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(54) **HOMOGENEOUS DOUBLE RECEPTOR
AGGLUTINATION ASSAY FOR
IMMUNOSUPPRESSANT DRUGS**

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(57) **ABSTRACT**

A homogeneous, non-competitive, double receptor agglutination assay for measuring immunosuppressant drugs is described. The assay employs at least two receptors wherein each receptor is specific for a separate binding site on the drug and wherein each receptor is bound to a detection particle. The immunosuppressant drug binds to the receptors and causes particle agglutination, which can be measured and correlated with the presence or amount of immunosuppressant drug in a sample.

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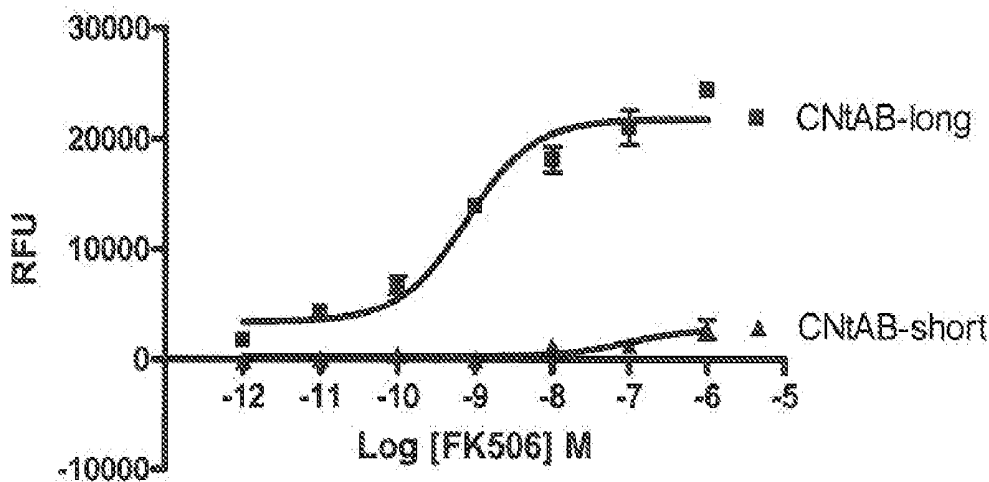


Figure 1

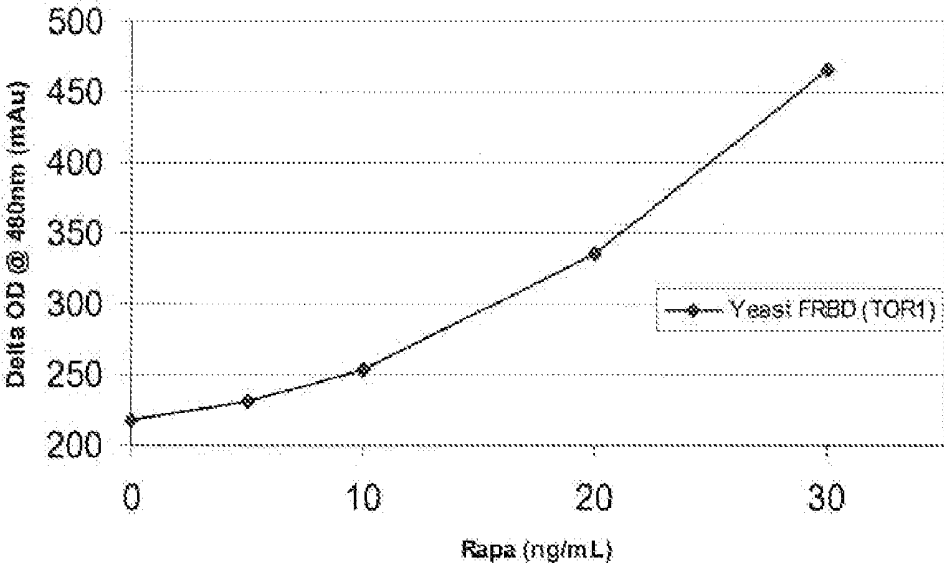


Figure 2

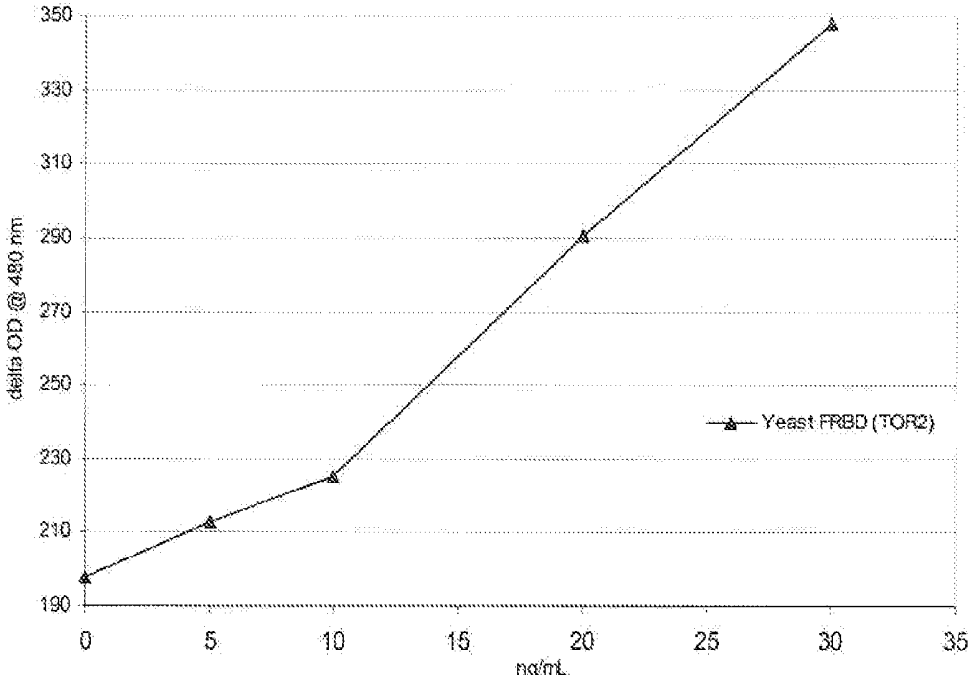


Figure 3

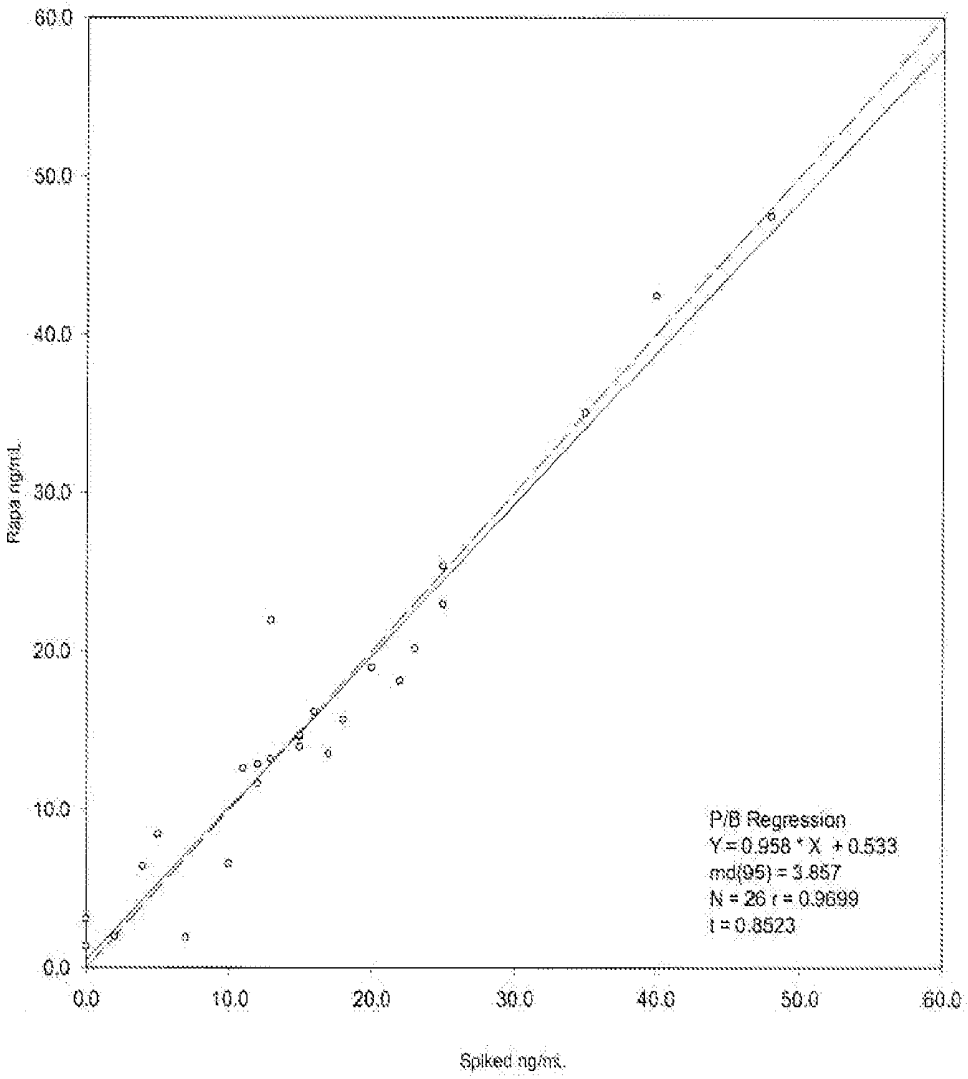


Figure 4

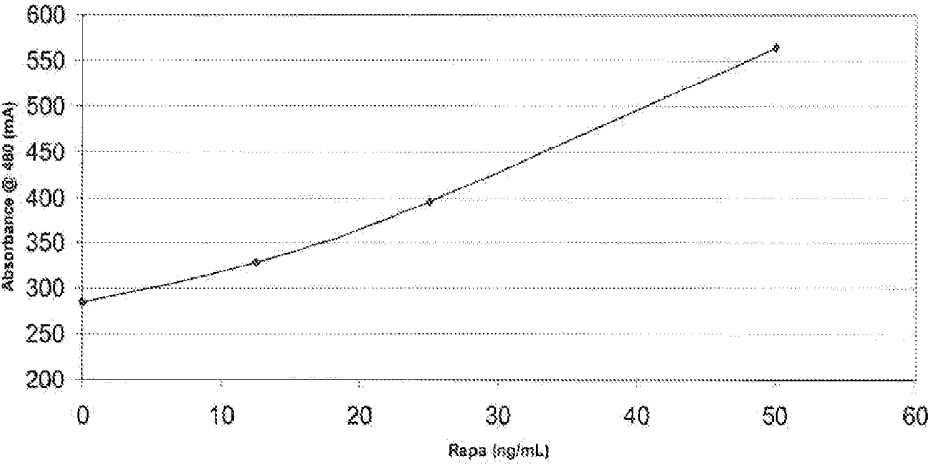
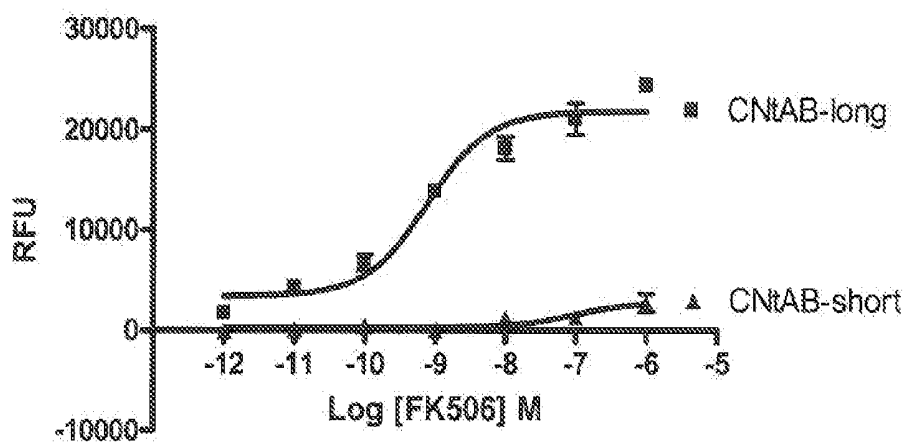


Figure 5



HOMOGENEOUS DOUBLE RECEPTOR AGGLUTINATION ASSAY FOR IMMUNOSUPPRESSANT DRUGS

RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S. S. §119 to U.S. Ser. No. 60/807,245 filed Jul. 13, 2006.

FIELD OF THE INVENTION

[0002] The present invention pertains to the field of therapeutic drug monitoring, and in particular, to immunoassay methods for determining the presence or amount of immunosuppressive drug substances in a sample. More particularly, the present invention relates to homogeneous, non-competitive, double receptor assays for measuring immunosuppressant drugs.

BACKGROUND

[0003] Several important immunosuppressive drugs act on immunophilins, cyclosporin, tacrolimus, rapamycin, and everolimus. Immunophilins are protein chaperones with peptidylprolyl isomerase activity that belong to one of the two large families, the cyclosporin-binding cyclophilins (CyPs) and the FK506 binding proteins (FKBPs).

[0004] Cyclosporin (also known as ciclosporine and cyclosporin A) is natural metabolite of a soil fungus, a peptide composed of 11 amino acids and, together with tacrolimus, is a calcineurin inhibitor. Cyclosporin is thought to bind to the cytosolic protein cyclophilin (an immunophilin) of immunocompetent lymphocytes, especially T-lymphocytes. This complex of cyclosporin and cyclophilin inhibits calcineurin, which under normal circumstances induces the transcription of interleukin-2. The drug also inhibits lymphokine production and interleukin release, leading to a reduced function of effector T-cells.

[0005] Tacrolimus (FK506) is also a fungal product (*Streptomyces isukubaensis*). It is a macrolide lactone and acts by inhibiting calcineurin. The drug is used particularly in liver, kidney, heart, and lung transplants. It binds to an immunophilin, followed by the binding of the complex to calcineurin and the inhibition of its phosphatase activity. In this way, it prevents the passage of G0 into G1 phase. Tacrolimus is more potent than cyclosporine and has less pronounced side effects.

[0006] Rapamycin (sirolimus) is an extremely potent immunosuppressive drug. It forms a tight complex with FKBP12 (a 12-kDa FK506 binding protein), and the rapamycin-FKBP12 complex in turn binds to mammalian target of rapamycin (mTOR) or yeast target of rapamycin (yTOR). There are two forms of yTOR, yTOR1 and yTOR2. As a member of the novel phosphatidylinositol kinase-related family, mTORs kinase activity is essential for its signaling function. The FKBP12-rapamycin binding domain (FRBD) in mTOR has been identified as an 11 kDa segment located N-terminal to the kinase domain.

[0007] Although cyclosporin and tacrolimus (FK506) have different molecular structures, their immunosuppressive properties and molecular mechanisms are similar. Both drugs bind intracellularly to abundant cytosolic proteins known as immunophilins. In the case of tacrolimus, the major binding protein appears to be FKBP12, while cyclosporin binds to a family of immunophilins called cyclophilins. These drug-immunophilin complexes can then

form a pentameric complex with calcineurin, calmodulin, and calcium causing a non-competitive inhibition of calcineurin-phosphatase activity.

[0008] Everolimus is an immunosuppressive macrolide bearing a stable 2-hydroxyethyl chain at position C-40 on the sirolimus structure. Everolimus, which has greater polarity than sirolimus, was developed in an attempt to improve the pharmacokinetic characteristics of sirolimus.

[0009] Calcineurin (CaN), a heterodimeric protein phosphatase, is composed of a 61 kDa catalytic subunit A (CaNA) and a 19 kDa regulatory subunit B (CaNB). It is known that the inhibitory effect of tacrolimus (FK506) on CaN enzyme activity is largely mediated through FKBP12. The fact that the FK506/FKBP12 complex binds CaN with high affinity in the presence of calcium makes it possible to develop a two-receptor "sandwich" assay to quantitatively measure tacrolimus in blood samples.

[0010] Since crystal structural analysis has revealed that both CaN A and B contribute interaction interfaces for binding to the FK506-FKBP12 complex, the desired fusion ought to span these two subunits. Recently, expression and purification of a 97 kDa single-chain fusion calcineurin A/B-calmodulin (scCN) polypeptide has been reported (Y. Qin et al, *Biochimica et Biophysica Acta*, 1747, 171-178, 2005). Although it was implicated that the single-chain CaN-CaM may exhibit phosphatase activity, the ability to bind FK506-FKBP12 complex has not yet been determined. In addition, the expression level of this 97 kDa scCN was quite poor. In an effort to produce a smaller and easier to manipulate protein, Schreiber and co-workers reported the generation of truncated CaN fusions. In one of such fusions, the amino acids 12-394 of CaNA were fused to the N-terminal of entire CaNB in order to express a functional CAB (fCAB). Similarly, a shorter version called minimal CAB (mCAB) and encompassing the amino acids 340-394 of CaNA and the entire CaNB, was also constructed. The binding activity of these fCAB and mCAB to FK506-FKBP12 complex was indicated by the transcriptional reporter assays in the transiently transfected Jurkat T cells (P. Clemons et al, *Chemistry & Biology*, 9, 49-61, 2002). However, no further studies have been reported since then regarding the expression and purification of the fCAB and mCAB to directly determine the interaction with FK506-FKBP12 in vitro.

[0011] For the adequate management of transplant patients on immunosuppressant drug therapy, it is important to obtain optimal blood concentration of the drug. Since immunosuppressant drugs are often given in combination with each other, specificity for the parent drug is critical.

[0012] Competitive immunoassays for immunosuppressant drugs in which a drug analog conjugate competes with free drug for a limited number of antibody binding sites are known and commercially available. Although these immunoassays show good selectivity for parent drug, they generally show significant cross-reactivity to one or more metabolites, thus giving rise to undesirable positive bias in the immunoassays when compared to reference methods. Current assays for immunosuppressant drugs also include high performance liquid chromatography coupled to mass spectrometry (LC/MS/MS). These methodologies require special equipment and skills.

[0013] A further problem is seen with competitive immunoassays of, e.g., rapamycin, which require the use of an analog derivative of rapamycin, which is inherently

unstable. A further problem with competitive immunoassays is that it is difficult to achieve sufficient sensitivity to measure very low therapeutic ranges, e.g., 5-20 ng/ml.

[0014] Direct covalent coupling of binding protein to particles has been demonstrated in the single receptor immunosuppressive drug assay format for detecting rapamycin in co-pending and commonly assigned U.S. Ser. No. 60/714, 712 filed Sep. 7, 2005 and U.S. Ser. No. 11, 468,940 filed Aug. 31, 2006, the content of which applications are herein incorporated by reference. The use of nanoparticles is also disclosed therein.

[0015] Armstrong et al., in *Clinical Chemistry* 44:12, 2516-2523 (1998) describe a pentamer formation assay for measurement of tacrolimus and its active metabolites after extraction from whole blood. They describe a microtiter plate-based assay using binding proteins.

[0016] Sagi-Eisenberg, in WO 2005/062708 published Jul. 14, 2005, teaches a heterogeneous assay for the detection of rapamycin utilizing immobilized FKBP12.

[0017] Huster, in US 2006/0099654 published May 11, 2006, describes an immunoassay method for determining sirolimus by combining a sample suspected of containing sirolimus with two latex bead reagents and a biotinylated analyte receptor. One bead reagent is coated with streptavidin and contains a photosensitive dye. A second bead reagent is coated with an antibody generated using a fragment of the sirolimus molecule and contains a chemiluminescent dye. The analyte receptor can be FKBP.

SUMMARY OF THE INVENTION

[0018] The present invention comprises a method for determining the presence or amount of an immunosuppressive drug in a sample comprising the steps of (a) providing a sample suspected of containing the immunosuppressive drug, (b) adding to the sample at least two receptors that are specific for the drug, wherein each of the receptors is specific for a separate binding site on the drug and wherein each of the receptors is bound to a detection particle, and further wherein the drug binds to the receptors and causes particle agglutination, (c) measuring the amount of particle agglutination, and (d) correlating the amount of particle agglutination with the presence or amount of immunosuppressive drug in the sample. In accordance with one embodiment, only one of the receptors is bound to a detection particle, and an antibody that specifically binds to the receptor protein not bound to a particle is used to agglutinate the particles. In another embodiment an antibody specific for the target immunosuppressant drug is substituted for one or both of the two receptors, wherein the antibody is bound to a detection particle.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG. 1. Rapamycin calibration curve according to the present invention as described in Example 8 using γ TOR1 FRBD. Rapamycin concentration in spiked whole blood samples is plotted on the x-axis and absorbance is plotted on the y-axis.

[0020] FIG. 2. Rapamycin calibration curve according to the present invention as described in Example 8 using γ TOR2 FRBD. Rapamycin concentration in spiked whole blood samples is plotted on the x-axis and absorbance is plotted on the y-axis.

[0021] FIG. 3. Chart comparing the measured rapamycin concentration (y-axis) vs. the spiked rapamycin concentration (x-axis) according to the present invention as described in Example 8. Rapamycin concentration in spiked whole blood samples is plotted on the x-axis and the measured rapamycin concentration is plotted on the y-axis.

[0022] FIG. 4. Rapamycin calibration curve according to the present invention as described in Example 15 using mTOR FRBD. Rapamycin concentration in spiked whole blood samples is plotted on the x-axis and absorbance is plotted on the y-axis.

[0023] FIG. 5. Plot showing interactions of FK506-bound FKBP12 with truncated calcineurin A/B fusions.

DETAILED DESCRIPTION OF THE INVENTION

[0024] As used herein, the term "detection particle" refers to any particle having a generally spherical shape with a diameter ranging from about 10 nm to about 1000 nm that allows one to distinguish between an aggregated state and a non-aggregated state in a homogeneous assay. The detection particle can be a nanoparticle or a microparticle, but for the purposes of convenience, all such particles are referred to herein as "microparticles". The surface of the detection particle may be functionalized, and various compounds may be attached to the surface, including for example, fluorophores, chemiluminescent entities, antibodies, biotin, or streptavidin.

[0025] An epitope is an area on the surface of an antigenic molecule that stimulates a specific immune response and against which that response is directed. Epitope tagging is a recombinant DNA method by which a protein encoded by a cloned gene is made immunoreactive to a known antibody. Development of fusion tag systems provides great flexibility for easy purification and detection of recombinant proteins using affinity matrices and specific antibodies. Epitope tags range from 10 to 15 amino acids in length and are designed to create a molecular handle for a protein. They are typically placed on either the amino or carboxyl terminus to minimize any potential disruption in tertiary structure and thus function of the protein. Because the tags are small, they are further unlikely to interfere with structure and function of the recombinant protein. Therefore, an epitope tag does not usually need to be removed before subsequent experiments are performed. A number of different tags have been used, for example, His₆ (a sequence of six histidines that have a strong affinity for matrices containing metal ions like Ni⁻² or Co⁻²), HA (a peptide sequence derived from the human influenza virus hemagglutinin protein), and AviTag, a sequence which can be enzymatically mono-biotinylated at its internal lysine residue by *E. coli* biotin protein ligase (BirA). The enzyme BirA can be added to the expression mix and the biotinylation performed in situ.

[0026] The term "FK506 binding protein", or "FKBP", as used herein refers to a receptor protein that binds to rapamycin to form a complex that will specifically bind to a protein selected from the group consisting of mTOR, γ TOR1, γ TOR2, and the FRBDs of mTOR, γ TOR1, and γ TOR2.

[0027] The term "specific binding" refers to a high avidity and/or high affinity binding between two paired species, including for example, such paired species as ligand/target moiety, enzyme/substrate, receptor/agonist, antibody/antigen, and lectin/carbohydrate. The binding interaction may

be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. In particular, the specific binding is characterized by the binding of one member of a pair to a particular species and to no other species within the family of compounds to which the corresponding member of the binding member belongs. Thus, for example an antibody preferably binds to a single epitope and to no other epitope within the family of proteins.

[0028] The term “receptor” or “binding moiety” as used herein, refers to a protein or glycoprotein that specifically binds to a target compound. An example of a receptor is an immunophilin that binds to an immunosuppressant drug.

[0029] The term “nanoshell” as used herein refers to a microparticle composed of a dielectric ore (for example, silica or calcium phosphate) coated with an ultra-thin metallic layer (for example, gold). Nanoshells suitable for use in the present invention have a diameter selected from a range of about 50 nm to about 250 nm. The surface of the microparticle may be functionalized, and various compounds may be attached to the surface, including for example, antibodies or streptavidin.

[0030] The term “microparticle” as used herein refers to a solid particle of about 10 nm to about 1000 nm in diameter. Typically for use in the present invention, the microparticles will have a diameter of about 100 nm to about 600 nm. Microparticles can be formed from a variety of materials and include polystyrene and latex microparticles, the surfaces of which are optionally functionalized, and various compounds may be attached to the surface, including, for example, antibodies or streptavidin.

[0031] As used herein, the term “antibody” refers to a polypeptide or group of polypeptides which are comprised of at least one binding domain, where an antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, which allows an immunological reaction with the antigen. Unless otherwise stated a general reference to an antibody encompasses polyclonal as well as monoclonal antibodies. The term “antibody” also includes recombinant proteins comprising the binding domains, as well as fragments of antibodies, including Fab, Fab', F(ab)₂, and F(ab')₂ fragments.

[0032] As used herein a “linker” is a bond, molecule, or group of molecules that binds two separate entities to one another. Linkers may provide for optimal spacing of the two entities or may further supply a labile linkage that allows the two entities to be separated from each other. Labile linkages include photocleavable groups, acid-labile moieties, base-labile moieties, and enzyme-cleavable groups.

[0033] As used herein the term “homogeneous assay” refers to a quantitative or qualitative test for determining the presence or concentration of a compound in a sample, wherein the unreacted test reagents do not need to be separated from the reagents that have reacted with the target compound in order to detect, or measure the concentration of, the target compound.

[0034] As used herein the term “sandwich assay” refers to a homogeneous assay for detecting a target compound wherein the assay utilizes two binding moieties that each specifically bind to the target compound but at two separate locations on the target compound.

[0035] The present invention relates to a homogeneous, particle-based, sandwich assay to detect an immunosuppressant drug in a biological fluid. More particularly, the present invention relates to a homogeneous particle-based assay using binding moieties that specifically bind to immunosuppressant drugs. The assay comprises at least two binding moieties, wherein each of the separate binding moieties binds to a different location on the target immunosuppressant drug, and each of the binding moieties is bound to one or more detection particles. Accordingly, during the assay a sample suspected of containing the immunosuppressant drug of interest is contacted with a composition comprising two binding moieties such that the degree of agglutination of the reactants, i.e., the number of sandwich complexes formed between the two binding moieties and the immunosuppressant drug, indicates the amount of immunosuppressant drug present in the sample.

[0036] Detecting the degree of agglutination is done using a homogeneous assay format wherein the unreacted reagents, i.e., the unbound binding moieties, do not need to be separated from the reacted reagents (i.e., sandwich complexes comprising two binding moieties and the immunosuppressant drug). Homogeneous assay systems are known to those skilled in the art and can be adapted for use in the present invention.

[0037] In accordance with one embodiment, the two binding moieties are bound to detection particles and the step of measuring the amount of agglutination occurring upon contact of a sample with the two binding moieties is conducted by measuring the turbidity of the sample relative to a reference. The effect on the degree of agglutination can be determined by absorbance measurement. In accordance with one embodiment the detection particle is a microparticle, and a microparticle agglutination assay is employed to determine the presence or quantity of an immunosuppressant drug in a sample. Such microparticle agglutination assays are considered to be homogeneous assays because a washing step is not employed prior to the step of measuring the amount of agglutination that has occurred. Microparticle agglutination assays are conveniently performed and measured using automated analyzers such as the Roche/Hitachi and COBAS analyzers (Roche Diagnostics, Indianapolis). In the microparticle agglutination assay of the present invention, a first binding moiety and a second binding moiety are bound, directly or indirectly, to microparticles. The first and second binding moieties are specific for different binding sites on the drug to be determined. In a reaction solution, these reagents (binding moieties bound to microparticles) are dispersed in the liquid medium and form a stable suspension. In the presence of the immunosuppressant drug, binding between the drug and the receptors (binding moieties) occurs, causing particle agglutination and a corresponding increase in turbidity of the solution, which is quantified by measuring absorbance of the solution. There is a direct correlation between the amount of drug present in a sample and the amount of absorbance measured.

[0038] In accordance with one embodiment the detection particle is a nanoshell. Nanoshells are a type of optically tunable detection particle composed of a dielectric (for example, silica, alginate, or calcium phosphate) core coated with an ultra-thin metallic (for example, gold) layer. Gold nanoshells possess physical properties similar to gold colloid, in particular a strong optical absorption due to the collective electronic response of the metal to light. The

optical response of gold nanoshells depends dramatically on the relative sizes of the nanoparticle core and the thickness of the gold shell. By varying the relative core and shell thicknesses, the color of gold nanoshells can be varied across a broad range of the optical spectrum that spans the visible and the near-IR spectral regions. Gold nanoshells can be made either to absorb or scatter light preferentially by varying the size of the particle relative to the wavelength of the light at their optical resonance. In one embodiment the nanoshell core has a diameter selected from the range of about 50 nm to about 200 nm, and in one embodiment about 120 nm, and the thickness of the nanoshell coating or shell is selected from a range of about 5 nm to about 30 nm.

[0039] A whole blood immunoassay has been described using gold nanoshells (Anal. Chem. 75:2377-2381, 2003). It is anticipated that binding proteins such as immunophilins could be substituted for antibodies in the nanoparticle assay described. The principle of nanoparticle aggregation is similar to that of microparticle agglutination technique. Blood's high turbidity and strong visible extinction complicate the detection of aggregates in an agglutination assay. However selection of the optimal wavelengths and pretreatments of a blood sample can overcome these complicating factors. Nanoshells also are advantageous in that they are quite stable with respect to aggregation in the whole blood and dimerization or aggregation of the nanoparticles by a sandwich assay format (immunosuppressive drug with binding proteins) can be detected via spectral red-shifting in the near infra-red, where whole blood is more transmissive, thus allowing detection of nanoshell aggregation in whole blood.

[0040] In accordance with one embodiment the immunosuppressant drug to be detected and quantitated is selected from the group consisting of cyclosporin, tacrolimus, rapamycin, and everolimus, and the binding moieties are immunosuppressant drug binding receptor proteins. In one embodiment the receptors represents native immunophilins of the target immunosuppressant drug, or binding fragments or derivatives of said native immunophilins. In one embodiment the target the immunosuppressant drug is rapamycin or everolimus and the first receptor comprises a FK506 binding protein (FKBP) and the second receptor comprises a target of rapamycin (TOR) protein. In a further embodiment the FKBP is FKBP12 or FKBP25 and the TOR is selected from the group consisting of mTOR, γ TOR1, γ TOR2, and the FRBDs of mTOR, γ TOR1, and γ TOR2.

[0041] In an alternative embodiment the immunosuppressant drug to be detected and quantitated in a sample is tacrolimus, wherein the first receptor comprises an FK506 binding protein (FKBP) and the second receptor comprises calcineurin, or a binding fragment or derivative thereof. In a further embodiment the second receptor further comprises calmodulin and a calcium ion bound to calcineurin. In yet another embodiment wherein the immunosuppressant drug to be detected and quantitated is a cyclosporin, wherein the first receptor comprises a cyclophilin selected from the group consisting of cyclophilin A (CypA), cyclophilin B (CypB), cyclophilin C (CypC), the structures of which are described in Mikol, et al. PNAS91:5183-5186 (1994), and the second receptor comprises an calcineurin. In a further embodiment the second receptor further comprises calmodulin and a calcium ion bound to calcineurin.

[0042] In accordance with one embodiment the immunosuppressant drug binding receptor protein is bound to a detection particle wherein the detection particle is a

nanoshell or microparticle. The detection particle can be bound to the receptor protein using any of the standard techniques known to those skilled in the art. For example the detection particle can be bound to the receptor protein through a direct covalent linkage using standard linkers or the protein can be bound to the detection particle through ionic, hydrogen bonding, hydrophobic/hydrophilic interactions or any combination thereof. In a one embodiment of the invention, receptor proteins are attached to the detection particle via direct coupling or through a tag.

[0043] As one example, detection particles can be attached to anti-tag antibodies and binding proteins can be attached to the corresponding tag. Alternatively, a first receptor protein can be attached to a tag (hapten), including for example biotin, and the detection particles can be bound to streptavidin or an antibody that binds to the tag (hapten). The second binding protein of the drug is also attached to the same tag or in an alternative embodiment to a different tag. The number of biotin or tagged molecules attached to the protein can be varied, but most preferably one biotin (or other tag) will be attached per molecule of binding protein to minimize spontaneous agglutination of the streptavidin microparticles (or ligand for said tag).

[0044] In accordance with one embodiment, streptavidin, avidin or an anti-biotin antibody (or an anti-hapten antibody) is attached to the detection particle. In the presence of immunosuppressant drug, a sandwich is formed simultaneously with the drug and two biotinylated binding proteins. In the presence of a streptavidin labeled detection particle, agglutination occurs, which can be measured by light scattering. The more drug present in the sample, the more agglutination is observed. When no drug is present, no sandwich is formed and the detection particles will not agglutinate. In accordance with one embodiment the detection particle is a streptavidin labeled microparticle and the biotinylated receptor proteins are pre-attached to the streptavidin microparticle prior to contacting the receptor proteins with the sample comprising the immunosuppressant drug. This procedure provides the most optimal results in the assay.

[0045] In a similar manner when the two receptor proteins are labeled with one member of a fluorescence resonance energy transfer (FRET) fluorophore pair, respectively the presence of the immunosuppressant drug can be detected. In the presence of the immunosuppressant drug, a sandwich is formed simultaneously with the drug and two biotinylated binding proteins. The formation of this complex brings the two fluorophores within sufficient proximity to one another that upon excitation of the donor fluorophore with excitation light, fluorescent energy transfer occurs. Such an event can be measured by detecting a decrease in donor fluorophore emissions or an increase in the acceptor fluorophore's emissions.

[0046] In accordance with one embodiment the receptor proteins can be expressed as recombinant fusion proteins that include a peptide sequence that either represents a ligand or tag or provides an optimal site for attachment of a molecule of biotin. For example the receptor protein can be a recombinant protein that comprises fusion partner with a biotinylation signal sequence. This fusion protein is co-expressed in *Escherichia coli* or in a cell-free system with biotin ligase, e.g., AviTag, which catalyses the covalent addition of a biotin moiety to a lysine residue in the signal sequence. In one embodiment the two different receptor

proteins used in the assay are AviTagged biotin labeled proteins. The AviTagged sequin can be attached through either the C-terminus or N-terminus. Models of the sandwich concept seem to suggest that at least one of the tags should be placed at the N-terminus to prevent steric hindrance. However, applicants have surprisingly found that the assay works well with both tags at the C-terminus.

[0047] In an alternative embodiment, antibodies to the receptor protein are bound to the surface of the detection particles, wherein the bound antibodies specifically bind to the receptor protein itself or to a tag that is bound to the receptor protein. In the presence of immunosuppressant drugs the sandwich forms and agglutination occurs. These antibodies are designed and or selected in such a way that the antibodies do not bind to the receptor protein binding site for the immunosuppressant drugs.

[0048] In accordance with one embodiment assay reagents are provided for detecting and quantifying the amount of an immunosuppressant drug present in a sample. The sample may be any liquid sample that is suspected of containing an immunosuppressant drug. More typically the sample is a biological bodily fluid recovered from a patient being administered an immunosuppressant drug. In one embodiment the sample is whole blood or a derivative product thereof. When whole blood is used as the sample and the assay is a microparticle agglutination detection method, the blood sample is typically pretreated prior to contacting the sample with the assay reagents in order to release cellular-bound drugs. This procedure includes combining the whole blood sample with a precipitating agent. The precipitating agent is selected from those known in the art including, for example, aqueous copper sulfate or zinc sulfate in methanol, ethanol, ethylene glycol, acetonitrile or similar water miscible organic solvents. The whole blood mixture is then mixed thoroughly and centrifuged. The clear supernatant is then transferred into the sample cup and placed on the analyzer for assay performance. In one embodiment the composition used to pretreat the whole blood sample comprises methanol and zinc sulfate. After pretreating the sample, the cellular debris is removed prior to adding the assay reagents. In one embodiment the cellular debris is removed by centrifugation at a low speed (approximately 12,000 rpm) for 5 minutes.

[0049] The assay reagents in accordance with one embodiment comprise a first receptor and a second receptor wherein the first and second receptors specifically bind to separate binding sites on the target drug to form a sandwich complex upon contact of the reagents with the drug. In accordance with one embodiment, the first and second receptors are each provided with a tag for binding the receptor to a detection particle. In another embodiment the first and second receptors will be provided with the detection particle already bound to the first and second receptors. In one embodiment the first and second receptors further comprise a biotinylated tag and the detection particle is bound to streptavidin. In a further embodiment each of the first and second receptors is provided with a single biotin molecule. In one embodiment the detection particle is a microparticle bound to streptavidin or to an antibody specific for biotin. In one embodiment the immunoassay reagents are provided as two complexes comprising a receptor bound to a microparticle through a biotin-streptavidin linkage.

[0050] In one embodiment an agglutination assay reagent is provided for quantifying the amount of rapamycin or

everolimus in a sample. In this embodiment, the first receptor comprises an FK506 binding protein bound to a detection particle, and said second receptor comprises a target of rapamycin (TOR) protein bound to a detection particle. In one embodiment the first receptor comprises FKBP12 or FKBP25 and the second receptor is selected from the group consisting of mTOR, γ TOR1, γ TOR2, and the FRBDs or mTOR, γ TOR1, and γ TOR2. In an alternative embodiment an agglutination assay reagent is provided for quantitating the amount of tacrolimus present in a sample, wherein the reagents comprise a first receptor comprising an FK506 binding protein (FKBP) bound to a detection particle and a second receptor comprising calcineurin, or a binding fragment or derivative thereof, bound to a detection particle. In a further embodiment the second receptor further comprises calmodulin and a calcium ion bound to calcineurin. In yet another embodiment an agglutination assay reagent is provided for quantitating the amount of cyclosporin, wherein the reagents comprise a first receptor comprising a cyclophilin bound to a detection particle selected from the group consisting of cyclophilin A (CypA), cyclophilin B (CypB), and cyclophilin C (CypC), and a second receptor comprising a calcineurin or fragment of calcineurin bound to a detection particle. In a further embodiment the second receptor further comprises calmodulin and a calcium ion bound to calcineurin. The detection particle is typically either a nanoshell or a microparticle.

[0051] In an alternative embodiment assay reagents are provided for detecting an immunosuppressant drug using a homogeneous FRET based assay. In this embodiment the assay reagents comprise a first receptor and a second receptor wherein the first and second receptors specifically bind to separate binding sites on the target immunosuppressant drug to form a sandwich complex upon contact of the reagents with the drug, wherein the first receptor is labeled with a donor fluorophore and the second receptor is labeled with an acceptor fluorophore.

[0052] In accordance with one embodiment a method is provided for determining the amount of an immunosuppressant drug in a sample. The method comprises the steps of isolating a sample, and optionally pretreating the sample to release the cellular bound immunosuppressant drug. The sample is then contacted with an assay reagent composition comprising a first and second receptor to form a suspension, wherein the first and second receptors specifically bind to separate locations on the target immunosuppressant drug. In one embodiment, the reagent composition will further comprise detection particles that bind to the first and second receptors. Alternatively, in one embodiment the detection particles are provided as a separate component that is added to the suspension before, during or after the addition of the first and second receptors. The assay reagents are mixed together, to induce agglutination in the presence of the target drug, wherein the first and second receptors each specifically bind to a separate binding site on said drug and the detection particles bind each of the receptors (if not already bound to the receptors) to form a detectable sandwich complex. The amount of agglutination is then measured directly, meaning that no further purification or washing steps are required, and thus the assay is a homogeneous assay. The amount of immunosuppressant drug is correlated to the detected amount of particle agglutination, using homogeneous assay techniques known to those skilled in the art. In accordance with one embodiment the extent of particle agglutination is

measured by an absorbance measurement and the amount of drug present is determined by reference to a standard curve.

[0053] In one embodiment the detection particles are either microparticles or nanoshells, and in one embodiment the detection particles are microparticles. The method can be used to measure the amount of an immunosuppressant drug selected from the group consisting of cyclosporin, tacrolimus, rapamycin, and everolimus by selection of the receptors used in the assay. To detect rapamycin or everolimus, the first receptor comprises a FK506 binding protein (FKBP) bound to a first detection particle and the second receptor comprises a target of rapamycin (TOR) protein bound to a second detection particle. To detect tacrolimus, the first receptor comprises an FK506 binding protein (FKBP) bound to a first detection particle, and the second receptor comprises a calcineurin bound to a second detection particle. To detect cyclosporine the first receptor comprises a cyclophilin bound to a first detection particle, and the second receptor comprises an calcineurin bound to a second detection particle. For methods directed to detecting and quantitating the presence of tacrolimus or cyclosporine the second receptor (calcineurin) may optionally be further complexed with calmodulin and a calcium ion.

[0054] The method for quantitating an immunosuppressant drug can be conducted on any aqueous sample that is believed to contain an immunosuppressant drug as a means of confirming the presence and determining the concentration of the drug in that sample. In one embodiment the sample is a bodily fluid isolated from a patient, including such fluids as blood, plasma, serum, urine, sperm, cerebral spinal fluid, saliva and the like. In one embodiment the sample is whole blood. Typically the blood sample is pretreated to release the cellular bound immunosuppressant drug before the sample is contacted with the assay reagents. In one embodiment the blood sample is extracted with a solution comprising methanol and zinc sulfate and cellular debris is removed prior to said mixing step. In one embodiment the detection particles are nanoshells and the blood sample is not pre-treated prior to the addition of the assay reagents.

[0055] In one embodiment, anti-immunosuppressant drug antibodies can also be used in an agglutination assay in place of one or both receptor proteins.

[0056] In one embodiment antibodies (and in one embodiment monoclonal antibodies) can be raised against a complex comprising an immunosuppressant drug and an immunophilin, wherein the antibody is selected based on its binding to the immunosuppressant drug. In this manner the specificity of the antibody for active immunosuppressant drugs may be enhanced relative to inactive metabolites of the immunosuppressant drug. As an example, an antibody could be raised against a portion of the rapamycin molecule specific of FKBP12 binding site (wherein the immunogen used comprises a complex of rapamycin and a target of rapamycin (TOR) protein). Alternatively, an antibody could be raised against a portion of the rapamycin molecule specific to FKBP-rapamycin associated protein binding site (wherein the immunogen used comprises a complex of rapamycin and FKBP12). A similar approach would be applicable to other immunosuppressant drugs including cyclosporin, tacrolimus, and everolimus. For example, an antibody is raised against FK-506 bound to FKBP12 and used to measure FK-506 in a sample. The binding of FK-506 is very tight to FKBP12 and the FK-506/FKBP12 complex

may be coupled to KLH as an immunogen to be injected into mice to elicit an immune response. (In this case the coupling would be done between FKBP12 and KLH through standard protein-protein coupling methods.) The isolation of suitable antibodies and selection of monoclonal antibodies would be done using standard methods known in the art. This approach can also be used to produce antibodies against cyclosporin A bound to cyclophilin.

[0057] In accordance with one embodiment a method for determining the amount of an immunosuppressant drug in a sample is provided, wherein the assay reagents are selected from the group consisting of receptor proteins bound to detection particles and antibodies bound to detection particles. In one embodiment the antibodies are monoclonal antibodies. In one embodiment the assay is conducted with two antibodies wherein the antibodies were raised against an immunosuppressant drug/immunophilin complex, but having specificity for two different sites on the immunosuppressant drug. In this embodiment the two antibodies are coupled to a detection particle. In another embodiment the assay is conducted with two antibodies wherein the antibodies were raised against an immunosuppressant drug/immunophilin complex, but having specificity for two different sites on the immunophilin. In this embodiment the two antibodies are coupled to a detection particle. In a further embodiment the assay reagents may comprise one receptor that specifically binds to the target immunosuppressant drug and one antibody that specifically binds to a different location of the immunosuppressant drug. In this embodiment both the receptor protein and the antibody are bound to detection particles.

[0058] In a further embodiment the assay reagents may comprise a first receptor, second receptor and an antibody that specifically binds to the second receptor. In this embodiment, the first receptor is bound to a detection particle, and the first receptor specifically binds to the target immunosuppressant drug. The second receptor protein binds to the target immunosuppressant drug at a different location than the first receptor protein. In this embodiment the second receptor protein is not bound to a detection particle and the antibody is typically not labeled. However, the antibody may be optionally bound to a detection particle. In one embodiment the antibody that specifically binds to the second receptor protein is generated against an immunogen comprising a second receptor/immunosuppressant drug complex. In one of the embodiments, one of the receptors, e.g., FKBP12 (for rapamycin) is labeled with a tag, e.g., biotin. When a sample is mixed with a limited amount of biotinylated first receptor protein and a second receptor protein, a sandwich complex is formed with the target drug present in the sample. The resultant biotinylated sandwich complex plus unreacted FKBP12 is then added to a second reagent containing streptavidin latex microparticles and an antibody which specifically binds to the second receptor protein. Agglutination takes place in proportion to the number of biotinylated sandwich complexes bound to streptavidin latex and agglutinated by the antibody, which in turn is directly related to the amount of drug present. Antibody reacting with free non-complexed second receptor partner does not result in any signal. It is important to note that in this alternative, the antibody binds to the second receptor at a site other than the receptor binding site for the drug. Anti

immunophilin antibodies that specifically bind to an FKBP/rapamycin-binding domain are described in U.S. Pat. No. 6,464,974.

[0059] In accordance with one embodiment a method of quantitating an immunosuppressant drug comprises providing a sample and mixing the sample with a reagent composition to form a suspension. The reagent composition comprises a receptor and an antibody, each of which is bound to a detection particle and wherein each of said receptor and an antibody specifically and simultaneously bind to the immunosuppressant drug. The amount of particle agglutination present in the suspension is then measured using a homogeneous assay technique, such as absorbance measurement, for example, and the amount of detected particle agglutination is then correlated with the amount of immunosuppressant drug present in the sample.

[0060] In accordance with another embodiment a method of quantitating an immunosuppressant drug comprises providing a sample, mixing the sample with a reagent composition to form a suspension, wherein the reagent composition comprises a first and second antibody, each of which is bound to a detection particle and wherein each of said first and second antibody specifically and simultaneously bind to the immunosuppressant drug. The amount of particle agglutination present in the suspension is then measured using a homogeneous assay technique, such as by absorbance measurement, for example, and the amount of detected particle

FRBD and FKBP12 peptide mutants are generated using site-directed mutagenesis in order to enhance the specificity of the assay for the parent drug of rapamycin. The concept to mutate the receptor proteins in order to enhance the specificity of the receptor complex for the parent drug of rapamycin could be applied to any of the receptor proteins disclosed herein. The formation of the γ TOR2 FRBD/rapamycin/FKBP12 complex can be monitored by several of the methods described herein and used to measure the rapamycin concentration in patient samples.

[0063] Publications of crystal structures can be used as an aid in the mutagenesis studies. The mutant binding proteins can be produced through recombinant methods well-known in the art and have affinity tags added through recombinant methods to aid in their purification, using methods that are also well known in the art. Accordingly, with reference to the following table, it is anticipated that the following amino acid residues can be modified to enhance the specificity of the molecules: the glutamic acid in position 54 of FKBP12, the asparagine in position 2038 of γ TOR2, the aspartic acid in position 1972 of γ TOR2, and the arginine in position 1976 of γ TOR2.

[0064] The following table shows C-C distances measured between rapamycin metabolites, FKBP12 and FRBD. The crystal structure 1 fap published by the RCSB PDB (Protein Data Base) was used to measure the C-C distances of the rapamycin metabolites (12).

PDB 1 fap C—C distances	C moiety	FKBP12 (Å)	FRB (Å)	TOR2
M1 12-hydroxy-Rapa	C12	H87 (7.12) I90 (6.90)	T2098 (7.07)	N2038
M2 24-hydroxy-Rapa	C24	E54 (7.69)	L2031 (6.53)	L1971
			E2032 (4.67)	D1972
M3 16-O-desmethyl-Rapa	C50		T2098 (4.00)	N2038
			Q2099 (5.73)	Q2039
			D2102 (4.04)	D2042
M4 39-O-desmethyl-Rapa	C52	E54 (6.79)	R2036 (4.54)	R1976
			G2040 (3.93)	G1980
M5 27,39-O-didesmethyl-Rapa	C51, (C52 see M4)		E2032 (5.18)	D1972
			E2033 (6.48)	D1973
			S2035 (5.45)	S1975
			R2036 (4.18)	R1976

agglutination is then correlated with the amount of immunosuppressive drug present in the sample.

[0061] In accordance with another embodiment a method of quantitating an immunosuppressant drug comprises providing a sample, mixing the sample with a reagent composition to form a suspension, wherein the reagent composition comprises a first and second receptor and antibody that specifically binds to the second receptor, wherein each of said first and second receptors specifically and simultaneously bind to the immunosuppressant drug, and the first receptor is bound to a detection particle. In one embodiment the antibody is also bound to a detection particle. The amount of particle agglutination present in the suspension is then measured using a homogeneous assay technique, such as by absorbance measurement, for example, and the amount of detected particle agglutination is then correlated with the amount of immunosuppressive drug present in the sample.

[0062] The present invention also anticipates the generation of receptor mutants that have enhanced selectivity for the parent drug. In accordance with one embodiment γ TOR2

[0065] In one embodiment, detection of complex formation is conducted using a fluorescence resonance energy transfer (FRET) pair of fluorophores. FRET is based upon the distance-dependent transfer of excited-state energy from a donor fluorophore to an acceptor fluorophore. The donor fluorophore is excited by incident light and if an acceptor is within 20 Å to 60 Å, the excited state energy from the donor can be transferred. Beyond the optimum range of intermolecular distances, the energy transfer efficiency falls off as the inverse sixth power of the distance. This transfer leads to a reduction in the donor's fluorescence intensity and excited-state lifetime, and an increase in the acceptor's emission intensity (Selvin, P. R., *Nature Structural Biology*, 2000, 7, 9, 730-734). This assay format requires that a pair of FRET fluorophores be coupled to a pair of binding receptors. A donor fluorophore such as europium and an acceptor fluorophore such as Cy5 have been used using light excitation at 340 nm and emission detection at 665 nm (Pulli, T., et al., *Analytical Chemistry*, 2005, 77, 2647-2642). Accordingly, first and second receptor proteins that specifi-

cally bind to an immunosuppressant drug can be labeled with the respective donor and acceptor fluorophores. For example, in an assay for detecting rapamycin, an europium donor fluorophore can be used to label a first receptor (for example, FKBP12) and a Cy5 acceptor fluorophore can be used to label a second receptor (for example γ TOR2 FRBD). The distances measured between selected amino acids in the mTOR FRBD-rapamycin-FKBP12 complex (1 fap, Protein Data Bank) are listed in the table below. One possible combination is to label the amino (NH_2) terminus of γ TOR2 (R1959) and label the amino terminus of FKBP12 (G1) which are 48.56 Å apart in the crystal structure, assuming that γ TOR2 adopts a conformation similar to mTOR FRBD in the mTOR FRBD-rapamycin-FKBP12 complex.

	mTOR FRBD (γ TOR2)	Distance	FKBP12
amino acid position	R2018 (R1959)(NH_2 terminus)	38.5 Å	E107 (COOH terminus)
	R2018 (R1959)(NH_2 terminus)	48.56 Å	G1 (NH_2 terminus)
	S2112 (G2052)(COOH terminus)	32.97 Å	G1 (NH_2 terminus)

[0066] Several commercial kits are available to label the amino acids of γ TOR2 and FKBP12 with the donor and acceptor fluorophores. For example, the LANCE Eu-W1024-ITC chelate (Perkin Elmer, AD0013) is optimized for the covalent labeling of proteins possessing at least one primary aliphatic amine (N-terminus or lysine residues) with europium. Additionally, the CyDye Cy5 mono-Reactive Dye pack (Amersham, Product Cod PA23500) can be used for the covalent labeling of amine groups on proteins with Cy5. Other chemistries are available to enable labeling via amine groups (using NHS ester dyes), thiol groups (using maleimide dyes), or aldehyde groups (using hydrazide dyes). The γ TOR2 and FKBP12 receptors can be labeled accordingly and the unlabeled fluorophores can be removed from the labeled receptors by column chromatography.

[0067] The assay can be performed by the addition of rapamycin to appropriately labeled γ TOR2 and labeled FKBP12. The presence of rapamycin will drive the formation of the γ TOR2/rapamycin/FKBP12 complex in a rapamycin dependent manner. Using a fluorometer one can measure the fluorescence resulting from the light excitation of the γ TOR2/rapamycin/FKBP12 complex formed. In this example, the fluorescence measured is directly proportional to the rapamycin concentration. One can construct a standard curve with known concentrations of rapamycin. The unknown rapamycin concentration in patient samples can thus be calculated based on the standard curve. Similarly, other immunosuppressant drugs can be detected by labeling the receptor proteins FKBP12 and calcineurin for detecting tacrolimus, or a cyclophilin and calcineurin for detecting cyclosporin, with a donor and acceptor fluorophore respectively.

[0068] The present invention also encompasses double receptor assays for tacrolimus (FK506) in which calcineurin A/B fusion proteins combined with FKBP12 are employed. The calcineurin A/B fusion proteins may be truncated sequences of the native protein with N- or C-terminal tags. Thus, in one embodiment, C-terminal his-tagged calcineurin A/B fusion proteins with molecular weights of 64 kDa

(CNtAB-long) and 26 kDa (CNtAB-short), respectively, were selected for binding activity assays, based on expression and solubility screen in *E. coli* as well as a cell-free system. When expressed in *E. coli*, both CNtAB-long and CNtAB-short fusions displayed a robust expression level and the expressed fusion proteins were stable and soluble. A >90% degree of purity was achieved for both fusion proteins by a simple affinity capture and size exclusion chromatography purification procedure. Importantly, a reference time-resolved fluorescence (TRF) assay showed an EC50 value of 0.77 nM for binding of the purified CNtAB-long to FKBP12 in an FK506-dependent manner. However, the binding of the purified CNtAB-short to FKBP12-FK506 was hardly detected in that assay. A Biacore Surface Plasmon Resonance (SPR) assay confirmed the results observed by the above TRF assay.

[0069] The present invention also encompasses kits containing the assay reagents for measuring immunosuppressant drugs in samples. The kit may further include a variety of containers, e.g., vials, tubes, bottles, and the like. Preferably, the kits will also include instructions for use. In one embodiment the kit comprises first and second binding moieties wherein the first and second binding moieties specifically bind to separate binding sites on the target drug to form a sandwich complex upon contact of the reagents with the drug. The first and second binding moieties represent compounds independently selected from the group consisting of receptor proteins and antibodies. In one embodiment the kit comprises a first and second receptor protein and an antibody, wherein the first receptor protein is bound to a detection particle and the antibody specifically binds to the second receptor protein. In this embodiment the antibody specific for the second receptor can optionally be bound to a second detection particle. The individual binding moieties can be provided in separate container or mixed together in a single container.

[0070] In an alternative embodiment the kit comprises a first receptor and a second receptor wherein the first and second receptors are each bound to at least one detection particle, and wherein the first and second receptors specifically bind to separate binding sites on the target drug to form a sandwich complex upon contact of the receptors with the drug. In one embodiment the receptor proteins of the kit are each provided with a tag for binding the receptor to a detection particle, and the detection particles are provided in a separate container. In another embodiment the first and second receptors will be provided with the detection particle already bound to the first and second receptors. The first and second receptor proteins can be provided in separate containers or mixed together in a single container. In one embodiment the first and second receptors comprise a biotinylated tag and the detection particle is bound to streptavidin. In one embodiment each of the first and second receptors is bound to a single biotin molecule. In one embodiment the detection particle is a microparticle bound to streptavidin or to an antibody specific for biotin. In one embodiment the kit comprises two complexes, each comprising a receptor bound to a microparticle through a biotin-streptavidin linkage.

[0071] In one embodiment a kit is provided for quantitating the amount of rapamycin or everolimus in a sample. In this embodiment, the kit comprises a first receptor comprising an FK506 binding protein bound to a detection particle, and a second receptor comprising a target of rapamycin

(TOR) protein bound to a second detection particle. In one embodiment the first receptor comprises FKBP12 or FKBP25 and the second receptor is selected from the group consisting of mTOR, γ TOR1, γ TOR2, and the FRBDs of mTOR, γ TOR1, and γ TOR2. In an alternative embodiment a kit is provided for quantitating the amount of tacrolimus present in a sample, wherein the kit reagents comprise a first receptor comprising an FK506 binding protein (FKBP) bound to a detection particle and a second receptor comprising calcineurin, or a binding fragment or derivative thereof, bound to a detection particle. In a further embodiment the kit further comprises calmodulin and a calcium ion, either contained in separate containers or bound to calcineurin.

[0072] In yet another embodiment a kit is provided for quantitating the amount of cyclosporin, wherein the reagents comprise a first receptor comprising a cyclophilin bound to a detection particle and a second receptor comprising a calcineurin bound to a detection particle. In a further embodiment the kit further comprises calmodulin and a calcium ion, either contained in separate containers or bound to calcineurin. The detection particle of the kits is typically either a nanoshell or a microparticle, and in one embodiment the detection particle is a latex microparticle.

[0073] The following examples are set forth to illustrate specific embodiments and should not be construed to limit the scope of the invention as defined by the claims.

SPECIFIC EMBODIMENTS

Example 1

Synthesis of cDNA Encoding γ TOR1 FRBD and γ TOR2 FRBD

[0074] The cDNAs encoding the 90-amino acid FKBP12 rapamycin binding domains (FRBD) of yeast TOR1 and TOR2 were chemically synthesized by Blue Heron Biotechnology (Seattle, Wash.) with the following optimized nucleotide acid sequence:

SEQ ID NO: 1 (γ TOR1 FRBD):
5' GAACCTTGGTATGAAGGACTCGAAGATGCATCTCGCCAATTTTTGGTTGAACACAATATC

GAAAAAATGTTTTCTACACTTGAACCCCTTACACAAACACCTTGGAAATGAACCACAAACCCCTC

TCTGAAGTCTCCTTTCAAAAATCCTTTGGCCGTGACCTTAACGACGCATATGAATGGCTTAAC

AACTACAAAAATCTAAAGATATTAATAACCTGAATCAAGCATGGGACATCTATTACAACGTA

TTTCGCAAAATTAAGTCTCAA 3'

SEQ ID NO: 2 (γ TOR2 FRBD):

5' GAACAATGGTATGAAGCCCTGGATGATGCCTCTCGTCAATTTTGGTGAACACAATACA

GAAAAAATGTTTTGAGCGCTCGAACCCCTTATATGAAATGTTAAAACGTGGTCCAGAACTTTA

CGGGAAATTTCTTTTCAAAATCTTTTGGTCGTGATCTCAATGATGCCTATGAATGGTTAATG

AATTATAAAAAAGCAAGATGTTAGCAACCTTAATCAAGCCTGGGATATTTACTATAACGTA

TTTCGCAAAATCGGTAAACAG 3'

Example 2

Construction of Expression Vectors

[0075] The synthetic cDNAs, along with two sets of oligonucleotide primers, were used to PCR-amplify approximately 300-bp DNA fragments, corresponding to γ TOR1 FRBD and γ TOR2 FRBD, and these DNA fragments were cloned into expression vector pIVEX2.7d or pIVEX2.8d (Roche Diagnostics GmbH, Germany). The resulting plasmids, confirmed by DNA sequencing, can express Avi-Tagged target proteins that are not only able to bind rapamycin-bound FKBP12 but also to be specifically biotin-labeled at the unique lysine residue by biotin ligase BirA.

SEQ ID NO: 3 (forward primer for γ TOR1 FRBD):
5' CTTTAAGAAGGAGATATACCATGGAACTTTGGTATGAAGGACTC 3'

SEQ ID NO: 4 (reverse primer for γ TOR1 FRBD):
5' AAGATGTCGTTTCAGGCCCTTGACGAGTAATTTTGCAGAA 3'

SEQ ID NO: 5 (forward primer for γ TOR2 FRBD):
5' CGCTTAATTAACATATGACCCGAACAATGGTATGAAGGCCTG 3'

SEQ ID NO: 6 (reverse primer for γ TOR2 FRBD):
5' TTAGTTAGTTACCGGATCCCTTACTGTTTACCGATTTTGCAGAA 3'

Example 3

Production of Biotinylated γ TOR1 FRBD and Biotinylated γ TOR2 FRBD in *E. coli*

[0076] After selection using the cell-free Rapid Translation System (RTS) from Roche Diagnostics Corporation, the optimal expression constructs were transformed into BL21-A1 cells, along with BirA expression vector, and induced in the presence of 50 μ M biotin, 0.2% arabinose and 0.2 mM isopropyl β -D-thiogalactopyranoside (IPTG) at 30° C. for 16 hours. The expressed recombinant proteins were purified to a purity of approximately 90% through the modified avidin beads and size exclusion chromatography.

[0077] Following is the amino acid sequence for recombinant yTOR1 FRBD (106 amino acids). Lys 101 is the specific site for BirA-dependent biotin labeling. This protein binds to rapamycin at the FKBP12 binding site. The AviTag sequence is indicated by bold type, and Lys 101 (K) is the specific site for BirA-dependent biotin labeling.

SEQ ID NO: 7 (AviTagged yTOR1 FRBD):
 1 10 20 30 40 50 60
 ELWYEGLEDASRQFFVEHNIKMFSTLEPLHKHLGNPQTLSEVSPQRSFGRDLNDAYEWL
 70 80 90 100
 NNYKSKDINNLNQAWDIYINVFRKITRQ**GLNDIFEAQKIEWHE**

[0078] Following is the amino acid sequence for recombinant yTOR2 FRBD (119 amino acids). Lys 12 is the specific site for BirA-dependent biotin labeling. This protein binds to rapamycin at the FKBP12 binding site. The AviTag sequence is indicated by bold type, and Lys 101 (K) is the specific site for BirA-dependent biotin labeling.

SEQ ID NO: 8: (AviTagged yTOR2 FRBD):
 1 10 20 30 40 50 60
 MS**GLNDIFEAQKIEWHE**IEGRGRLIKHMTEQWYEGLDASRQFFGEHNTEKMFAALEPLYE
 70 80 90 100 110 120
 MLKRGPETLREISFQNSFGRDLNDAYEWLMNYKSKDVSNNLNQAWDIYINVFRKITGKQ

Example 4

Construction of Expression Vectors

[0079] Two oligonucleotide primers were used to PCR-generate a DNA fragment, encoding FKBP12 with multiple glycine and histidine amino acids at the carboxyl end, were cloned into the Nco I/Sma I sites of the expression vector

purified to approximately 95% by sequential affinity purifications using Ni-NTA agarose and Mono-Avidin beads (Pierce), respectively.

[0081] Following is the amino acid sequence for this recombinant rapamycin binding protein (134 amino acids). Lys 129 is the specific site for BirA-dependent biotin labeling. This protein binds to rapamycin.

SEQ ID NO: 9: (AviTagged FKBP12):
 1 10 20 30 40 50
 MGVQVETISPGDGRTPPKRGQTCVVHYTGMLEDGKKFDSSDRNKPFKFMGLGKQEVIRG
 60 70 80 90 100 110
 WEEGVAQMSVQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLKLEGGGSHHHHHHG
 120 130
GLNDIFEAQKIEWHE

pIVEX2.7d. The resulting plasmid, confirmed by DNA sequencing, can express a fusion protein with an expected molecular weight of 15.0 kDa that is able to bind rapamycin, nickel NTA matrix and to be biotin-labeled at the AviTag specific Lys by biotin ligase BirA.

Example 5

Production of Biotinylated FKBP12 (MW 15 kDa) in *E. coli*

[0080] Transformed BL21-A1 cells harboring the above expression vector and a vector expressing BirA were induced by 0.2% arabinose and 0.4 mM isopropyl IPTG at 30° C. for 16 hours. The expressed recombinant protein was

Example 6

Preparation of FKBP12-coated Microparticles

[0082] Streptavidin microparticles (127 nm) (SA-Latex Universalreagenz ZLF, Mat, No 03140431001) were obtained from Roche Diagnostics GmbH, Germany. Final concentration was 0.3% (w/v) in 50 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.9.

[0083] Biotinylated FKBP12 from Example 5 (32 µL of 3.27 mg/mL) was added to 5.2 mL of 50 mM MOPS buffer (pH 7.9). This biotinylated protein solution was added to 5.2 mL of stirred suspension 0.3% (w/v) of streptavidin microparticles (127 nm) in 50 mM MOPS (pH 7.9) all at once at room temperature. The reaction was allowed to stir at room temperature for 2 hours and overnight at 4° C., and then centrifuged at 16,000 rpm for 1 hour 45 minutes. The

microparticles were resuspended in 50 mM MOPS buffer (pH 7.9) and then centrifuged at 16,000 rpm. The process of resuspension and centrifugation was repeated one more time, and the final concentration of FKBP12-coated microparticles was adjusted to 0.15% (w/v).

Example 7

Preparation of γ TOR1 FRBD- and γ TOR2 FRBD-coated Microparticles

[0084] Biotinylated γ TOR1 FRBD from Example 3 (300 μ L of 0.35 mg/mL) was added to 50 mM MOPS buffer (pH 7.9). This biotinylated protein solution was added to 5.2 mL of stirred suspension 0.3% (w/v) of streptavidin microparticles (127 nm) in 50 mM MOPS (pH 7.9) all at once at room temperature. The reaction was allowed to stir at room temperature for 2 hours and overnight at 4° C., and then centrifuged at 16,000 rpm for 1 hour 45 minutes. The microparticles were resuspended in 50 mM MOPS buffer (pH 7.9) and then centrifuged at 16,000 rpm. The process of resuspension and centrifugation was repeated one more time, and the final concentration of γ TOR1 FRBD-coated microparticles was adjusted to 0.15% (w/v).

[0085] Preparation of γ TOR2 FRBD-coated microparticles using the γ TOR2 FRBD from Example 3 was made as described above.

allowed to stand at room temperature for 5 minutes, and then spun down at 12,000 rpm for 5 minutes. The supernatant was transferred into the sample cup and loaded onto the analyzer. **[0090]** The first working reagent (reaction buffer) was placed in the R1 reagent rotor and the second working reagent (microparticle reagent) in the R2 reagent rotor. An assay was performed using a Roche/Hitachi 917 automated analyzer (Roche Diagnostics Corporation, Indianapolis) using a 35 μ L sample volume, 150 μ L of first working reagent and 80 μ L of second working reagent (measurement at wave length 480 nm) for 10 minutes. The calibration curve for the rapamycin assay using γ TOR1 FRBD is shown in FIG. 1. The calibration curve for the rapamycin assay using γ TOR2 FRBD is shown in FIG. 2.

[0091] In another assay, thirteen individual whole blood samples obtained from 13 volunteers were spiked at various levels of rapamycin covering a range of 0-48 ng/mL (26 samples total were used). All 26 samples were measured according to the procedure described above using a Roche/Hitachi 917 automated analyzer. The data obtained is shown in FIG. 3 and is for assay made using γ TOR2 FRBD.

Example 9

Synthesis of cDNA Encoding mTOR FRBD

[0092] The cDNA encoding 90 amino acid FKBP12-rapamycin binding domain of mTOR (FRBD) was chemically synthesized by Blue Heron Biotechnology with the following optimized nucleotide acid sequence:

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SEQ ID NO: 10 (mTOR FRBD):
5' gaaatgtggcatgaaggacttgaagaagctagccgtctctattttggtgaacgcaacgta
aaaggaatgtttgaagtaacttgaacctttacacgcaatgatggaacgtggaccccaaacctta
aaagaaacctccttaatacaagcatatggtcgtgacttaaggaagctcaagaatggtgctcgt
aaatatatgaaatctggtaatgtaaaagatttaactcaagcctgggatttatactatcacggt
ttccgccgtatctcaaaaaca 3'
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Example 8

Rapamycin Calibration Curves Using Spiked Whole Blood Samples

[0086] A reaction buffer reagent was prepared by making a 175 mM PIPES buffer, pH 7.4, containing 0.5% polyacrylic acid (PAA). This was a first working reagent.

[0087] Mixing a 1:1 ratio (v/v) of FKBP12-coated microparticles and γ TOR1 FRBD-coated microparticles provided a second working reagent. An alternative second working reagent was prepared by mixing a 1:1 ratio (v/v) of FKBP12-coated microparticles and γ TOR2 FRBD-coated microparticles.

[0088] Calibrators were made from an EDTA whole blood pool by spiking rapamycin at the levels of 0, 5, 10, 20, and 30 ng/mL using a rapamycin stock of 1 μ g/mL in methanol. The pretreatment of blood samples was done using a protein precipitation reagent of a 4:1 mixture of methanol to aqueous zinc sulfate (300 mM).

[0089] Two hundred μ L of whole blood was added to 200 μ L of precipitation reagent and vortexed for 15-30 seconds,

Example 10

Construction of Expression Vectors

[0093]

```
SEQ ID No: 11 (primer 1 for mTOR FRBD):
5' CTTTAAGAAGGAGATATACCATGGAAATGTGGCATGAAGGACTTGAA
3'
```

```
SEQ ID NO: 12 (primer 2 for mTOR FRBD):
5' AAGATGTCGTTTCAGGCCCTTGTGTTTGAGATACGGCGGAA 3'
```

[0094] The above two oligonucleotide primers and the above-described cDNA encoding mTOR FRBD were used to PCR-amplify a DNA fragment of 310 bp and was cloned into the Nco I/Sma I sites of the expression vector pIVEX2.7d. The resulting plasmid, confirmed by DNA sequencing, can express a fusion protein that is able to bind rapamycin-bound FKBP12 and can be biotin-labeled using biotin ligase BirA.

Example 11

Production of Biotinylated mTOR FRBD in *E. coli*

[0095] The cell-free Rapid Translation System (RTS) from Roche was used to produce the biotin-labeled, AviTagged mTOR FRBD. Purified plasmid DNA was incubated with the both RTS reaction and biotinylation reagents in an RTS 500 ProteoMaster reaction device for 18 hours. The generated recombinant protein was purified to a purity of approximately 95% through the modified avidin beads.

[0096] The amino acid sequence for this recombinant FRBD (107 amino acids) is as follows (Lys 102 is the specific site for BirA-dependent biotin labeling):

```
SEQ ID NO: 13 (AviTagged mTOR FRBD):
1      10      20      30      40      50
MEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSPNQAY
GRDLMEAQ

60      70      80      90      100
EWCRKYMKSGNVKDLTQAWDLYYHVFRRIKQGGGLNDIFEAQKIEWHE
```

Example 12

Preparation of FKBP12-coated Microparticles

[0097] FKBP12 coated microparticles were prepared following a procedure similar to that described in Example 6. Streptavidin microparticles (SA-Latex Universalreagenz ZLF, Mat. No 0314041001) were obtained from Roche Diagnostics GmbH, Germany (127 nm). The final concentration was 0.3% (w/v) in 50 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.9.

[0098] Biotinylated FKBP12 (40 μ L of 2.5 mg/mL) was added to 5 mL of 50 mM MOPS buffer (pH 7.9). This biotinylated protein solution was added to 5 mL of a stirred suspension of 0.3% streptavidin microparticles (127 nm) in 50 mM MOPS (pH 7.9) all at once at room temperature. The reaction was allowed to stir at room temperature for 2 hours and overnight at 4° C., and then centrifuged at 16,000 rpm for 1 hour 45 minutes. The microparticles were resuspended in 50 mM MOPS buffer (pH 7.9) and then centrifuged at 16,000 rpm. The process of resuspension and centrifugation was repeated one more time, and the final concentration of FKBP12-coated microparticle was adjusted to 0.15% (w/v).

Example 13

Preparation of mTOR FRBD-coated Microparticles

[0099] Biotinylated m-TOR FRBD from Example 11 (50 μ L of 2 mg/mL) was added to 5 mL of 50 mM MOPS buffer (pH 7.9). This biotinylated protein solution was added to 5 mL of stirred suspension, 0.3%, of streptavidin microparticles (127 nm) in 50 mM MOPS (pH 7.9) all at once. The reaction was allowed to stir at room temperature for 2 hours,

then centrifuged at 16,000 rpm for 1 hour 45 minutes, and the final concentration of protein-streptavidin microparticles was adjusted to 0.15% (w/v).

Example 14

Rapamycin Calibration Curve Using Spiked Whole Blood Samples

[0100] Mixing a 1:1 ratio (v/v) of FKBP12-coated microparticles and mTOR FRBD-coated microparticles provided a first working reagent. This reagent was used at a final concentration of 0.15% in 50 mM MOPS buffer at pH 7.9.

[0101] A second working reagent was prepared by making a 175 mM PIPES buffer, pH 7.4, containing 0.8% polyacrylic acid (PAA).

[0102] Calibrators were made by using EDTA whole blood samples spiked with 12.5, 25, and 50 ng/mL of rapamycin (from a stock of 10 μ g/mL of rapamycin in methanol). The pretreatment of blood samples was done using protein precipitation with methanol and aqueous CuSO₄ (copper sulfate) solution.

[0103] 200 μ L of whole blood was added to 200 μ L of methanol, followed immediately by 50 μ L of 300 mM CuSO₄. This was vortexed for 15-30 seconds, allowed to stand at room temperature for 5 minutes, and then spun down at 12,000 rpm for 5 minutes. The supernatant was transferred to a sample cup and loaded onto a Roche/Hitachi 917 automated analyzer (Roche Diagnostics Corporation, Indianapolis).

[0104] An assay was performed using a 20 μ L sample volume, 80 μ L of the first working reagent, 180 μ L of the second working reagent, and a measurement at wave length of 480 nm. The results are shown in FIG. 4.

Example 15

Rapamycin Assay Using Whole Blood Sample

[0105] A human blood sample was spiked with 25 ng/mL of rapamycin, extracted following the procedure described above, and the concentration was determined using the calibration curve shown in FIG. 4 as 24.7 ng/mL and 24.6 ng/mL in duplicate runs.

Example 16

Production of Truncated Recombinant Calcineurin A and B Fusions (CNtABs) in *E. coli*

Truncated Calcineurin A and B cDNAs

[0106] The cDNA encoding the truncated human calcineurin A (amino acids 12-394) and wild-type chain B (amino acids 1-170) was chemically synthesized by Blue Heron Biotechnology (Seattle, Wash.) with the following optimized nucleotide acid sequence (SEQ ID NO: 14):

fluorescence assay (data not shown). Taken together, these data indicate that the recombinant CNtAB-long is functional but that functionality of CNtAB-short is unlikely.

What is claimed is:

1. An assay method for determining a presence or an amount of an immunosuppressant drug in a sample comprising the steps of

providing a sample;

mixing said sample with a first receptor and a second receptor to form a suspension, wherein said first and second receptors are bound to detection particles and each of said first and second receptors specifically bind to a separate binding site on said drug, wherein the presence of the drug results in agglutination of the detection particles;

directly detecting or measuring an amount of particle agglutination in said suspension; and

correlating the amount of particle agglutination with the presence or amount of the immunosuppressant drug in the sample.

2. The method of claim 1 wherein the immunosuppressant drug is selected from the group consisting of cyclosporin, tacrolimus, rapamycin, and everolimus, and said first receptor is an immunophilin or a binding fragment thereof.

3. The method of claim 1 wherein the detection particles are selected from the group consisting of microparticles and nanoshells.

4. The method of claim 1 wherein

the immunosuppressant drug is rapamycin or everolimus; the first receptor comprises an FK506 binding protein (FKBP); and

the second receptor comprises a target of rapamycin (TOR) protein.

5. The method of claim 4 wherein the FKBP comprises one of FKBP12 or FKBP25 and the second receptor is selected from the group consisting of mTOR, the FKBP binding domain of mTOR, γ TOR1, the FKBP binding domain of γ TOR1, γ TOR2, and the FKBP binding domain of γ TOR2.

6. The method of claim 1 wherein

the immunosuppressant drug is tacrolimus;

the first receptor comprises an FK506 binding protein (FKBP); and

the second receptor comprises calcineurin or a binding fragment thereof.

7. The method of claim 6 wherein the calcineurin has both calmodulin and a calcium ion bound thereto.

8. The method of claim 1 wherein

the immunosuppressant drug is cyclosporin;

the first receptor comprises a cyclophilin; and

the second receptor comprises calcineurin.

9. The method of claim 8 wherein the calcineurin has both calmodulin and a calcium ion bound thereto.

10. The method of claim 3 wherein the sample is a blood sample obtained from a patient being administered at least one immunosuppressant drug.

11. The method of claim 10 wherein the blood sample is processed prior to said mixing step.

12. The method of claim 3 wherein the detection particles are nanoshells.

13. The method of claim 3 wherein the detection particles are bound to said first and second receptors via a biotin-streptavidin or biotin-avidin linkage.

14. The method of claim 3 wherein the detection particles are covalently bonded either directly or indirectly through a linker to said first and second receptors.

15. The method of claim 14 wherein a linker comprises an antibody to the first receptor and an antibody to the second receptor.

16. The method of claim 13 wherein the first and second receptors are recombinant fusion proteins comprising an attachment site for a molecule of biotin.

17. The method of claim 16 wherein the attachment site comprises a biotinylation signal sequence.

18. The method of claim 17 wherein the fusion proteins are co-expressed with, or expressed in the presence of, a biotin ligase, which catalyzes covalent addition of a biotin molecule to a residue in the signal sequence.

19. The method of claim 18 wherein the biotin ligase comprises AviTag and the residue is lysine.

20. The method of claim 17, wherein one fusion partner of the fusion protein is a recombinant receptor protein having a C-terminus and an N-terminus and one fusion partner comprises the biotinylation signal sequence, further wherein when the attachment site is at the C-terminus for the first receptor, it is at either the N-terminus or the C-terminus for the second receptor.

21. A reagent for determining an immunosuppressant drug in a sample, said reagent comprising

a first drug binding complex comprising a first receptor bound to a first detection particle;

a second drug binding complex comprising a binding moiety selected from the group consisting of a second receptor bound to a detection particle, an immunosuppressant drug specific antibody bound to a second detection particle, and a second receptor specific antibody complexed with a second receptor, wherein each drug binding complex specifically binds to a separate binding site on said immunosuppressant drug.

22. The reagent of claim 21 wherein the binding moiety is a second receptor bound to a detection particle.

23. The reagent of claim 22 wherein the immunosuppressant drug to be detected is rapamycin, said first drug binding complex comprises an FK506 binding protein, and said second drug binding complex comprises a target of rapamycin (TOR) protein.

24. The reagent of claim 21 wherein the detection particle is a microparticle.

25. The reagent of claim 21 wherein the second drug binding complex comprises a second receptor specific antibody complexed with a second receptor.

26. A method for determining a presence or an amount of an immunosuppressant drug in a sample comprising the steps of:

providing a sample;

mixing said sample with a receptor bound to a first detection particle and a binding moiety selected from the group consisting of an immunosuppressant drug specific antibody bound to a second detection particle, and a second receptor specific antibody complexed with a second receptor to form a suspension, wherein said receptor specifically binds to said immunosuppressant drug at a location different from where the immunosuppressant drug specific antibody binds, wherein the presence of the drug results in agglutination of the detection particles;

measuring an amount of particle agglutination in said suspension by absorbance measurement; and correlating the amount of particle agglutination with the presence or amount of the immunosuppressant drug in the sample.

27. The method of claim **26** wherein the immunosuppressant drug is selected from the group consisting of cyclosporin, tacrolimus, rapamycin, and everolimus and the binding moiety is an antibody specific for the immunosuppressant drug, wherein the antibody is bound to a detection particle.

28. The method of claim **26** wherein the immunosuppressant drug is rapamycin or everolimus; the first receptor comprises an FK506 binding protein (FKBP); and the binding moiety comprises a target of rapamycin (TOR) protein or antibody that specifically binds to a target of rapamycin (TOR) protein.

29. The method of claim **26** wherein the immunosuppressant drug is tacrolimus; the first receptor comprises an FK506 binding protein (FKBP); and the second receptor comprises a calcineurin or antibody that specifically binds to calcineurin.

30. The method of claim **24** wherein the immunosuppressant drug is cyclosporin; the first receptor comprises a cyclophilin; and the second receptor comprises a calcineurin or antibody that specifically binds to calcineurin.

31. A kit for detecting immunosuppressant drugs, said kit comprising a first receptor and a second receptor that specifically bind to a target immunosuppressant drug at two separate

locations on the immunosuppressant drug, said first and second receptors each further comprising a tag; and a plurality of detection particles, each of said particles being bound to an agent that binds to said tag.

32. A kit for detecting an immunosuppressant drug in a sample, said kit comprising

a first reagent comprising a suspension of an immunophilin bound to a first detection particle;

a second reagent comprising a suspension of a receptor protein selected from the group consisting of FKBP-rapamycin associated protein, calcineurin and an antibody to calcineurin, said second receptor protein bound to a detection particle, wherein the detection particles are selected from the group consisting of microparticles and nanoshells.

33. A kit for detecting active rapamycin, said kit comprising

a first reagent comprising an FK506 binding protein (FKBP) linked to a first detection particle;

a second reagent comprising a target of rapamycin (TOR) protein linked to a second detection particle, wherein the first and second detection particles are independently selected from the group consisting of microparticles and nanoshells.

34. The kit of claim **33** wherein the FKBP protein is FKBP12 or FKBP25, and wherein the FKBP-rapamycin associated protein is selected from the group consisting of mTOR, the FKBP binding domain of mTOR, yTOR1, the FKBP binding domain of yTOR1, yTOR2, and the FKBP binding domain of yTOR2.

* * * * *

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摘要(译)

描述了用于测量免疫抑制药物的均相，非竞争性双受体凝集试验。该测定使用至少两种受体，其中每种受体对药物上的单独结合位点具有特异性，并且其中每种受体与检测颗粒结合。免疫抑制药物与受体结合并引起颗粒凝集，可以测量颗粒凝集并将其与样品中免疫抑制药物的存在或量相关联。

