
1600
1400
1200
1000
800
600
400
200
0
AChR-MG (diluted to 0.05nM)
Ambi-guous
Normal
Lupus
anti-MuSK
Positive/total:
30/30
8/13
0/32
0/10
0/11
Sera as classified by the classical assay

Fig. 4

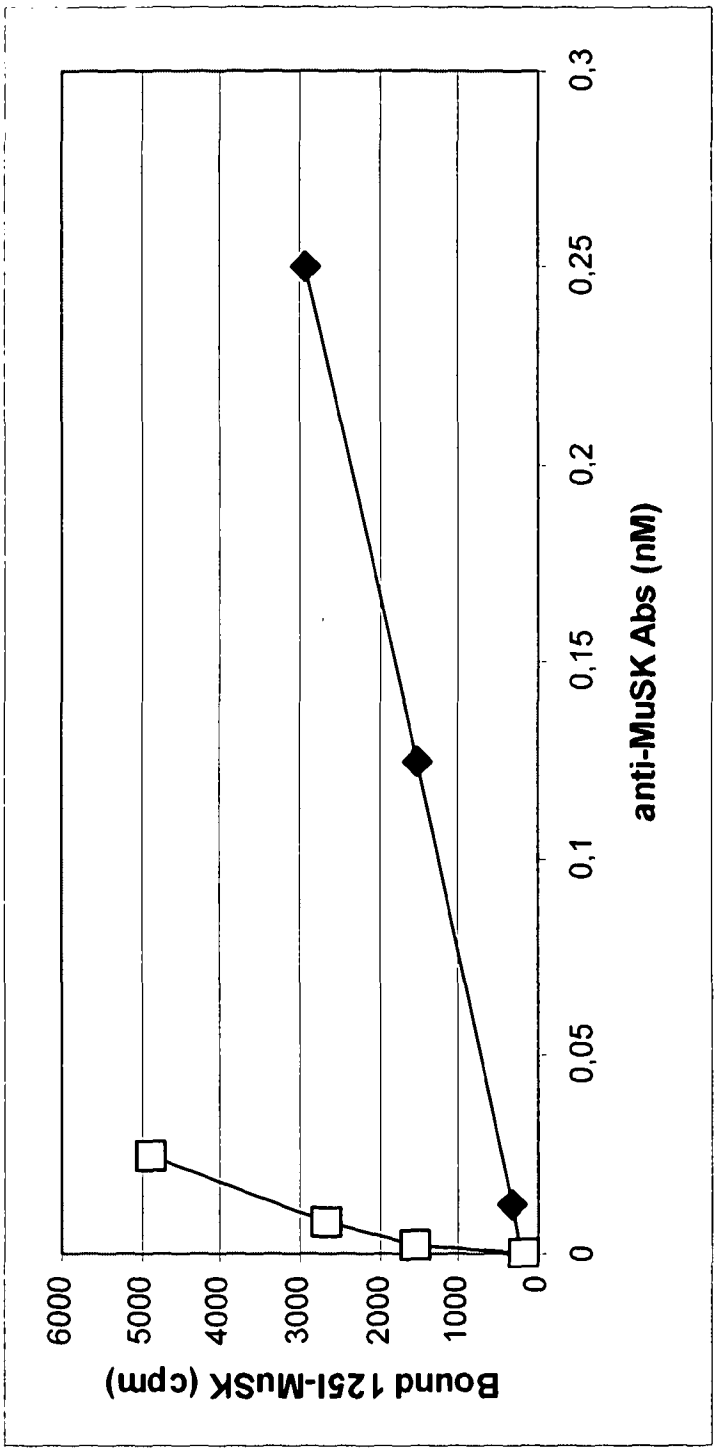

Bound 125I-MuSK (cpm)
6000
5000
4000
3000
2000
1000
0
0
0,05
0,1
0,15
0,2
0,25
0,3
anti-MuSK Abs (nM)

Fig. 5

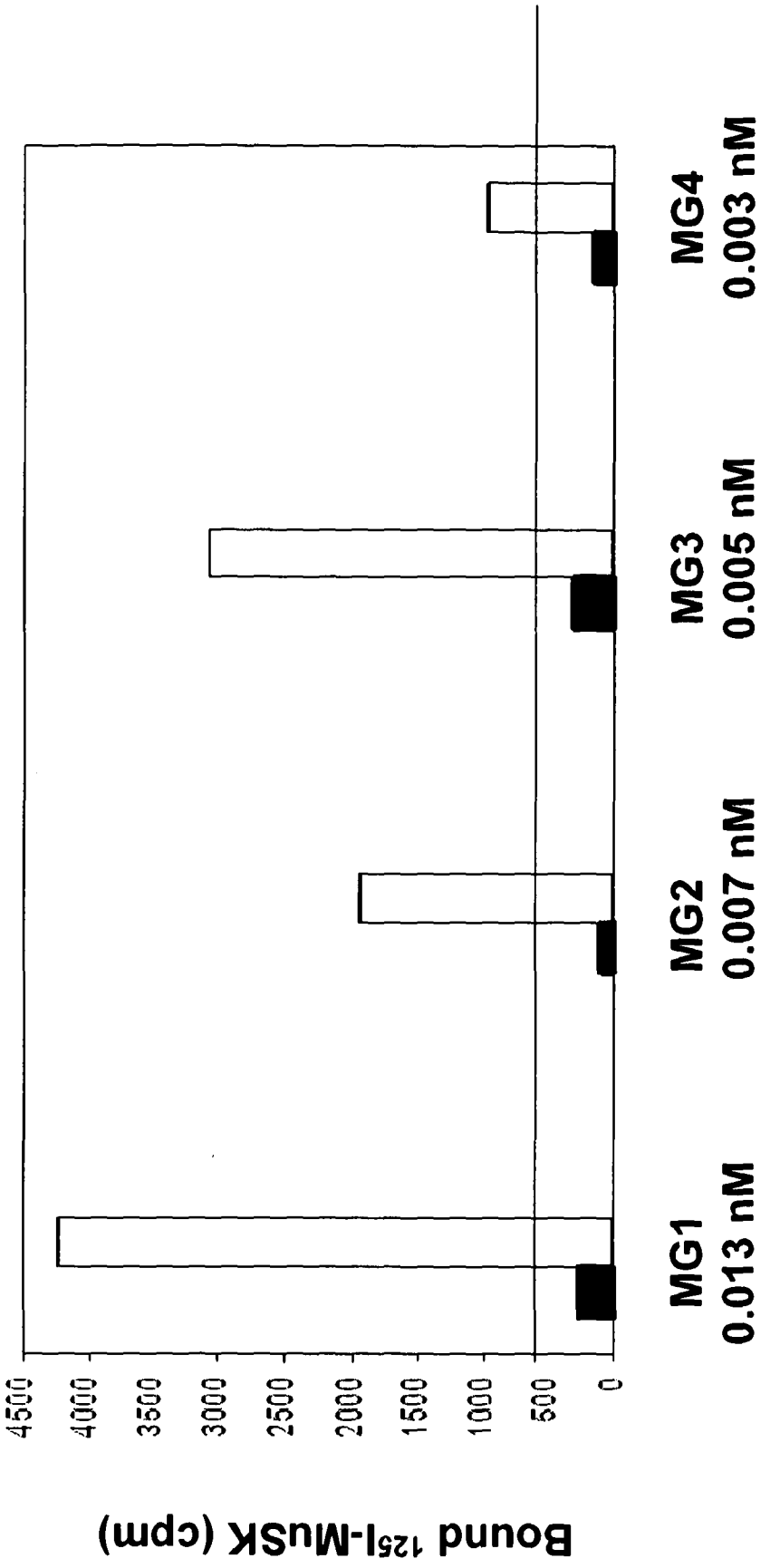

Bound 125I-MuSK (cpm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
MG1
0.013 nM
MG2
0.007 nM
MG3
0.005 nM
MG4
0.003 nM

Fig. 6

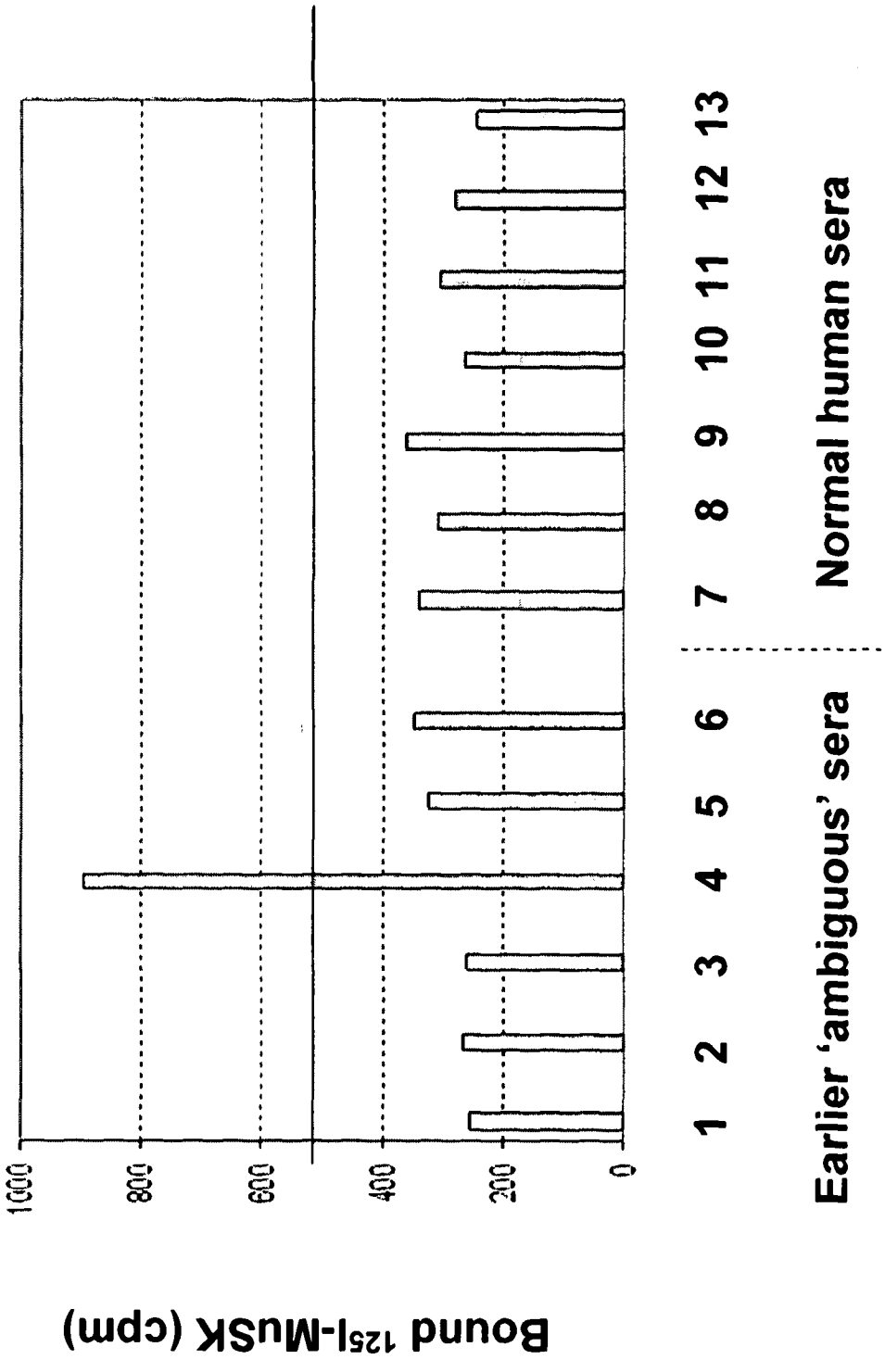
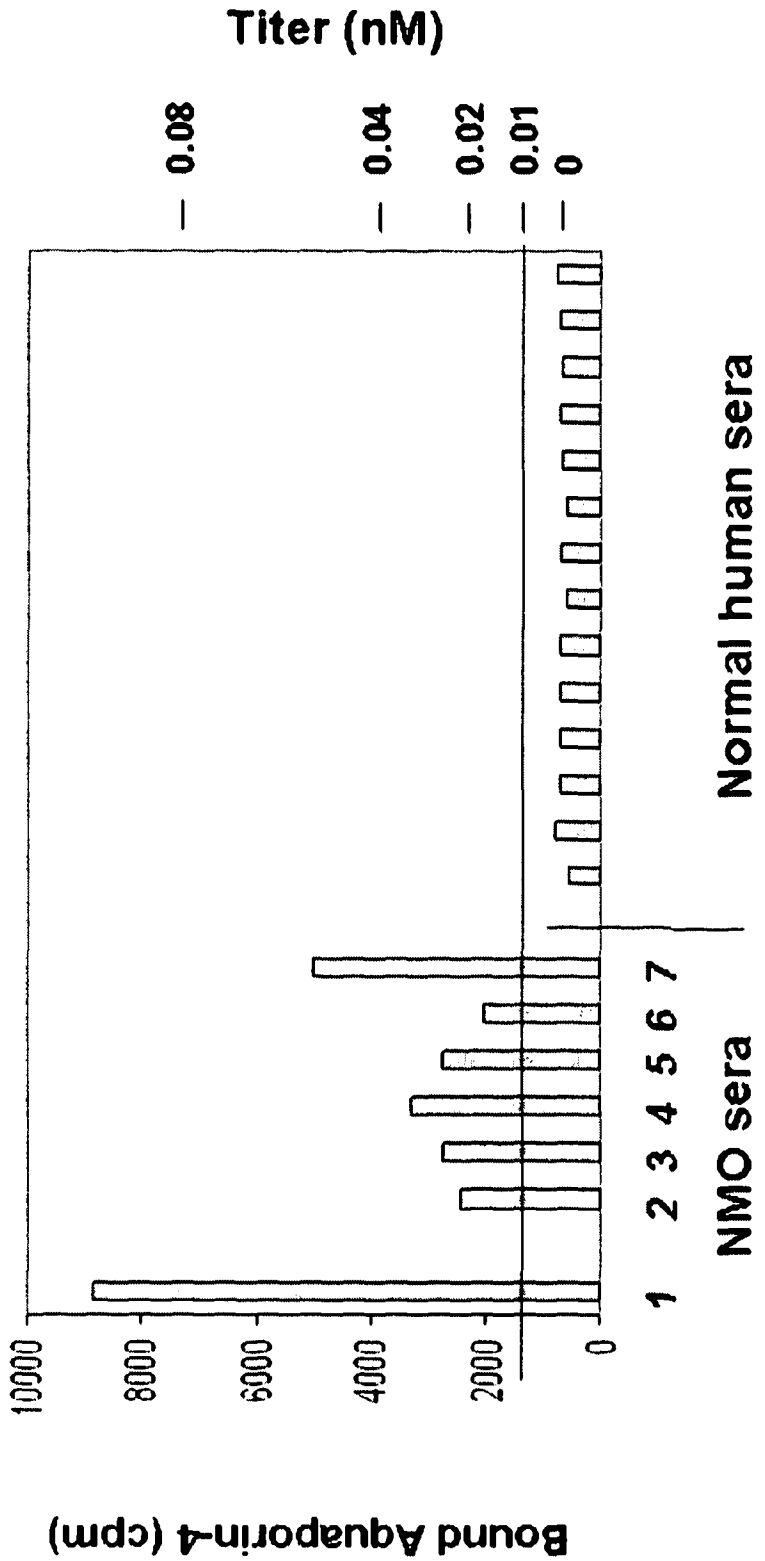

Bound 125I-MuSK (cpm)
1000
800
600
400
200
0
1 2 3 4 5 6
Earlier 'ambiguous' sera
7 8 9 10 11 12 13
Normal human sera

Fig. 7


Titer (nM)
0.08
0.04
0.02
0.01
0
Bound Aquaporin-4 (cpm)
10000
8000
6000
4000
2000
0
1 2 3 4 5 6 7
NMO sera
Normal human sera

专利名称(译)	诊断分析		
公开(公告)号	EP2561358A2	公开(公告)日	2013-02-27
申请号	EP2011724450	申请日	2011-04-18
[标]申请(专利权)人(译)	HELLENIC INST巴斯德 TZARTOS苏格拉底 tzartos约翰 tzartou elissavet		
申请(专利权)人(译)	HELLENIC巴斯德研究所 tzartos，SOCRATES		
当前申请(专利权)人(译)	HELLENIC巴斯德研究所 tzartos，SOCRATES		
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摘要(译)

一种检测来自受试者的生物样品中靶分子的存在的方法，其中所述靶分子能够结合结合分子，其中所述方法包括以下步骤：a）使所述生物样品经历纯化步骤至提供富集的靶分子样品，其中纯化步骤包括：i）使结合分子与生物样品接触以使靶分子与结合分子结合，和ii）从结合分子中除去靶分子以提供富集的靶分子样品; b）对富集的靶分子样品进行测定以检测靶分子的存在。