

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
16 December 2004 (16.12.2004)

PCT

(10) International Publication Number
WO 2004/108762 A1

- (51) International Patent Classification⁷: **C07K 14/72**, G01N 33/78
- (21) International Application Number: PCT/EP2004/005967
- (22) International Filing Date: 2 June 2004 (02.06.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/475,821 4 June 2003 (04.06.2003) US
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: IMMOBILIZED MEMBRANE PROTEINS AND METHODS OF USE THEREFOR

(57) Abstract: The present invention provides compositions, kits and methods for the preparation of membrane proteins immobilized on solid supports. Such immobilized membrane proteins e.g. thyroid stimulating hormone receptor, are useful in solid phase assays for the detection of molecules that bind to the membrane protein, e.g. autoantibodies associated with Graves' disease.

WO 2004/108762 A1

IMMOBILIZED MEMBRANE PROTEINS AND METHODS OF USE THEREFOR

Related Applications

This application claims priority to prior filed U.S. Provisional Application Serial No. 60/475,821, filed June 4, 2003. The entire contents of this provisional patent application are hereby incorporated herein by reference.

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Work described herein was supported under DGTRE convention 41005, awarded by the Region Wallonne Convention.

Background of the Invention

Autoimmune thyroid disease causes cellular damage and alters thyroid gland function by humoral and cell-mediated mechanisms. Cellular damage occurs when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes causing cell lysis and inflammatory reactions. Alterations in thyroid gland function result from the action of stimulating or blocking autoantibodies on cell membrane receptors. Three principal thyroid autoantigens are involved in autoimmune thyroid disease: thyroperoxidase (TPO), thyroglobulin (Tg) and the thyroid stimulating hormone (TSH) receptor. TSH receptor autoantibodies are heterogeneous and either mimic the action of TSH and cause hyperthyroidism as observed in Graves' disease, or may antagonize the action of TSH and cause hypothyroidism. Graves' disease is a common organ-specific autoimmune disease with an incidence of approximately 4 in 10,000 people per year (Vanderpump, M.P.J. *et al.* (1995) *Clin. Endocrinol.(Oxf.)*, **43**, 55-68). A syndrome including goiter and thyrotoxicosis, the disease differs from all other autoimmune diseases in being associated with target organ hyperfunction rather than organ damage. Thyrotoxicosis is directly caused by autoantibodies that activate the thyrotropin (TSH) receptor (TSHR) (reviewed in Rapoport, B. *et al.* (1998) *Endocr. Rev.* **19**, 673-716). Genetic factors play an important role in Graves' disease (for example, see Tomer, Y. *et al.* (1999) *J. Clin. Endocrinol. Metab.* **84**, 4656-4664). A concordance rate of 0.35 between identical twins (Brix, T.H. *et al.* (2001) *J. Clin. Endocrinol. Metab.* **86**, 930-934), however, indicates that a substantial contribution derives from nongenetic factors.

Tests of the humoral response, *i.e.*, thyroid autoantibodies, can be assessed in most clinical laboratories. Unfortunately, the diagnostic and prognostic use of thyroid autoantibody measurements has been hampered by technical problems, including irreproducibleability and lack of sensitivity. Thus, while autoantibody tests have inherent clinical utility, these tests have, to date, been selectively employed. Accordingly, there is a clear need in the art for new and improved methods for detecting autoantibodies such as thyroid autoantibodies.

Summary of the Invention

The present invention is based at least in part on the discovery of a method by which membrane proteins can be directly adsorbed on a solid support such that the functional and epitopic properties of the membrane proteins are conserved. In particular, the instant inventors have developed a method of preparing detergent solubilized membranes containing thyroid membrane proteins, *e.g.*, thyroid stimulating hormone (TSH) receptor, wherein a salt fractionation step provides a resulting supernatant fraction that is enriched for thyroid membrane proteins. This method also facilitates soluble membrane fraction adsorption directly onto a solid support, to function in detection assays for molecules which bind to the membrane protein, *e.g.*, autoantibodies for TSH receptor.

A significant advantage of a solid phase TSH receptor assay is that, given that the TSH receptor is attached to a solid phase, separation of molecules that bind to the immobilized receptor from free molecules is greatly facilitated. In contrast, in a liquid phase receptor assay, receptor-bound molecules are separated from unbound molecules by precipitation of the molecule/receptor complex, a subsequent centrifugation step, and aspiration of the supernatant. As the centrifugal pellets usually require washing, the centrifugation step must be repeated. The solid phase assays of the invention eliminate the requirement for centrifugation, thus greatly facilitating automation of the assay.

Accordingly, the instant invention features a method for the production of soluble cell membrane fractions suitable for direct immobilization on a solid support. The instant invention further features methods for the production of immobilized membrane proteins (also referred to herein as solid phase membrane proteins), such as thyroid membrane proteins, *e.g.*, TSH receptor. These solid phase membrane proteins

are used in binding assays for the detection of molecules that bind to the solid phase membrane proteins, such as autoantibodies associated with an auto-immune disease. In a preferred embodiment, instant invention features a method for the production of a solid phase TSH receptor for use in a receptor binding assay for the detection of molecules that bind to the TSH receptor, *e.g.*, molecules that bind to the TSH binding site on the TSH receptor. This method may be used to detect and/or quantify the hormone thyrotropin (TSH) or autoantibodies that bind to the TSH receptor. The assay is therefore useful in the identification of patients suffering from autoimmune thyroid diseases or disorders whose blood may contain autoantibodies, *e.g.*, patients suffering from Graves' disease or Hashimoto's disease.

In a preferred embodiment, the invention features a solid phase assay in which TSH receptor is bound to a solid phase, *e.g.*, the inside of a polystyrene test tube or to the surface of superparamagnetic beads. Such tubes are referred to herein as "receptor coated tubes" and such superparamagnetic beads as "receptor coated beads". Whether bound to tubes or to beads, such immobilized receptors may be referred to as a "solid phase receptor". In one embodiment, the receptor coated tubes or beads are incubated with a sample from a subject, *e.g.*, bodily fluid, *e.g.*, patient sera, that either contains or lacks autoantibodies to the TSH receptor. The receptor coated tubes or beads are additionally incubated with an agent that binds to the TSH receptor, *e.g.*, TSH, *e.g.*, iodine 125 labelled bovine TSH (^{125}I -bTSH). Following the incubation, the receptor coated tubes or beads are washed with buffer and the radioactivity (cpm) in the tubes or beads is measured using standard techniques, *e.g.*, by using a gamma counter. In the case where the sample contains autoantibodies to the TSH receptor, *e.g.*, anti-TSH receptor autoantibodies that bind to the TSH binding site on the TSH receptor, binding of the ^{125}I -bTSH to the receptor coated tubes or beads is inhibited. As a consequence, a lower amount of radioactivity is detected in the tubes used to assay a sample containing autoantibodies to the TSH receptor than in tubes used to assay a sample that does not contain autoantibodies to the TSH receptor. The solid-phase assay of the invention can be performed in one step, wherein the receptor coated beads or tubes are incubated simultaneously with a sample from a subject and an agent that binds competitively to the receptor. The assay of the invention can also be performed in two steps, *i.e.*, the receptor coated tubes are first incubated with a sample, the tubes are then washed with a buffer to remove unbound sample, and the washed receptor coated tubes are then incubated in a subsequent step with the agent,

e.g., ^{125}I -bTSH. The radioactivity present in the tubes is then measured in a gamma counter as in the one-step assay.

Brief Description of the Drawings

Figure 1 depicts the effects of detergent on the protein content of soluble membrane preparations containing TSH receptor.

Figure 2 depicts the effect of fractionation on the protein content of soluble membrane preparations containing TSH receptor.

Figure 3 depicts the effect of physical treatment of the soluble membrane preparation containing TSH receptor.

Figure 4 depicts the optimal reaction conditions for the solid phase TRAb competitive binding assay.

Figure 5 depicts a comparison between porcine and bovine TSH as radiolabeled tracers in the solid phase TRAb competitive binding assay.

Detailed Description of the Invention

The present invention is based, at least in part, on the discovery of a method by which membrane proteins can be directly adsorbed on a solid support such that the functional and epitopic properties of the membrane proteins are conserved. In particular, a method is provided for preparing detergent solubilized membranes containing thyroid membrane proteins, *e.g.*, thyroid stimulating hormone (TSH) receptor, wherein a salt fractionation step provides a resulting supernatant fraction that is enriched for the thyroid membrane proteins. This method further provides a soluble membrane fraction that is able to adsorb directly onto a solid support and to function in detection assays for molecules which bind to the membrane protein, *e.g.*, autoantibodies for TSH receptor.

Accordingly, the instant invention features methods for the preparation of soluble cell membrane fractions suitable for direct immobilization on a solid support. The invention further features methods for the production of immobilized membrane proteins, such as thyroid membrane proteins. These immobilized membrane proteins may be used in binding assays for the detection of molecules that bind to the solid phase membrane proteins, such as autoantibodies associated with an auto-immune diseases and disorders. The assays of the invention are thus useful in the identification

of autoantibodies in a subject and in the diagnosis of associated auto-immune diseases or disorders.

Accordingly, in one aspect, the invention features a method for preparing soluble cell membranes suitable for direct immobilization on a solid support, comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt.

In a related aspect, the invention features a method for preparing a soluble cell membrane fraction enriched for one or more membrane proteins, wherein the one or more membrane proteins are capable of adsorbing directly on a solid support, comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt ions.

In another related aspect, the invention provides a soluble cell membrane fraction suitable for directly coating a solid support prepared according to the methods of the invention.

In one aspect, the invention provides a method for immobilizing a membrane protein on a solid support, comprising contacting a solid support with a soluble cell membrane fraction of the invention under conditions sufficient to permit direct adsorption of the membrane protein to the solid support.

In another aspect, the invention provides a membrane protein immobilized on a solid support prepared according to the methods of the invention.

In one aspect, the invention features a method for preparing a solid support on which one or more membrane proteins are immobilized, wherein the solid support is suitable for detecting molecules that bind the membrane protein, comprising: (a) mixing a detergent solubilized cell membrane solution containing one or more membrane proteins with salt ion, such that a resulting supernatant fraction is enriched for the membrane proteins; (c) contacting a solid support with the supernatant fraction under conditions such that the membrane proteins adsorb directly on the solid support; thereby providing membrane proteins immobilized on a solid support suitable for detecting molecules that bind the membrane proteins.

In various embodiments, the salt is selected from the group consisting of ammonium sulfate, ammonium phosphate, potassium phosphate, rubidium phosphate, sodium phosphate, other Hofmeister salts, and combinations thereof. In one embodiment, the salt is ammonium sulfate. In one embodiment, the ammonium sulfate is added to a final molarity of about 1.

In one embodiment, the membrane protein is a thyroid membrane protein. In one embodiment, the thyroid membrane protein is thyroid stimulating hormone (TSH) receptor or thyroid peroxidase.

In one embodiment, the detergent solubilized cell membranes are derived from cultured cells. In one embodiment, the cultured cells express a recombinant membrane protein. In another embodiment, the detergent solubilized cell membranes are derived from animal tissue. In various embodiments, the animal is selected from the group consisting of a human, cow, pig, rat, mouse, dog, monkey and guineau pig.

In various embodiments, the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material or a non-magnetic material. In other embodiments, the solid support is selected from the group consisting of test tubes, microwell titer plates and magnetic beads.

In one aspect, the invention features a method for preparing thyroid stimulating hormone (TSH) receptor or a fragment thereof immobilized on a solid support, wherein the immobilized TSH receptor is suitable for detecting human TSH receptor autoantibodies or TSH in a sample, comprising: (a) mixing a detergent solubilized cell membrane solution containing human thyroid stimulating hormone receptor or a fragment thereof with ammonium sulfate under conditions such that the resulting supernatant fraction is enriched for TSH receptor; (c) contacting a solid support with the supernatant fraction under conditions to permit direct adsorption of the TSH receptor on the solid support; thereby providing immobilized TSH receptor suitable for detecting TSH receptor autoantibodies or TSH.

In one embodiment, the ammonium sulfate precipitation step comprises incubating about one quarter volume of saturated ammonium sulfate solution with about one volume solubilized cell membranes.

In various embodiments, the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material and a non-magnetic material. In other various embodiments, the solid support is selected from the group consisting of test tubes, microwell titer plates and magnetic beads.

In one aspect, the invention features a method for detecting a molecule in a sample that specifically binds to a membrane protein, comprising: (a) contacting a membrane protein immobilized on a solid support according to the methods of the invention with a sample; (b) contacting the immobilized protein with an agent capable of binding to the membrane protein in a competitive reaction with the molecule; (c)

detecting the agent bound to the immobilized membrane protein in the absence of the sample; (d) detecting the agent bound to the immobilized membrane protein in the presence of the sample; wherein a decreased amount of agent bound to the immobilized membrane protein in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of a molecule that specifically binds to the membrane protein.

In one embodiment, the molecule in the sample is an autoantibody to the membrane protein. In various embodiments, the autoantibody is associated with an autoimmune disease or the effect of a drug.

In one embodiment of the invention, the agent capable of binding to the membrane protein is an agonist or antagonist of the membrane protein. In one embodiment, the antagonist or agonist is an antibody or fragment thereof.

In one aspect, the invention features a method for detecting autoantibodies to human thyroid stimulating hormone (TSH) receptor in a sample, comprising: (a) contacting TSH receptor immobilized on a solid support prepared according to the methods of the invention with a sample; (b) contacting the immobilized TSH receptor with an agent capable of binding to the TSH receptor in a competitive reaction with TSH receptor autoantibodies; (c) detecting the agent bound to the immobilized TSH receptor in the absence of the sample; (d) detecting the agent bound to the immobilized TSH receptor in the presence of the sample; wherein a decreased amount of agent bound to the immobilized TSH receptor in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of autoantibodies to human thyroid stimulating hormone receptor.

In various embodiments, the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material and a non-magnetic material. In other embodiments, the solid support is selected from the group consisting of tubes, microwell titer plates and magnetic beads.

In one embodiment, the TSH receptor autoantibodies to be detected are associated with autoimmune diseases or disorders. In one embodiment, the TSH receptor autoantibodies to be detected are associated with a drug reaction. In another embodiment, the TSH receptor autoantibodies to be detected are associated with Graves' disease.

In one embodiment of this aspect, the immobilized TSH receptor is contacted with the sample and agent simultaneously. In another embodiment, the immobilized

TSH receptor is first contacted with the sample and subsequently contacted with the agent.

In one embodiment of these aspects, the sample is bodily fluid from a subject. In a preferred embodiment, the subject is a human. In various embodiments, the bodily fluid is selected from the group consisting of blood, plasma, serum, urine, cerebrospinal fluid, serosal fluid and tissue extract.

In various embodiments of these aspects, the agent is human immunoglobulin or thyroid stimulating hormone (TSH). In certain embodiments, the thyroid stimulating hormone (TSH) is porcine TSH or bovine TSH. In one embodiment, the agent is radiolabeled. In a preferred embodiment, the radiolabel is iodide 125. In yet other embodiments, the agent is detected by a means selected from the group consisting of fluorescence, luminescence, a dye and an enzyme.

In another aspect, the invention features a method for diagnosing a disease or disorder associated with an autoantibody to a thyroid membrane protein in a subject, comprising the steps of: (a) mixing a detergent solubilized cell membrane solution containing a thyroid membrane protein with salt to obtain a membrane fraction; (b) contacting a solid support with the membrane fraction under conditions sufficient to permit direct adsorption of the membrane protein to the solid support; (c) contacting the membrane protein with a sample; (d) contacting the membrane protein with an agent capable of binding to the membrane protein in a competitive reaction with the molecule; (e) detecting the agent bound to the membrane protein in the presence of the sample; and (f) detecting the agent bound to the membrane protein in the absence of the sample; wherein a decreased amount of agent bound to the membrane protein in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of a molecule that specifically binds to the membrane protein.

In another aspect, the invention features a method for diagnosing Graves' disease in a subject, comprising the steps of: (a) mixing a detergent solubilized cell membrane solution containing human thyroid stimulating hormone (TSH) receptor or a fragment thereof with ammonium sulfate under conditions such that the resulting supernatant fraction is enriched for TSH receptor; (b) contacting a solid support with the supernatant fraction under conditions to permit direct adsorption of the TSH receptor on the solid support; (c) contacting the TSH receptor with a sample; (d) contacting the TSH receptor with an agent capable of binding to the TSH receptor in a

competitive reaction with TSH receptor autoantibodies; (e) detecting the agent bound to the TSH receptor in the presence of the sample; (f) detecting the agent bound to the TSH receptor in the absence of the sample; wherein a decreased amount of agent bound to the TSH receptor in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of autoantibodies to human thyroid stimulating hormone receptor.

In yet another aspect, the invention features a kit comprising a soluble membrane fraction suitable for direct immobilization on a solid support, wherein the soluble membrane fraction is prepared according to a method comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt.

In another aspect, the invention features a kit comprising a soluble cell membrane fraction enriched for one or more membrane proteins, wherein the one or more membrane proteins are capable of adsorbing directly on a solid support.

In yet another aspect, the invention features a kit comprising one or more membrane proteins immobilized on a solid support, wherein the one or more membrane proteins immobilized on the solid support are prepared according to a method comprising the steps of: (a) mixing a detergent solubilized cell membrane solution containing one or more membrane proteins with salt to obtain a membrane fraction enriched for the one or more membrane proteins; and (b) contacting a solid support with the membrane fraction under conditions sufficient to permit direct adsorption of the one or more membrane proteins to the solid support.

Definitions

Before further description of the present invention, and in order that the invention may be more readily understood, certain terms are first defined and collected here for convenience:

The term "membrane" refers to a thin sheet of tissue that covers, lines or connects organs, cells or cell organelles, *e.g.*, a lipid bilayer that acts as a barrier within or around a cell. A preferred membrane is a membrane embedded with proteins. Cell membranes can include those of any order, such as plasma membranes, endoplasmic reticulum membranes, microsomal membranes and myelin membranes. The term "soluble cell membrane" refers to membrane which has been rendered soluble, *e.g.*, by the addition of detergent.

The term "membrane protein" refers to a protein molecule (or assembly of molecules) that is either embedded in or weakly attached to a biological membrane, *e.g.*, the plasma membrane. Membrane proteins can include integral membrane proteins, peripheral membrane proteins and lipid anchored proteins. "Integral membrane protein" refers to proteins that are sufficiently embedded to remain with the membrane during the initial steps of biochemical purification. "Peripheral membrane protein" refers to membrane proteins that separate into the water-soluble phase or which are anchored, *e.g.* lipid anchored proteins, such that the proteins remain with the membrane during purification. The term "thyroid membrane protein" refers to a membrane protein associated with or derived from thyroid tissue, *e.g.*, TSH receptor.

The term "enriched" and/or "enriched for one or more membrane proteins" is intended to mean that the preparation, *e.g.*, the fraction, contains a higher concentration of the membrane proteins than usual, *e.g.*, greater than before fractionation. Enriched is further intended to mean that the preparation, *e.g.*, the fraction, has a greater capacity to attach, fix or adsorb directly, *e.g.*, without an intervening substance, on a solid surface.

As used herein, the term "thyroid stimulating hormone receptor" or "TSH receptor" refers to naturally-occurring TSH receptor, natural and unnatural functional analogs and fragments and derivatives thereof, capable of binding to TSH receptor binding molecules, *e.g.*, thyroid stimulating hormone (TSH) or anti-TSH receptor antibodies, such as autoantibodies to TSH receptor. Human TSH receptor can comprise the nucleotide sequence as provided in Genbank Accession No. NM_000369, GI:4507700, and the amino acid sequence as provided in Genbank Accession No. NP_000360, GI:4507701, herein incorporated by reference.

As used herein, the term "fragment" means a subset of the conserved amino acid sequence of a protein, *e.g.*, TSH receptor protein, that retains the ability to bind its naturally binding partners, *e.g.*, TSH receptor binding molecules, *e.g.*, TSH or anti-TSH receptor antibody. The term is intended to include such fragments in conjunction with or combined with additional sequences or moieties, as for example where the peptide is coupled to other amino acid sequences or to a carrier. The terms "fragment" and "peptide" can, therefore, be used interchangeably since a peptide will be the most common fragment of the TSH receptor protein. Reference herein to a "fragment," "portion" or "segment" of an TSH receptor peptide does not mean that the

composition must be derived from the intact TSH receptor protein. Such "fragments," "portions" or "segments" can be produced by various means well-known to those skilled in the art, such as, for example, manual or automatic peptide synthesis, enzymatic treatment of a whole TSH receptor protein and cloning. In certain embodiments, the TSH receptor peptide comprises at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700 or 750 amino acids. In certain embodiments, the TSH receptor peptide of the present invention is capable of binding to an anti-TSH receptor autoantibody of a subject.

The term "peptide" or "polypeptide" is used in its broadest sense, *i.e.*, any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "peptide" includes proteins, oligopeptides, protein fragments, muteins, fusion proteins and the like. The term "protein" is used herein to designate a naturally occurring polypeptide. Peptides of the present invention can be made synthetically, using techniques that are known in the art, or encoded by a nucleic acid, such as DNA or RNA.

The term "solid support" refers to any surface upon which a molecule of interest, *e.g.*, a membrane protein, may be attached, fixed or restrained. A solid support may comprise, for example, a plastic material, magnetic material or non-magnetic material.

The term "adsorb" is intended to mean to attract and bind so as to form a thin layer on the surface, *e.g.*, to adsorb molecules of a substance in a fluid on a surface, or to the accumulation of a liquid, *e.g.*, soluble membranes, on the surface of a solid, *e.g.*, a plastic, magnetic or non-magnetic material.

The term "immobilization" is intended to mean the fixation or attachment to a solid support so as to restrain or limit movement, *e.g.*, fixation of a molecule, *e.g.*, a membrane protein, to a solid support such that it cannot move freely in solution. The term "direct" immobilization, including "direct immobilization of a membrane protein on a solid support", is intended to mean that the membrane protein becomes fixated or attached to the solid support without an intervening substance, *e.g.*, so that the membrane protein and solid support are in direct contact, *e.g.*, without an antibody or other binding molecule to fix or attach the membrane protein to the solid support.

A molecule is "fixed" or "affixed" to a substrate if it is covalently or non-covalently associated with the substrate such that the substrate can be rinsed with a

fluid (*e.g.* standard saline citrate, pH 7.4) without a substantial fraction of the molecule dissociating from the substrate.

The terms “induce” “inhibit,” “potentiate,” “elevate,” “increase,” “decrease,” or the like, denote quantitative differences between two states, and refer to at least statistically significant differences between the two states. For example, “a decreased amount of agent bound to immobilized TSH receptor in the presence of a sample” means that the bound agent will be at least significantly less when the assay is carried out in the presence of the sample as compared to the amount of agent bound in the absence of the sample.

The term a “patient sample”, “body fluid” or “bodily fluid” is intended to mean the liquid components of the body, *e.g.*, any bodily fluid in which a binding molecule of the immobilized membrane protein is found and is indicative of a disease or disorder associated with that binding molecule. Body fluids can include blood, plasma, sera, cerebrospinal fluid, synovial fluid, peritoneal fluid, pleural fluid, pericardial fluid, aqueous humour, saliva, sweat, tears, lymph, chyme, chyle, bile, urine, stool water, semen, amniotic fluid, milk and pancreatic juice. Particularly suitable bodily fluids include blood, a blood fraction, urine, saliva, tears, and cerebrospinal fluid. In a preferred embodiment, the bodily fluid is blood or a fraction thereof, such as serum or plasma. More preferably, the bodily fluid is serum.

As used herein, the term “subject” is intended to include all vertebrates, *i.e.*, human and non-human animals. The term “non-human animals” of the invention includes, but is not limited to, mammals, rodents, mice and non-mammals, such as non-human primates, sheep, dog horse, cow, chickens, amphibians, reptiles and the like. In certain embodiments, the subject is a mammal, *e.g.*, a primate, *e.g.*, a human. In other embodiments, human subjects include those at risk for or suffering from a thyroid disorder, *e.g.*, Graves’ disease, or a disorder associated with the aberrant expression, quantity or localization of a molecule that binds to a membrane protein, *e.g.*, an autoimmune disease or disorder wherein the autoantibody associated with the autoimmune disorder is specific for an antigen, wherein the antigen is a membrane protein.

The term “treatment” or “treating” as used herein refers to the prevention of a disease or disorder (prophylaxis), and to the reduction or elimination of symptoms of the disease or disorder (therapy).

The terms "prevention," "prevent," or "preventing" as used herein refers to inhibiting, averting or obviating the onset or progression of a disease or disorder (prophylaxis).

As used herein, the terms "immune" and "immunity" refers to the quality or condition of being able to resist a particular infectious disease.

The terms "immunize" and "immunization," as used herein, refer to the act of making a subject not susceptible to a disease or disorder, less responsive to a disease or disorder, and/or have an increased degree of resistance to a disease or disorder.

A chemical entity, such as a protein, polypeptide or antibody, is "isolated" if a composition comprising the entity is substantially free of other macromolecules, such as other proteins. A chemical entity is "purified" in a composition in which the entity is present in substantially greater relative concentration than it exists in its natural state, for example in a body fluid of a subject. Preferably the chemical entity comprises at least 80%, more preferably at least 90%, and even more preferably at least 95% by weight of the macromolecular species present in the composition. Most preferably, the chemical entity is purified to homogeneity, *i.e.*, other macromolecular species are not significantly detectable using standard techniques, such as polyacrylamide gel electrophoresis and high performance liquid chromatography.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in an organism found in nature.

A "kit" is any manufacture (*e.g.* a package or container) comprising at least one reagent, *e.g.* a solid support, a preparation of soluble membrane containing one or more membrane proteins of interest, an agent that binds competitively to the membrane protein, a standard or an internal standard, for specifically determining the presence or abundance in a sample of a molecule that binds to an immobilized membrane protein of the invention. The kit may include instructions for use. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention.

Unless otherwise specified herein, the terms "antibody" and "antibodies" broadly encompass naturally-occurring forms of antibodies (*e.g.*, IgG, IgA, IgM, IgE) and recombinant antibodies such as single-chain antibodies, chimeric and humanized antibodies and multi-specific antibodies, as well as functional fragments and

derivatives thereof. Antibody derivatives may comprise a protein or chemical moiety conjugated to an antibody.

I. Existing assays for thyroid stimulating hormone receptor

A number of solid phase TSH radioreceptor assays have been described. PCT Patent Application No. 99/64865 teaches a solid phase radioreceptor assay for TSH receptor autoantibodies. European Patent Application No. EP 0975970 describes a solid phase receptor binding assay for detecting TSH receptor autoantibodies. Both of these patent documents describe an assay in which the TSH receptor is attached to the solid phase by means of an antibody that specifically recognizes the TSH receptor, *i.e.*, an antibody is first attached to the interior of the test tube and this antibody then specifically binds the TSH receptor. PCT Patent Application No. 99/64865 teaches methods comprising porcine TSH receptor attached to an antibody fragment (4E31 (Fab)₂ previously coated onto plastic tubes. The use of antibodies to immobilize the TSH receptor to a solid phase is required in both patent documents, and both patent documents fail to teach a method for directly immobilizing a functional TSH receptor.

A solid-surface ¹²⁵I-labeled TSH binding assay has been described by Yavin *et al.* (1981 *Proc. Natl. Acad. Sci. USA* 78 (5) 3180-3184). Yavin *et al.* teach an assay in which microtiter plates were precoated with poly L lysine and then a dilute solution of thyroid membranes were added to the wells. Unabsorbed membranes were removed and the wells washed with buffer. Each well was then incubated with ¹²⁵I labelled TSH. After two hours of incubation at 4°C, the microtiter plate was centrifuged and unbound iodine ¹²⁵I-bTSH was decanted. The radioactivity in each well was then determined. The assay disclosed in Yavin *et al.* is fundamentally different from the methods of the present invention. Firstly, Yavin *et al.* teach a method for coating solid particles of thyroid membranes on a plastic surface that has been precoated with poly L lysine. In the methods of the present invention, TSH receptor is adsorbed directly from a solution of detergent. The detergent solubilised receptor solution of the present invention does not contain solid particles of plasma membrane, since the receptor solution it is subjected to ultracentrifugation (*e.g.*, at 100,000g for one hour) and the pellet discarded. Secondly, Yavin *et al.* teach an assay comprising a centrifugation step before decanting the unbound ¹²⁵I-bTSH. The methods of the present invention do not comprise a centrifugation step to separate

bound from unbound ^{125}I -bTSH. Indeed, one advantage of a solid phase assay is the elimination of the need for such a centrifugation step.

The present invention is based, at least in part, on the discovery of a method to attach a functional TSH receptor to a solid support without the need for an antibody. The present invention provides advantages over the methods currently taught in the art. For example, obtaining an antibody to immobilise the TSH receptor on a solid phase is a difficult and expensive process, as is the production and quality control of such an antibody. No such antibody is available commercially. The antibody must be able to bind to the TSH receptor in such a way that the TSH hormone and the autoantibodies that bind to the TSH binding site can still bind freely to their respective binding sites. The antibody must also be of high affinity or the receptor will dissociate during the assay incubation or washing steps. For these reasons, only a small subset of antibodies raised against the TSH receptor would fulfill the above requirements. In addition, there is no obvious, direct method to screen mixtures of hybridomas for a clone that produces such a monoclonal antibody. To test an antibody for suitability, the antibody must be coated on a solid support at relatively high concentration and tested for its ability to bind a functional TSH receptor. It is not feasible to perform this type of assay with the low concentrations of monoclonal antibodies obtained during the screening of hybridomas. It is therefore necessary to screen the antibodies in two steps, where monoclonal antibodies specific for the TSH receptor are first identified by a suitable technique, and are then tested individually for their ability to immobilize the TSH receptor. Thus, the process for obtaining a suitable antibody is laborious and expensive. The present invention provides the significant advantage that such an antibody is not needed. Further, preparation of immobilized TSH receptor by using an antibody involves first incubating the solid phase with antibody and then washing the solid phase to remove any unbound antibody. In the instant methods for preparing immobilized membrane proteins, this incubation is eliminated, rendering the assay less time consuming, less labour intensive and less expensive to produce. Additionally, the methods of the instant invention, but not requiring a monoclonal antibody to immobilize the receptor, is a simpler system. As a consequence, there is less variation in the assay (*e.g.*, due to lot to lot variation in the biological activity of the immobilising antibody). Moreover, the methods of the invention can be easily automated.

II. Soluble Cell Membranes

A. Sources

The instant invention features preparations of solubilized cell membrane, *e.g.*, solubilized cell membrane preparations suitable for directly adsorbing onto a solid support. Solubilized cell membrane preparations of the invention can be prepared from tissues or cells derived from numerous sources. In one embodiment, detergent solubilized cell membrane solutions are prepared from tissues derived from an animal, *e.g.*, a non-human animal. The animals from which the tissues are derived can be any animal commonly used in scientific, medical, veterinary, pharmacological and toxicological experiments. Examples of animals from which tissues are used to prepare solubilized cell membrane include, but are not limited to, cow, pig, rat, mouse, dog, monkey and guinea pig.

Solubilized cell membrane preparations of the invention can also be prepared from cells, *e.g.*, cells grown and maintained in culture. Cells particularly useful in the invention are cells expressing a membrane protein that is to be immobilized on a solid surface for use in a solid phase assay. In one embodiment, cultured cells express the native form of the membrane protein of interest. In another embodiment, the cultured cells express a recombinant form of the protein. The recombinant form of the protein may be a native, naturally occurring form, or may be an analog or derivative of the membrane protein. Preferably, analogs or derivatives of the membrane protein expressed in cultured cells retain their native activity or function, *e.g.*, their ability to bind their natural ligands or binding partners.

A multitude of protected, unprotected, and partially protected natural and unnatural functional analogs and derivatives of TSH receptor are also intended to be within the scope of the invention. Also within the scope of the invention are derivatives of naturally-occurring TSH receptor protein which are engineered and expressed in a cell based upon a knowledge of the sequence of the naturally-occurring amino acid sequence of the genetic material (DNA or RNA) which encodes the sequence. As used herein, the term "altered peptide" refers to a chemical modification of natural or unnatural proteins or peptides, *e.g.*, TSH receptor peptides and analogs of TSH receptor peptides, in which one or more amino acids which are not present in the original sequence are added, deleted, or otherwise changed, and derivatives thereof.

It will be appreciated that a portion or segment of the described sequence can also be used in the invention so long as it is sufficiently characteristic of the desired TSH receptor protein or fragment thereof to bind TSH receptor binding molecules such as TSH or anti-TSH receptor antibodies. Such variations in the sequence can easily be made, for example by synthesizing an alternative sequence. The alternate sequence can then be tested, for example, in an in vitro system to ascertain its ability to bind antibody or TSH.

The present invention also includes a recombinant molecule comprising a nucleic acid sequence encoding a TSH receptor peptide, operatively linked to a vector capable of being expressed in a host cell. A DNA "coding sequence" or a "nucleotide sequence encoding" a particular peptide or protein, is a DNA sequence which is transcribed and translated into a polypeptide in a host cell when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

As used herein, "operatively linked" refers to insertion of a nucleic acid sequence into an expression vector in such a manner that the sequence is capable of being expressed when transformed into a host cell. As used herein, an "expression vector" is an RNA or DNA vector capable of transforming a host cell and effecting expression of an appropriate nucleic acid sequence, *e.g.*, replicating within the host cell. An expression vector can be either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A*

Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding an TSH receptor peptide of the present invention or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

The regulatory sequences include those required for transcription and translation of the nucleic acid, and may include promoters, enhancers, polyadenylation signals and sequences necessary for transport of the molecule to the appropriate cellular compartment. When the nucleic acid is a cDNA a recombinant expression vector, the regulatory functions responsible for transcription and/or translation of the cDNA are often provided by viral sequences. Examples of commonly used viral promoters include those derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40, and retroviral LTRs.

Regulatory sequences linked to the nucleic acid can be selected to provide constitutive or inducible transcription, by, for example, use of an inducible enhancer. Thus, in a specific embodiment of the invention the nucleic acid molecule, which encodes for an TSH receptor peptide of the present invention, is under control of an inducible control element, such that expression of the gene can be turned on or off, by contacting or not contacting, respectively, the host cell(s) containing the nucleic acid with an agent which affects the inducible control element. In other embodiments of the invention, the nucleic acid molecule, which encodes for a TSH receptor peptide of the present invention, is under the control of a promoter which constitutively drives the expression of the nucleic acid molecule. Regulatory elements which drive constitutive expression of nucleic acid molecules to which they are operably linked

can be viral elements (*e.g.*, derived from polyoma, Adenovirus 2, cytomegalovirus, Simian Virus 40 or retrovirus).

Suitable host cells can be any cells that are capable of producing the membrane proteins of the present invention. Such host cells include, but are not limited to, bacterial, fungal, insect and mammalian cells. Host cells of the present invention can include, but are not limited to, fibroblasts, epithelial cells and endothelial cells.

A “promoter sequence” is a DNA regulatory region capable of binding RNA polymerase in a cell and initiation transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain “TATA” boxes and “CAT” boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the –10 and –35 consensus sequences.

B. Primary soluble membrane preparation

Soluble membrane preparations of the invention can be produced according to any method known in the art. For example, total cell membranes can be isolated according to the method of Nagamatsu et al. (Nagamatsu, S., et al. (1992) J. Biol. Chem. 267, 467-472) or numerous other published protocols. The resulting solution contains soluble membrane proteins of interest as well as other inert plasma membrane proteins. The solution contains no particulate matter because, during its production, the detergent solubilized membranes are centrifuged, *e.g.*, at about 100,000g for about one hour, and the supernatant containing the soluble proteins is retained while the pellet containing the particulate matter is discarded. In a standard protocol, cells are homogenized in homogenization buffer (*e.g.*, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, 1 mM phenylmethylsulfonyl fluoride). The nuclei and cell debris are removed from the homogenate by centrifugation (*e.g.*, 900 × g for 10 min at 4 °C) and the resulting supernatant is ultra-centrifuged (*e.g.*, 110,000 ×

g for 75 min at 4 °C in a SW40 rotor, Beckman ultracentrifuge). The membrane pellet is solubilized in a buffer, usually containing a detergent (*e.g.*, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), for a minimum of 1 h at 4 °C. Insoluble material is removed by centrifugation (*e.g.*, 14,000 × g for 10 min at 4 °C) and 1 µg/ml aprotinin added to solubilized membrane samples prior to storage at 70 °C. In a preferred embodiment, the soluble membrane is prepared using a detergent. Preferred detergents include Thesit (Roche) (*e.g.*, 1% Thesit) or Triton X-100 (*e.g.*, 1% Triton X-100).

In a preferred embodiment of the invention, the soluble membrane preparation is derived from thyroid tissue. In one embodiment, the thyroid membrane preparation contains thyroid membrane proteins, *e.g.*, TSH receptor. In an exemplary protocol, porcine thyroid glands are minced, treated by polytron in ice cold Tris-HCL buffer, pH 7.5, and the resulting suspension centrifuged. The supernatant is removed and the pellet resuspended, ground on ice and recentrifuged for 30 minutes at 7500 rpm. The pellet is then resuspended in a detergent solution to solubilize the membranes. A preferred detergent is Thesit detergent (836630 Roche), which is used at a concentration of 4.5g/600 ml in Tris buffer. These conditions limit the polytron phase and utilize lower detergent concentrations as compared to conventional protocols standardly used in the art, such as the protocol used for TRAb BC1007 in an IVD kit (Biocode). The solubilized membrane is centrifuged at 7500 rpm and the resulting supernatant is collected and filtered over cotton. The filtrate is then subjected to ultracentrifugation for 180 minutes at 35,000 rpm at 4°C. The top lipid layer and the pellet are eliminated, and the supernatant is collected. These conditions produce a primary membrane preparation of lower total protein concentration than conventional protocols, but which advantageously controls the protein contents of the membrane preparation containing the receptor. The conditions consistently yield a preparation with a predictable and constant TSH receptor: total protein ratio that ultimately functions well following a further fractionation step in subsequent solid phase assays.

The detergent solubilized membrane preparation can be used immediately or frozen and stored until ready to use. Alternatively, the solubilized membrane preparation can be lyophilised to give a preparation of low humidity and be stored lyophilised at 4°C. In one embodiment, the TSH receptor solution is lyophilised and the lyophilised material is rehydrated using a high ionic strength buffer, *e.g.*, 50 mM phosphate buffer containing between about 0.9 and 4% NaCl, pH 7.5, to give a

solution with a total protein concentration of about 10-40 mg per millilitre of buffer. In one embodiment, the lyophilised receptor solution contains a high concentration of TSH receptor per milligram of total protein.

C. Fractionation

In a preferred embodiment, the instant invention features a fractionation step to enrich the solubilized membrane for the membrane protein of interest. The fractionation step also provides the membrane protein in an optimized environment which facilitates the direct adsorption of the membrane protein fraction onto a solid support. Fractionation of primary solubilized membrane preparations can be carried out according to methods commonly known in the art, and are preferably achieved by the addition of a salt. Such fractionation procedures, commonly referred to as "salting out", are standard in the art. Salting out is based upon the principle that the solubility of protein depends on, among other things, the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as "salting-in". As the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as "salting-out".

Many types of salts can be employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulfate is a commonly utilized in the art because it has high solubility and is relatively inexpensive. Particularly useful salts in the instant invention are those that produce complex anions in solution, *e.g.*, ammonium sulfate and ammonium phosphate. In one embodiment, ammonium sulfate is used to fractionate the primary preparation of soluble membrane. Other salts useful in the fractionation step include the salts of the Hofmeister series, including, but not limited to, ammonium phosphate, potassium phosphate, rubidium phosphate and sodium phosphate. One of skill in the art will recognize that any salt may be used which allows the particular membrane proteins of interest to be successfully adsorbed onto a solid surface and retain its functional or epitopic properties, *e.g.*, retain its ability to bind to a natural binding partner. It is also

within the scope of the invention that organic molecules, *e.g.*, guanidine or urea, which, when in solution, provide ions having salting out properties for proteins or complex membranes, may be useful in the fractionation method of the invention. The usefulness of a particular salt or organic molecule in the fractionation method of the invention can be easily assessed by carrying out standard binding assays as those described in the exemplification herein.

The fractionation step can be carried out by adding a saturated salt solution slowly to the membrane protein mixture to raise the salt concentration of the mixture. The fractionation step can alternatively be carried out by adding powdered salt crystals slowly to the membrane protein mixture to bring up the salt concentration of the mixture. For example, the salt concentration reaches 25% saturation when 1 ml of the saturated salt solution is added to 3 ml of the salt-free protein solution; 50% for 3 ml added; 75% for 9 ml added; and so on. Methods for preparation of saturated salt solutions are standardly known in the art. To prepare a saturated solution of ammonium sulfate, for example, 750 g of ammonium sulfate is added to 1000 ml of water in a beaker or flask and stirred at room temperature for 15 minutes or until saturation. The clear supernatant solution is decanted after the undissolved solids settle on the bottom of the flask. In the fractionation methods of the invention, ammonium sulfate is added to the soluble membrane solution so that the salt concentration reaches about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 60%. In the fractionation method of the invention, ammonium sulfate is preferably added to the soluble membrane solution until the salt concentration reaches 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29% and 30%. Ammonium sulfate used in the fractionation method can be added to a final molarity of about 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 M. Ammonium sulfate used in the fractionation method can be added to a final molarity of about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 and 2.0. Ammonium sulfate used in the fractionation method can be added to a final molality of about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 3.5 and 4.0. Molality is independent of temperature, and is therefore a useful measure of salt concentration in the fractionation methods of the invention. It will be understood by one of ordinary skill in the art that the optimal final concentration of a particular salt or organic molecule to be used in the fractionation step can be determined without undue

experimentation by conducting standard binding assays as those described herein over a range of final salt concentrations, and determining at which concentration maximal binding is achieved.

To assure maximum yield and to avoid unnecessary denaturation of proteins, the fractionation is usually carried out at low temperatures, *i.e.*, between 0 and 4°C. In a typical fractionation step carried out on a small scale, a saturated ammonium sulfate solution is routinely used. In a large scale commercial process, it is preferable to add ammonium sulfate directly into the protein mixture as powdered solids so that the effect of dilution by the salt solution is minimized.

D. Membrane proteins

The solubilized membrane preparations featured in the instant invention preferably contain a membrane protein of interest that is to be immobilized on a solid support. A membrane protein of the instant invention is any protein molecule (or assembly of molecules) that is either embedded in or weakly attached to a biological membrane, especially the plasma membrane. Proteins that are sufficiently embedded to remain with the membrane during the initial steps of biochemical purification are commonly referred to as integral membrane proteins whereas peripheral membrane proteins generally separate into the water-soluble phase. A possible exception is that if the latter are anchored (*e.g.*, lipid anchored protein), the proteins will remain with the membrane during purification. An integral membrane protein is a protein molecule (or assembly of proteins) that in most cases spans the biological membrane with which it is associated (especially the plasma membrane) or which, in any case, is sufficiently embedded in the membrane to remain with it during the initial steps of biochemical purification (compare peripheral membrane protein). Peripheral membrane proteins are proteins that adhere only loosely to the biological membrane with which they are associated. These molecules do not span the lipid bilayer core of the membrane, but attach indirectly, typically by binding to integral membrane proteins, or by interactions with the lipid polar head. Therefore the so-called regulatory protein subunits of many ion channels and transmembrane receptors, for example, may be defined as peripheral membrane proteins. These proteins, in contrast to integral membrane proteins, tend to collect in the water-soluble fraction during protein purification. An exception to this rule is that cells may attach an "anchor" moiety, such as the fatty-acid anchor of lipid anchored proteins, which makes the

purification properties of these proteins the same as integral membrane proteins, although researchers tend not to classify them as such. In lipid anchored proteins, a covalently attached fatty acid, such as palmitate or myristate, serves to anchor the protein to the cytoplasmic face of the cell membrane. Examples include G proteins and certain kinases. It is believed that the fatty acid chain inserts and assumes a place in the bilayer structure of the membrane alongside the similar fatty-acid tails of the surrounding lipid molecules. Potential points of attachment include the terminal amino group of the protein backbone and the side chain of cysteine residues.

Membrane proteins of the invention can be proteins from any biological membrane, including without limitation, cell membranes of any order, such as plasma membranes, endoplasmic reticulum membranes, microsomal membranes and myelin membranes. The instant invention features, in a preferred embodiment, soluble membrane preparations containing thyroid membrane proteins, e.g., membrane proteins from thyroid plasma membranes or microsomal membranes. The invention encompasses any thyroid membrane protein that is wished to be immobilized on a solid surface, e.g., for use in a solid-phase assay to detect molecules which bind to the thyroid membrane protein. Examples of thyroid membrane proteins include, but are not limited to, thyroid stimulating hormone receptor from plasma membrane and thyroid peroxidase from microsomal membrane. Preferably, a natural binding partner of the thyroid membrane protein is a molecule to be assayed or detected in a solid-phase assay. Examples of natural binding partners of TSH receptor include, but are not limited to, TSH and autoantibodies to TSH receptor. Examples of natural binding partners of thyroid peroxidase include autoantibodies to thyroid peroxidase. In a particularly preferred embodiment, the natural binding partner of the thyroid membrane protein is associated with a thyroid disease or disorder, e.g., an autoimmune disease such as Graves' disease or Hashimoto's disease.

III. Solid phase assays

A. Assay

An exemplary method for detecting the presence, absence or abundance of a molecule in a sample, e.g., biological sample, involves obtaining a biological sample from a test subject and contacting the biological sample with an immobilized membrane protein. In one embodiment, the biological sample is contacted with the

immobilized membrane protein in the presence of a competitive binding agent capable of binding in a competitive manner with the molecule in the sample. A competitive binding agent is employed to detect the presence, absence or abundance of a molecule in a sample which similarly binds to the immobilized protein.

In one embodiment of the invention, a method is provided for detecting a molecule in a sample that specifically binds to a membrane protein, comprising (a) contacting a membrane protein immobilized on a solid support according to the methods of the invention; (b) contacting the immobilized protein with an agent capable of binding to the membrane protein in a competitive reaction with the molecule; (c) detecting the agent bound to the immobilized membrane protein in the absence of the sample; (d) detecting the agent bound to the immobilized membrane protein in the presence of the sample; wherein a decreased amount of agent bound to the immobilized membrane protein in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of a molecule that specifically binds to the membrane protein. In a preferred embodiment, the invention provides a method for detecting autoantibodies to human thyroid stimulating hormone (TSH) receptor in a sample, comprising: (a) contacting TSH receptor immobilized on a solid support prepared according to the method of claim 27 with a sample; (b) contacting the immobilized TSH receptor with an agent capable of binding to the TSH receptor in a competitive reaction with TSH receptor autoantibodies; (c) detecting the agent bound to the immobilized TSH receptor in the absence of the sample; (d) detecting the agent bound to the immobilized TSH receptor in the presence of the sample; wherein a decreased amount of agent bound to the immobilized TSH receptor in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of autoantibodies to human thyroid stimulating hormone receptor.

In order to conduct assays with the above-mentioned approaches, the non-immobilized component, *e.g.*, sample and/or competitive binding agent, is added to the solid phase upon which the membrane protein is fixed. After the reaction is complete, uncomplexed components may be removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized upon the solid phase. The detection of complexes anchored to the solid phase can be accomplished in a number of methods outlined herein. In a preferred embodiment, the competitive binding agent, *e.g.*, also referred to herein as a tracer, can be labeled for the purpose

of detection and readout of the assay, either directly or indirectly, with detectable labels discussed herein well-known to one skilled in the art. The solid-phase assays of the invention can be carried out such that the steps of contacting are performed simultaneously. For example, in one embodiment, the immobilized membrane protein is contacted with the sample and the competitive binding agent at the same time and a competition for binding is allowed to proceed. The solid-phase assay of the invention can alternatively be carried out such that the steps of contacting are performed sequentially. For example, in one embodiment, the immobilized membrane protein is first contacted with the sample, the solid support is washed and the immobilized membrane protein is then contacted with the competitive binding agent. In another embodiment, the immobilized membrane protein is first contacted with the competitive binding agent, the solid support is washed and the immobilized membrane protein is then contacted with the sample. It will be understood by one skilled in the art that the amounts of components and sequence of contacting steps are carried out in the methods of the invention according to biochemical principles such that a competition in binding can be observed. The presence, absence or abundance of molecule in a sample can be detected by determining the amount of competitive binding agent bound in the presence of the sample as compared to the amount of agent bound in the absence of the sample. One of skill in the art will recognize that the presence, absence or abundance of molecule in a sample can also be detected by determining the amount of competitive binding agent bound in the presence of the sample as compared to the amount of agent bound in the presence of a suitable control, e.g., sera that is known not to contain the molecule. The presence, absence or abundance of molecule in a sample can also be detected by determining the amount of competitive binding agent bound in the presence of the sample as compared to that bound according to a standard curve that is prepared with standards for the molecule, e.g., standard preparations containing known amounts of the molecule. It will be understood that the amount of competitive agent bound can be assessed by any appropriate normalizing or standardizing method standardly known in the art.

The present invention is further intended to encompass the detection of the molecule bound to the immobilized membrane protein by direct, rather than competitive, means, e.g., by any convenient agent, e.g., antibody or small molecule, that is capable of recognizing and binding to the molecule of the sample when bound to the immobilized membrane protein. Such agents can be detected in the assay by

any means known in the art or as described herein, *e.g.*, by radiolabel, chemiluminescence or fluorescence. Such agents can be labeled prior to addition to the assay system or may be labeled after addition to the assay, *e.g.*, once bound to the molecule.

B. Immobilization

A method of the present invention for conducting an assay comprises anchoring the membrane protein onto a solid phase support, also referred to as a substrate, and detecting binding agent/membrane protein complexes anchored on the solid phase at the end of the reaction. In a preferred embodiment of such a method, the membrane protein can be anchored to a solid phase and a sample from a subject can be allowed to react as an unanchored component of the assay, *e.g.*, in the presence of a labeled competitive binding agent. Preferably, proteins immobilized on a solid support retain their functional and epitopic properties, *e.g.*, retain their ability to bind to their natural binding partners.

There are many established methods for anchoring assay components to a solid phase. These include, without limitation, membrane proteins which are immobilized by direct adsorption onto the solid support, through conjugation of biotin and streptavidin or through an antibody to the membrane protein. Biotinylated assay components can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).

In a preferred embodiment of the invention, methods are provided for directly immobilizing a membrane protein on a solid support, comprising contacting a solid support with a soluble cell membrane fraction prepared according to the methods of the invention, under conditions sufficient to permit direct adsorption of the membrane protein to the solid support. In one embodiment, the invention features a method for preparing a solid support on which one or more membrane proteins are immobilized, wherein the solid support is suitable for detecting molecules that bind the membrane protein, comprising: (a) mixing a detergent solubilized cell membrane solution containing one or more membrane proteins with salt ion, such that a resulting supernatant fraction is enriched for the membrane proteins; (b) contacting a solid support with the supernatant fraction under conditions such that the membrane proteins adsorb directly on the solid support; thereby providing membrane proteins

immobilized on a solid support suitable for detecting molecules that bind the membrane proteins. Examples of suitable conditions include, without limitation, constant shaking or agitation throughout the coating step and incubation at 4°C.

To immobilize the membrane protein, *e.g.*, TSH receptor, directly on a solid support, *e.g.*, a tube, a suitable volume of the soluble membrane solution (*e.g.*, from about 100 to 1000 microlitres) is brought into contact with the solid support, *e.g.*, aliquotted into tubes (*e.g.*, polystyrene Nunc Star tubes), and incubated overnight at room temperature with agitation. The solid support, *e.g.*, tubes, are then washed with buffer, *e.g.*, 50 mM phosphate, 0.9 % NaCl, pH 7.5, to remove all membrane proteins that are not bound to the tube. Following this wash, the solid support, *e.g.*, tubes, are ready for use or alternatively can be dried and stored at 4°C until ready for use. In one embodiment of the invention, membrane protein, *e.g.*, TSH receptor, is directly coated onto superparamagnetic beads. Magnetic beads are commercially available from companies such as Dynal in Norway. The same methodology can be used to immobilize membrane proteins, *e.g.*, TSH receptor, on beads as that described above for immobilizing membrane proteins, *e.g.*, TSH receptor, on polystyrene tubes. The superparamagnetic beads can then be used in a receptor binding assay to detect, *e.g.*, TSH or TSH receptor autoantibodies in a sample. Separation of bound agent from free agent is carried out by using a magnet to collect the superparamagnetic beads on the wall of the tube while the liquid containing unbound tracer is aspirated. The beads are resuspended with buffer following removal of the magnetic field. Collection and resuspension of the beads can be repeated as often as is necessary to wash the beads.

Solid supports of the invention can comprise a plastic material, a magnetic material or a non-magnetic material. In one embodiment, the solid support is a plastic material, *e.g.*, polystyrene. In one preferred embodiment, the solid support is a magnetic material, *e.g.*, a magnetic bead. Other suitable carriers or solid phase supports for such assays include any material capable of binding the class of molecule to which the membrane protein belongs. Other well-known supports or carriers include, but are not limited to, glass, polystyrene, nylon, polypropylene, nylon, polyethylene, dextran, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. Solid supports can be of any form convenient to allow contact between the immobilized membrane protein and the sample containing the binding molecule. Solid supports useful in the invention include, without limitation, test tubes, microwell titer plates and beads. In certain embodiments of the invention,

the surfaces with immobilized assay components can be prepared in advance and stored.

C. Competitive binding agents

The methods of the invention provide binding agents, *e.g.*, competitive binding agents, which are able to bind the membrane protein of interest in a competitive manner with the molecule to be detected in a sample. Any binding agent that binds to the immobilized membrane protein in a competitive manner with the molecule to be detected in a sample can be used in a solid phase assay to detect the presence or aberrant abundance of the molecule (*e.g.*, in a bodily fluid). In one embodiment, a binding agent is a protein or peptide. In one embodiment, the binding agent is an agonist or antagonist of the immobilized membrane protein. In one embodiment, the binding agent is a ligand of an immobilized membrane receptor, *e.g.*, a hormone and hormone receptor. In one embodiment, TSH is used as a competitive binding agent in the solid phase TSH receptor assays of the invention. TSH can be purified from natural sources or can be chemically synthesized. TSH can be from any species such that the TSH binds to the immobilized TSH receptor. In one embodiment, TSH is human, bovine or porcine TSH.

A multitude of protected, unprotected, and partially protected natural and unnatural functional analogs and derivatives of the competitive binding agent are also intended to be within the scope of the invention. Also within the scope of the invention are derivatives of naturally-occurring forms of the binding agent which are engineered and expressed in a cell based upon a knowledge of the sequence of the naturally-occurring amino acid sequence of the genetic material (DNA or RNA) which encodes the sequence. As used herein, the term "altered peptide" refers to chemical modifications of natural or unnatural proteins or peptides, *e.g.*, proteins or peptides in which one or more amino acids which are not present in the original sequence are added, deleted, or otherwise changed, and derivatives thereof.

It will be appreciated that a portion or segment of the described sequence can also be used in the invention so long as it is sufficiently characteristic of the desired protein or fragment thereof to bind the immobilized membrane protein in a competitive manner with the molecule, *e.g.*, molecule in a sample. Such variations in the sequence can easily be made, for example by synthesizing an alternative sequence.

The alternate sequence can then be tested, for example, in an in vitro system to ascertain its ability to bind the immobilized membrane protein.

As known in the art, amino acid residues may be in their (1) protected form in which both amino and carboxy groups possess appropriate protecting groups, (2) partially-protected form in which either amino or carboxy groups possess appropriate protecting groups, or (3) unprotected form in which neither amino nor carboxy groups possess an appropriate protecting group. Numerous reactions for the formation and removal of such protecting groups are described in a number of standard works including, for example, "Protective Groups in Organic Chemistry", Plenum Press (London and New York, 1973); Green, T. H., "Protective Groups in Organic Synthesis", Wiley (New York, 1981); and "The Peptides", Vol. I, Schroder and Lubke, Academic Press (London and New York, 1965). All such forms are encompassed within the present invention. Representative amino protecting groups include, for example, formyl, acetyl, isopropyl, butoxycarbonyl, fluorenylmethoxycarbonyl, carbobenzyloxy, and the like. Representative carboxy protecting groups include, for example, benzyl ester, methyl ester, ethyl ester, t-butyl ester, p-nitro phenyl ester, and the like.

In addition to protected forms in which both amino and carboxy groups possess appropriate protecting groups, the binding agent of the present invention may also be modified. As used herein, the term "modified" refers to those proteins or peptides which have been modified, *e.g.*, altered, varied and the like, in which to increase the stability or half-life of the protein or peptide. Such modifications include, but are not limited to, PEGylation, complexing the IGF-2 peptide with an MHC tetramer, *e.g.*, an MHC class II tetramer, and the like. Processes for preparing these modified peptides are well known to those of ordinary skill.

Other competitive binding agents are also encompassed by the instant invention. An immunoglobulin or antibody that binds to the immobilized membrane protein in a competitive manner with the molecule to be detected in a sample can be used in a solid phase assay to detect the presence or aberrant abundance of the molecule (*e.g.*, in a bodily fluid). In one embodiment, human immunoglobulin is used as a competitive binding agent in the solid phase assays of the invention. In one embodiment, an antibody, *e.g.*, a purified antibody, to TSH receptor is used as a competitive binding agent of the invention. Detection can be facilitated by the use of

an antibody derivative, which comprises an antibody of the invention coupled to a detectable substance.

Accordingly, in one aspect, the invention provides substantially purified antibodies, antibody fragments and derivatives, all of which specifically bind to a membrane protein of the invention. In various embodiments, the substantially purified antibodies of the invention, or fragments or derivatives thereof, can be human, non-human, chimeric and/or humanized antibodies. In another aspect, the invention provides non-human antibodies, antibody fragments and derivatives, all of which specifically bind to a polypeptide of the invention and preferably, a marker polypeptide. Such non-human antibodies can be goat, mouse, sheep, horse, chicken, rabbit, or rat antibodies. Alternatively, the non-human antibodies of the invention can be chimeric and/or humanized antibodies. In addition, the non-human antibodies of the invention can be polyclonal antibodies or monoclonal antibodies. In still a further aspect, the invention provides monoclonal antibodies, antibody fragments and derivatives, all of which specifically bind to a polypeptide of the invention and preferably, a marker polypeptide. The monoclonal antibodies can be human, humanized, chimeric and/or non-human antibodies.

Determining the amount of competitive binding agent bound to an immobilized membrane protein can be accomplished, for example, by coupling the competitive binding agent with a radioisotope or enzymatic label such that binding of the competitive binding agent to the immobilized membrane protein can be determined by detecting the labeled binding agent in the solid phase. For example, binding agents (*e.g.*, TSH) can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, assay components can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. The competitive binding agents of the invention can be detected using any of the means for molecule detections standardly known in the art. In one embodiment, the binding agent, *e.g.*, TSH, is radiolabeled. In a preferred embodiment, the radiolabel is ^{125}I . Examples of suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , ^{32}P or ^3H ; examples of suitable enzymes

include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of bioluminescent materials include luciferase, luciferin, and aequorin.

D. Kits

The invention includes a kit for assessing the presence, absence or abundance (*e.g.*, aberrant abundance) of a molecule that binds to the immobilized membrane protein (*e.g.* in a sample such as a patient sample). A kit of the invention, in its simplest form, provides a soluble membrane preparation, *e.g.*, membrane fraction enriched for one or more membrane proteins, suitable for direct immobilization on a solid support. A kit of the invention may provide a soluble membrane preparation, *e.g.*, containing one or more membrane proteins of interest, *e.g.*, TSH receptor, immobilized on a solid support, *e.g.*, coated on test tubes, microtiter wells or magnetic beads. A kit could additionally provide a labeled competitive binding agent, *e.g.*, TSH. The kit of the invention may optionally comprise additional components useful for performing the methods of the invention. By way of example, the kit may comprise fluids (*e.g.* buffer) suitable for binding a competitive binding agent with immobilized membrane protein, one or more sample compartments, instructional material, such as an instruction manual, which describes performance of a method of the invention, and a positive and or negative control. A complete kit could further include, without limitation, test tubes containing a suitable amount of immobilized membrane protein, *e.g.*, TSH receptor; labeled competitive binding agent, *e.g.*, TSH, in either liquid or lyophilised form; non-specific binding solution, *e.g.*, a solution suitable for measuring non-specific binding, *i.e.*, binding to anything other than the TSH binding site; a solution of serum that does not effect inhibition in the competitive binding assay; a standard curve, *e.g.*, consisting of a series of sera samples containing predetermined increasing quantities of binding agent, *e.g.*, TSH, so that increasing inhibition of competitive agent, *e.g.*, tracer, binding would be measured when these sera are included in the test; a control sample, *e.g.*, a sample of serum with a defined percentage of inhibition, to allow the correct performance of the test to be confirmed;

buffer solution(s) suitable for re-hydrating any lyophilised reagents, or suitable for washing the tubes; and a package insert containing the "instructions for use" of the kit. In kits providing membrane proteins pre-immobilized on a solid support, sufficient membrane protein, *e.g.*, TSH receptor, would preferably be coated so as to provide high specific binding but with a concentration sufficient to allow inhibition of binding of competitive binding agent, *e.g.*, TSH tracer, by the low concentrations of autoantibodies present in patients sera.

The compositions, kits and diagnostic methods of the invention are useful in the automation of solid-phase assays for the detection of molecules which bind to a particular membrane protein, *e.g.*, for the detection of autoantibodies associated with a thyroid disease or disorder, *e.g.*, autoantibodies to TSH receptor associated with Graves' disease. One of skill in the art will recognize that the methods described herein can be automated without undue experimentation.

V. Diagnostic and Clinical Applications

The compositions, kits and methods of the invention can be used to detect any disease or disorder associated with the absence, presence or abundance, *e.g.*, aberrant abundance, of a molecule that binds to a membrane protein, *e.g.*, an autoantibody to a membrane protein or a ligand for a membrane receptor. In a preferred embodiment, the disease or disorder is a thyroid disease, *e.g.*, an autoimmune thyroid disease (AITD). The invention thus includes a method of assessing whether a patient is afflicted with a disease or disorder associated with a molecule that binds to an immobilized membrane protein, *e.g.*, a thyroid disorder, *e.g.*, autoimmune thyroid disease. This method comprises detecting the molecule in a patient sample by using a solid phase assay of the invention, *e.g.*, solid phase TSH receptor assay. The presence, absence, or aberrant abundance of the molecule in the patient sample as compared to the normal abundance is an indication that the patient is afflicted with the particular disease or disorder.

The compositions, kits, and methods of the invention have the following additional uses, among others:

- 1) assessing whether a patient is afflicted with a disease or disorder, *e.g.*, a thyroid disorder;

- 2) assessing the stage of a disease or disorder, *e.g.*, a thyroid disorder, in a human patient;
- 3) predicting the clinical outcome of a patient diagnosed with disease or disorder, *e.g.*, a thyroid disorder;
- 6) assessing the efficacy of one or more test compounds for inhibiting the disease or disorder, *e.g.*, a thyroid disorder, in a patient;
- 7) assessing the efficacy of a therapy for inhibiting the disease or disorder, *e.g.*, thyroid disorder, in a patient;
- 8) monitoring the progression of the disease or disorder, *e.g.*, thyroid disorder, in a patient; and
- 9) selecting a composition or therapy for inhibiting the disease or disorder, *e.g.*, thyroid disorder, in a patient.

Autoimmune thyroid disease causes cellular damage and alters thyroid gland function by humoral and cell-mediated mechanisms. Cellular damage occurs when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes causing cell lysis and inflammatory reactions. Alterations in thyroid gland function result from the action of stimulating or blocking autoantibodies on cell membrane receptors. In one embodiment of the invention, primary preparations of soluble membrane or membrane fractions contain one of the three principal thyroid autoantigens involved in AITD, *e.g.*, thyroperoxidase (also referred to herein as thyroid peroxidase) (TPO), thyroglobulin (Tg) and the TSH receptor. Other thyroid autoantigens, such as the Sodium Iodide Symporter (NIS) are also encompassed in the instant invention. As yet, however, no diagnostic role in thyroid autoimmunity for NIS has been established.

The compositions, kits and methods of the invention are thus useful in detecting or diagnosing a disease or disorder associated with an autoantibody to a thyroid autoantigen. For example, TSH receptor autoantibodies (TRAb) are heterogeneous and may either mimic the action of TSH and cause hyperthyroidism as observed in Graves' disease or alternatively, antagonize the action of TSH and cause hypothyroidism. The latter occurs most notable in the neonate as a result of a mother with antibodies due to AITD. TPO antibodies (TPOAb) have been involved in the tissue destructive processes associated with the hypothyroidism observed in

Hashimoto's and atrophic thyroiditis. The appearance of TPOAb usually precedes the development of thyroid dysfunction. Some studies suggest that TPOAb may be cytotoxic to the thyroid. The pathologic role of autoantibody to Tg (TgAb) remains unclear. In iodide sufficient areas, TgAb is primarily determined as an adjunct test to serum Tg measurement, because the presence of TgAb can interfere with the methods that quantitate Tg. In iodide deficient areas, serum TgAb measurements may be useful for detecting autoimmune thyroid disease in patients with a nodular goiter and for monitoring iodide therapy for endemic goiter. Serum TgAb measurements may also be useful in the detection, diagnosis or monitoring the progression of thyroid cancer, *e.g.*, thyroid carcinomas.

Thyroid diseases or disorders for which the compositions, kits and diagnostic methods are useful include, without limitation, hyperthyroidism, hypothyroidism, Graves' disease, Hashimoto's disease (*e.g.*, Hashimoto's thyroiditis), atrophic thyroiditis, nodular giter, endemic goiterand thyroid cancer, *e.g.*, thyroid carcinomas. It will be understood by one skilled in the art that any autoimmune disease or disorder involving the appearance of autoantibodies specific for the thyroid antigens set forth above in the bodily fluids of a subject are diseases or disorders for which the compositions, kits and diagnostic methods are useful. In one embodiment, the compositions, kits and diagnostic methods of the invention can be used for family or population screening for the detection of subjects at risk for developing an autoimmune disease or disorder, *e.g.*, an autoimmune disease affecting the thyroid gland. In various embodiments, detection of autoantibodies to TPO using the methods of the invention are useful in the following applications, without limitation: in the diagnosis of autoimmune thyroid disease; as a risk factor for autoimmune thyroid disease, hypothyroidism during Interferon alpha, Interleukin-2 therapy, Lithium therapy, thyroid disfunction during amiodarone therapy, hypothyroidism in Down's Syndrome patients, thyroid disfunction during pregnancy, post-partum thyroiditis and for miscarriage and in-vitro fertilization failure. In other various embodiments, detection of autoantibodies to TSH receptor (TRAb) are useful in the following applications, without limitation: diagnosis of Graves' disease; for relating TRAb values to a treatment algorithm for Graves' disease; to investigate the etiology of hyperthyroidism when the diagnosis is not clinically obvious; to detect remission of hyperthyroidism; to evaluate patients suspected of "euthyroid Graves' ophthalmopathy"; to monitor pregnant women with a past or present history of Graves'

disease; to monitor euthyroid pregnant women who have had prior radioiodide treatment for Graves' disease and during the third trimester to evaluate the risk of neonatal hyperthyroidism; to monitor pregnant woman who take antithyroid drugs for Graves' disease to maintain a euthyroid state during pregnancy; assessment of the risk of fetal and neonatal thyroid dysfunction; to identify neonates with transient hypothyroidism due to the presence of TSH receptor blocking antibodies.

It will be appreciated that a variety of patient samples may be used in the methods of the present invention. In these embodiments, the presence or aberrant abundance of the binding molecule can be assessed by assessing the amount (*e.g.* absolute amount or concentration) of the molecule in the patient sample, *e.g.*, a bodily fluid, such as a serum sample. The bodily fluid can be obtained from the subject using any available method, which may be selected on the basis of the amount of fluid required. In certain cases, a collected blood sample may be used in a variety of tests and only a portion or aliquot of the sample drawn will be required for use in the methods described herein. The amount of the marker(s) of the invention can be determined in whole blood or in a fraction of the blood. Preferably, the amount of the binding molecule is determined for a cell-free fraction of the blood, such as the plasma or the serum. It is particularly preferred to determine the amount of marker(s) in the serum. In embodiments in which only a fraction of the blood is used in the analysis, the method also includes the steps of separating the desired blood fraction from the whole blood acquired from the subject. This separation of blood fractions can be achieved using methods which are well-known in the art.

The bodily fluid may be further processed, as is known in the art, prior to the measurement of the marker. For example, the fluid can be processed to remove a particular protein, such as serum albumin, a set of proteins, or cells or cell components which are present in the fluid and which may interfere with the analysis. The processing can include steps such as precipitation, chromatography, centrifugation, ultrafiltration and dialysis.

The cell sample can, of course, be subjected to a variety of well-known post-collection preparative and storage techniques (*e.g.*, nucleic acid and/or protein extraction, fixation, storage, freezing, ultrafiltration, concentration, evaporation, centrifugation, *etc.*) prior to assessing the amount of the marker in the sample.

The invention is further illustrated by the following examples which in no way should be construed as being further limiting.

EXAMPLES

Example I: Solid-phase assay to detect anti-TSH receptor antibodies in buffer

A solid phase assay was first carried out to demonstrate that TSH receptor directly immobilized on a solid support can bind and thereby detect molecules that bind TSH receptor, such as an antibody to TSH receptor.

To immobilize the TSH receptor, a suitable volume of the receptor solution (*e.g.*, from about 100 to 1000 microlitres) was aliquoted into polystyrene Nunc Star tubes and the tubes were incubated overnight at room temperature with agitation. The tubes were then washed with buffer, *e.g.*, 50 mM phosphate, 0.9 % NaCl, pH 7.5, to remove all TSH receptor that was not bound to the tube. Following this wash, the tubes were used directly, or alternatively, the tubes were dried and stored at 4°C until ready for use.

The assay was carried out in buffer with TSH receptor bound to a tube and by using radiolabeled bovine TSH tracer (^{125}I -bTSH) to monitor binding. Non specific binding was measured by incubating the tracer and solid phase receptor in the presence of an excess of unlabeled, bovine TSH (bTSH). Binding of the TSH tracer was measured by incubating the radiolabeled tracer in the absence of unlabeled bTSH. The results of this solid phase assay are provided in Table 1.

Table 1: Solid phase assay performed in buffer.

<u>Tube</u>	<u>Cpm</u>	<u>Sample</u>
1	57188	Total counts (cpm)
2	63051	Total counts (cpm)
3	2570	^{125}I -bTSH binding in the presence of 1 μg of bTSH (NSB)
4	7214	^{125}I -bTSH binding in the presence of 1 μg of bTSH (NSB)
5	14787	^{125}I -bTSH binding in absence of bTSH (B0)
6	15601	^{125}I -bTSH binding in absence of bTSH (B0)

Specific binding (*i.e.*, binding in the absence of bTSH (B0) minus the non-specific binding observed in the presence of an excess of bTSH (NSB)) was observed to be, on average, 10,302 cpm, which represented approximately 17.1% of the total radioactivity added to the tub (60,119 cpm on average). These data showed that the solid phase receptor was capable of binding the radiolabeled TSH tracer and that it was possible to effectively inhibit the binding using 1 μ g unlabeled TSH. This data indicated that the solid phase assay could be used to detect any molecule that binds to the TSH binding site, since if the molecule is incubated with labeled TSH and solid phase receptor, binding of the radiolabeled TSH to the receptor will be inhibited and this inhibition can be successfully measured.

Example II: Solid-phase assay to detect autoantibodies to TSH receptor in sera from patients with Graves' disease

A solid phase assay was next carried out to demonstrate that a solid phase TSH receptor assay could be used to detect autoantibodies in the serum of patients afflicted with Graves' disease. The assay was performed using sera from patients with clinically diagnosed Graves' disease, and thus containing autoantibodies to TSH receptor. The "B0" and "NSB" samples contained sera from a serum pool that came from a healthy population with no evidence of thyroid disease. The assay was carried out in two steps, as described above, in a total volume of 100 microliters and was performed in duplicate. The extent to which autoantibodies present in the serum from patients with Graves' disease inhibited the binding of 125 I-bTSH was calculated and presented in Table 2.

Table 2: Solid phase assay performed with serum from patients with Graves' disease

<u>Sample</u>	<u>cpm</u>	<u>Mean cpm</u>	<u>[cpm- NSB]</u>	<u>% Inhibition</u>
Total counts (cpm)	60115	60028		
Total counts (cpm)	59941			
non specific binding (NSB)	1904	1952		
non specific binding (NSB)	1999			
125 I-bTSH binding in absence of bTSH (BO)	5788	6091	4139	00.0

¹²⁵ I-bTSH binding in absence of bTSH (BO)	6393			
Graves' disease patient No 1	3217	3187	1235	70.2
Graves' disease patient No 1	3157			
Graves' disease patient No 2	2291	2402	450	89.1
Graves' disease patient No 2	2513			

These data showed that the presence of sera from Graves' disease patients inhibited the binding of the ¹²⁵I-bTSH to the solid phase receptor, thus indicating the presence of autoantibodies to the TSH receptor in these patients. This assay is not restricted to Graves' disease patients, as the serum from any patient with autoimmune thyroid disease which contains autoantibodies to the TSH receptor (*e.g.*, antibodies that bind to the TSH binding site of the TSH receptor) will be detected in a like manner.

Example III: TSH receptor immobilized on beads to detect anti-TSH receptor antibodies

An assay was next carried out with TSH receptor immobilized on superparamagnetic beads (Dynal) to demonstrate that TSH receptor bound to beads can bind ¹²⁵I-bTSH. To prepare the TSH receptor immobilized on beads, approximately 1 ml of beads (6.5×10^8 beads) was washed three times with phosphate buffered saline (PBS) using a magnet to collect the beads on the tube wall and the buffer removed by aspiration. The beads were incubated with the solution of soluble receptor for 15 hours with agitation. Using a magnet to collect the beads from solution, the beads were washed three times with PBS. Approximately 100 μ l aliquots of the beads were then incubated with ¹²⁵I-bTSH in the presence of 50 μ l of PBS containing an excess of bTSH (NSB) or 50 μ l of PBS (BO). The tubes were incubated with agitation for 3 hours. The reaction was terminated by collecting the beads on the side of the tubes with a magnet and aspirating the liquid reaction mixture. The beads were then washed three times with PBS. Following the last wash, the tubes containing the beads were counted in a gamma counter for 1 min. The amount of radioactivity (cpm) recorded is presented in Table 3 below.

Table 3: Solid phase assay using TSH receptor immobilized on beads.

<u>Sample</u>	<u>cpm</u>	<u>Mean cpm</u>	<u>[B0-NSB]</u>	<u>Bound/total (%)</u>
Total counts	115549			
Total counts	115327	115438		
NSB	5149			
NSB	5139	5144		
B0	17523			
B0	13710	15617	10437	8.9

The data in Table 3 indicated that 10473 cpm of ^{125}I -bTSH bound specifically to the TSH receptor-coated beads, and that this 10473 cpm was displaceable by TSH. These results indicated that any molecule which can bind to the TSH binding site of the TSH receptor, *e.g.*, autoantibody to the TSH receptor, would inhibit binding of the ^{125}I -bTSH tracer and therefore be detected in the assay. These results further indicated that TSH receptor immobilized on superparamagnetic beads could be used to detect autoantibodies to the TSH receptor in patients with thyroid autoimmune disease.

In the Examples set forth herein, the bTSH was labeled with radioactive iodine (^{125}I -bTSH). Similar assays can be carried out using other radiolabels commonly used in the art. Non-radioactive labels could also be employed. Examples of non-radioactive labels include, but are not limited to, fluorescent, luminescent, or chemiluminescent molecules. Additionally, the TSH could be labeled with an enzyme, *e.g.*, horseradish peroxidase, alkaline phosphatase or beta lactamase. When an enzyme is used to detect the bound TSH, an appropriate substrate for the particular enzyme is added following separation of bound and free TSH. The enzymatic reaction is allowed to proceed in order to quantify the concentration of enzyme-labeled TSH in the bound and/or the free fraction. The TSH can also be labeled with molecules such as biotin, and following separation of bound from free fractions, streptavidin (or avidin or neutravidin) coupled to an enzyme is added to the tubes or beads in an appropriate buffer. After incubation for a length of time sufficient to allow the biotin and streptavidin to bind, the tubes or beads are washed and the enzymatic

reaction can then be used as described above to quantify the biotinylated TSH in the bound fraction.

Example IV: Optimal conditions for preparation of soluble membrane fraction containing TSH receptor

Conditions were next optimized for preparation of the primary preparation of detergent solubilized cell membranes. Porcine thyroid glands were minced, treated by polytron in ice cold Tris-HCL buffer, pH 7.5, and the resulting suspension was centrifuged. The supernatant was removed and the pellet was resuspended, ground on ice and recentrifuged for 30 minutes at 7500 rpm. The pellet was then resuspended in a detergent solution to solubilize the membranes. The optimal detergent was found to be Thesit detergent (836630 Roche), which was used at a concentration of 4.5g/600 ml in Tris buffer. These conditions limited the polytron phase and utilized lower detergent concentrations as compared to conventional protocols standardly used in the art, such as the protocol used for TRAb BC1007 in an IVD kit (Biocode). The solubilized membrane was centrifuged at 7500 rpm and the resulting supernatant was collected and filtered over cotton. The filtrate was then subjected to ultracentrifugation for 180 minutes at 35,000 rpm at 4°C. The top lipid layer and the pellet were eliminated, and the supernatant was collected.

As seen in Figure 1, the conditions set forth above produced a primary membrane preparation of lower total protein concentration than the conventional protocol, but which advantageously controlled the protein contents of the membrane preparation containing the receptor. The conditions consistently yielded a preparation with a predictable and constant TSH receptor: total protein ratio that ultimately functioned well following a further fractionation step in subsequent solid phase assays. This primary preparation of detergent solubilized membrane was either used directly in the next fractionation step, or was frozen and stored until ready for use. The primary preparation of detergent solubilized membrane was alternatively subjected to lyophilisation and the lyophilized material stored until ready for use. The lyophilized material was redissolved in buffer and subjected to fractionation as described below.

The primary preparation of detergent solubilized membrane was then subjected to a physicochemical fractionation, yielding a membrane fraction enriched

for TSH receptor and in a milieu that allowed the membrane proteins to be directly adsorbed on a solid support. Specifically, 450 μ l of ammonium sulfate (70g/100ml) was added to 1.8 ml of the primary membrane preparation and mixed for 2 hours at 4°C. The mixture was then centrifuged for 10 minutes at 4°C and the soluble supernatant was recovered. This membrane fraction was then used to directly adsorb the membrane proteins contained in the fraction onto the surface of tubes or beads. Several membrane preparations, both before and after the fractionation step, were coated onto the inside surface of tubes and tested for binding of radiolabeled bTSH as described above. As presented in Figure 2, greater TSH binding indicating greater amounts of immobilized TSH receptor was observed following fractionation of the membrane preparation for three different primary membrane preparations. The fractionation step facilitated efficient coating of a solid support when the primary membrane preparation was prepared according to the optimized method as set forth above (RD001), and additionally was observed to allow efficient coating if the primary receptor preparation was prepared according to less optimal protocols and yielded primary preparations of varying protein concentrations (91 and 95). It was additionally observed that lyophilisation of the primary membrane preparation prior to fractionation provided a slightly more efficient coating. For example, as provided in Figure 3, greater binding of radiolabeled bTSH was observed when the tube was coated with a membrane fraction which was lyophilized before the fractionation step as compared to a tube coated with a membrane fraction that was not lyophilized before the fractionation step.

Example V: Further optimization of conditions for solid phase TRAb competitive binding assay

Tubes (Nunc maxisorb) or wells (Starwell C8 maxisorb) were coated with the membrane fraction containing TSH receptor as prepared in Example IV. Coating was achieved by directly contacting the inner surface of the tubes or wells with the membrane fraction and incubating for 18-24 hours with gentle rotation or agitation at 25°C. The tubes or wells were emptied and rinsed extensively with PBS buffer, coated with milk proteins to limit non-specific binding, washed again and dried.

The coated tubes as prepared above were then tested for sensitivity in a competitive binding assay. Binding by a standard antibody against TSH receptor was

measured by competition with ^{125}I -bTSH. The standard used was WHO 90/672 (TSAb), which is a standard prepared from a pool of patients' sera containing autoantibodies to TSH receptor. To initiate a standard assay, 100 μl of biological sample, or in this case, 100 μl volume containing the standard antibody WHO 90/672, was dispensed in the coated tubes and incubated for 2 hours at room temperature with constant agitation to allow the antibodies in the sample to bind to the immobilized receptor. Following incubation, the solution was removed, the tubes were washed three times and radiolabeled TSH was then added to the tubes. Either purified native bovine TSH or purified native porcine TSH was used, and both have been used successfully. Approximately 30,000 cpm of radiolabeled TSH was added to each tube. The tubes were incubated with the radiolabeled TSH for 2 hours with constant agitation at room temperature. The solution was then removed by aspiration, the tubes washed three times and then counted. Control samples for nonspecific binding contained an excess (approximately 1 μg) of purified unlabeled TSH.

As shown in Figure 4, the WHO 90/672 standard was able to displace the radiolabeled bTSH tracer over a range of concentrations of input standard. The sensitivity observed was comparable to the international standard for analytical sensitivity of 3.19 IU/L. As shown in Figure 5 and summarized in Table 4 below, both porcine and bovine TSH yielded comparable sensitivity in the assay.

Table 4: Porcine vs. bovine TSH tracer in solid phase assay

	<u>bTSH</u>	<u>pTSH</u>
Total input counts	30418	31613
NSB/Total measured	3.8%	8.1%
B0/Total measured	30.8%	52.8%

The assay was then used to measure TSH tracer displacement effected by four quality control samples. The quality control samples were pools of serum samples with known increasing amounts of tracer binding inhibition potency as previously determined using TRAb BC1007, a standard liquid phase TSH receptor radioligand assay used as a reference for accuracy and sensitivity. The TRAb titers of the quality control samples are expressed in terms of percent inhibition of TSH tracer binding.

Table 5: TRAb titers of quality control samples as measured by reference liquid-phase and experimental solid-phase assays.

	<u>QC</u> <u>Sample 1</u> (% Inhibition)	<u>QC</u> <u>Sample 2</u> (% Inhibition)	<u>QC</u> <u>Sample 3</u> (% Inhibition)	<u>QC</u> <u>Sample 4</u> (% Inhibition)
QC specifications (TRAb BC1007)	5.0-14.0	15.4-26.2	29.1-40.3	59.1-77.1
bTSH tracer	11.0	20.4	30.5	52.2
pTSH tracer	16.9	25.3	20.1	44.7

Finally, the solid-phase assay was used to measure TSH tracer displacement effected by serum samples from patients suffering from Graves' disease and who were selected according to data yielded by using the reference TRAb BC1007 liquid phase assay. The results are presented in Table 6 below.

Table 6: TRAb titers of human sera as measured by reference liquid-phase and experimental solid-phase assays.

	<u>Human</u> <u>serum 1</u> (% Inhibition)	<u>Human</u> <u>serum 2</u> (% Inhibition)	<u>Human</u> <u>serum 3</u> (% Inhibition)	<u>Human</u> <u>serum 4</u> (% Inhibition)
Titers measured in TRAb BC1007 liquid phase assay	26.0	32.0	42.0	78.0
bTSH tracer	14.5	17.7	27.1	45.6
pTSH tracer	7.2	8.4	12.4	41.9

The solid-phase assay yielded results that accurately reflect the content of antibody titer against TSH receptor in the patients' sera, as measured by the reference assay, and the data is directly comparable to the data obtained using the standard liquid phase assay TRAb BC1007. These results thus demonstrate that the solid-phase assay developed in the instant invention can be used to accurately detect autoantibodies present in a patients' sera. The solid phase assay of the instant

invention can thus be used in diagnostic assays for Graves' disease and can equally be extended to the diagnosis of other thyroid autoimmune diseases.

Incorporation by Reference

The contents of all references (including GenBank references, literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated herein in their entireties by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. In addition, the entire contents of all patents and publications cited herein is hereby incorporated by reference.

What is claimed is:

1. A method for preparing soluble cell membranes suitable for direct immobilization on a solid support, comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt.
2. A method for preparing a soluble cell membrane fraction enriched for one or more membrane proteins, wherein the one or more membrane proteins are capable of adsorbing directly on a solid support, comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt ions.
3. The method of claim 1 or 2, wherein the salt is selected from the group consisting of ammonium sulfate, ammonium phosphate, potassium phosphate, rubidium phosphate, sodium phosphate, other Hofmeister salts, and combinations thereof.
4. The method of claim 1 or 2, wherein the salt ions are ammonium sulfate.
5. The method of claim 4, wherein the ammonium sulfate is added to a final molarity of about 1.
6. The method of claim 2, wherein the membrane protein is a thyroid membrane protein.
7. The method of claim 6, wherein the thyroid membrane protein is thyroid stimulating hormone (TSH) receptor or thyroid peroxidase.
8. The method of claim 1 or 2, wherein the detergent solubilized cell membranes are derived from cultured cells.
9. The method of claim 8, wherein the cultured cells express a recombinant membrane protein.

10. The method of claim 1 or 2, wherein the detergent solubilized cell membranes are derived from animal tissue.
11. The method of claim 10, wherein the animal is selected from the group consisting of a human, cow, pig, rat, mouse, dog, monkey and guineau pig.
12. A soluble cell membrane fraction suitable for directly coating a solid support prepared according to the method of claim 1 or 2.
13. A method for immobilizing a membrane protein on a solid support, comprising contacting a solid support with a soluble cell membrane fraction of claim 12 under conditions sufficient to permit direct adsorption of the membrane protein to the solid support.
14. The method of claim 13, wherein the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material or a non-magnetic material.
15. The method of claim 13, wherein the solid support is selected from the group consisting of test tubes, microwell titer plates and magnetic beads.
16. The method of claim 13, wherein the membrane protein is a thyroid membrane protein.
17. The method of claim 16, wherein the thyroid membrane protein is thyroid stimulating hormone (TSH) receptor or thyroid peroxidase.
18. A membrane protein immobilized on a solid support prepared according to the method of any one of claims 13- 17.
19. A method for preparing a solid support on which one or more membrane proteins are immobilized, wherein the solid support is suitable for detecting molecules that bind the membrane protein, comprising:

(a) mixing a detergent solubilized cell membrane solution containing one or more membrane proteins with salt ion, such that a resulting supernatant fraction is enriched for the membrane proteins;

(c) contacting a solid support with the supernatant fraction under conditions such that the membrane proteins adsorb directly on the solid support;

thereby providing membrane proteins immobilized on a solid support suitable for detecting molecules that bind the membrane proteins.

20. The method of claim 19, wherein the salt is selected from the group consisting of ammonium sulfate, ammonium phosphate, potassium phosphate, rubidium phosphate, sodium phosphate, other Hofmeister salts, and combinations thereof.

21. The method of claim 19, wherein the salt is ammonium sulfate.

22. The method of claim 21, wherein the ammonium sulfate is added to a final molarity of about 1.

23. The method of claim 19, wherein the membrane protein is a thyroid membrane protein.

24. The method of claim 19, wherein the thyroid membrane protein is thyroid stimulating hormone (TSH) receptor or thyroid peroxidase.

25. The method of claim 19, wherein the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material and a non-magnetic material.

26. The method of claim 19, wherein the solid support is selected from the group consisting of test tubes, microwell titer plates and magnetic beads.

27. A method for preparing thyroid stimulating hormone (TSH) receptor or a fragment thereof immobilized on a solid support, wherein the immobilized TSH receptor is suitable for detecting human TSH receptor autoantibodies or TSH in a sample, comprising:

(a) mixing a detergent solubilized cell membrane solution containing human thyroid stimulating hormone receptor or a fragment thereof with ammonium sulfate under conditions such that the resulting supernatant fraction is enriched for TSH receptor;

(c) contacting a solid support with the supernatant fraction under conditions to permit direct adsorption of the TSH receptor on the solid support;

thereby providing immobilized TSH receptor suitable for detecting TSH receptor autoantibodies or TSH.

28. The method of claim 27, wherein the ammonium sulfate precipitation step comprises incubating about one quarter volume of saturated ammonium sulfate solution with about one volume solubilized cell membranes.

29. The method of claim 27, wherein the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material and a non-magnetic material.

30. The method of claim 27, wherein the solid support is selected from the group consisting of test tubes, microwell titer plates and magnetic beads.

31. A method for detecting a molecule in a sample that specifically binds to a membrane protein, comprising:

(a) contacting a membrane protein immobilized on a solid support according to the method of claim 13 with a sample;

(b) contacting the immobilized protein with an agent capable of binding to the membrane protein in a competitive reaction with the molecule;

(c) detecting the agent bound to the immobilized membrane protein in the absence of the sample;

(d) detecting the agent bound to the immobilized membrane protein in the presence of the sample;

wherein a decreased amount of agent bound to the immobilized membrane protein in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of a molecule that specifically binds to the membrane protein.

32. The method of claim 31, wherein the molecule in the sample is an autoantibody to the membrane protein.
33. The method of claim 32, wherein the autoantibody is associated with an autoimmune disease or the effect of a drug.
34. The method of claim 31, wherein the agent capable of binding to the membrane protein is an agonist or antagonist of the membrane protein.
35. The method of claim 34, wherein the antagonist or agonist is an antibody or fragment thereof.
36. A method for detecting autoantibodies to human thyroid stimulating hormone (TSH) receptor in a sample, comprising:
- (a) contacting TSH receptor immobilized on a solid support prepared according to the method of claim 27 with a sample;
 - (b) contacting the immobilized TSH receptor with an agent capable of binding to the TSH receptor in a competitive reaction with TSH receptor autoantibodies;
 - (c) detecting the agent bound to the immobilized TSH receptor in the absence of the sample;
 - (d) detecting the agent bound to the immobilized TSH receptor in the presence of the sample;
- wherein a decreased amount of agent bound to the immobilized TSH receptor in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of autoantibodies to human thyroid stimulating hormone receptor.
37. The method of claim 31 or 36, wherein the solid support comprises a material selected from the group consisting of a plastic material, a magnetic material and a non-magnetic material.

38. The method of claim 31 or 36, wherein the solid support is selected from the group consisting of tubes, microwell titer plates and magnetic beads.
39. The method of claim 36, wherein the TSH receptor autoantibodies to be detected are associated with autoimmune diseases or disorders.
40. The method of claim 36, wherein the TSH receptor autoantibodies to be detected are associated with a drug reaction.
41. The method of claim 36, wherein the TSH receptor autoantibodies to be detected are associated with Graves' disease.
42. The method of claim 36, wherein the immobilized TSH receptor is contacted with the sample and agent simultaneously.
43. The method of claim 36, wherein the immobilized TSH receptor is first contacted with the sample and subsequently contacted with the agent.
44. The method of claim 31 or 36, wherein the sample is bodily fluid from a subject.
45. The method of claim 44, wherein the subject is a human.
46. The method of claim 44, wherein the bodily fluid is selected from the group consisting of blood, plasma, serum, urine, cerebrospinal fluid, serosal fluid and tissue extract.
47. The method of claim 36, wherein the agent is human immunoglobulin or thyroid stimulating hormone (TSH).
48. The method of claim 47, wherein the thyroid stimulating hormone (TSH) is porcine TSH or bovine TSH.
49. The method of claim 36, wherein the agent is radiolabeled.

50. The method of claim 49, wherein the radiolabel is iodide 125.
51. The method of claim 36, wherein the agent is detected by a means selected from the group consisting of fluorescence, luminescence, a dye and an enzyme.
52. A method for diagnosing a disease or disorder associated with an autoantibody to a thyroid membrane protein in a subject, comprising the steps of:
- (a) mixing a detergent solubilized cell membrane solution containing a thyroid membrane protein with salt to obtain a membrane fraction;
 - (b) contacting a solid support with the membrane fraction under conditions sufficient to permit direct adsorption of the membrane protein to the solid support;
 - (c) contacting the membrane protein with a sample;
 - (d) contacting the membrane protein with an agent capable of binding to the membrane protein in a competitive reaction with the molecule;
 - (e) detecting the agent bound to the membrane protein in the presence of the sample; and
 - (f) detecting the agent bound to the membrane protein in the absence of the sample;
- wherein a decreased amount of agent bound to the membrane protein in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of a molecule that specifically binds to the membrane protein.
53. A method for diagnosing Graves' disease in a subject, comprising the steps of:
- (a) mixing a detergent solubilized cell membrane solution containing human thyroid stimulating hormone (TSH) receptor or a fragment thereof with ammonium sulfate under conditions such that the resulting supernatant fraction is enriched for TSH receptor;
 - (b) contacting a solid support with the supernatant fraction under conditions to permit direct adsorption of the TSH receptor on the solid support;
 - (c) contacting the TSH receptor with a sample;
 - (d) contacting the TSH receptor with an agent capable of binding to the TSH receptor in a competitive reaction with TSH receptor autoantibodies;

(e) detecting the agent bound to the TSH receptor in the presence of the sample;

(f) detecting the agent bound to the TSH receptor in the absence of the sample;

wherein a decreased amount of agent bound to the TSH receptor in the presence of the sample as compared to the amount of agent bound in the absence of the sample indicates the presence of autoantibodies to human thyroid stimulating hormone receptor.

54. A kit comprising a soluble membrane fraction suitable for direct immobilization on a solid support, wherein the soluble membrane fraction is prepared according to a method comprising a step of fractionating a detergent solubilized cell membrane solution by the addition of salt.

55. A kit comprising a soluble cell membrane fraction enriched for one or more membrane proteins, wherein the one or more membrane proteins are capable of adsorbing directly on a solid support.

56. A kit comprising one or more membrane proteins immobilized on a solid support, wherein the one or more membrane proteins immobilized on the solid support are prepared according to a method comprising the steps of:

(a) mixing a detergent solubilized cell membrane solution containing one or more membrane proteins with salt to obtain a membrane fraction enriched for the one or more membrane proteins; and

(b) contacting a solid support with the membrane fraction under conditions sufficient to permit direct adsorption of the one or more membrane proteins to the solid support.

FIG. 1

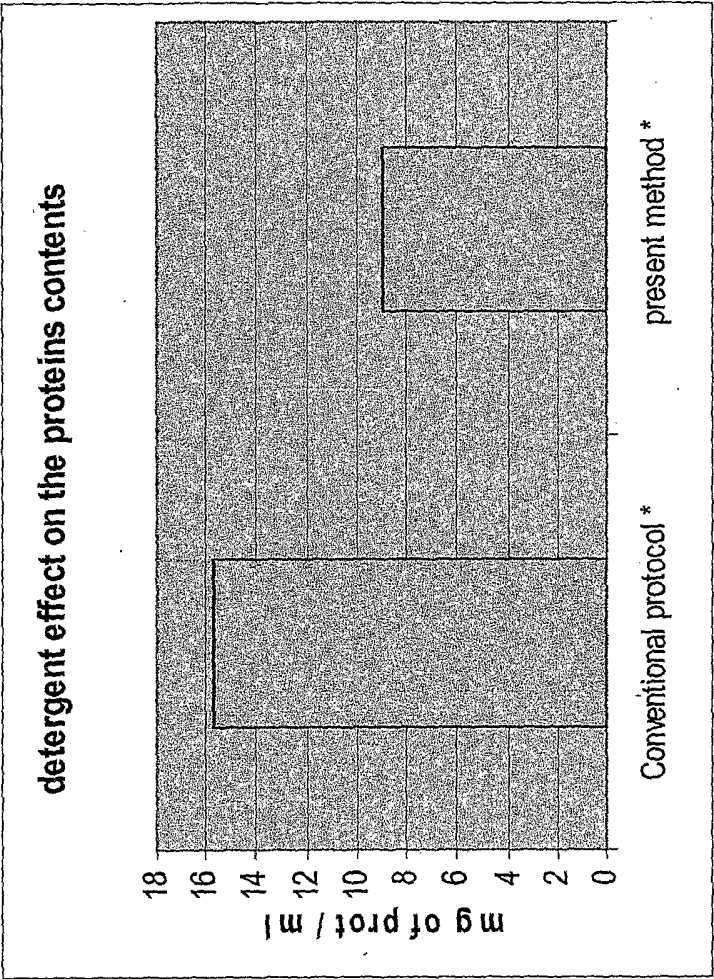


FIG. 2

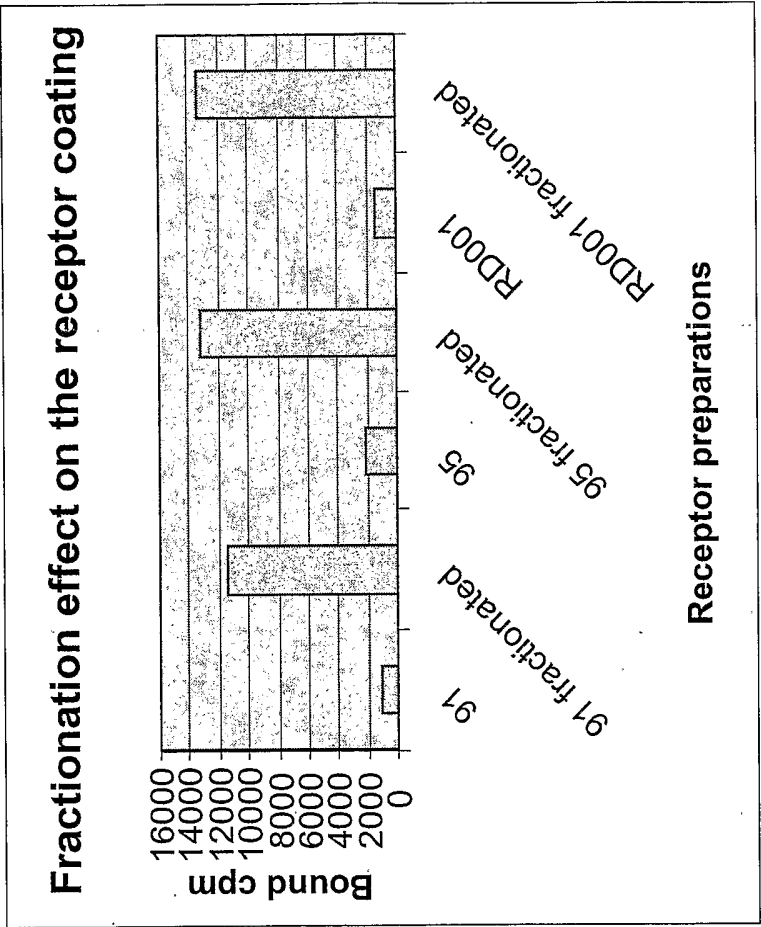


FIG. 3

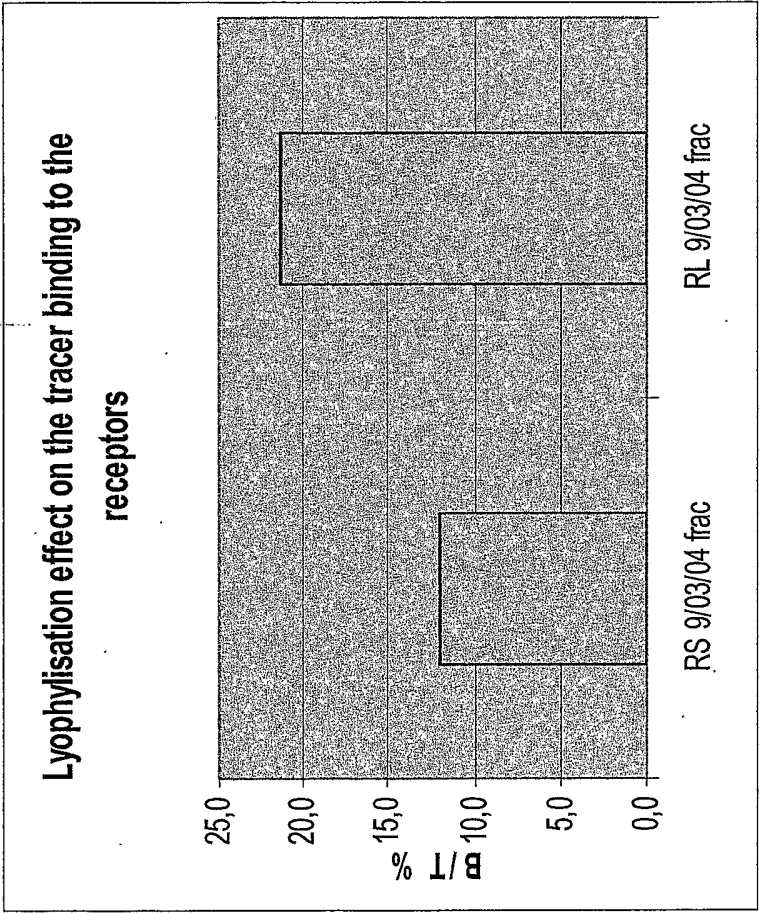


FIG. 4

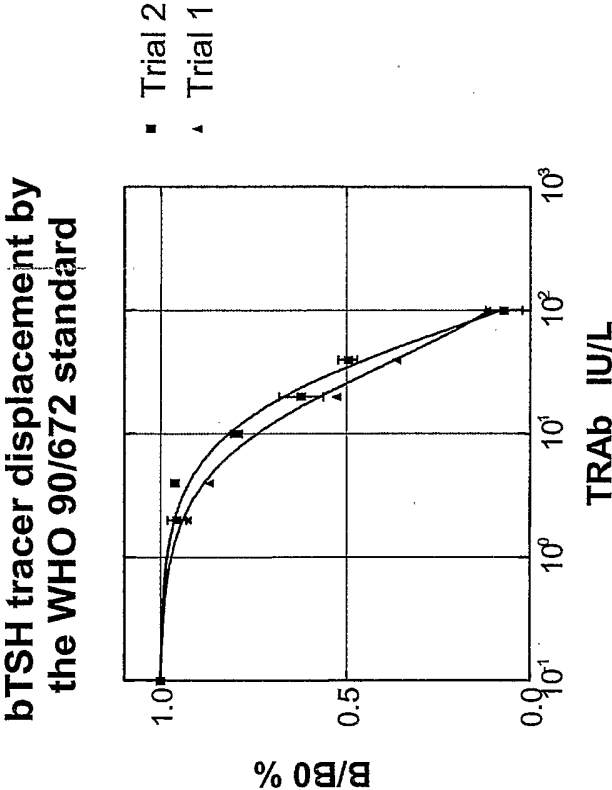
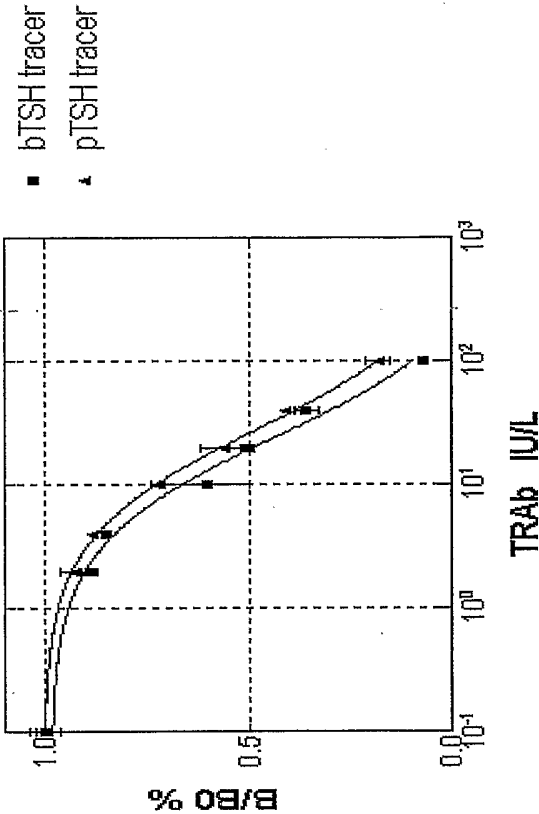


FIG. 5

Porcine or bovine tracer
displacement by the WHO
90/672 standard



INTERNATIONAL SEARCH REPORT

National Application No
PCT/EP2004/005967

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/72 G01N33/78

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

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

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

EPO-Internal, BIOSIS, PAJ, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

25 August 2004

Date of mailing of the international search report

09/09/2004

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005967

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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