US 20100009394A1

(19) **United States**
(12) **Patent Application Publication**
(10) **Pub. No.: US 2010/0009394 A1**
(43) **Pub. Date: Jan. 14, 2010**

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(54) **UNIVERSAL TANDEM SOLID-PHASES BASED IMMUNOASSAY**

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(21) Appl. No.: **12/172,403**

(22) Filed: **Jul. 14, 2008**

Publication Classification

(51) **Int. Cl.**
G01N 33/53 (2006.01)

(52) **U.S. Cl.** **435/7.94**

(57) **ABSTRACT**

Universal tandem solid-phases based immunoassay (UTSIA) is a sandwich-ELISA equivalent assay for low abundance antigen determination that overcomes limitations of sandwich-ELISA (antibody inactivation by solid phase and strict requirement of a pair of primary and secondary antibodies) by using an affinity binding solid phase to capture antigen specifically from a fluid sample, sequentially dissociating the antigen, transferring, and coating the antigen to a non-affinity binding solid phase for specific antigen determination. Cell-based UTSIA is a cell-based ELISA equivalent assay that overcomes limitations of image method for determining an antigen in the cells or tissue immobilized on a solid phase by dissociating and transferring the detection antibody bound on the antigen of the cells or tissue immobilized on the solid phase to a second solid phase and immobilizing the detection antibody there for specific detection of the antigen via the detection of the detection antibody.

Antigen Detecting Mechanism of Cell-based UTSIA

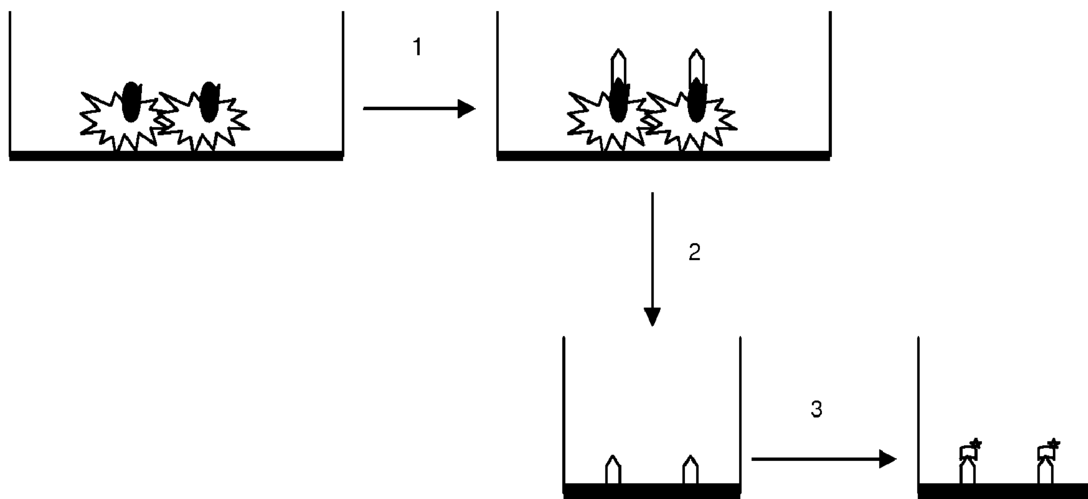


Fig 1A. Analyte Capture and Detection Mechanism in UTSIA

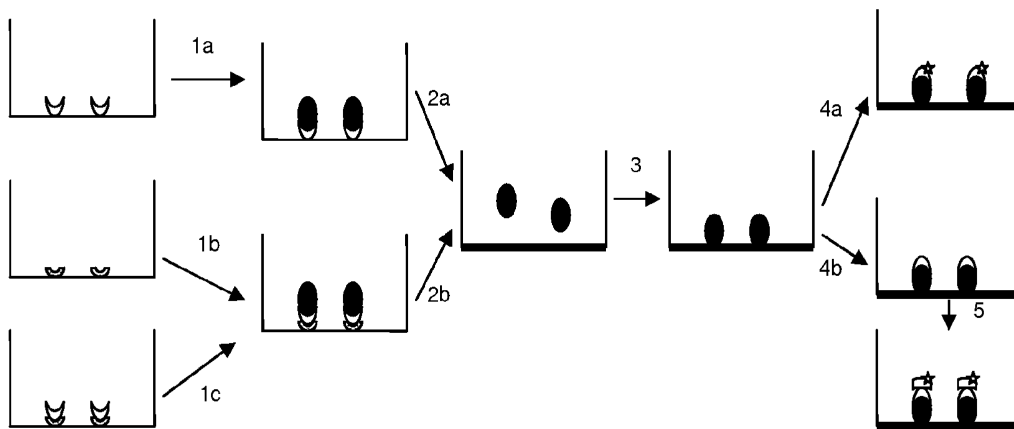


Fig 1B. Keys for Figure 1A

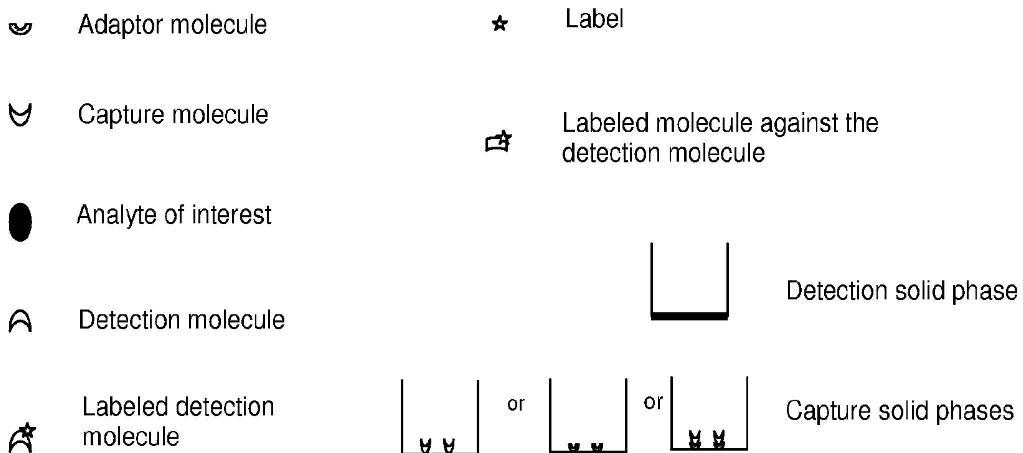


Fig 2: Specific Detection and Quantification of Target Antigen (rabbit IgG) by Universal Tandem Solid-phases Based Immunoassay
(Adaptor Molecule: Protein G; Capture Molecule: anti-Rabbit IgG from donkey)

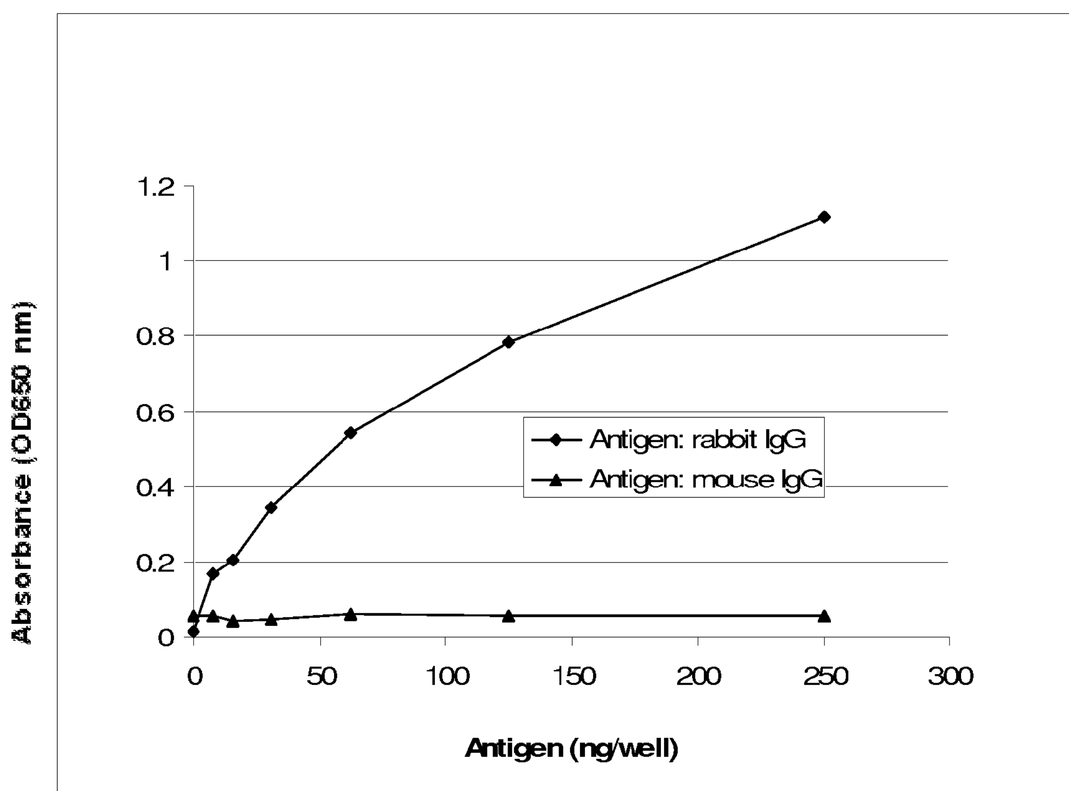


Fig 3A: Antigen Detecting Mechanism of Cell-based UTSIA

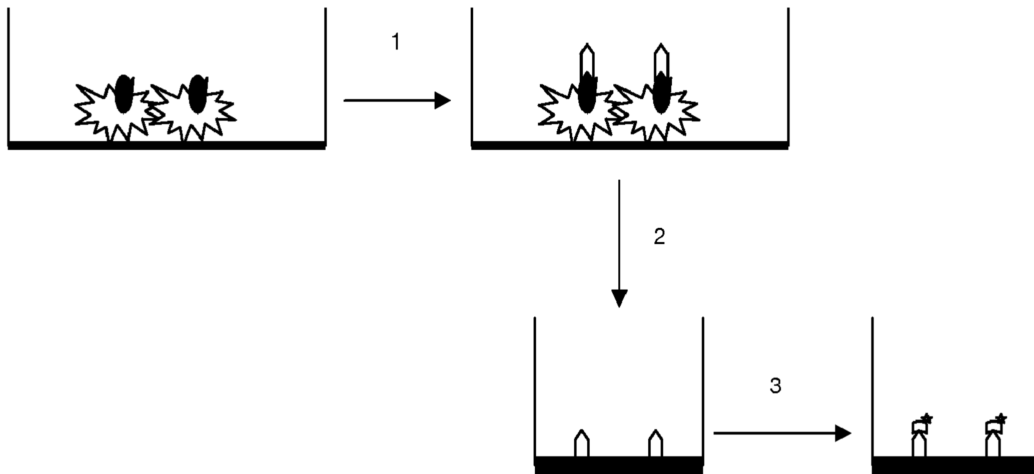
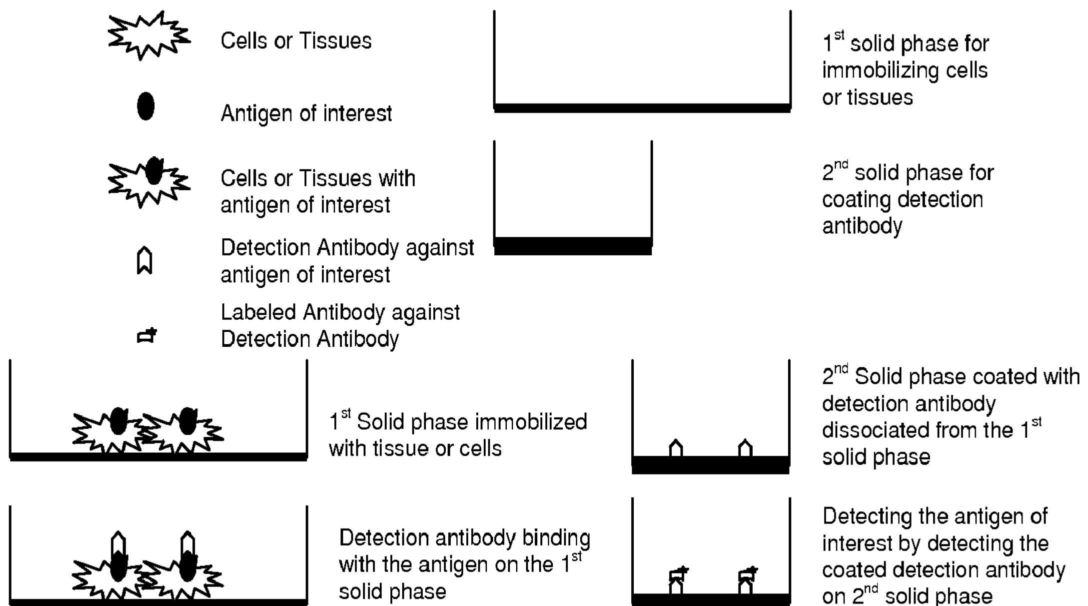


Fig 3B: Keys for fig 3A



UNIVERSAL TANDEM SOLID-PHASES BASED IMMUNOASSAY

FIELD OF THE INVENTION

[0001] The present invention relates to immunoassay methods for detecting and/or quantitating analyte(s) in a fluid sample and detecting and/or quantitating analyte(s) in cells or tissue immobilized on solid support(s).

BACKGROUND OF THE INVENTION

[0002] In this post-genomics era, it is critically important to develop a convenient and robust method for detection and quantification of low abundance proteins for biological and biomedical researches, clinical diagnostics, environmental and food monitoring, and biotech-pharmaceutical industries (Duncan et al 2005, Srinivas et al 2002, and Albala 2001).

[0003] Traditionally sandwich-ELISA is a popular immunoassay to detect and quantitate analyte in biological and non-biological samples (Zangar et al, 2006, Zhou et al 2005). Its applications in clinical diagnostics, biomedical research, food and environmental monitoring, and biotech and pharmaceutical industries are depended on its advantages: simplicity, sensitivity, and specificity. However, setting up a sandwich-ELISA for most of analytes is a challenge until current invention.

[0004] Sandwich-ELISA uses specific analyte-antibody binding reaction (affinity binding) to detect its analyte (Voller et al 1978, Lequin 2005, and Zangar et al 2006). The basic requirements for the method to detect analyte include having an analyte with at least two binding sites for specific analyte antibody binding reactions, having a pair of perfect matched capture and detection antibodies which can be used to form a sandwich-like complex with an analyte for the analyte's capture and detection, and having a capture antibody with capacity to maintain its analyte binding activity after immobilized on a solid phase. The general procedure for analyte detection includes that the analyte first reacts with excess solid-phase antibody (the capture antibody) immobilized on a solid phase (affinity binding solid phase), after incubation and followed by washing, the bound analyte on the solid phase is reacted with excess labeled antibody (the detection antibody). After further washing, the label is measured, and the signal of the label is directly correlated with the amount of analyte present. A modification of the sandwich-ELISA is the double sandwich-ELISA, which involves a third antibody. The third antibody carries a label and reacts with an unlabeled detection antibody already bound to the analyte. Then the signal of the label is measured, and like before, the signal of the bound label is directly correlated with the amount of the analyte present. The advantages of sandwich-ELISA are that, by using a capture antibody on a solid phase, the method can enrich and purify an analyte from a fluid sample containing complicated components (this is especially important for the detection of a low abundance protein), and that, by using a pair of capture antibody and detection antibody, the double affinity bindings significantly increase the specificity of the detection.

[0005] However, as mentioned above, in this post-genomics era, to develop a sandwich-ELISA is still a big challenge for scientists. Why is it so difficult to develop a sandwich-ELISA? Because it is hard to find a capture antibody which maintains the analyte binding capacity after its coating on a solid phase, as more than 75% of polyclonal antibodies and 90% of monoclonal antibody are denatured after coating on the solid phase (Butler 1993); it is even more hard to find a pair of matched capture antibody and detection antibody which form a sandwich complex with an analyte for the successful analyte capture and detection. If a protein without sandwich-ELISA available, it may take years to develop successfully a sandwich-ELISA for its quantitation. All of these problems of sandwich-ELISA lead to the assay only can be used in limited number of proteins discovered.

[0006] Since Engvall and Perlmann published their first paper on ELISA in 1971 (Lequin 2005), there are many inventions and literatures in the field of immunoassay for low abundance protein determination. However, most of these researches are focused on improvements of sandwich-ELISA, not worked on inventing an alternative convenient, and robust immunoassay which not only overcomes the technical difficulties of sandwich-ELISA but also preserves the advantages of sandwich-ELISA. For example, U.S. Pat. No. 5,236,849 worked on reducing the label signal background and increasing sensitivity of sandwich-ELISA by adding additional steps of dissociating the analyte-antibody complex from the affinity binding solid phase and then rebinding the complex to a new affinity binding solid phase which attached with a reactive group which was capable of specifically binding with the complex; although U.S. Pat. No. 5,236,849 used two affinity binding solid phases and a dissociating step in its patent claims, the invention does not solve the technical limitations of sandwich-ELISA by following facts: U.S. Pat. No. 5,236,849 used sandwich complex mechanism of sandwich-ELISA for analyte or antibody's capture and detection; and in dissociating step of U.S. Pat. No. 5,236,849 the complex of analyte and antibody was dissociated from the solid phase in a form of the immuno-complex, not breaking the complex to elute un-complexed analytes into the liquid phase of the dissociating buffer; in addition, both solid phases of U.S. Pat. No. 5,236,849 used were affinity binding solid phases (in which there is at least an affinity binding component immobilized). Thus, the method is not an universal method, as the setting up of two different affinity binding solid phases for each analyte analysis is very difficult. Finally, the detection step of U.S. Pat. No. 5,236,849 focuses on the detection of complex of analyte and antibody, not on the single analyte or single antibody, that is "assaying for the complex, bound to the second solid carrier" (Copied from claim IC of U.S. Pat. No. 5,236,849). Another example is the patent of U.S. Pat. No. 5,236,830; the inventor also used two affinity binding solid phases and a dissociating step for analyte assay. The goal of the invention is to increase the sensitivity of the analyte detection, and the mechanism of the analyte's capture and detection is sandwich-ELISA, and the two solid phases used are all affinity binding solid phases to increase detection sensitivity, and the analyte is required to be modified first before be captured by the first solid phase. One more example needs to be mentioned is the invention patented by Brust (U.S. Pat. No. 5,989,806). The inventor also used two affinity binding solid phases and a dissociating step for analyte assay. Again the goal of the invention (U.S. Pat. No. 5,989,806) was to increase the sensitivity of the analyte detection but not to invent an alternative assaying mechanism that different from sandwich-ELISA; the mechanism of the analyte's capture and detection in Brust invention was sandwich-ELISA, as two solid phases used were all affinity binding solid phases which used sandwich-complex, capture molecule—analyte—detection molecule, for analyte capture and detection.

For example, in column 3 line 6 to 54 of the invention U.S. Pat. No. 5,989,806 disclosed that two assaying mechanisms in the two affinity binding solid phases, one was R1-A-R2 and another was R4-R3-A-R2; the R1 or R3 (capture molecule) was used to capture analyte A, and R2 (detection molecule) was used to detect analyte A; and R1-A-R2 and R3-A-R2 were all sandwich-complex. Therefore, both of these two assaying mechanisms are same as the assaying mechanism of sandwich-ELISA. In column 3 line 8-9 Brust disclosed that the R1 was immobilized on a solid support for capture of analyte A. Therefore, the first solid phase of U.S. Pat. No. 5,989,806 is an affinity binding solid phase. In column 3 line 31-33 and line 48-51 Brust disclosed that the R4 was immobilized on a solid support for capture of R3-A-R2, the complex containing analyte A. Therefore, the second solid phase of U.S. Pat. No. 5,989,806 is an affinity binding solid phase too. As Brust's invention is based on sandwich-ELISA mechanism to capture and detect the analyte of interest, Brust hasn't disclosed an immunoassay method that is capable of replacing sandwich-ELISA. In addition, even though Brust disclosed the influence of dissociation buffer on the affinity bindings, he didn't disclose the dissociation buffer's influence on the non-affinity binding (physical adsorption): in column 7 line 56-62 and column 9 line 3-6 Brust disclosed that for affinity bindings, dissociation buffer (5 mM glycine-HCl pH2.5) increased detection sensitivity for low affinity analytes if capture molecule coated on the solid phase does not changed (Novel system I); and dissociation buffer also increased detection sensitivity for high affinity analytes when the adaptor molecule and capture molecule changed (Novel system II). These showed that both high affinity and low affinity bindings could be dissociated by the dissociation buffer {for Novel system I, the capture molecule was the coating antigen gp41 (column 6 line 3-10), and analyte (sample) was HIV 1 antibody (see column 7 line 8-15); for Novel system II, the capture molecules were coating antigen gp41 before dissociation and monoclonal antibody against human immunoglobulin G labeled with biotin after dissociation (column 6 line 3-10 and column 8 line 33-35), and analyte (sample) was HIV 1 antibody (see column 8 line 20-27)}. In summary, all of these inventions (U.S. Pat. No. 5,236,830, U.S. Pat. No. 5,236,849, and U.S. Pat. No. 5,989,806) are modified sandwich-ELISA, and not inventions which disclose an alternative assaying mechanism other than sandwich-ELISA. Therefore, they are all sandwich-ELISA based immunoassay. The goal of these inventions is to improve sensitivity of sandwich-ELISA but not to find an alternative assay mechanism that different from the sandwich-ELISA. Similarly, all other disclosed patents and literatures in the field of immunoassay before current invention have not solved the technical limitations of sandwich-ELISA successfully; specifically, they continue using sandwich-ELISA mechanism, the formation of antibody-analyte-antibody complex in an affinity binding solid phase or in two affinity binding solid phases, to capture and detect analyte of interest specifically from a complicated fluid sample. For dissociation step, they are focused on its affects on the affinity bindings, but not on their influences on the non-affinity bindings or physical adsorption.

[0007] Before current invention of UTSIA, if the sandwich-ELISA or modified sandwich-ELISA for an analyte is not available, then alternative way for the determination of the analyte from the sample comprises two methods: first using immunoprecipitation or affinity chromatography to isolate the analyte from the fluid sample, then using western blot, mass-spectrometry, or other methods to detect the analyte isolated. As these methods are not integrated together in a single assay format as sandwich-ELISA, and each of the methods self is a complicated procedure, they are not efficient and very time consuming. Thus, there is a need to invent a convenient and robust method to replace sandwich-ELISA with a novel alternative assaying mechanism other than sandwich complex for the analyte capture and detection.

[0008] This invention discloses a novel immunoassay method (UTSIA) to replace traditional sandwich-ELISA for protein or other analytes' detection and quantification. Using the novel immunoassay technology, a sandwich-ELISA equivalent immunoassay for low abundance protein quantitation can be easily set up using commercial antibodies available, the antibodies routinely only can be used for western blot, immunoprecipitation, immunohistochemistry, and/or flow cytometry. The basic idea of the invention is that by separating the process of analyte capture and detection of sandwich-ELISA in an affinity binding solid phase into two different solid phases-based processes (one affinity binding solid phase for specific analyte capture and one non-affinity binding solid phase for specific analyte detection) to break the strict requirement of a pair of perfect matched two antibodies required for formation of sandwich-like antibody-analyte-antibody complex in sandwich-ELISA. The first solid phase (the affinity binding solid phase) of present invention is for the capture of the analyte by affinity binding, and the second solid phase (non-affinity binding solid phase) of present invention is for immobilizing the analyte for further specific detection. As the invention needs an affinity binding solid phase and a non-affinity binding solid phase (which may be located on two different solid supports or located on a same solid support) and can be used to set up immunoassays for unlimited number of proteins (because the invention can use many commercial antibodies as capture molecule and detection molecule), and is capable to up scale to formats of microarray for multiple analytes detection and quantitation, the invention is named as universal tandem solid-phases based immunoassay (UTSIA). The application of UTSIA comprises protein analyte determination, especially for low abundance protein analyte determination.

[0009] Another important immunoassay is immunohistochemistry (IHC) and immunocytochemistry (ICC). IHC is a technique developed by Coons six decades ago (Coons AH CH et al., 1941). Today IHC has a central role in the field of diagnostic medicine pathology. IHC's variation ICC also has important role in biomedical research, clinical diagnostics, and drug discovery. Both techniques of IHC and ICC are using a detection antibody to detect an analyte immobilized on a solid phase. The major difference is that the analyte detected by IHC is located in tissue immobilized on a solid phase, while the analyte detected by ICC is located in cells fixed on a solid phase.

[0010] The major challenge of ICC and IHC is that it is difficult to set up quantitative ICC and IHC. Traditionally ICC and IHC are qualitative methods or semiquantitative methods (Kaczmarek E et al 2004, Lang et al 2006, Dodson 2002, Ramos-Vara 2005, Guardigli et al 2005). The presence and amount of the analyte on the tissue or cells immobilized on a solid phase is judged by assigning scores by an skilled observer such as pathologist by using a proper microscope based on certain criteria, or by cellular or tissue image method that involves image capture of labeled detection molecule that

bound on the analyte in the cells or tissue and then using computer to run image data analysis to get numerical data. The image method usually is fluorescence based method. It is well known that the scores method is not objective; the results from the scores method for quantification can be varied significantly with different observers. And the image method also is not an ideal quantitative method as it is not a direct measurement of analyte quantity, needs expensive equipment, consists of complicated manipulation procedures, is very time consuming, and produces data with big variation due to background interference from cells and tissue. Thus, there is an urgent need to develop better method to replace image method for quantitative ICC or quantitative IHC.

[0011] The invention discloses an important variant of UTSIA, cell-based UTSIA, for quantitative IHC and ICC. It is a cell-based ELISA equivalent immunoassay which is used to detect and quantitate analyte(s) of interest in cells or tissue immobilized on solid phase(s). The mechanism of the cell-based UTSIA is that dissociating, transferring, and immobilizing the specific antibody that bound on the analyte of interest on the cells or tissues immobilized on a solid phase to another non-affinity binding or affinity binding solid phase for detection. The antibody on the late solid phase(s) is directly correlated with the amount of the analyte on the first solid phase. The cell-based UTSIA can be used in the situation that a cell-based ELISA can't be set up as lack high quality specific antibodies, and the image method is not robust for the quantitation of the analyte in cells or tissue immobilized on a solid phase. The applications of the cell-based UTSIA may comprise cell based or tissue based biomarker evaluation, detection, and quantification for biomedical research, clinical diagnosis, and drug discovery.

SUMMARY OF THE INVENTION

[0012] The major goal of current invention of universal tandem solid-phases based immunoassay (UTSIA) is to develop an alternative protein analyte quantifying method other than sandwich-ELISA with properties of simple, robust, and easier setting up, because practically the sandwich-ELISA is not possible to be set up if there is no a pair of perfect matched capture antibody and detection antibody available for forming sandwich-like immunocomplex on a solid phase, even though there are many other antibodies commercial available to the analyte of interest.

[0013] Different from sandwich-ELISA, in which the capture and detection of the analyte depend on forming a sandwich like antibody-analyte-antibody immunocomplex on a solid phase, UTSIA captures and detects the analyte of interest by two isolated processes, comprises an affinity binding solid phase which comprises solid phase—Protein G—capture antibody (if the capture antibody is not inactivated by the solid phase, then the affinity binding solid phase is solid phase—capture antibody) for capturing the analyte from a fluid sample, and a non-affinity binding solid phase which comprises hydrophobic solid support such as polystyrene plate for coating the analyte after dissociating the analyte from the affinity binding solid phase for further detection by a labeled or unlabeled detection antibody.

[0014] This novel while simple immunoassay mechanism of UTSIA provides properties of more flexibility and easier setting up than sandwich-ELISA: 1. there are more antibodies available for acting as capture antibody, as the adaptor molecule Protein G on the solid phase prevents the inactivation of the capture antibody by the solid phase; 2. there are more antibodies available for acting as detection antibody, as the analyte coated on a polystyrene plate (non-affinity binding solid phase) can be recognized by most of antibodies commercial available if these antibodies are screened and selected by using the analyte coated on a same kind solid phase. The easier setting up of UTSIA is an especially important advantage as it may take years to develop a sandwich-ELISA for an analyte of interest, while using commercial available antibodies to set up UTSIA may take only days. As UTSIA also can use two antibodies that target on different epitopes of an analyte, the specificity of the UTSIA is equivalent as sandwich-ELISA. The sensitivity of UTSIA, the prototype example of UTSIA demonstrates that the ng level of IgG protein can be detected in a concentration-dependent manner, and the sensitivity may be further improved by optimizing the assay system or combining with high sensitivity detection assay commercially available. In addition, as two solid phases of UTSIA can be located on a same solid support, such as half of a 96 well polystyrene plate is affinity binding solid phase, and another half is non-affinity binding solid phase, the assay can be up scaled to automation assay from manual assay using commercial ELISA automation robots available.

[0015] The major inventive step of current invention (UTSIA) is the combination of an affinity binding solid phase and a non-affinity binding solid phase for specific analyte capture and determination from a fluid sample. Inventing the novel combination the current inventor successfully discovers a novel assaying mechanism to replace or to substitute sandwich-ELISA. Prior arts disclosed that the combination of two affinity binding solid phases can increase sensitivity of sandwich-ELISA, but the teachings of using two affinity binding solid phases are not possible to let learner find an assaying mechanism that can replace or substitute sandwich-ELISA, as the sandwich-ELISA is an affinity binding solid phase based assay too. Prior arts never disclosed an immunoassay that combines an affinity binding solid phase with a non-affinity binding solid phase for low abundance protein or analyte determination.

[0016] In addition, it should be emphasized that it is not obvious to discover an immunoassay (UTSIA) that using the combination of an affinity binding solid phase with a non-affinity binding solid phase for determining an analyte from a fluid sample. Because the physical adsorption used in the non-affinity binding solid phase is a major problem that causes un-wanted, non-specific detection noise signals in previous immunoassays such as western blot and sandwich-ELISA; therefore, the non-affinity binding or physical adsorption is always avoided in setting up detection step of immunoassays in prior arts.

[0017] It also should be emphasized that it is not trivial to set up a novel immunoassay (UTSIA) that using the combination of an affinity binding solid phase and a non-affinity binding solid phase for determining an analyte from a fluid sample. Because this involves how to release analyte bound by affinity binding from an affinity binding solid phase and then to let the released analyte successfully further coated on the non-affinity solid phase for further specific detection. From literatures published, it said that both affinity binding and physical adsorption involve binding mechanisms of ionic interaction, hydrogen bonding, hydrophobic interaction, and conformational matching; it therefore is difficult and confusing to select which buffer components and conditions that may reach the goal of specifically release analyte bound by affinity binding while not blocking the analyte's further

physical adsorption. In addition, people usually misunderstand that for physical adsorption (coating), the concentration of analyte protein of interest should be high, and therefore prior arts are usually not try to coat a protein analyte with very low concentration on a solid phase for further specific detection. Further more, people usually misunderstand that the physical adsorption of protein on a solid phase takes long time (many hours) incubation, and therefore, prior arts are not to use the physical adsorption in the step of protein determination. Anyway, after through many failures and frustrations, finally I figured out that, for non-affinity binding (physical adsorption) on a polystyrene support, the buffer contains any components with blocking power such as tween-20, BSA or other proteins prevents physical adsorption, while extreme pH of a buffer does not affects the physical adsorption significantly on the physical adsorption; and in contradictory, for affinity bindings on a polystyrene support, the buffer containing any of above blocking components actually promotes specific affinity binding in certain ranges, while the extreme pH of a buffer prevents the affinity binding. Therefore, I used a buffer with extreme pH that does not contain any blocking components to specifically release protein analyte bound on an affinity binding solid phase, and further coating the protein analyte on a non-affinity binding solid phase. And further more, I demonstrate that the protein analyte at ng level can be efficiently coated on a polystyrene support and can be further specifically quantified in a concentration dependent manner; and I also show that the physical adsorption of analyte protein on the non-affinity binding solid phase (polystyrene support) only take 15 min to half hours to get satisfactory coating, this finding is very useful for assay - - - time saving!

[0018] Another major inventive step of current invention is figuring out a way to use an adaptor molecule in the affinity binding solid phase to set up a protein quantitation immunoassay while avoids the modifications of the capture molecule, the detection molecule, the analyte, or other molecules that interacting with adaptor molecule and capture molecule, that the modifications are usually required in the one or two affinity binding solid phases based modified sandwich-ELISA. In UTSIA, an adaptor molecule or adaptor molecules which bind(s) capture molecule in both extreme (such as pH 2.5) and regular pH (such as pH 7.0) could provide the novel assay system with maximum flexibility to use commercial antibodies as the capture molecule and detection molecule, as the capture molecule on the affinity binding solid phase does not release to the liquid phase of dissociating buffer, and therefore, does not interfere with the analyte coating on the solid phase, and does not interfere with the analyte specific determination on the non-affinity binding solid phase by the detection molecule. This inventive step greatly simplifies the burden of setting up assay and reducing the difficulty of setting up assay, and provides the UTSIA with universal property to use any combination of antibodies that from a variety of animal sources as capture molecule and/or detection molecule.

[0019] One more inventive step of UTSIA needs to mention is that the dissociation process is different in affinity binding solid phase(s) based modified sandwich-ELISA assay and in non-affinity binding solid phase based UTSIA. For affinity binding solid phase(s) based assay (modified sandwich-ELISA), the analyte dissociated by extreme pH must be neutralized to let the analyte re-binding with original capture molecule, or another capture molecule. But for non-affinity binding solid phase based assay (UTSIA), the analyte dissociated by extreme pH is optional to be neutralized, as even without neutralizing, the analyte still can be coated on the non-affinity solid phase efficiently.

[0020] Finally and most important inventive achievement of current invention (UTSIA), of course, is figuring out an easier setting up assay system for low abundance protein determination other than the system of sandwich-ELISA, this has never been disclosed in prior arts of immunoassay field and proteomics field.

[0021] The major application of UTSIA includes determining a low abundance protein analyte in a fluid sample. It can be used in basic and clinical medicines, biomedical researches, biotech-pharmaceutical industries, and other fields. It is especially useful when a sandwich-ELISA is not available for quantitation of protein analyte. UTSIA may also be used to set up antibody-based microarray for detection of multiple protein analytes.

[0022] Cell-based UTSIA is a modification of UTSIA for determining an analyte that located on cells or tissue immobilized on a solid phase. The assay comprises releasing a detection antibody bound on the analyte to a liquid phase by using a dissociation buffer, and then immobilizing the antibody released on a non-affinity binding or affinity binding solid phase for further detecting and quantifying; if the detection antibody is labeled, it can be directly measured; if the detection antibody is unlabeled, it is further determined by a labeled antibody against the detection antibody. One advantage of the cell-based UTSIA is that it increases specificity via several mechanisms: it only detect the detection antibody bound by affinity binding (as the detection antibody bound nonspecifically on the cells or tissue can't be released by dissociation buffer), it avoids the non-specific background interference of tissue or cells. Another advantage is that it does not need microscopy and image taking and image analysis, and therefore, it is more simple and less time consuming, and more robust. The cell-based UTSIA is especially useful for cell-based drug discovery in biotech-pharmaceutical industries. It is also useful for cell-based or tissue-based biomarker detection, evaluation, and quantification in basic and clinical medicines, biomedical researches, and other fields. The cell-based UTSIA may also be used in microarray format for detection of multiple analytes, and can be up-scaled to automation assay.

THE DETAIL DESCRIPTION OF THE INVENTION

[0023] The following definitions, description of figures, general descriptions, steps, figures, preferred embodiments, and prototype example are offered by way of illustration of present invention, and not by way of limitation of present invention.

DEFINITIONS

[0024] Adaptor molecule: as used herein, is a affinity binding molecule which can be immobilized on a solid phase (the first solid phase) to be part of a capture system or capture solid phase, wherein the adaptor molecule immobilized on the solid phase is capable of binding a capture molecule and is capable of preventing the inactivation of the capture molecule's analyte binding ability by the solid phase; the adaptor molecule is selected from a group comprising Protein G, Protein A, Protein A/G, Protein L, antibodies, proteins, recombinant proteins, recombinant antibodies, small organic

molecules, poly-peptide, or nucleic acid aptamers; adaptor molecule comprises site(s) for attaching or coating to a solid phase and site(s) for binding of a capture molecule; the ideal adaptor molecule is the one that binds the capture molecule at both neutral pH and extreme acidic pH, or at both neutral pH and extreme basic pH.

[0025] Affinity binding: as used herein, is a binding reaction same as or similar to the specific binding interactions between an antigen and its antibody or between a ligand and its receptor; the affinity binding between capture molecule and analyte usually can be disrupted by the buffer comprising extreme pH.

[0026] Affinity binding solid phase: as used herein, is a solid phase which already immobilized with affinity binding molecule(s), wherein the affinity binding molecule(s) on the solid phase is used to capture a target analyte or other substance(s) via affinity binding(s).

[0027] Affinity binding molecule: as used herein, comprises molecule that is capable of binding another molecule via affinity binding.

[0028] Analyte: as used herein, is a substance of interest in a fluid sample or a sample; it may have same meaning of target analyte, analyte of interest, antigen, or antigen of interest; the analyte comprises proteins or other components which have binding site(s) for a capture molecule and a detection molecule as well as have hydrophobic structures that can be used to coat the analyte on a non-affinity binding detection solid phase; if the capture molecule or the detection molecule is an antibody, then the analyte may be named as antigen.

[0029] Antibody: as used herein, the antibody is a protein encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically bind and recognize an antigen; the antibody comprises the intact antibody or the fragment antibody produced by animal, tissue culture, and recombinant DNA technology; if the adaptor molecule is an Fc-domain binding protein (such as Protein G), then the capture antibody is an antibody comprising Fc-domain which binds with the Fc-domain binding protein specifically.

[0030] Antigen: as used herein, is an analyte that can be captured by a capture antibody and/or that can be detected by a detection antibody.

[0031] Aptamer: as used herein, is a polymer molecule that has high specificity and affinity for its target molecule; it may replace the antibody for UTSIA or UTSIA based assays; it comprises nucleic acid polymer.

[0032] Assaying: as used herein, refers to qualitative or quantitative analysis of a substance.

[0033] Blocking buffer: as used herein, is a buffer with blocking components which blocks a solid phase's non-specific adsorption to reduce the non-specific signals; the blocking components comprise BSA, Tween-20, and the like; usually the blocking buffer is used after the immobilization of the adaptor molecule and/or the capture molecule on a capture solid phase, or the immobilization of analyte of interest on a detection solid phase; the blocking buffer is also used in steps of affinity binding such as the bindings between adaptor molecule (Protein G) and the capture molecule (antibody), the capture antibody and the antigen, the antigen and the detection antibody, the detection antibody and the labeled third anti-detection-antibody, and the like; the blocking buffer is capable of blocking nonspecific binding reaction of solid phases to reduce background noise signals; the blocking buffer can't be used before and during the steps of physical adsorption on a solid phase, because the blocking buffer will block or reduce the adsorption of the molecule (adaptor molecule, capture molecule, or analyte) onto the solid phase; if the adaptor molecule immobilized on a capture solid phase is Protein G or Protein A, then after binding with a capture antibody, the un-occupied Fc-binding sites of Protein G or Protein A is preferred to be blocked by a blocking buffer comprising serum, Fc-domains, or immunoglobulins.

[0034] Capture molecule: as used herein, is a molecule used to capture an analyte being assayed via a specific affinity binding reaction between the capture molecule and the analyte in a liquid phase or on a solid phase; the capture molecule comprises antibody, recombinant antibody, protein, recombinant proteins, small or big organic molecules, and peptide or nucleic acid aptamers; if the capture molecule is an antibody, then it is named as capture antibody.

[0035] Capture system and capture solid phase: Capture solid phase is an affinity binding solid phase, and capture system is the system of UTSIA to capture analyte(s) from a fluid sample and the system comprises a capture solid phase which comprises a first solid phase; the capture solid phase refers to the solid phase immobilized with affinity binding molecule(s) for specific analyte capturing; wherein the affinity binding molecules for the capture system or capture solid phase comprise adaptor molecule and/or capture molecule.

[0036] Cell-based ELISA: as used herein, is an immunoassay for detecting an antigen of interest on cells immobilized on a solid phase; its major steps comprise growing the cells on a solid phase, then fixing the cells on the solid phase, penetrating the cells to expose antigen of interest, binding with a labeled detection antibody or an unlabeled detection antibody which recognizes the antigen of interest on the solid phase, finally detecting the antigen of interest on the solid phase by detecting the signal of the label on the detection antibody bound on the solid phase; or detecting the unlabeled detection antibody by adding a labeled antibody against the unlabeled detection antibody and then detecting the signal from the label of the labeled antibody bound; the label comprises an enzyme.

[0037] Cell-based UTSIA: as used herein, is a modified UTSIA for determining an analyte of interest on samples (such as cells or tissues) immobilized on a solid phase; its essential detecting mechanism comprises transferring the detection molecule bound on the analyte of the sample immobilized on the solid phase to a non-affinity binding solid phase or an affinity binding solid phase, and immobilizing there, then detecting the detection molecule on the solid phases directly or indirectly. Its major goal is to replace current imaging method (or image method) to a non-imaging based immunoassay for determining an analyte of interest on samples (such as cells or tissues) immobilized on a solid phase.

[0038] Chromogen: as used herein, is a substance that lacks definite color but may be transformed into a pigment.

[0039] Coating: as used herein, is same as physical adsorption, used to immobilize substance on a solid phase; in contrast to the affinity binding, the substance immobilized on a solid phase or a solid support by the coating or the physical adsorption is nearly not dissociable from the solid phase by buffer with extreme pH.

[0040] Detection molecule: as used herein, is a molecule used to detect an analyte via a specific affinity binding between a detection molecule and the analyte; the detection molecule comprises antibody, recombinant antibody, protein, recombinant proteins, small or big organic molecules, and

peptide or nucleic acid aptamers; if the detection molecule is an antibody, then it is named as detection antibody and its target analyte is an antigen.

[0041] Detection solid phase: in UTSIA, detection solid phase is the non-affinity binding solid phase used for coating analyte dissociated in a dissociation buffer; the detection solid phase comprises of hydrophobic surface such as polystyrene plate. In cell-based UTSIA, the detection solid phase is the one to immobilize the detection molecule dissociated in a dissociation buffer, it comprises affinity binding solid phase or non-affinity binding solid phase; in both UTSIA and cell-based UTSIA the detection solid phase is also named as second solid phase.

[0042] Determining and determination: as used herein, determining refers to qualitative, quantitative, or qualitative and quantitative analysis of a substance; determination has same meaning of the determining; assaying also has same meaning of the determining.

[0043] Dissociating buffer: as used herein, is a buffer comprising extreme low or high pH (e.g., less than 2.5), which is capable of releasing, eluting, or dissociating analyte(s) bound on a capture solid phase into liquid phase of the dissociating buffer; the dissociating buffer can't contain blocking components which might interfere with the analyte's coating process onto a detection solid phase.

[0044] ELISA: as used herein, is an enzyme linked immuno-sorbent assay.

[0045] Fc-domain: as used herein, is the protein or fragment of protein which can bind with Fc-binding protein by affinity binding; one example is the antibody, which contains Fc-domain.

[0046] Fc-binding protein: as used herein, is a protein which is capable of binding Fc-domain protein, such as an antibody with a Fc-domain; the Fc-binding protein comprises protein-A, Protein G, protein or protein fragment derived from protein-A and/or Protein G, antibodies specifically targeting on Fc-domain of an antibody, and any other proteins or other molecules which can specifically bind with Fc-domain of a protein or an antibody; Fc-binding protein can be used as adaptor molecules in UTSIA if the capture molecule is a molecule with Fc-domain such as antibody.

[0047] First solid phase: as used herein, refers to the solid phase for making capture solid phase in UTSIA's capture system, which is usually immobilized with affinity molecule (s) for capturing an analyte or antigen from a fluid sample; in case of cell-based UTSIA it is the first solid phase for immobilizing the cells or tissues containing an analyte or antigen of interest, but the solid phase does not capture an antigen from the sample (cells or tissue) via affinity binding.

[0048] Fluid sample: as used herein, fluid sample is a fluid that might contain an analyte of interest to be analyzed, comprising biological fluids, extracts or lysates of cells or tissues, biological samples such as blood, serum, plasma, cerebrospinal fluid, lymphatic fluid, semen, urine, sputum, synovial fluid, saliva, lacrimal tears, nipple aspirate, and eye fluid; fluid samples may be of animal, plant, bacterial, viral, prion, or other life species; in this invention there are many descriptions such as the fluid sample, target sample, sample of interest, the sample, and biological material; all of them have same or similar meaning that they all may contain an analyte of interest; for analysis, if a sample is not a fluid, such as the tissue and the organ, the sample can be changed to fluid sample by adding suitable buffer and elements such as proteinase inhibitors and by processing with suitable procedures such as vortex and ultrasonic disruption under proper temperature and other proper conditions; the control sample is the one does not contain the analyte of interest, it is equal to the negative control sample; for UTSIA, side by side to run a sample and a control sample can make sure that the detection of an analyte is not a false positive result.

[0049] Hole punch: as used herein, is a cutting instrument to make sections with defined area and defined shape.

[0050] HRP: as used herein, is horseradish peroxidase; it is tagged on an antibody for signal production and detection; it is an enzyme that needs a substrate such as TMB to produce a colorful product for the signal detection.

[0051] IHC: as used herein, is immunohistochemistry.

[0052] ICC: as used herein, is immunocytochemistry.

[0053] Image method or imaging method: as ued herein, is a semiquantitation method to determine an analyte in cells or tissue immobilized on a solid phase; the method comprises taking pictures of the fluorescence labeled detection molecule that bound on the analyte and then transferring the image data to numerical data. The major disadvantages of the image method comprise the interference of autofluorecence from cells or tissue, noise signal from non-specific antibody binding, requiring specific expensive equipments, and very time consuming.

[0054] Immuno-complex: as used herein, is the complex of antigen and antibody formed by specific antigen antibody affinity binding.

[0055] Immobilize, immobilizing, and immobilization: as used herein, is a process or an action of fixing a substance onto a part of solid phase which comprises surface of the solid phase; the process comprises physical adsorption (coating), affinity binding, chemical covalent attachment, or combination of any of the three processes.

[0056] IP: as used herein, is a method of using a solid phase immobilized with an Fc-binding protein to precipitate or capture an immuno-complex of antibody and antigen from a fluid sample; the antigen obtained from the IP is usually further processed and detected by SDS-PAGE and Western Blot; IP is the abbreviation of immunoprecipitation.

[0057] Label: as used herein, is a molecule with a physical property or biochemical activity that is analyzable by a detector via the label's physical property or the label's catalyzed activity; it is usually tagged on a detection molecule or a third antibody against an unlabeled detection molecule for an analyte detection and quantification; the label comprises dye, fluorescent tag, radioactive isotope, or enzyme; the signal produced from the label itself or mediated by itself, can be fluorescent, chemiluminescent, light-scatting, nano-crystalline, calorimetric, or radioactive, or any combination thereof; in special case, an unlabeled detection molecule bound on an analyte on a detection solid phase can be detected by an instrument, and the unlabeled detection molecule is acted as a detection molecule and a label molecule simultaneously.

[0058] Labeled: as used herein, it refers that a substance is tagged with a label molecule which is detectable directly or indirectly by adding additional molecules.

[0059] Liquid phase: as used herein, comprises the phase of a fluid sample, a buffer, or a solution; it has same meaning of fluid, solution, and fluid phase; and the essential component of liquid phase comprises water.

[0060] Microarray or array: as used herein, is a solid phase containing multiple numbers of immobilized probe elements, in a linear or two-dimensional array of discrete regions, each is in a finite area; this may also be referred to as antibody array

or microarray, protein-array or microarray, antibody chip, or protein chip; the goal of antibody microarray is to detect multiple analytes in a fluid sample in a same time manner.

[0061] Neutralizing buffer: as used herein, is a buffer with opposite extreme high or low pH of a dissociating buffer; the neutralizing buffer neutralizes the dissociating buffer's extreme pH and brings back the pH to a level that is suitable for an analyte's adsorption and determination on a detection solid phase; the neutralizing buffer can't contain blocking components which might interfere with the analyte's coating process and detection process on the detection solid phase.

[0062] Non-affinity binding solid phase: as used herein, is a solid phase which does not immobilized with affinity binding molecule(s), wherein the non-affinity binding solid phase is used to bind its target substance via non-affinity binding comprising physical adsorption.

[0063] Physical adsorption and coating: as used herein, physical adsorption and coating has same meaning, is a physical process of immobilizing a molecule on a solid phase without involving chemical covalent coupling reactions and without involving affinity binding; usually the solid phase used for coating or physical adsorption comprises a hydrophobic surface such as polystyrene plate, and the molecule for coating comprises a macromolecule containing hydrophobic residues; in the buffer condition without blocking elements such as BSA, tween-20, or serum, the molecule is adsorbed onto the surface of the hydrophobic solid phase.

[0064] Primary antibody and secondary antibody: as used herein, primary antibody is the antibody used directly to bind an antigen being assayed, it is same as the capture antibody; secondary antibody is the antibody used to bind an antigen for its detection, it is same as the detection antibody.

[0065] Protein or polypeptides or peptides: as used herein, are molecules which comprise multiple amino acid residues linked via peptide bonds.

[0066] Quantitate: as used herein, is to determine the amount(s) or the concentration(s) of analyte(s) of interest in a fluid sample; it has same meaning of quantify; its noun is quantitation or quantification.

[0067] Quantification: as used herein, is the noun of measuring the amount or concentration of a substance; it has same meaning of quantitation.

[0068] Sandwich-ELISA: as used herein, is an immunoassay for capturing and determining an analyte in a fluid sample by using a capture molecule and a detection molecule to bind the analyte to form a complex of capture molecule—analyte—detection molecule, a sandwich-like immunocomplex; wherein the capture molecule can be immobilized on a solid phase directly; traditional sandwich-ELISA is a single affinity binding solid phase based method, while modified sandwich-ELISA may has additional affinity binding solid phase (s); the essential parts of both sandwich-ELISA and modified sandwich-ELISA are that all of them need a sandwich-like complex of capture molecule—analyte—detection molecule for capturing and detecting the analyte, and all of them only use affinity binding solid phase(s) for analyte capture and detection.

[0069] (s): as used herein, refers single or multiple; for example, molecule(s) refer to a molecule or many molecules, antigen(s) refer to an antigen or many antigens, analyte(s) refers to an analyte or many analytes, site(s) refer to one site or many sites, biomarker(s) refer to a biomarker or many biomarkers, phase(s) refer to a phase or many phases, compound(s) refer to a compound or many compounds, pool(s) refer to a pool or many pools, library(s) refer to a library or many libraries, and candidate(s) refer to a candidate or many candidates, proteins refer to one protein or many proteins.

[0070] Sample: for cell-based UTSIA, the sample is cells or tissue immobilized on a solid phase; for UTSIA, sample is the fluid or non-fluid that may contain the analyte of interest that need to be detected and/or quantitated; and the non-fluid sample is changed to fluid via mixing with water-based buffer.

[0071] Separated defined region: as used herein, refers to a region with border, so different regions are separated with each other; for example, the well in 96 well polystyrene plate for ELISA is a separated defined region.

[0072] Sheet: as used herein, is 2-dimensional solid phase comprising membrane, leaf, surface, pane, or slide; wherein the 2-dimensional solid phase is suitable for immobilizing cells or tissue for IHC or ICC.

[0073] Solid phase: as used herein, the solid phase comprises plastic, glass, metal, or other insoluble materials containing a number of regions wherein adaptor molecule, capture molecule, analyte, detection molecule, or sample (antigen, protein, or other macromolecule having hydrophobic structure) can be immobilized on its surface, and the solid phase can be used to carry out the steps of UTSIA or cell-based UTSIA; the solid phase may have same meaning as the solid surface and solid support.

[0074] Signal generating system: as used herein, is a system for detecting a substance of interest bound on second solid phase (it is also named as detection solid phase or non-affinity binding solid phase in UTSIA); the system comprises a labeled detection molecule against the substance, or an unlabeled detection molecule against the substance and a labeled molecule against the unlabeled detection molecule.

[0075] TMB solution: as used herein, is the chromogenic reagent for peroxidase, designed for ELISA and UTSIA; it contains 3,3',5,5'-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2), and other stabilizing agents.

[0076] Un-complexed: as used herein, is equal to the meaning of not binding with each other by affinity binding(s).

[0077] UTSIA: as used herein, is the abbreviation of universal tandem solid-phases based immunoassay; UTSIA is a novel immunoassay, which captures an analyte from a fluid sample on an affinity binding solid phase and then detects the analyte on a non-affinity binding solid phase; the essential differences of analyte capturing and detecting mechanism between the UTSIA and sandwich-ELISA are that UTSIA doesn't need sandwich-like complex of capture molecule—analyte—detection molecule, while sandwich-ELISA does need the sandwich-like complex; and that UTSIA is the combination of affinity binding solid phase and non-affinity binding solid phase based method, while the sandwich-ELISA is sole affinity binding solid phase based method.

DESCRIPTION OF THE FIGURES

[0078] FIG. **1**A. Analyte Capture and Detection Mechanism in UTSIA: 1a, incubating a fluid sample with a capture solid phase (the solid phase immobilized with a capture molecule) to immobilize an analyte on the capture solid phase; 1b, pre-incubating a fluid sample with a capture molecule to form a complex of the analyte and the capture molecule, then contacting the complex with a capture solid phase (the solid phase immobilized with an adaptor molecule) to immobilize the analyte; 1c, incubating a fluid sample with a capture solid phase pre-immobilized with the complex of an adaptor mol-

ecule and a capture molecule to immobilize the analyte on the capture solid phase; 2a, adding a dissociating buffer to the capture solid phase to release the analyte to liquid phase and then transferring the liquid phase containing the analyte to a detection solid phase; 2b, adding a dissociating buffer to the capture solid phase to release the analyte to liquid phase and then transferring the liquid phase containing the analyte to a detection solid phase; 3, immobilizing the analyte to the detection solid phase by physical adsorption; 4a, adding a labeled detection molecule to recognize the analyte coated on the detection solid phase for specific detection and quantification of the analyte; 4b, adding a unlabeled detection molecule to recognize the analyte coated on the detection solid phase; 5, then adding a labeled molecule against the unlabeled detection molecule for the detection and quantification of the analyte.

[0079] FIG. **1**B: Keys for FIG. **1**A.

[0080] FIG. **2**. Specific Detection and Quantification of Target Antigen (rabbit IgG) by Universal Tandem Solid-phases Based Immunoassay: a prototype example of UTSIA, which demonstrates that the UTSIA specifically detects and quantitates a target analyte (rabbit IgG), but not a control analyte (mouse IgG), in concentration dependent manner.

[0081] FIG. **3**A. Antigen Detecting Mechanism of Cell-based UTSIA: 1. a first solid phase immobilized with cells or tissue is incubated with a detection antibody against an antigen of interest to bind the detection antibody on the first solid phase; 2. then the unbound detection antibody is removed and the first solid phase is incubated with a dissociating buffer (with extreme pH) to release the detection antibody bound on the solid phase into a liquid phase of the dissociating buffer; then the liquid phase containing the detection antibody dissociated from the first solid phase is transferred to a second solid phase which may pre-contain a neutralizing buffer (with opposite pH of the dissociating buffer, the mixture of the dissociating buffer and the neutralizing buffer will get a pH optimal for coating the detection antibody on the second solid phase) to immobilize the detection antibody on a second solid phase (the second solid phase comprises non-affinity binding solid phase for detection antibody coating or affinity binding solid phase for capture of the detection antibody); 3. finally the determination of the antigen of interest in the cell or tissue immobilized on the first solid phase is decided by detecting the detection antibody immobilized on the second solid phase via using a labeled antibody against the detection antibody; the labeled antibody is incubated with the second solid phase for proper time, then the unbound is removed, and then the bound labeled antibody is used for signal detection; the signal from the labeled antibody on the second solid phase is correlated with the presence and the amount of the antigen of interest in the cells or tissue immobilized on the first solid phase. Please note: if the detection antibody is labeled with a detectable tag, then it is preferred to be immobilized by affinity binding solid phase which has an affinity binding molecule capable of capture the labeled detection antibody and then detected directly on the solid phase instead of the step 3 of FIG. **3**A.

[0082] FIG. **3**B. Keys for FIG. **3**A.

DETAIL DESCRIPTION OF THE INVENTION

(I) General Description

[0083] As mentioned in background of invention, in traditional sandwich-ELISA, the inactivation of the capture antibody by the solid phase is a major problem. There are prior inventions that further modify sandwich-ELISA, such as bridge immunoassay (U.S. Pat. No. 5,296,347, George B. LaMontte), that teaches using adaptor molecule (such as avidin) to modify the affinity binding solid phase of sandwich-ELISA to prevent the inactivation of the capture molecule by the solid phase; but essential capture and detection of the analyte in the modified sandwich-ELISA still needs sandwich antibody-analyte-antibody complex, plus needs additional multiple affinity bindings besides the affinity bindings in the sandwich complex. The modified sandwich-ELISA like bridge immunoassay is not convenient and not universal, as for every analyte, its capture molecule needs to be further modified for further affinity bindings that are required in the assay system; plus, it still faces the technical limitation, the requirement of a pair of perfect matched capture antibody and detection antibody for forming sandwich-like immunocomplex, as mentioned previously in the background of the invention. Therefore, it is ideal to invent an assay system that can use an adaptor molecule that the capture molecule doesn't need to be modified, like Protein G or Protein A in the immunoprecipitation (IP), to prevent the inactivation of the capture molecule by the solid phase while the capture molecule or detection molecule does not need to be modified. The difficult goal can be realized only by breaking the capture and detection of the analyte into two isolated steps, therefore, the adaptor molecule on a solid phase can bind the capture molecule while not affect the detection molecule. For example, if the adaptor molecule is Protein G, then all capture molecules, like antibodies with Fc-domain, can bind with the adaptor molecule Protein G naturally, without any additional modification; this provides the capture process with properties of convenience, flexibility, and universality. Please note, in sandwich-ELISA, the capture antibody (capture molecule) is physically adsorbed onto the solid phase (this is the coating process). It is convenient to use physical adsorption (coating) to immobilize a capture molecule (e.g., antibody) or an analyte on the solid phase. The greatest advantage of the physical adsorption of antibody or analyte to a solid phase is that the coated molecules such as antibody and analyte on the solid phase are nearly not dissociable (U.S. Pat. No. 5,009,998) and the process of the coating is very convenient. However, as the physical adsorption is a random immobilization process, most of the antibodies usually lose most of their analyte binding capacities (78-82%) after coated on the solid phase (Subramanian, A. and Velander, W.H. 1996). In another experiment, Lu (1996) reported that the immobilization of antibody using Protein A resulted in increasing sensitivity 10 times higher than that of random immobilization. Why antibodies inactivated after adsorption on the solid phase? My opinion is that most of the antibodies used today are not screened and selected from the antibodies that already adsorbed on a solid phase and not inactivated by the solid phase. In contradictory, the antibodies are usually screened from the analytes coated on a solid phase. The three dimensional structures of these antibodies selected may change to a structures not optimal for analyte binding after coating on a solid phase and may lead to lose of analyte binding activity. Therefore, it is relatively easier to find antibodies that bind to an analyte coated on a solid phase, but it is difficult to find antibodies that maintain the analyte binding activity after directly coated on a solid phase. In consequence, immobilizing an adaptor molecule such as Protein A or Protein G on a solid phase first, then using the adaptor molecule immobi-

lized to bind a capture antibody in a liquid phase overcomes the technical challenge of solid phase inactivation of the capture antibody while does not require the modification of the capture molecule, and naturally this leads to find more antibodies available for capture of analytes from their fluid samples to a solid phase. This capture process using protein G or the like as adaptor molecule in the assay step is named as IP like analyte capture process. One of the IP like analyte capture process was disclosed by Fahnestock et al (U.S. Pat. No. 4,977,247) in early 1990. He described that a Protein G variant called Protein G type 10 could be immobilized on a solid phase, and then using the solid phase to bind an antibody which is specifically against an analyte; this affinity binding solid phase could be used to isolate analyte of interest in a sample; further more, he found that the Protein G variant type 10 was irreversibly bound with IgG antibody, thus, the analyte eluted from the affinity binding solid phase does not contain IgG. However, Fahnestock didn't have idea of using his affinity binding solid phase and his useful Protein G type 10 to create a novel immunoassay to replace/substitute the traditional sandwich-ELISA for determining low abundance protein analyte in a fluid sample; he only used the protein G in his affinity chromatography.

[0084] Another technology to review is indirect ELISA, an immunoassay specific for an antibody detection (Voller et al 1978 and Lequin 2005). In this method, an antigen purified is directly immobilized onto a solid phase via physical adsorption. The target antibody in a fluid sample reacts with the antigen bound on the solid phase to form an immuno-complex, and thus the target antibody is captured on the solid phase from a fluid sample. Then a labeled detection antibody with specificity for the target antibody is added and incubated. Following washing off unbound labeled detection antibody, the amount of the target antibody is measured by detecting the signal produced by the label of the bound detection antibody on the solid phase. Similar to sandwich-ELISA, indirect ELISA also has a useful modification, the double indirect ELISA, which involves a third antibody. The third antibody carries a label and reacts with the unlabeled detection antibody already bound to the target antibody. After washing to remove unbound substances, the signal of the label is measured, and the signal of the label is directly correlated with the amount of the target antibody present in the fluid sample.

[0085] Unlike sandwich-ELISA, the indirect ELISA is not used to detect the presence of an analyte (Voller et al 1978) of low abundance. This is because that the analyte or protein in a sample such as cell lysate usually exists in a mixture of million of other proteins and other biomolecules such as DNA, RNA, lipids, and carbohydrates; in addition, the analyte usually exists in a very low concentration. Therefore, it is impossible to directly bind enough amount of the target analyte in the sample onto the solid phase of indirect ELISA specifically for further detection. However, I contemplated that it is possible to detect protein analyte of low abundance using indirect ELISA if the method is combining with the analyte capture steps of IP like analyte capture process mentioned above; specifically, is to using an affinity binding solid phase to capture the analyte from a fluid sample, then using a non-affinity binding solid phase to coat the analyte for further specific detection. Actually and fortunately, the idea is really working. Specifically, the analyte separated from the IP like analyte capture process is dissociated and then coated onto the solid phase of indirect ELISA for further analyte detection by using a detection antibody. As the analyte separated from the IP like analyte capture process is enriched and is relative pure, and in a water based buffer system without blocking agents such as Tween-20 and BSA, it can be adsorbed to a hydrophobic solid phase of indirect ELISA efficiently (at ng level) for further specific detection. This novel combination or integration of IP like analyte capture process (capture analyte by affinity binding using an affinity binding solid phase) with indirect ELISA analyte detection system (immobilizing the analyte for specific detection by physical adsorption using a non-affinity binding solid phase) forms the core elements of present invention UTSIA. It must point out, even though previously there are people trying to run ELISA such as sandwich-ELISA after getting an analyte from immunoprecipitation or affinity chromatography, they never integrated the immunoprecitation assay or affinity chromatography with indirect ELISA into a novel immunoassay like present invention UTSIA.

[0086] Obviously, to make the invention works well, it needs to design an efficient and convenient analyte capture solid phase system to obtain an analyte from a fluid sample, an simple analyte dissociating system to release the analyte from the capture solid phase, and a easy detection solid phase to immobilize the released analyte, and a robust signal generating system to detect the presence and the amount of the analyte bound on the detection solid phase.

[0087] To capture an analyte from a fluid sample, the best way is to immobilize the analyte on a solid phase via specific affinity binding reaction between the analyte and affinity binding molecule(s) (adaptor molecule, capture molecule, or complex of adaptor and capture molecules) attached on the solid phase. Therefore, the choices of solid phase and the affinity binding molecules are critical for setting up the analyte capture solid phase system (capture system or affinity binding solid phase). Now let me talk about the solid phase for the capture system of present invention. The solid phase is insoluble in water based buffer or solution or samples, and stable in extreme low or high pH conditions. Also the solid phase has capacity to attract and immobilize the capture affinity binding molecule directly, has chemical functional groups which can be used to couple the affinity molecules on the solid phase, or has combinations of these properties. Alternatively, the solid phase can attain additional coating materials which can be used to physically adsorb or chemically couple the affinity binding molecules to immobilize them on the solid phase. The solid phase comprises plastic, derivative plastic, magnetic or non-magnetic materials, glass, or silicon materials. The solid phase can be in any suitable shape such as plate, membrane, sheet, dipstick, bead, well, chip, and any proper configurations that known to those of ordinary skill in the art. A preferred embodiment of the solid phase for the capture solid phase comprises polystyrene 96 well plate. The polystyrene plate has hydrophobic surface which is ideal for protein adsorption or coating. Usually the hydrophobic adsorption is the most convenient method to immobilize an affinity binding molecule on a solid phase comparing with other methods such as the covalent chemical coupling. Of course the hydrophobic surface such as polystyrene can be further modified by radiation or other techniques that alter the chemistry of the surface, such as adding hydrophilic groups (carboxyl and amine groups) onto the hydrophobic surface that may optimize the process of physical adsorption or coating of biomolecules such as protein onto the solid phase

surface, or adding other reactive groups that can be used for the covalent immobilization of biomolecules onto the surface of the solid phase.

[0088] One embodiment of the affinity binding molecule on the capture solid phase comprises a capture molecule or capture antibody. The capture molecule is coated on a hydrophobic polystyrene solid phase by physical adsorption or coating process. Before and during the coating process, the buffer containing the capture molecule should avoid blocking components such as Tween-20, BSA, and serum, because these blocking components will reduce or prevent the adsorption of the capture molecule on the solid phase. After the solid phase immobilized with the capture molecule, it should be blocked by a buffer containing blocking components such as Tween-20 and BSA to reduce nonspecific binding in late experimental procedures. The advantage of coating the capture molecule directly on the solid phase is that it is the simplest analyte capture system. The disadvantage of the coating is that it usually inactivates the capture molecule or capture antibody by the solid phase during the adsorption or coating process.

[0089] In another embodiment of the affinity binding molecule on the capture solid phase comprises an adaptor molecule. The adaptor molecule is immobilized on a solid phase by physical adsorption (coating process), or covalently coupling reaction. The function of the adaptor molecule is acting as an intermediate molecule between the solid phase and the capture molecule to prevent the inactivation of the capture molecule by the solid phase. Therefore, at least one part of the adaptor molecule is capable of immobilizing onto the solid phase by physical adsorption or by chemical coupling reactions, and at least part of the adaptor molecule can bind with the capture molecule by affinity binding reaction. The solid phase comprises hydrophobic solid support such as polystyrene plate. Before and during the coating process to immobilize the adaptor molecule on the solid phase, the buffer containing the adaptor molecule should avoid blocking components such as Tween-20, BSA, and serum, because these blocking components reduce or prevent the adsorption of the adaptor molecule onto the solid phase. However, after the solid phase immobilized with the adaptor molecule, it should be blocked by a buffer containing blocking components such as Tween-20 and BSA to reduce nonspecific binding in late experimental procedures. The advantage of coating the adaptor molecule on the solid phase is that it prevents the inactivation of the capture molecule on the solid phase by providing proper orientation of the capture molecule or capture antibody on the solid phase. The alternative method of physical adsorption to immobilize an adaptor molecule to a solid phase is covalent coupling reaction. The method is generally more complicated, and therefore, is not preferred for current invention if the physical adsorption working well. There are many publications that describe the chemical coupling reactions to couple affinity binding molecules to a solid phase, the papers to begin with are by Nisnevitch et al 2001 and by Qoronfleh et al 2003. Alternatively, there are commercial solid phase immobilized with adaptor molecule such as Protein G or Protein A/G available for ordering, which may cost more from your budget; examples of these commercial solid phases are Reacti-Bind™ Protein G coated 96 well strip plate and Reacti-Bind™ Protein A/G coated 96 well strip plate from Pierce Biotechnology, Inc, Rockford, Ill., USA.

[0090] Even though the adaptor molecule is critical for present invention, there are only two ways available to find the adaptor molecules. One is to screen naturally available proteins such as Protein A, Protein G, Protein A/G, and protein L immobilized on a solid phase and test whether they bind the capture antibody in different pH and protect the capture antibody from inactivation by the solid phase; another is to make the adaptor molecule by recombinant molecular cloning technology or other technologies and then screen whether they bind the capture antibody in different pH and protect the capture antibody from inactivation by the solid phase. Tanaka G et al (2006) reported that a recombinant Fc-binding protein (E72G3) with self-adhering ability on hydrophobic solid phase is produced by linking the Fc-binding domain of Protein G (G3) and hydrophobic domain of elastin (E72) by a recombinant molecular cloning technology; the E72G3 retains the antibody binding activity after coating on a hydrophobic solid phase. Therefore, the E72G3 and E72G3 like recombinant proteins can be tested to use as the adaptor molecule for current invention. The ideal adaptor molecule like the protein G type 10 discovered by Fahnestock can be found by screen whether the adaptor molecule immobilized on a solid phase can bind the capture molecule or other substance of interest in pH of interest and whether the capture molecule bound by the adaptor molecule can bind analyte in the pH of interest.

[0091] Ideally adaptor molecule is the one that binds the capture molecule either in neutral pH and extreme acidic pH or in neutral pH and basic extreme pH. This kind of adaptor molecule could provide maximum flexibility of UTSIA to select capture molecule and detection molecule, as the capture molecule will not dissociate into the dissociation buffer, and thus the capture molecule will not interfere with analyte coating on the detection solid phase and will not interfere with the detection of the detection molecule if it is not pre-labeled.

[0092] If the adaptor molecule such as protein G immobilized on the capture solid phase does not bind the capture antibody in the dissociation buffer, then the capture antibody bound originally in neutral pH buffer also dissociates into the dissociation buffer, and its further coating on the detection solid phase not only decreases the coating surface of the analyte dissociated, but also eliminate the selection of detection antibody from same animal species of the capture antibody if the detection antibody is not pre-labeled. These will be further described in following paragraphs.

[0093] After immobilizing the adaptor molecule on the solid phase and blocking the solid phase with proper blocking buffer (i.e., if the adaptor molecule is a protein G, then the blocking buffer can't contain the substance with Fc domain such as serum.), there are three ways to capture an analyte from a fluid sample. One way is that a capture molecule reacts with the analyte in the fluid sample first to form a complex of the capture molecule and the analyte in liquid phase; then the liquid phase containing the complex is incubated with the solid phase immobilized with the adaptor molecule to bind the complex via affinity binding interaction between the adaptor molecule and the capture molecule of the complex. This way is especially useful when the fluid sample doesn't contain substances that may interfere with the affinity binding reaction between the adaptor molecule and the capture molecule. For example, if the adaptor molecule is Protein G and the capture molecule is a capture antibody, then a fluid sample such as cell lysate is mixed with the capture antibody first to form an immuno-complex of the analyte and the capture antibody. Because the cell lysate doesn't contain the antibody-like molecules that may directly binds with Protein G,

the immuno-complex will efficiently bind to Protein G immobilized on the solid phase. As the interaction between the immuno-complex and Protein G is an affinity binding, it's ideal to including blocking components Tween-20 and/or BSA in the affinity binding process to reduce non-specific and non-affinity bindings. However, the some blocking components such as serum can't be used in this step, because it contains immunoglobulin which will block the affinity binding between the immuno-complex and Protein G. The maximum amount of the capture molecule added to the fluid sample is that the maximum amount that can be bound by the adaptor molecule immobilized on the solid phase. Another way is that a capture molecule is incubated with the solid phase immobilized with the adaptor molecule first to bind the capture molecule on the solid phase. After binding the capture molecule to the adaptor molecule immobilized on the solid phase, the solid phase is blocked with a blocking buffer comprising components such as Tween-20 and BSA. If the adaptor molecule is Protein G and the capture molecule is a capture antibody, then the solid phase immobilized with the complex of Protein G and the capture antibody, the antibody immobilized is ready to capture the analyte in a fluid sample without the immunoglobulin-like molecules (the molecule containing Fc-domain) which will bind with Protein G. But if the analyte of interest is an IgG, then the solid phase immobilized with the complex of Protein G and the capture antibody is not ready to capture the analyte yet. The solid phase must be further blocked with a blocking buffer which contains serum/immunoglobulins to completely block the unoccupied Fc-binding sites of Protein G on the solid phase to prevent un-wanted binding reactions. It is important to select blocking serum/immunoglobulins (for blocking the capture solid phase) from an animal species that will not interfere with the late detection steps if the adaptor molecule protein G immobilized on the solid phase does not bind the capture antibody and the blocking immunoglobulins in dissociation buffer (a buffer comprises with extreme pH). For example, if the detection molecule is an unlabeled polyclonal antibody from rabbit, then the blocking serum/immunoglobulins should not get from rabbits, because it interferes with late detection step that use anti-rabbit-HRP and rabbit polyclonal detection antibody. At the moment, the solid phase immobilized with the complex of Protein G and the capture antibody, after being blocked with proper serum/immunoglobulin, is ready to capture the analyte in the fluid samples. The third way is that a capture molecule, a fluid sample, and a solid phase immobilized with an adaptor molecule are mixed together to form a complex of analyte-capture molecule-adaptor molecule-solid phase to capture the analyte from the fluid sample. Similar to the first way, the fluid sample should not contain substances that interfere with the affinity binding reaction between the adaptor molecule and the capture molecule. For example, if the adaptor molecule is Protein G and the capture molecule is a capture antibody, then a fluid sample (like cell lysate) is mixed with the capture antibody and the solid phase to form an immuno-complex of the analyte, the capture antibody, and Protein G on the solid phase. Because the cell lysate doesn't contain the antibody-like molecules that may directly binds with Protein G, the complex of analyte-capture molecule-adaptor molecule will be efficiently formed on the solid phase. As the interactions among the components of the complex are affinity bindings, it's ideal to including blocking components Tween-20 and/or BSA to reduce non-specific and no-affinity bindings. However, the blocking component such as serum/immunoglobulins can't be used in this step if the adaptor molecule is a Fc-binding protein such as Protein G, because it contains immunoglobulins which block the affinity binding between the capture antibody and Protein G, thus, it inhibits the capture of the analyte from the fluid sample.

[0094] Now the analyte is on the solid phase of the capture systems. As mentioned previously, the direct detection of the analyte with a detection antibody on the capture solid phase is the technique of the sandwich-ELISA, which is difficult due to the difficulty in finding a perfectly matched pair of the capture antibody and the detection antibody to form a sandwich complex. Therefore, I am not using the sandwich-ELISA to continue the analyte detection; instead, I dissociate the analyte from the capture solid phase (affinity binding solid phase) by using a dissociating buffer to release the analyte bound, and then transfer the analyte and adsorb the analyte to another solid phase (non-affinity binding solid phase or physical adsorption) for further specific detection. The detail procedure is described further in following paragraphs.

[0095] After the analyte is immobilized on the capture solid phase, the solid phase is washed by PBS or other water based buffers (the buffers do not contain blocking components such as Tween-20, BSA, and serum) to remove unbound substances and to clean unbound blocking components such as BSA and Tween-20. Then the solid phase is incubated with a dissociating buffer to release the analyte into the liquid phase of the dissociating buffer from the solid phase. The dissociating buffer comprises a buffer with extreme pH (e.g., 0.2 M Glycine pH 2.5), which is capable of separating the analyte bound on the solid phase into the liquid phase of the dissociating buffer. The mechanism of the dissociation may be that the extreme pH will redistribute the charges of hydrophilic functional groups of amino acid residues in the analyte and in the capture molecule, and this may interrupt the affinity bindings between the capture molecule and the analyte; and therefore, the analyte is released into the liquid phase of the dissociating buffer. Please note, as the analyte dissociated from the solid phase needs to be further coated onto the hydrophobic detection solid phase for further detection, the dissociating buffer can't contain the blocking components which might interfere with the analyte's coating process, and these blocking components comprise BSA, serum, tween-20, and the like. There may be other mechanisms besides extreme pH that may be helpful to release the analyte bound into the liquid phase, these include salt concentrations, organic solvents, and detergents. Even though present inventor has not tested whether these mechanisms are compatible with present invention UTSIA, the obvious disadvantages of some of the mechanisms such as organic solvents and detergents are that they are not easily to be neutralized and, in addition, some of them may disrupt hydrophobic bindings (physical adsorption) which is critical for present invention. Therefore, their applications in UTSIA should be further cautiously investigated.

[0096] Then the dissociating buffer containing the analyte is transferred to a detection solid phase (non-affinity binding solid phase) which contains a neutralizing buffer (e.g., 1M Tris/HCl pH 9.0) with opposite extreme pH of the dissociating buffer. The mixing of the dissociating buffer and the neutralizing buffer neutralizes the dissociating buffer's extreme pH and brings the pH to a level that is suitable for the analyte's adsorption onto the detection solid phase, and the pH is suitable for the late steps for the detection of the analyte

on the detection solid phase. This can be achieved by using different ratio of the dissociating buffer to the neutralizing buffer to see which combination results in maximum analyte coating on the solid phase and getting best signal detection. The neutralizing buffer also can't contain blocking components such as BSA, serum, tween-20, and the like. The detection solid phase (non-affinity binding solid phase) comprises a solid phase for immobilizing the analyte dissociated in the dissociating buffer via physical adsorption, it comprises hydrophobic solid phase such as 96 well polystyrene plates (the hydrophobic surface like polystyrene can be further modified by radiation or other techniques that alter the chemistry of the surface such as adding hydrophilic groups of carboxyl and amine groups onto the hydrophobic surface, that may optimize the process of physical adsorption or coating of biomolecules such as protein onto the detection solid phase surface). The detection solid phase does not require the affinity binding molecule(s) immobilized for the capture of the analyte from the liquid phase, so it is different from the capture solid phase, which requires at least an affinity binding molecule (such as the adaptor molecule or the capture antibody) for the capture of the analyte. The detection solid phase immobilizes the analyte by a simple physical adsorption or coating process preferably. Before and during the analyte adsorption or coating process, the detection solid phase can't be incubated with substances that have blocking capacity, such as Tween-20, BSA, and serum, as these substances block the sites of the solid phase that are critical for the analyte's adsorption or coating. As mentioned earlier, the dissociating buffer containing the analyte and the neutralizing buffer in the detection solid phase do not contain these blocking substances. In addition, it is reported that pH 9.6 of carbonate coating buffer is ideal for protein coating on the radiation modified polystyrene plate, even though other pH ranges such as neutral pH (7-7.5) the protein also can be coated onto the solid phase. These special buffer conditions promote the physical adsorption of the analyte onto the detection solid phase. Therefore, after proper time of incubation in these conditions, the analyte dissociated in the liquid phase is immobilized onto the detection solid phase for further specific detection.

[0097] You may ask why the analyte dissociated in liquid phase does not coat back to the capture solid phase (the affinity binding solid phase), but it is coated onto the non-affinity binding detection solid phase? It is because that the capture solid phase was blocked previously and there is no way to coat the analyte back on the capture solid phase by physical adsorption, while the detection solid phase is not blocked and its hydrophobic surface is easily to be coated with analytes (like proteins) which also have hydrophobic residues in their molecules. In addition to above reason, the capture solid phase immobilizes the analyte by affinity binding, but the detection solid phase immobilizes the analyte by physical adsorption (coating process); under extreme pH of the dissociating buffer, the analyte does not bind with the affinity molecule such as capture antibody immobilized on the capture solid phase.

[0098] In case there are other molecules co-precipitated (co-capture) with the analyte by the capture system, then these molecules, if they are complexed with the analyte by affinity binding, they are also released to the liquid phase of the dissociating buffer with the analyte; and the molecules are also coated to the detection solid phase with the analyte. These molecules can be detected on the detection solid phase by their specific detection molecules. If there are multiple molecules need to be coated on a detection solid phase, then a suitable area of the detection solid phase for proper adsorption need to be experimentally optimized. You can image, if the area is too narrow, it may be only part of the molecules can be coated on the solid phase; the coated part of the molecules may be not optimal enough for further detection.

[0099] After the immobilization of the analyte on the detection solid phase, the next step is to remove the unbound substances and then washing the detection solid phase with PBS or other water-based buffers, and to block the detection solid phase by using blocking buffer comprising blocking components such as Tween-20, BSA, and serum. It is important to select the blocking serum from an animal species that will not interfere with the detection steps. For example, if the detection molecule is an un-labeled antibody from rabbit, then the blocking serum/immunoglobulins should not get from the rabbit. After blocking, the analyte on the detection solid phase is detected by a signal generating system. The signal generating system is selected from the group comprising a labeled detection antibody or an unlabeled detection antibody and a labeled antibody against the unlabeled detection antibody. If the signal generating system comprises a labeled detection antibody, the analyte immobilized on the detection solid phase is incubated with a solution containing the labeled detection antibody and the blocking components such as BSA, serum, and Tween-20, to let the labeled detection antibody specifically binds on the analyte coated on the detection solid phase. After removing and washing to remove unbound substances from the detection solid phase, the signal from the label on the detection antibody bound on the detection solid phase is detected by an instrument or other suitable means. The label is selected from the group comprising enzyme, chromogens, luminescent compounds, chemiluminescent compounds, radioactive elements, and direct visual labels such as colored microparticles; the label is capable of producing a signal for detection either by itself or in conjunction with one or more additional substances. For example, if the label is enzyme HRP, then the signal is produced by adding its substrate TMB and other substances required for the enzyme reaction under proper time and temperature to form a blue color product, and then the intensity of the color product (thus, the signal of the label) is measured by a spectrometry via detecting its absorbance at 650-655 nm wavelength. The signal is correlated with the presence of the analyte. To determine the amount or the concentration of the analyte, a standard curve of concentrations or amounts vs absorbances is produced by measuring absorbances produced from serial different concentrations or amounts of the analyte on the assay. Comparing with the standard curve, then the concentration or the amount of the analyte in a fluid sample is decided. If the signal generating system comprises an unlabeled detection antibody and a labeled antibody against the unlabeled detection antibody, the analyte immobilized on the detection solid phase is incubated with a solution containing the unlabeled detection antibody and blocking components such as BSA and Tween-20, to let the unlabeled detection antibody specifically binding on the analyte coated on the detection solid phase. After removing and washing to remove unbound substance from the detection solid phase, the labeled antibody against the unlabeled detection antibody is incubated with the solid phase in the presence of blocking components such as Tween-20, BSA, and serum to let the labeled antibody specifically binding on the unlabeled detection anti-

body bound on the detection solid phase. After removing and washing to remove unbound substance from the detection solid phase, the labeled antibody immobilized on the solid phase is detected by a detector or other means via the label's physical property or the label's catalyzed activity. The label is selected from the group comprising enzyme, chromogen, luminescent compound, chemiluminescent compound, radioactive element, and direct visual label such as colored micro-particle; the label is capable of producing a signal for detection either by itself or in conjunction with one or more additional substances. To determine the amount or the concentration of the analyte, a standard curve of concentrations or amounts vs absorbances is produced by measuring absorbances produced from serial different concentrations or amounts of the analyte on the assay. Comparing with the standard curve, then the concentration or the amount of the analyte in a fluid sample is decided. The advantage of the signal generating system comprising an unlabeled detection antibody and a labeled antibody against the unlabeled detection antibody is that it provides a universal detecting platform for detection of multiple different analytes in multiplex immunoassay or in microarray immunoassay. For example, multiple different analytes immobilized on different spots of a detection solid phase can be detected by their corresponding different detection antibodies from a same animal species, and then the unlabeled detection antibodies are detected by a common labeled antibody specifically against these unlabeled detection antibodies. For example, if these unlabeled detection antibodies are monoclonal mouse IgGs, then a common HRP-labeled anti-mouse IgG can be used to detect these monoclonal IgG antibodies.

[0100] In case the capture molecule is not very specific to the analyte that leads to the capture of un-wanted cross-reacting substances, the situation can be improved by additional loading the neutralized dissociating buffer which contains the analyte and the unwanted cross-reacting substances to a new capture system which uses another capture molecule against the target analyte, then followed by dissociating, transferring, neutralizing, coating, and detecting. Repeating the capture steps may provide much clean enrichment of the target analyte from a fluid samples, that is, increasing specificity of the assay by repeating the capture steps.

[0101] In case the analyte level in the sample is very low, such as in pg level, its detection on the detection solid phase can be carried out by the technology of high sensitivity detection system which is disclosed in U.S. Pat. No. 5,731,158. Basically, the HRP-detection antibody or HRP-antibody against the unlabeled detection antibody on the detection solid phase is reacted with H_2O_2 and biotinyl-tyramide to covalently attach many biotin residues on the detection solid phase, and then using streptavidin-HRP to bind with the biotins on the solid phase, and then reacts with HRP substrate TMB to amplify the detection signal. Currently there is commercial high sensitivity detection kit available.

[0102] Previous several paragraphs described the case of the adaptor molecule protein G which does not bind the capture antibody in the dissociation buffer, that lead to specific rule to select detection antibody; this greatly reduces the flexibility of selection of capture antibody and detection antibody at assay setting up, one of the major goal of UTSIA. To overcome this issue in UTSIA is to use an adaptor molecule which binds the capture antibody in both neutral pH and in extreme pH. Fahnestock et al (U.S. Pat. No. 4,977,247) disclosed a Protein G variant called Protein G type 10 could be immobilized on a solid phase to bind an antibody to capture an analyte, and the Protein G variant type 10 was irreversibly bound with IgG antibody, thus, the analyte eluted from the affinity binding solid phase does not contain IgG. Thus the Protein G type 10 is an ideal adaptor molecule candidate for UTSIA. If the adaptor molecule which binds the capture antibody in both neutral pH and in extreme pH (acidic pH or basic pH) is used in the capture solid phase, then the detection antibody can be from any animal species no matter what kind of capture antibody is used. Alternatively, another solution to the issue is to use two adaptor molecules on the capture solid phase, for example, one variant of protein G is used to bind the capture antibody on neutral pH, and another variant of protein G is used to bind the capture antibody in extreme pH which is used to dissociate analyte from the capture solid phase.

[0103] Now it is clear that UTSIA is a novel and convenient immunoassay which overcomes the technical limitation of sandwich-ELISA in the capture and detection of an analyte from a fluid sample. The UTSIA is not only a sandwich-ELISA equivalent immunoassay, but also an immunoassay with more flexible powers and wider applications. You may wonder whether the UTSIA quantification can be used in the situation that the analyte of interest is located in the cells or tissue immobilized on a solid phase, such as IHC and ICC. The answer is yes. In IHC and ICC, there are many other substances of tissue or cells immobilized on the solid phase, these substances on the solid phase are not only interfering with the detection of the analyte of the interest, but also diluting the analyte of interest in a relative low density, all these lead to that it is difficult to measure the analyte of interest by methods such as enzyme based assay of cell-based ELISA if without high quality detection antibody with super specificity. Therefore, currently routine quantitative method of the analyte in IHC and ICC is by image method (usually it is fluorescence based image method) that involving the image capture and image analysis (Warford et al 2004 and Kaczmarek E et al 2004). As the image method is a fluorescent based method it needs specific equipments for image capture and software for image analysis, therefore, it is not convenient, robust, and simple method. In addition, as the image method is directly worked on the cells or tissue, it is always have interference from background noise signal such as autofluorecence that from the cells or tissue background, and always there are noise signals from nonspecific bindings of the detection antibody on the cells or tissue. It should be pointed out that in the image method the processes of image taking and image transferring to numerical data usually leads to big variations at final results. Even though the cell-based ELISA is a very nice cells-based analyte quantification method, it is very difficult to set up for routine applications if there is no a very high quality antibody available for an analyte of interest and if the expression level of the analyte is not high enough in the cells or tissue (Versteeg et al 2000, Yang et al 2006). This bottleneck problem in quantitative IHC and ICC is solved by cell-based UTSIA, a variant technology of UTSIA. Specifically, first using an detection antibody (labeled or unlabled) binding on the analyte of interest in the cells or tissue immobilized on a first solid phase, then using a dissociating buffer to release the detection antibody bound into a liquid phase of the dissociating buffer, then immobilizing the detection antibody dissociated in the liquid phase onto a second solid phase which is non-affinity binding solid phase or affinity binding solid phase; finally, the labeled detection antibody is detected on the second solid phase, or the unla-

beled detection antibody is detected by an labeled antibody against the unlabeled detection antibody coated on the second solid phase. The presence and the amount of the analyte of interest on the first solid phase is directly correlated with the detection antibody immobilized on the second solid phase(s), and thus directly correlated with the signal from the label of the labeled detection antibody or the labeled antibody bound on the second solid phase(s). Therefore, the analyte of interest on the cells or tissue immobilized on the first solid phase is detected by measuring the presence and the amount of the detection antibody immobilized on the second solid phase(s). One of the advantage of cell-based UTSIA for quantitation of the analyte on the cells or tissue immobilized on a solid phase is that the dissociation step only releases the detection antibody which binds on the cells or tissue via affinity binding, but not the one binds on the cells or tissue via non-affinity bindings; therefore, the advantage is to reduce noise background. The detail of the UTSIA based analyte detection and quantification in cells or tissue immobilized on a solid phase will be further described in late paragraphs.

[0104] Followings are major applications of UTSIA in different fields. Please note, in following preferred embodiments of the invention, some detail steps or processes may be missed in their descriptions, such as blocking, dissociating, neutralizing, transferring, coating, washing, or detecting, it should refer back to above paragraphs of general description and refer forward to prototype example for experimental guidance. As indicated before, they are offered by way of illustration of present invention, and not by way of limitation of present invention.

(II) The Preferred Embodiments of the Invention

1. Detecting and Quantitating an Analyte in a Fluid Sample (FIG. 1A, FIG. 1B, and FIG. 2):

[0105] Like sandwich-ELISA, UTSIA can be used to detect and quantitate an analyte of interest in a fluid sample. As there are three different capture solid phases (affinity binding solid phases) that are based on affinity bindings to capture target analyte, there are several ways to capture and detect an analyte from a fluid sample. A. using a first solid phase immobilized with a capture molecule: the fluid sample is first incubated with the first solid phase to let the analyte immobilized on the first solid phase via the affinity binding between the analyte and the capture molecule immobilized on the first solid phase; after removing and washing to get rid of the unbound substances, the first solid phase is incubated with a dissociating buffer to release the analyte into the liquid phase of the dissociating buffer; then transferring the dissociating buffer containing the analyte to a non-affinity binding detection solid phase (second solid phase) which may be pre-incubated with a neutralizing buffer to coat the analyte onto the detection solid phase; after removing and washing to remove the unbound buffer mixture, the detection solid phase is blocked, and then incubated with either a labeled detection molecule to detect the analyte bound via the label of the labeled detection molecule in the presence of blocking components, or an unlabeled detection molecule and a labeled molecule against the unlabeled detection molecule to detect the analyte bound via the label of the labeled molecule in the presence of blocking components; the signal strength of the detection is correlated with the amount or concentration of the analyte in the fluid sample; B. using a first solid phase immobilized with an adaptor molecule: the fluid sample is first incubated with a capture molecule to form a complex of the analyte and the capture molecule in a liquid phase, and then the complex in the liquid phase is incubated with the first solid phase in the presence of blocking components to immobilize the complex via the affinity binding between the capture molecule of the complex and the adaptor molecule immobilized on the first solid phase; after removing and washing to get rid of the unbound substances, the first solid phase is incubated with a dissociating buffer to release the analyte into the liquid phase of the dissociating buffer; then transferring the liquid phase of the dissociating buffer containing the analyte to a non-affinity binding detection solid phase (second solid phase) (which may be pre-incubated with a neutralizing buffer) to coat the analyte onto the detection solid phase; after removing and washing to remove the unbound buffer mixture, the detection solid phase is blocked, and then incubated with either a labeled detection molecule to detect the analyte bound via the label of the labeled detection molecule in the presence of blocking components, or an unlabeled detection molecule and a labeled molecule against the unlabeled detection molecule to detect the analyte bound via the label of the labeled molecule in the presence of blocking components; the signal strength of the detection is correlated with the amount or concentration of the analyte in the fluid sample; C. using a first solid phase immobilized with an complex of an adaptor molecule and a capture molecule (the capture molecule is binding with the adaptor molecule pre-immobilized on the first solid phase): the fluid sample is first incubated with the first solid phase to obtain the analyte from the fluid sample in the presence of blocking components, and then after removing and washing to get rid of the unbound substances, the first solid phase is incubated with a dissociating buffer to release the analyte into the liquid phase of the dissociating buffer; then transferring the liquid phase of dissociating buffer containing the analyte to a non-affinity binding detection solid phase (second solid phase) (which may be pre-incubated with a neutralizing buffer) to coat the analyte onto the detection solid phase; after removing and washing to remove the unbound buffer mixture, the detection solid phase is blocked, and then incubated with either a labeled detection molecule to detect the analyte bound on the detection solid phase via the label of the labeled detection molecule in the presence of blocking components, or an unlabeled detection molecule and a labeled molecule against the unlabeled detection molecule to detect the analyte bound on the detection solid phase via the label of the labeled molecule in the presence of blocking components; the signal strength of the detection is correlated with the amount or concentration of the analyte in the fluid sample.

2. Detecting Multiple Analytes (UTSIA Based Antibody Microarray):

[0106] The antibody based microarray is a relative new concept that intents to detect multiple proteins in a same assay. However, except in rare case such as cytokines, it is suggested that it is nearly impossible to setting up an antibody-based microarray to detect hundreds of proteins in same time and in same assay with current methods (Zangar et al 2006). It is because every protein has its unique amino sequence and unique 3-dimensional structure, and thus, it is thought that every protein will need a special set of assay conditions such as antibodies, buffer and incubation time for its detection. Indeed, until today the availability of commercial antibody-based microarray is limited, only sandwich-

based antibody microarray for the detection of a few proteins such as cytokines available (please note, in this case, the microarray is developed from the commercial available sandwich-ELISA for cytokines analysis). Therefore, to make the antibody-based microarray for multiple proteins detection possible, an alternative capture and detection mechanism other than the sandwich-ELISA is required.

[0107] UTSIA, different from the sandwich-ELISA, has many universal properties that make the setting up of antibody-based microarray for multiple proteins detection practical. One is that an adaptor molecule such as Protein G on a capture solid phase prevents the capture antibodies' inactivation by the solid phase while does not require the modification of the capture antibodies; this leads to that there are more capture antibodies (such as rabbit polyclonal antibodies) available for analytes' capture from fluid sample. Another is that UTSIA uses a dissociating buffer with extreme pH which is capable of disrupting all or most of all of the affinity bindings between the capture antibodies and the analytes of interest on the capture solid phase and release the analytes into liquid phases; the third is that the analytes in the liquid phases can be coated to non-affinity binding detection solid phase by physical adsorption, because the analytes such as proteins are capable of to be coated on a hydrophobic solid phase (non-affinity binding solid phase); the fourth is that analytes coated on the detection solid phase can be bound with their specific detection antibodies that come from a same animal species such as mouse monoclonal antibodies (IgG), then all of these mouse monoclonal antibodies bound on the detection solid phase are detected by a common labeled anti-mouse IgG for signal measurement. Therefore, these universal steps of capture, dissociating, coating, and detection make the UTSIA realistic for multiple analytes detections, such as scaled up to antibody-based microarray format to detect and quantitate multiple analytes in a same time and in a same assay.

[0108] UTSIA based antibody microarray comprises a capture solid phase immobilized with an adaptor molecule such as Protein G, wherein the adaptor molecule is coated on discrete separated regions on the solid phase. To capture multiple analytes from a fluid sample, the discrete separated regions of the solid phase pre-coated with the adaptor molecule are incubated with fluid mixtures of the fluid sample and the different capture antibodies which are from a same animal species targeting on different analytes in the presence of blocking components, wherein each discrete separated region of the solid phase pre-coated with the adaptor molecule is incubated with a mixture of the fluid sample and a capture antibody. The multiple analytes can be immobilized on the discrete separated regions via the affinity binding interactions between the capture antibodies in the fluid mixture and the adaptor molecule Protein G immobilized on the solid phase. After washing the solid phase to remove unbound substances, a dissociating buffer is added to these different regions on the solid phase to elute these analytes into liquid phases, with each liquid phase contains an analyte released from a discrete region of the capture solid phase; and then the analytes in the liquid phases are transferred and coated to discrete regions of a detection solid phase (which may pre-contain neutralizing buffer on these regions). After washing and blocking, the coated analytes on the discrete regions of the detection solid phase are then detected by different detection antibodies (each analyte in each region incubated with its corresponding detection antibody) in the presence of blocking components. It is preferred that all detection antibodies are from a same animal species and are different from the animal species that making the capture antibodies to avoid cross immuno-reactions in final detection step (using a common labeled third antibody against the detection antibodies). Then, proper amounts of a common labeled third antibody against these different detection antibodies (such as anti-mouse IgG-HRP if the detection antibodies are mouse monoclonal antibodies) are added to these regions of the detection solid phase for the analytes' detections in the presence of blocking components. The strengths of the signals of the label on the discrete regions of the detection solid phase are correlated with the amount of these different analytes in the fluid sample.

3. Cell-Based UTSIA (FIG. 3A and FIG. 3B):

[0109] As mentioned in background, there is an urgent need to develop better method for quantitative ICC or quantitative IHC than the score method and the image method. The cell-based UTSIA is a solution to the urgent need. Instead of taking images from the labeled antibody bound on tissue or cells immobilized on the solid phase of IHC or ICC to quantify the analyte of interest, cell-based UTSIA uses a dissociating buffer to release the detection antibody which bound on cells or tissue immobilized on the solid phase (first solid phase) via affinity bindings to a liquid phase of the dissociating buffer, then transfers the liquid phase to a non-affinity binding second solid phase (that may contain a neutralizing buffer) to coat the detection antibody dissociated in the liquid phase on the non-affinity binding second solid phase; alternatively, the liquid phase of the dissociating buffer containing a labeled detection antibody is transferred to an affinity binding solid phase pre-immobilized with an affinity binding molecule which is capable of binding the labeled detection antibody in the liquid phase to immobilize the labeled detection antibody in the liquid phase on the second solid phase; the affinity binding molecule comprises protein G, preferred the one that can bind the labeled detection antibody at extreme acidic or basic pH; if the protein G only bind the labeled detection antibody at neutral pH, then the liquid phase of the dissociation buffer containing the labeled detection antibody must be first neutralized by a neutralizing buffer to bring the extreme pH to neutral pH, and then the buffer mixture is added to the affinity binding solid phase. On the second solid phase, the detection antibody originally bound on the cells or tissue of the first solid phase is purified from the background cells or tissue on the first solid phase, and this will increase final detection specificity and avoid background noise signal interference from the cells and the tissue; since that the detection of the detection antibody on the second solid phase avoids most of non-specific bindings originated from the cells or tissue immobilized on the first solid phase, and that the nonspecific binding of the detection antibody on the tissue or cells is not dissociated by the dissociation buffer with extreme pH, therefore, only the affinity binding detection antibody bound on the cells or tissue is dissociated and further immobilized on the second solid phase; this reduces the non-specific binding signals. In addition, if the area of the first solid phase immobilized with cells or tissue is bigger than area of the second solid phase, the detection antibody is also enriched on the second solid phase and this increases final detection sensitivity (since the bigger area of the first solid phase, it will immobilize more cells, so it will have more analyte of interest, thus it will bind with more detection antibody; when the detection antibody is coated to a smaller area of a second solid

phase, it is enriched). If the detection antibody is labeled with a detectable tag, it can be directly determined on the second solid phase; if the detection antibody is unlabeled, it can be indirectly determined on the second solid phase by using a labeled antibody against the detection antibody. The signal strength of the detection antibody on the second solid phase is directly correlated with the amount of the analyte of interest on the cells or tissue immobilized on the first solid phase.

[0110] Please note, on the first solid phase, before and during the binding of the detection antibody to its analyte, the blocking components should be selected from the one that without significant cross-reaction with the anti-detection antibody used in late on the second solid phase. For example, if the detection antibody is a mouse monoclonal antibody, then the blocking serum should not get from mouse or other animal species that may have cross-reactions with the mouse monoclonal antibody on the second solid phase when detected by a labeled antibody against the mouse monoclonal detection antibody.

[0111] Followings are further guidance of cell-based UTSIA for your reference which comprises: growing cells in a 96 well tissue culture plate (first plate or first solid phase) until the cells are near completely confluent; removing the medium from the well; washing with PBS; fixing the cell with 4% paraformaldehyde PBS for proper time; removing the paraformaldehyde and washing the fixed cells with PBS; permeabilizing the fixed cells with cold methanol or PBS with 0.5% Triton X-100; blocking the cells with blocking buffer which comprises Tween-20, BSA, and serum; the serum is not from the animal species which the detection antibody was made and the serum is preferred from the animal species which the secondary antibody was taken; incubating the cells with an detection antibody against an analyte of interest in a blocking buffer comprises Tween-20 and BSA; removing the unbound detection antibody and washing the cells with PBS; adding a dissociating buffer with extreme pH to release the detection antibody bound on the analyte of interest on the cells fixed in the well of 96 well plate into the liquid phase of the dissociating buffer; transferring the liquid phase to a well of another 96 well polystyrene plate (second plate or second solid phase) which may pre-contain a neutralizing buffer with opposite pH of the dissociating buffer to make buffer mixture with pH optimal for the detection antibody to be detected in late steps of the assay; gentle rotating the plate to coat the detection antibody on the well of the plate; removing the unbound buffer mixture from the well and washing with PBS; blocking the well with a blocking buffer with Tween-20, BSA, and serum which will not interfere with the detection of the detection antibody; incubating the well with an antibody-HRP against the detection antibody bound in the well of the plate in the blocking buffer comprising Tween-20 and BSA; removing unbound antibody-HRP and washing the well with PBS; adding TMB substrate solution to the well of the second plate and incubating without light for proper time; the signal of the analyte of interest in the cells fixed on the first plate is determined by detecting the absorbance of the second plate by reading the OD650 nm.

[0112] If the detection antibody is prelabeled, it can be determined directly after immobilizing on an affinity binding solid phase (second solid phase), for example, after fixed cells with 4% paraformaldehyde, permealized with 0.5% triton-100 or cold methanol, and blocked with blocking buffer, the cells is incubated with an labeled detection antibody against an analyte of interest in the blocking buffer; then removing the unbound labeled detection antibody and washing the cells with PBS; then adding a dissociating buffer with extreme pH to release the labeled detection antibody bound on the analyte of interest on the cells fixed into the liquid phase of the dissociating buffer; transferring the liquid phase to a well of another 96 well polystyrene plate immobilized with/without protein G (second solid phase) which may pre-contains a neutralizing buffer with opposite pH of the dissociating buffer to make buffer mixture with pH optimal for the labeled detection antibody to be immobilized and be detected; gentle rotating the plate to immobilized the labeled detection antibody on the well of the plate; removing the buffer mixture from the well and then washing with PBS; the signal of the analyte of interest in the cells fixed on the first plate is determined by detecting the presence and the amount of the labeled detection antibody immobilized on the second plate (second solid phase). Please note, the success of the alternative way of cell-based UTSIA depends on whether the label of the labeled detection antibody maintains its activity after the antibody coated on the second plate.

[0113] For frozen tissue, mounting the frozen tissue block on a cryostat holder and cutting the tissue to 5 μm thick slice at minus 18 to minus 20° C.; loading the tissue slice to a surface of polystyrene sheet that may be coated with poly-L-Lysine or other materials which promote tissue adhesion on the surface; letting the section dry for at 5-30 minutes at room temperature; then cutting the surface immobilized with tissue to a round small section with defined area (first solid phase) such as with diameter of 4.5 mm, 9 mm, or 13.5 mm which is capable to be put into the well of 96 well plate, 48 well plate, or 24 well plate (the cutting section to round small pierce with defined area can be done with a hole punch); then putting the section with defined area to a well of one of the plates (such as 4.5 mm diameter tissue section to well of 96 well plate, or 13.5 mm diameter section to well of 24 well plate) to fix the tissue with 2-4% paraformaldehyde 10-15 min (or with acetone for 2 min) (if the analyte expression is weak in the tissue, it prefers to use larger section with bigger diameter), washing with PBS to remove paraformaldehyde or acetone; permeabilizing the fixed tissue with cold methanol or PBS with 0.5% Triton X-100; washing the tissue with PBS 2 times; blocking the section with blocking buffer comprising Tween-20, BSA, and serum (the serum should be from an animal species that is different from the animal species making the unlabeled detection antibody, to avoid non-specific cross reactions; but if the detection antibody is pre-labeled, then the selection of blocking agents has no such limitations), then incubating the section with an detection antibody diluted in PBST with 2.5% serum (the serum should be from an animal species that is different from the animal species making the unlabeled detection antibody, to avoid non-specific cross reactions) 30 min to 1 hour; removing and washing the section with PBS to get rid of unbound labeled or unlabeled detection antibody; adding a dissociating buffer with extreme pH to release the labeled or unlabeled detection antibody bound on the analyte of interest on the fixed tissue on the section in the well of the plate; then transferring the liquid phase containing the dissociated labeled or unlabeled detection antibody to a well of another polystyrene plate (pre-immobilized or un-immobilized with protein G)(second plate or second solid phase) which may pre-contain a neutralizing buffer with opposite pH of the dissociating buffer to make buffer mixture with pH optimal for the detection antibody to be immobilized on the solid phase and be detected in late

steps of the assay; gentle rotating the plate for proper time to immobilize the labeled or unlabeled detection antibody on the well of the second plate; removing the buffer mixture from the well and washing with PBS; detecting the labeled detection antibody directly; or blocking the well with a blocking buffer with Tween-20, BSA, and serum; incubating the well with an labeled secondary antibody (such as antibody-HRP) against the unlabeled detection antibody bound in the well of the second plate in the blocking buffer comprising Tween-20 and BSA; removing unbound labeled secondary antibody (antibody-HRP) and washing the well with PBS; adding TMB substrate solution to the well of the second plate and incubating without light for proper time if the labeled secondary antibody is the antibody-HRP; the signal of the analyte of interest in the tissue fixed on the first plate is determined by detecting the absorbance of the second plate by reading the OD650 nm.

[0114] Similarly, the formalin-fixed and paraffin-embedded tissues also can run cell-based UTSIA. After immobilizing the tissue on a glass slide, and followed by regular procedures of deparaffinizing, rehydrating, analyte retrieval, and blocking, the tissue on the glass slide is then incubating with an detection antibody diluted in PBST with 2.5% serum (the serum should be from an animal species that is different from the animal species making the unlabeled detection antibody, to avoid non-specific cross reactions) 30 min to 1 hour; removing and washing the section with PBS to get rid of unbound labeled or unlabeled detection antibody; adding a dissociating buffer with extreme pH to release the labeled or unlabeled detection antibody bound on the analyte of interest on the tissue immobilized on the glass slide; then transferring the liquid phase containing the dissociated labeled or unlabeled detection antibody to a well of another polystyrene plate (pre-immobilized or un-immobilized with protein G)(second plate or second solid phase) which may pre-contain a neutralizing buffer with opposite pH of the dissociating buffer to make buffer mixture with pH optimal for the detection antibody to be immobilized on the solid phase and be detected in late steps of the assay; gentle rotating the plate for proper time to immobilize the labeled or unlabeled detection antibody on the well of the second plate; removing the buffer mixture from the well and washing with PBS; detecting the labeled detection antibody directly; or blocking the well with a blocking buffer with Tween-20, BSA, and serum; incubating the well with an labeled secondary antibody (such as antibody-HRP) against the unlabeled detection antibody bound in the well of the second plate in the blocking buffer comprising Tween-20 and BSA; removing unbound labeled secondary antibody (antibody-HRP) and washing the well with PBS; adding TMB substrate solution to the well of the second plate and incubating without light for proper time if the labeled secondary antibody is the antibody-HRP; the signal of the analyte of interest on the tissue immobilized on the glass slide is determined by detecting the absorbance of the second plate by reading the OD650 nm.

[0115] All above steps of cell-based UTSIA can be run on room temperature if the analyte fixed and antibodies used are stable in room temperature; you can optimize the protocols by modifying any steps of the protocols or by adding steps or by reducing steps that lead to better sensitivity and specificity to assay your analyte of interest. In summary, the detail steps and the experimental procedures for detection of each analyte of interest should be tested by experiments and referenced with the general guidance in this chapter. The dissociating buffer is 0.2 M Glycine pH 2.5, and neutralizing buffer is 1M Tris/HCl pH 9.0 (the mixture of the dissociating buffer and the neutralizing buffer at volume ratio of 5:1 bring the pH to around 7, the neutral pH); PBS is phosphate buffered saline pH 7.0, PBST is PBS with 0.05% Tween-20; blocking buffer is PBST with 1% BSA (bovine serum albumin), or blocking buffer is PBST with 10% goat serum if the detection antibody is not an antibody from a goat; and TMB solution (Sigma T8665) is the substrate for HRP tagged on an antibody. The first solid phase comprises the polystyrene plate for tissue culture or the polystyrene sheet such as Permanox and Polystyrene Microscope Slides from Electron Microscopy Sciences for tissue immobilization; and the second solid phase comprises the polystyrene plates for ELISA (Nunc Maxisorp or Sigma #6562, high protein binding ELISA plate). The polystyrene sheet can be treated with poly-L-lysine (Electron Microscopy Sciences catalog#19320-A/19320-B) to promote slice of frozen tissue adhesion on the sheet if it is necessary.

[0116] Again, if the level of the analyte in the cells or tissue is very low, such as in pg level, its detection via detection antibody on the detection solid phase can be carried out by the technology of high sensitivity detection system which is disclosed in U.S. Pat. No. 5,731,158. Basically, the HRP-detection antibody or HRP-antibody against the unlabeled detection antibody on the detection solid phase is reacted with H_2O_2 and biotinyl-tyramide to covalently attach many biotin residues on the detection solid phase, and then using streptavidin-HRP to bind with the biotins on the solid phase, and then reacts with HRP substrate TMB to amplify the detection signal.

[0117] After determining the presence and the amount of the analyte of interest on the first plate (first solid phase), it is now critical to get an internal normalization control data from a substance in the tissue or cells immobilized on the first solid phase that the substance is directly correlated with the presence and the amount of the cells immobilized on the first solid phase. The substance comprises proteins, carbohydrates, and DNA/RNA. There are many methods to detect the presence and the amount of these substances, one of the convenient methods is using Bicinchoninic Acid (BCA) protein assay (Stoscheck, C M. 1990) to detect the presence and the amount of the proteins on the cells or tissue immobilized on the first solid phase.

[0118] The detail steps of the BCA assay for cells or tissue immobilized on a solid phase (the solid phase is load in a well of 96 well plate) are described here: the cells/tissue immobilized on a solid phase (96 well tissue culture plate) is washed with PBS to clean the dissociating buffer left, then after removing PBS from the cells or the tissue immobilized, 100 ul/well BCA mixtures (A:B=50:1) (PIERCE #23225 BCA Protein Assay kit) is added to the well immobilized with cells/tissue on the first plate; the plate is incubated at 37° C. for proper time to develop purple product solution which the color density is directly correlated with the presence and the amount of the proteins, and thus correlated with the amount of cells/tissue immobilized on the first solid phase. The color product solution of the BCA Protein Assay on the first plate is detected by measuring absorbance at OD562 nm.

[0119] Now we get the amount of proteins as the internal normalization control and the amount of the analyte of interest. Dividing the amount of the analyte by the amount of the proteins gets a number that can be used to compare with other

data of the analyte got from other samples, if the protein amounts per cell from these samples are comparable.

[0120] Besides above internal normalization control, it may also need a negative control for cell-based UTSIA; this can be done by using a non-specific IgG which is from a same animal species of the labeled or unlabeled detection antibody (IgG) to stain the tissue or cells, then follows above described steps to dissociate, transfer, immobilize the non-specific IgG on a second plate for determination of the non-specific IgG affinity binding (bound on the off targets); this non-specific affinity binding IgG level on the second plate acts as a negative control for the labeled or unlabeled detection antibody that bound on the analyte of interest on tissue or cells that immobilized on a solid phase.

[0121] Cell-based UTSIA is especially useful for cell-based drug discovery, because the method comprises a simple and robust enzyme and cell-based assay, and can be scaled up to medium or high through put by adapting to automation. For lead drug candidate screening, the compound is added in the cells growing on the 96 well plate for proper time, and then removing the treatment medium, and running the cell-based UTSIA as above instructed to detect the analyte of interest. The cells without treatment or only treated with the vehicle is also running cell-based UTSIA as instructed above as a negative control. If there is a significant signal difference between the control and the treatment cells on the analyte of interest, then the compound deserves further validations.

[0122] Similarly, cell-based UTSIA is also useful in animal-based drug discovery {in vivo drug discovery or pharmacodynamic (PD) analysis}, because the method comprises a simple and robust enzyme and tissue-based assay, and has format of tissue-based multiplex analyte detection assay (see paragraph below). For lead drug candidate screening, the compound is dosed to animal for proper time with a proper schedule, then collecting tissue sample of interest, making the formalin fixed tissue sections or frozen tissue sections, then running the cell-based UTSIA as above instructed. The animal without treatment or only treated with the vehicle is also running the cell-based UTSIA as instructed above as a negative control. If there is a significant signal difference between the control and the treatment animals on the analyte of interest, then the compound deserves further validations. If the analyte signal responses well to the drug treatment in the animal experiment, it may be further validated and used as a biomarker for clinical treatment evaluation.

[0123] Cell-based UTSIA also can be used in detection and quantification of multiple clinical biomarkers. For example, a frozen tissue can be used to make multiple tissue slices which were fixed on many round polystyrene supports with defined area, such as the round sections with diameter **4.5**mm; these many tissue sections from a same tissue block can be used to detect multiple analytes (or biomarkers) by using different detection antibodies which are made from a same animal species, and then a common labeled antibody against the detection antibodies is used to detect the detection antibodies by running cell-based UTSIA. This novel detection (quantification) of multiple analytes from a same tissue sample will increase detection efficacy significantly. The detection and quantification of multiple analytes in a same tissue sample by cell-based UTSIA is an advance in microarray or multiplex methods. The detail procedure please refers to above paragraphs for technique detail.

[0124] Similarly, the cell-based UTSIA can be used in multiple analytes determination in cell-based assay. For example, growing cells in a 96 well tissue culture plate (first plate or first solid phase) until the cells are near completely confluent; removing the medium from the wells; washing with PBS; fixing the cell with 4% paraformaldehyde PBS for proper time; removing the paraformaldehyde and washing the fixed cells with PBS; permeabilizing the fixed cells with cold methanol or PBS with 0.5% Triton X-100; blocking the cells with blocking buffer which comprises Tween-20, BSA, and serum; the serum is not from the species from which the detection antibodies was made and the serum is preferred from the species from which the secondary antibody was taken; incubating the plate with different specific detection antibodies that from a same animal species, that cells in each well of the plate is incubated with a specific detection antibody against an analyte of interest in a blocking buffer comprises Tween-20 and BSA; removing the unbound detection antibodies and washing wells of the plate with PBS; adding a dissociating buffer with extreme pH to every well of the plate to release the bound every detection antibody in each well of the plate into the liquid phase of the dissociating buffer in each well; transferring the liquid phase of each well of the first plate to corresponding well of another 96 well polystyrene plate (second plate or second solid phase) which pre-contains a neutralizing buffer with opposite pH of the dissociating buffer to make pH optimal for the detection antibody to be coated on the well of the second plate; gentle rotating the plate to coat the detection antibodies on the wells of the plate; removing the unbound buffer mixture from the wells and washing with PBS; blocking the wells with a blocking buffer with Tween-20, BSA, and serum which will not interfere with the detection of the detection antibody; incubating the wells with an antibody-HRP against the detection antibodies bound in the wells of the plate in the blocking buffer comprising Tween-20 and BSA; removing unbound antibody-HRP and washing the wells with PBS; adding TMB substrate solution to the wells of the second plate and incubating without light for proper time; the signal of the analytes of interest in the cells fixed on the first plate are determined by detecting the absorbance of wells of the second plate by reading the OD650 nm.

[0125] In this post-genomic era, there are many discussions that the future medicine will be a "personalized medicine". The main idea of "personalized medicine" is that the diagnosis, treatment, prognosis, and prevention will all depend on the exact target that causing the disease. That suggests that the detection and quantification of the target related biomarker(s) on cells/tissue will be critical for future medicine. As described above, the cell-based UTSIA has unique property that compliments with current routine IHC and ICC, specifically on the field of quantification, it will has critical roles in current and future medicine.

EXAMPLE OF THE INVENTION

[0126] UTSIA invention prototype example: to detect and quantitate an analyte in a fluid sample (FIG. **2**) (Note: the prototype example's steps of dissociating, transferring, and coating also can be used in cell-based UTSIA to immobilize the detection antibody on the second or detection solid phase for specific detection):

[0127] I. Materials and Agents:

[0128] The capture solid phase (first solid phase) and the detection solid phase are transparent 96 well polystyrene plates for ELISA (Nunc maxisorp plate or Sigma #6562, high protein binding ELISA plate), the adaptor molecule is Protein

G (BioVision #6510-5), dissociating buffer is 0.2 M Glycine pH 2.5, and neutralizing buffer is 1M Tris/HCl pH 9.0, PBS is phosphate buffered saline pH 7.0, PBST is PBS with 0.05% Tween-20, 1st blocking buffer is PBST with 1% BSA (bovine serum albumin), and 2nd blocking buffer is PBST with 10% goat serum, and TMB solution (Sigma T8665) is the substrate for HRP tagged on the detection antibody. The fluid sample is PBS with 1% BSA containing an antigen rabbit IgG (Santa cruz # sc-577) and a background control antigen mouse IgG (Santa cruz # sc-46680). The capture molecule to the antigen rabbit IgG is anti-rabbit IgG from donkey (Chemicon #AP182). The labeled detection molecule is an anti-rabbit IgG-HRP from donkey (Amersham Biosciences # NA934V). The detection molecule to the control antigen mouse IgG is anti-mouse IgG-HRP from sheep (Amersham Biosciences # NA931V).

[0129] II. Procedures:

[0130] **1**. Immobilizing the capture molecule Protein G to the capture solid phase: In the well of 96 well polystyrene plates (first solid phase), 100 μl of (200 ng) Protein G per well in PBS is loaded and incubated for **1** hour at room temperature. Then the unbound fluid is washed away by two time 300 μl/well PBS washing, and then the solid phase is blocked by 1% BSA in PBS 200 ul/well for 15 min.

[0131] 2. Immobilizing a capture molecule to the capture solid phase coated with Protein G: A capture molecule, anti-rabbit IgG from donkey (Chemicon # AP182), is diluted 10 times (1:10 dilution) with 1st blocking buffer, then the diluted capture molecule solution 100 ul/well is incubated in the capture solid phase immobilized with Protein G at room temperature for 30 min, then unbound is removed; then the solid phase is blocked with 10% goat serum PBST for 10 min in room temperature; and then the solid phase is washed by PBST 1 time;

[0132] 3. Capturing an antigen of interest from a fluid sample onto the capture solid phase: a fluid sample 100 μl/well containing an antigen rabbit IgG or containing a control antigen mouse IgG is added to the capture solid phase, and incubated for 30 min at room temperature. The unbound fluid sample is removed and washed by PBS 300 μl/well for 2 times.

[0133] 4. Dissociating the antigen from the capture solid phase into the liquid phase: the dissociating buffer 110 μl/well is added to the capture solid phase immobilized with the antigen and incubated in room temperature for 10 min with gentle rotation to separate the bound antigen from the capture solid phase into the liquid phase of the dissociating buffer.

[0134] 5. Transferring the dissociated antigen and coating it to a detection solid phase: 100 ul dissociating buffer with the dissociated antigen is transferred to the detection solid phase having 20 μl of the neutralizing buffer, then the buffer mixture containing the dissociated antigen is mixed and incubated in the detection solid phase for 30 min at room temperature with gentle rotation to immobilize the antigen onto the detection solid phase. After removing the buffer mixture, the detection solid phase is blocked with 200 μl 2nd blocking buffer each well for 15 min, then the blocking buffer was removed.

[0135] 6. Detecting the antigen on the detection solid phase with a detection antibody against the antigen: a labeled detection antibody (anti-rabbit IgG-HRP) against the antigen rabbit IgG 1:5000 at 100 μl/well in 2 blocking buffer, or a labeled detection antibody (anti-mouse IgG-HRP) against the control antigen mouse IgG 1:5000 at 100 μl/well in 2nd blocking buffer, is incubated with the detection solid phase immobilized with the antigen for 30 min. The unbound is removed by PBST 300 μl/well washing 3 times. Then the TMB solution 100 μl/well is added to the solid phase and is incubated without light for 30 min. The signal of the antigen on the detection solid phase is detected by reading the OD655 nm.

[0136] III. Results (FIG. **2**): The analyte of interest (rabbit IgG), but not control protein mouse IgG, is specifically detected and quantitated by UTSIA in a does-dependent manner.

I claim:

1. An alternative method of sandwich-ELISA for detecting and quantifying an analyte in a fluid sample, the universal tandem solid-phases based immunoassay (UTSIA), which comprises:

(a) an affinity binding solid phase based specific analyte capture process to capture the analyte from the fluid sample via contacting the fluid sample with the affinity binding solid phase;

(b) a dissociation buffer based process to release the analyte captured on the affinity binding solid phase into a liquid phase via incubating the affinity binding solid phase bound with the analyte with the dissociation buffer;

(c) a non-affinity binding solid phase based analyte physical adsorption process to coat the analyte released in the liquid phase of the dissociation buffer on the non-affinity binding solid phase for further specific analyte detection and quantitation via incubating the dissociation buffer containing the analyte released with the non-affinity binding solid phase; and

(d) a specific analyte determining process via incubating the analyte coated on the non-affinity binding solid phase with a labeled detection molecule capable of specific binding with the analyte coated, or with an unlabeled detection molecule capable of specific binding with the analyte coated and with a labeled molecule capable of specific binding with the unlabeled detection molecule bound.

2. The method of claim **1**(a), wherein said affinity binding solid phase comprises (i) a solid phase immobilized with an adaptor molecule, wherein the adaptor molecule immobilized is capable of binding with a capture molecule pre-added in said fluid sample which is capable of specifically binding with said analyte, (ii) a solid phase immobilized with a capture molecule, wherein the capture molecule immobilized on the solid phase is capable of binding with said analyte in said fluid sample specifically, or (iii) a solid phase immobilized with a capture molecule via an adaptor molecule wherein the capture molecule is capable of binding with said analyte in said fluid sample specifically.

3. The method of claim **1**, wherein said solid phase comprises polystyrene support.

4. The method of claim **2**, wherein said adaptor molecule comprises molecules capable of protecting the capture molecule from inactivation by a solid phase and immobilizing the capture molecule on the solid phase.

5. The method of claim **2**, wherein said adaptor molecule comprises Protein G.

6. The method of claim **2**, wherein said capture molecule comprises antibody.

7. The method of claim **1**, wherein said analyte comprises protein.

8. The method of claim **1**, wherein said fluid sample comprises cell lysate, blood, serum, plasma, salvia, urine, spinal fluid, and other biological fluids or fluids made from biological materials.

9. The method of claim **1**(b), wherein said dissociation buffer comprises acidic or basic buffer which is capable of breaking the affinity binding between the capture molecule and the analyte and releasing the analyte into liquid phase of the buffer from solid phase, while the buffer is not capable of releasing the adaptor molecule immobilized on solid phase into the liquid phase, and not capable of blocking the analyte's physical adsorption on non-affinity binding solid phase.

10. The method of claim **1**(d), wherein said labeled means that a molecule is tagged with another molecule which is detectable directly, or indirectly by adding additional components.

11. The method of claim **1**, wherein said detection molecule comprises antibody.

12. The method of claim **1**(d), wherein said labeled molecule comprises labeled antibody.

13. An alternative method of image method for detecting and quantifying an analyte in a tissue or cells immobilized on a solid phase, the cell-based UTSIA, which comprises:

(a) a specific analyte detection process via incubating the tissue or cells immobilized on said solid phase with a labeled detection molecule capable of specific binding with the analyte, or with an unlabeled detection molecule capable of specific binding with the analyte;

(b) a dissociation buffer based process to release the labeled detection molecule or unlabeled detection molecule bound on the analyte in the tissue or cells immobilized on the solid phase into a liquid phase of the dissociation buffer via incubating the solid phase bound with the labeled detection molecule or unlabeled detection molecule with the dissociation buffer;

(c) (1) a non-affinity binding solid phase based physical adsorption process that the labeled detection molecule or unlabeled detection molecule released in the liquid phase of the dissociation buffer is coated on the non-affinity binding solid phase for further specific detection and quantitation, via incubating the liquid phase of the dissociation buffer containing the labeled detection molecule or unlabeled detection molecule released with the non-affinity binding solid phase; or (2) an affinity binding solid phase based labeled detection antibody capture process, in which the solid phase is pre-immobilized with a molecule which is capable of binding and capturing the labeled detection molecule in the dissociation buffer, and the labeled detection molecule released in the liquid phase of the dissociation buffer is immobilized on the affinity binding solid phase for further detection and quantitation, via incubating the liquid phase of the dissociation buffer containing the labeled detection molecule released with the affinity binding solid phase;

(d) a specific analyte determining process via detecting and quantifying the labeled detection molecule on the non-affinity binding solid phase or on the affinity binding solid phase directly or indirectly; or via incubating the unlabeled detection molecule coated on the non-affinity binding solid phase with a labeled molecule capable of specific binding with the unlabeled detection molecule coated and then detecting and quantifying the labeled molecule bound on the non-affinity binding solid phase directly or indirectly; wherein the amount of analyte is correlated with the amount of labeled or unlabeled detection molecule measured.

14. The method of claim **13**(c)(1), wherein said molecule which is capable of binding and capturing the detection molecule in the dissociation buffer comprises protein G.

15. The method of claim **13**, wherein said analyte comprises protein.

16. The method of claim **13**, wherein said solid phase comprises glass and plastic support.

17. The method of claim **13**, wherein said detection molecule comprises antibody.

18. The method of claim **13**(b), wherein said dissociation buffer comprises acidic or basic buffer which is capable of breaking the affinity binding between the labeled detection molecule or unlabeled detection molecule and the analyte, and releasing the labeled detection molecule or unlabeled detection molecule that bound on the analyte in tissue or cells immobilized on solid phase into liquid phase of the buffer, while the buffer is not capable of releasing the tissue or cells immobilized on the solid phase into the liquid phase of the buffer.

19. The method of claim **13**(c), wherein said solid phase comprises hydrophobic solid phase such as polystyrene support.

20. The method of claim **13**(d), wherein said labeled molecule comprises labeled antibody.

21. The method of claim **13**, wherein said labeled means that a molecule is tagged with another molecule which is detectable directly, or indirectly by adding additional components.

* * * * *

patsnap

专利名称(译)	基于通用串联固相的免疫测定		
公开(公告)号	US20100009394A1	公开(公告)日	2010-01-14
申请号	US12/172403	申请日	2008-07-14
[标]申请(专利权)人(译)	郭RIBO		
申请(专利权)人(译)	郭RIBO		
当前申请(专利权)人(译)	郭RIBO		
[标]发明人	GUO RIBO		
发明人	GUO, RIBO		
IPC分类号	G01N33/53		
CPC分类号	G01N33/54306 G01N33/5082		
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外部链接	Espacenet USPTO		

摘要(译)

基于通用串联固相的免疫分析（UTSIA）是用于低丰度抗原测定的夹心ELISA等效分析，其克服了夹心ELISA（固相抗体灭活和对一对一抗和二抗的严格要求）的限制。亲和结合固相以特异性地从流体样品中捕获抗原，顺序解离抗原，将抗原转移和涂覆到非亲和性结合固相以进行特异性抗原测定。基于细胞的UTSIA是基于细胞的ELISA等效测定，其克服了图像方法的限制，其通过解离和转移结合在固定在细胞或组织的组织的抗原上的检测抗体来确定固定在固相上的细胞或组织中的抗原。固相到第二固相并通过检测检测抗体将检测抗体固定在那里以特异性检测抗原。