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(71) Applicant *(for all designated States except US)*: **SEATTLE BIOMEDICAL RESEARCH INSTITUTE** [US/US]; 307 Westlake Avenue North, Suite 500, Seattle, WA 98109 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GRAY, Sean, A.** [US/US]; 957 E. Maple Street, Kent, WA 98030 (US). **WEIGEL, Kris** [US/US]; 503 13th Avenue E., Apt. 206, Seattle, WA 98102 (US). **CANGELOSI, Gerard, A.** [US/US]; 314 NW 72nd Street, Seattle, WA 98117 (US).

(74) Agent: **NOWAK, Thomas, S.**; Christensen O'connor Johnson, Kindness PLLC, 1420 Fifth Avenue, Suite 2800, Seattle, WA 98101 (US).

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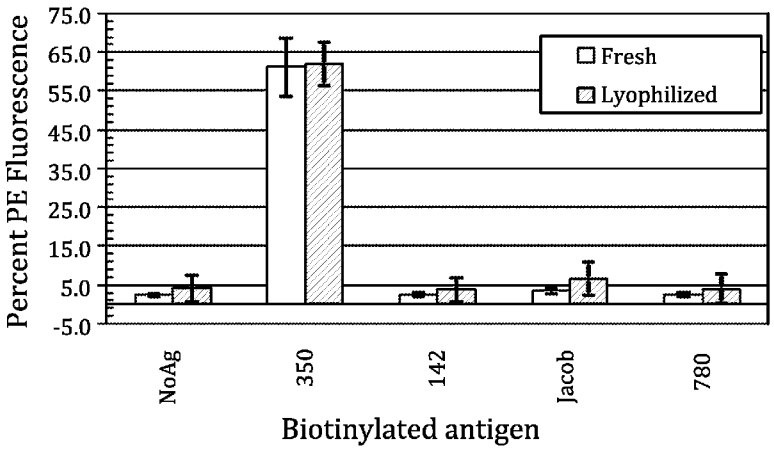


Fig.1A.

(57) Abstract: The present invention relates to bioprobe compositions for selectively binding an antigen of interest in a sample, and related methods and kits. The bioprobe compositions comprise lyophilized particles displaying antigen-binding molecules on the outer surface, which maintain their structural and functional integrity upon lyophilization and subsequent reconstitution.

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BIOPROBE COMPOSITIONS AND THEIR METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/487,903 filed September May 19, 2011, which is expressly incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT LICENSE RIGHTS

This invention was made with Government support under AI0744571 and AI082186 awarded by the United States National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

Affinity reagents that bind to specific antigens of interest are critical tools in biomedical research, biomarker discovery, and diagnostic testing. Generation of monoclonal antibodies (MAbs) by traditional methods, typically by the mouse hybridoma route, is a significant bottleneck in biomedical research and development. MAbs in diagnostic tests often require significant optimization before being usable for diagnostic assays, and licensing costs can exceed all other test costs combined. Cheaper and more available affinity reagents would greatly facilitate biomedical research and empower developers of new diagnostic tests.

Recombinant antibody-like molecules such as single-chain Fragment variable (scFv) and fragment antigen binding (Fab) are potentially appealing alternatives to MAbs. Libraries of these molecules have been displayed on the surfaces of organisms including *Escherichia coli*, phages, yeast, and on ribosomes. Although these methods have existed for many years, few such fragments have proven useful as molecular probes in diagnostic tests. Methods for rapidly selecting antigen-binding yeast-displayed scFv clones were described nearly ten years ago. Yeast display libraries express scFv on the surface of *Saccharomyces cerevisiae* cells. Using a combination of magnetic bead enrichment and fluorescent-activated cell sorting (FACS), yeast clones that bind specifically to antigens can be selected from naïve libraries in 2–3 weeks. This selection process is much faster and less expensive than the mouse hybridoma route. However, scFv selected by this method rarely perform well when secreted into solution. Like natural antibodies,

yeast-displayed scFv are products of selection, in this case for activity in the environment of a yeast cell surface. Hence, many yeast-displayed scFv perform poorly in other environments, especially when secreted. Although functional soluble scFv have been reported, in practice the great majority of scFv culled from yeast display libraries have exhibited unsatisfactory activity in standard immunoassay formats, thus requiring additional rounds of optimization. Therefore, the reliable and efficient production of scFv for use as effective affinity reagents remains unpredictable.

Despite the advances in the art regarding production of affinity reagents, such as monoclonal antibodies and antibody-like molecules, a need remains for fast and cost-effective production of versatile reagents useful for reliable detection of antigens of interest. The invention set forth in this disclosure addresses this need and provides further related advantages.

SUMMARY

This summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This summary is not intended to identify key features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

In one aspect, the present invention provides a bioprobe composition for selectively binding an antigen of interest in a sample. The bioprobe composition comprises a lyophilized bioparticle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the bioparticle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

In some embodiments, the bioparticle is selected from the group consisting of a cell, a cellular organelle, or a virus. In some embodiments, the cell is selected from the group consisting of a yeast, bacterium, plant, or animal cell. In some embodiments, the yeast is selected from the genera *Saccharomyces* or *Pichia*. In some embodiments, the bacterium is selected from the genera *Escherichia* or *Bacillus*. In some embodiments, the cellular organelle is a ribosome. In some embodiments, the the virus is a bacteriophage.

In some embodiments, the heterologous antigen-binding molecule is selected from the group consisting of an antibody-like molecule and a T cell receptor (TCR). In some embodiments, the antibody-like molecule comprises an antigen-binding fragment of an

antibody or T cell receptor. In some embodiments, the antibody-like molecule is a single-chain antibody, a bispecific antibody, an Fab fragment, or an $F(ab)_2$ fragment. In some embodiments, the single-chain antibody is a single-chain variable fragment (scFv), single-chain Fab fragment (scFab), V_HH fragment, V_{NAR}, or nanobody.

In some embodiments, the lyophilized bioparticle is capable of being maintained between about 4 and 40 degrees Celsius before reconstitution of the bioparticle. In some embodiments, the lyophilized bioparticle is capable of being maintained for at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months, or more before reconstitution of the bioparticle.

In some embodiments, the at least one heterologous antigen-binding molecule is displayed on the bioparticle surface prior to lyophilization by inducing expression and translocation of the molecule to, or assembly of the molecule on, the bioparticle surface.

In another aspect, the present invention provides a bioprobe composition for selectively binding an antigen of interest in a sample, comprising a lyophilized particle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the particle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

In another aspect, the present invention provides a method of detecting the presence of an antigen of interest in a biological sample. The method comprises A) contacting a biological sample with a bioprobe composition described herein under conditions sufficient to permit the binding of the bioprobe with an antigen of interest; and B) detecting the binding of the bioprobe to the antigen of interest.

In some embodiments, the method further comprises contacting the biological sample with a detection reagent that binds to the antigen of interest. In some embodiments, the detection reagent comprises a reagent selected from the group consisting of a polyclonal antibody, polyclonal antiserum, a monoclonal antibody, a TCR, a lectin, and a natural binding partner to the antigen. In some embodiments, the method further comprises contacting the biological sample with a detectably-labeled reporter reagent and separating the bioprobe from unbound reporter. In some embodiments, the binding of the antigen of interest to the bioprobe is detected using an antibody sandwich flow cytometric assay, cell bioprobe immunofluorescence microscopy, or an ELISA-like

assay. In some embodiments, the binding of the antigen of interest present in the biological sample to the bioprobe is detected by using a competitive inhibition assay.

In some embodiments, the biological sample is selected from the group consisting of blood, urine, sputum, mucus, saliva, cerebral spinal fluid, tissues, stool, nutrient sources, or processed derivatives thereof.

In another aspect, the present invention provides a method of detecting the presence of an antigen of interest in a biological sample. The method comprises A) contacting a biological sample with a capture reagent that binds to the antigen of interest; and B) contacting the biological sample with a detection reagent under conditions sufficient to permit the binding of the detection reagent with an antigen of interest, wherein the detection reagent comprises a bioprobe composition described herein.

In some embodiments, the detection reagent further comprises a detectable label. In some embodiments, the method further comprises contacting the biological sample with a detectably-labeled reporter agent that specifically binds to the bioprobe composition, and separating the capture reagent from unbound reporter agent.

In another aspect, the present invention provides a method for validating a candidate biomarker as a biomarker of a biological state. The method comprises A) contacting a library comprising a plurality of bioparticles displaying heterologous antigen-binding molecules with a detectably-labeled candidate biomarker of a biological state under conditions sufficient to permit the binding of a displayed heterologous antigen-binding molecule with the labeled candidate biomarker; B) isolating the bioparticles displaying heterologous antigen-binding molecules bound to the candidate biomarker; C) propagating the bioparticles isolated in step (B); D) inducing expression and surface display of the heterologous antigen-binding molecules in the bioparticles propagated in step (C); E) preserving the bioparticles displaying the heterologous antigen-binding molecules induced in step (D); and F) validating the candidate biomarker as a biomarker for the biological state by contacting biological samples obtained from a plurality of individuals exhibiting and not exhibiting the biological state with reconstituted bioparticles displaying the heterologous antigen-binding molecules, the plurality being sufficiently large to statistically associate the candidate biomarker with the biological state.

In another aspect, the present invention provides a kit for selectively binding an antigen of interest in a sample. The kit comprises A) the bioprobe composition in any

one of Claims 1-15; and B) reconstitution buffer. In some embodiments, the kit further comprises detection reagent, detectably-labeled reporter reagent, instructions for use, and/or at least one detectably-labeled antigen of interest as a control.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings.

FIGURE 1 graphically illustrates the pre- and post-lyophilization antigen binding and specificity of yeast-scFv bioprobe clone *350-E2*. Fresh, non-lyophilized *350-E2* yeast and lyophilized, rehydrated *350-E2* yeast were split into 10 aliquots. In Panel A, each aliquot received either no antigen, or 100 nM each of antigens 350, 142, Jacob, or 780. Biotinylated antigens were used in every case, and antigen binding was detected by incubating with SA-PE. Assays illustrated in Panel B were performed as in Panel A, except that non-biotinylated antigens were used and antigen binding was detected by incubation with rabbit-anti-350 antiserum followed by goat-anti-rabbit-phycoerythrin (GaR-PE). The percent of PE-positive yeast was determined by gating the PE fluorescence relative to the "no antigen" control. Values are means of three experiments plus or minus the standard deviation. The clear bars indicate binding of the fresh yeast to each antigen, and the bars with diagonal lines indicate binding of the lyophilized/reconstituted yeast to the antigens.

FIGURE 2 graphically illustrates the lower limit of detection of 350 antigen by lyophilized/reconstituted yeast-scFv bioprobe clone *350-E2*. Lyophilized *350-E2* yeast cells were reconstituted in 1 mL YWB and split into 11 equal aliquots. Each aliquot was incubated with 10 concentrations of 350-biotin antigen ranging from 20 nM to 0.04 nM (plus the 0 nM control) in twofold serial dilutions in 2.5 mL (total volume) YWB. Bound 350 antigen was detected by incubating with a rabbit anti-350 antiserum followed by GaR-PE detection. Samples were analyzed by flow cytometry. The entire experiment was repeated three independent times, and each replicate was performed with an individually grown, induced, and lyophilized *350-E2* clone. Each data point shows the average mean percent PE-positive yeast plus and minus the standard deviation.

FIGURE 3 illustrates the immunofluorescence assay using lyophilized/reconstituted yeast-scFv bioprobe reagents. Panel A is a photomicrograph showing the lyophilized/reconstituted *350-E2* yeast bioprobe binding to cognate 350 antigen at 500 nM concentration. Bound antigen was detected by incubating with a rabbit anti-350 antiserum followed by GaR-FITC detection and photographed at 90 × magnification and digitally zoomed 3× to visualize staining patterns. Panel B graphically illustrates results of an experiment in which 350-biotin was tested in triplicate using 11 concentrations ranging from 160 nM down to 0.14 nM in twofold serial dilutions. For each concentration, three fields were photographed twice, first in visible mode, and then in FITC mode. The percent of FITC-positive yeast were calculated and graphed relative to the concentration of 350 antigen.

FIGURE 4 graphically illustrates the ELISA-like assay using lyophilized/reconstituted yeast-scFv bioprobe reagents. Binding of cognate 350 antigen to lyophilized/reconstituted *350-E2* yeast bioprobes was tested in triplicate in eight concentrations from 100 nM to 0.78125 nM, in addition to no antigen control and to 100 nM each of non-cognate antigens 142, 780, and Jacob. The binding was detected by staining with rabbit anti-350 polyclonal antiserum followed by GaR conjugated to horseradish peroxidase (GaR-HRP), and ascertaining the absorbance spectrophotometrically at 450 nm.

FIGURE 5 graphically illustrates results of a lyophilization time course assay using yeast-scFv bioprobe clone *780-23*. One aliquot of lyophilized yeast was removed at intervals over the course of 86 days and stained first for c-*myc*-FITC expression followed by staining with cognate 780-biotin or three non-cognate antigens (Jacob-biotin, 350-biotin, or 142-biotin, plus a no antigen control). Following detection of antigen binding with SA-PE, as quantified by flow cytometry, the percent of PE-positive yeast were determined using the no-antigen stain as a gating reference. The graph depicts the percent positive yeast (y-axis) at each of the time points tested (x-axis) for each of the four antigens. The assay was terminated when binding to cognate 780-biotin antigen had dropped below 15%.

FIGURE 6 graphically illustrates results of a lyophilization time course assay using yeast-scFv bioprobe clone *350-12*. Three individual colonies of clone *350-12* were lyophilized separately on different days. Every 3 to 5 days, one aliquot of each was tested for binding to cognate antigen (100 nM 350-biotin) and to a non-cognate control

antigen (100 nM 780-biotin) as described for FIGURE 5. The graph depicts the binding to the cognate or non-cognate antigens, as determined by percent of PE-positive yeast in flow cytometric analysis (y-axis), for each of the time periods tested (x-axis). Because of the staggered lyophilization dates, data were grouped into groups of 2 to 3 days, and each point represents the average and standard deviation for the day range indicated in the graph.

DETAILED DESCRIPTION

Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Press, Plainsview, New York (2000) and Ausubel, et al., *Current Protocols in Molecular Biology* (Supplement 47), John Wiley & Sons, New York (1999), which are incorporated herein by reference, for definitions and terms of the art. Additionally, all other references cited herein are hereby expressly incorporated by reference in their entireties.

Additionally, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth. As used in this specification, the term "about" refers to a range of 10% above or below the stated figure.

One solution to problems associated with the generation of monoclonal antibodies and antibody-like molecules for affinity reagents is to forgo the attempt to generate soluble scFv and instead use the yeast-displayed scFv (yeast-scFv) directly as detection reagents. In this strategy, the yeast cell provides a surface on which to present the scFv, which is similar in nature to a plastic or ceramic bead particle that has been coupled to an antibody. An example of this strategy was reported by Cho et al., who used yeast-displayed scFv to immunoprecipitate (IP) brain endothelial proteins from a complex cell lysate (Cho, Y.K., et al., "A Yeast Display Immunoprecipitation Method for Efficient Isolation and Characterization of Antigens," *J Immunol Methods 341*:117–126, 2009). In this method, bound proteins were eluted from the yeast cell surface by mild reduction, and the eluted proteins subjected to mass spectrometry to reveal their identity [this

technique was termed Yeast Display Immunoprecipitation (YDIP) (Cho, Y.K., et al., "A Yeast Display Immunoprecipitation Method for Efficient Isolation and Characterization of Antigens," *J Immunol Methods 341*:117–126, 2009)]. Another example is a competitive-inhibition flow cytometry (CIFC) assay (Gray, S.A., et al., "Flow Cytometry-Based Methods for Assessing Soluble scFv Activities and Detecting Antigens in Solution," *Biotechnol Bioeng 105*:973-981, 2010). CIFC is an indirect assay in which antigen capture is detected and quantified by competitive inhibition of yeast-scFv binding to fluorescently labeled antigen.

However, disadvantages of both YDIP and CIFC include that yeast-scFv probes must be cultured and induced to express scFv before each assay. In addition, YDIP requires mass spectrometry to detect captured antigens, a potentially cumbersome procedure requiring significant laboratory instrumentation. CIFC also has its own limitations, being an indirect format that is susceptible to false-positive results due to non-specific inhibition of fluorescent antigen binding.

The present invention, which overcomes the limitations described above, is based in part upon the inventors' development of novel bioprobe compositions and related assays. As described below, bioprobes were generated incorporating whole yeast cells displaying scFv on their surfaces (yeast-scFv bioprobes). In the assays, the yeast-scFv bioprobes were used in place of soluble purified scFv or traditional monoclonal antibodies. Specifically, in this study, a nonimmune library of human scFv displayed on the surfaces of yeast cells was screened for clones that bind to recombinant cyst proteins of *Entamoeba histolytica*, an enteric pathogen of humans. Additionally, it was surprisingly found that the yeast-scFv clones could be stabilized by lyophilization without loss of the structural and receptor integrity. Thus, the lyophilized yeast-scFv bioprobes were capable of later use in detection assay formats in which the yeast-scFv served as substitutes for solid support-bound monoclonal antibodies. Specific binding of antigen by the reconstituted yeast-scFv bioprobes was detected by staining with rabbit polyclonal antibodies. In flow cytometry-based assays, lyophilized yeast-scFv reagents retained full binding activity and specificity for their cognate antigens after 4 weeks of storage at room temperature in the absence of desiccants or stabilizers. Because flow cytometry is not available to all potential assay users, alternative assays, such as an immunofluorescence assay and an enzyme-based ELISA-like assay, were also successfully developed that

incorporate the yeast-scFv bioprobes to detect antigen with similar sensitivity and specificity as the flow cytometry-based assay.

The successful use the yeast-scFv bioprobes demonstrates that the bioprobes of the present invention are sufficiently stable to maintain structural and functional integrity during the lyophilization/storage/reconstitution process. Additionally, the complex nature of the cell surface and cytoplasm do not interfere in reporter and/or detection reagents, such as polyclonal antibodies and horseradish peroxidase. Moreover, the antigen-specific bioprobe reagents (e.g., yeast-scFv bioprobes) provide the additional advantage of being easily selected from nonimmune libraries in 2–3 weeks, produced in vast quantities, and packaged in lyophilized form for extended shelf life before utilization in various assay applications.

In accordance with the foregoing, in one aspect, the present invention provides a bioprobe composition for selectively binding an antigen of interest in a sample. The bioprobe composition comprises a lyophilized bioparticle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the bioparticle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

As used herein, the term "bioparticle" is used to refer to any particle derived from a biological system while retaining the integrity of heterologous antigen-binding molecules displayed on the surface of the bioparticle. Thus, the term "bioparticle" can encompass intact cells, cellular organelles, viruses, or other biological constructs that can be assembled, propagated, or generated to display on the outer surface at least one heterologous antigen-binding molecule. For any embodiment, there is no requirement that the bioparticle be living or demonstrate metabolic integrity post-lyophilization. However, the bioparticle must be amenable to lyophilization and subsequent reconstitution. This requires sufficient structural integrity such that the bioparticle remains intact, including retention of the functional heterologous antigen-binding molecule(s) displayed on the outer surface of the bioparticle, after lyophilization and subsequent reconstitution. In this context, the term "functional" is used to indicate that the displayed heterologous antigen-binding molecule(s) retain the capacity to selectively bind the antigen of interest.

In some embodiments, the bioparticle is a cell. As will be apparent to persons of ordinary skill in the art, the bioparticle can be any cell that can be made to express and/or

display a heterologous antigen-binding molecule on its surface and that can retain its structural integrity upon lyophilization and subsequent reconstitution. A determination of the cell's integrity after reconstitution can be made with any number of simple assays. Such assays can include simple observation using microscopy or flow cytometry in combination with the binding of labeled antigen, as described herein (see e.g., Example 5). In some embodiments, the cell is a microbial cell, such as a yeast or bacterial cell. In alternative embodiments, the cell is a plant or animal cell. In some embodiments, the cells that contain rigid cell walls are selected for use as bioparticles because the rigid cell wall can enhance the retention of integrity after the lyophilization and subsequent reconstitution processes.

In some embodiments, the bioparticle cell is a yeast cell. Exemplary yeasts include yeast selected from the genera *Saccharomyces* or *Pichia*, for example, *Saccharomyces cerivisiae* or *Pichia pastoralis*. For example, as illustrated in the description below, yeast bioprobes were generated by screening libraries of *Saccharomyces cerivisiae* displaying human-derived single-chain variable antibody fragments (scFv) on their surface. However, persons of skill in the art will recognize that the present compositions are not limited to such embodiments, but rather encompass any yeast amenable to display of heterologous antigen-binding molecule on its surface using common recombinant DNA techniques. See, e.g., Sambrook, et al. (2000) or Ausubel, et al. (1999).

In other embodiments, the bioparticle cell is a bacterium. The bacterium can be Gram positive or Gram negative. Exemplary bacteria are selected from the genera *Bacillus* (Gram positive) and *Escherichia* (Gram negative). Specific examples include *Bacillus megaterium* and *Escherichia coli*. Transgenic bacteria have been used to display library of heterologous polypeptides, including scFv antibodies, which are retained on the surface for subsequent cell sorting. See, e.g., Georgiou, G., et al., "Display of Heterologous Proteins on the Surface of Microorganisms: From the Screening of Combinatorial Libraries to Live Recombinant Vaccines," *Nat. Biotechnol. 15*:29-34, 1997. As above, persons of skill in the art will recognize that the present compositions are not limited to such embodiments, but rather encompass any bacterium amenable to display of heterologous antigen-binding molecule on its surface using common recombinant DNA techniques. See, e.g., Sambrook, et al. (2000) or Ausubel, et al. (1999).

In yet other embodiments, the bioparticle is a non-microbial cell, such as an animal cell or a plant cell. For example, scFvs have been displayed and isolated from human embryonic kidney 293T (HEK-293T) cells (see, e.g., Ho, M. et al., "Isolation of Anti-CD22 Fv With High Affinity by Fv Display on Human Cells," *Proc. Natl. Acad. Sci. USA 103*:9637–9642, 2006.

In other embodiments, the bioparticle is a cellular organelle. An exemplary organelle is the ribosome, which has been previously used to display large repertoires of antibodies and antibody-like molecules. See, e.g., He, M. and Khan, F., "Ribosome Display: Next-Generation Display Technologies of Antibodies *In Vitro*," *Expert Rev. Proteomics 2*:421-430, 2005.

In other embodiments, the bioparticle is a virus. An illustrative example is a bacteriophage. Use of bacteriophages to display libraries of exogenous polypeptides has been established. For example, using filamentous M13-derived bacteriophages, many display libraries have been made by expressing the exogenous polypeptides as fusions with the bacteriophage coat protein pIII. As the virus is assembled within the host bacterium, the fusion protein is transported to the bacterial periplasm and incorporated into the phage particle. Additional M13 filamentous proteins that have been utilized for fusion with the exogenous polypeptides include pVI, pVII, pVIII, and pIX. In the context of antibody-like polypeptides, the result is a library of diverse antibody-like polypeptides with intact binding properties linked to the DNA that encodes them, which enables the subsequent selection and propagation of polypeptides exhibiting particular binding properties.

As used herein, the term "heterologous antigen-binding molecule" is used to refer to any molecule that binds to an antigen. The term "heterologous" specifically refers to the characteristic that the antigen-binding molecule is not naturally occurring in or on the bioparticle, but rather is caused to be expressed and/or displayed on the bioparticle by experimental manipulation. In preferred embodiments, the heterologous antigen-binding molecule selectively binds to its cognate antigen (e.g., the antigen of interest) as compared to a non-cognate antigen. The term "selectively binds" is used to refer to the enhanced affinity and avidity binding characteristics of the heterologous antigen-binding molecule for the cognate antigen as compared to the non-cognate antigen. For example, in some embodiments, the heterologous antigen-binding molecule can bind to its cognate antigen with 5%, 10%, 25%, 50%, 75% or greater efficiency as compared to any non-

cognate antigen. In preferred embodiments, the heterologous antigen-binding molecule can bind to its cognate antigen with an efficiency that is more than 2, 5, 10, 20 times or more as compared to binding to its non-cognate antigen.

In some embodiments, the heterologous antigen-binding molecule is an antibody-like molecule. As used herein, the term "antibody-like molecule" encompasses antibodies or fragments thereof, derived from any antibody-producing animal (e.g., fish, reptiles, birds, and mammals, including mice, rats, rabbits, camelids, and primates, including human). Unless otherwise stated, exemplary antibody-like molecule can include antibodies such as monoclonal, multispecific antibodies (e.g., bispecific antibodies), chimeric antibodies, anti-idiotype antibodies, and may be any intact molecule or fragment thereof, and of any isotype.

As used herein, the term "antibody fragment" refers to a portion derived from or related to a full-length antibody, generally including the antigen-binding or variable region thereof. Illustrative examples of antibody fragments include Fab, Fab', $F(ab)_2$, $F(ab')_2$ and Fv fragments, scFv fragments, diabodies, linear antibodies, single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

As used herein, the term "single-chain antibody" refers to an antibody fragment that contains at least one antigen-binding region in a single polypeptide molecule. For example, as used herein, the term "single-chain Fv" or "scFv" specifically refers to an antibody fragment that comprises the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Additionally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains, which enables the scFv to form the desired structure for antigen binding. An scFv can also be generated to be multivalent, namely to contain multiple pairings of V_H and V_L domains in a single polypeptide chain, where each pairing can bind to the same or different antigen.

Other exemplary single-chain antibodies include single-chain Fab fragments (scFab) and nanobodies. The term single-chain Fab fragments (scFab) refers to an antibody fragment that comprises a single fragment antigen-binding region (Fab) of an antibody in a single polypeptide chain, typically with a peptide linker between the H and L chain fragments. The term "nanobody" refers to antibody fragment that consists of a single polypeptide monomeric variable antibody domain that is able to specifically bind antigen. Nanobodies can include V_HH fragments, which refers to fragments of heavy

chain antibodies from camelids, and V_{NAR} fragments, which refers to fragments of heavy-chain antibodies derived from cartilaginous fish.

In other embodiments, the heterologous antigen-binding molecule is a T-cell receptor. Like antibodies, T-cell receptors (TCR) are members of the immunoglobulin superfamily of proteins and function to recognize and/or bind to antigens. In vertebrate immune system, TCRs recognize antigens presented on the MHC. Native TCRs exist in αβ and γδ dimeric forms, which are structurally similar. When dimerized, the αβ and γδ forms appear very similar to an Fab fragment of a typical antibody. Accordingly, in the present embodiments, the heterologous antigen-binding molecule comprises antigen-binding portions of a TCR. As with antibodies, the antigen-binding portions of a TCR include engineered single-chain TCRs and TCR fragments comprising the variable regions of α, β, γ and δ chains, alone or paired in various combinations. Display of TCRs in single-chain and dimeric form on ribosomes, bacteriophage, cell, or proteinaceous particle is described in International Publication No. WO 2004/044004.

The bioparticles of the present invention display at least one heterologous antigen-binding molecule on their surface. As used herein, the term "display" refers to the position of the antigen-binding molecules in the environment immediately beyond the outer surface of the bioparticle. As such, the antigen-binding domains of the molecules are in communication with the surrounding environment and are thus capable of coming into physical contact with and binding their cognate antigens. However, as implied by the term "display," the antigen-binding molecules remain anchored or connected to the bioparticle. In some embodiments, the heterologous antigen-binding protein is a fusion with a domain of a protein that is endogenous to the bioparticle and that spans the outer surface (e.g., plasma membrane, cell wall, capsid, and the like) of the bioparticle. Such domains are known in the art for various bioparticles. For example, as described above, the display of exogenous proteins on M13 filamentous bacteriophages can utilize fusions with coat proteins pIII, pVI, pVII, pVIII, and pIX. Additionally, as described below, libraries of yeast cells displaying human scFv utilized Aga1-Aga2 to tether the human scFv to the yeast cell walls.

As described herein, the inventors generated bioprobes comprising bioparticles with the surprising property of being capable of lyophilization and subsequent reconstitution, wherein the reconstituted bioparticles retained the display of functional antigen-binding molecules for specific binding to the antigen of interest. Lyophilization

can be performed according to any of various well-known lyophilization protocols. In some embodiments, the lyophilization protocol simply involves flash freezing of the bioprobes, followed by drying in a suitable lyophilization apparatus. In additional embodiments, a lyophilization reagent can be used in which the bioprobes are first immersed and/or suspended. Suitable lyophilization reagents include, but are not limited to, phosphate-buffered saline or specialized reagents such as 2X Concentrate Lyophilization Reagent (OPS Diagnostic, LLC). In some embodiments, a freeze-drying indicator reagent may also be used, such as, for example, the Freeze Drying Indicator from OPS Diagnostic, which includes a colorimetric reagent that turns from red in the unlyophilized state to blue in the lyophilized state. As described below in Example 4, yeast bioprobes displaying human scFv were lyophilized by concentrating the yeast bioprobes by centrifugation, flash-freezing the concentrated yeast in liquid nitrogen for approximately 15 seconds, followed by application of a vacuum overnight in a Millrock Technology Model BT48A lyophilizer.

The lyophilization process permits the prolonged storage of the bioprobes in a preserved state that does not require refrigeration or other cumbersome storage conditions. Accordingly, the present invention provides bioprobe compositions wherein the lyophilized bioparticle is capable of being maintained in the preserved state for a prolonged period of time, such as 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 365 days, or more, or any time period therein. Stated otherwise, the lyophilized bioparticle is capable of being maintained in the preserved state for at least about 1 month to a year or more, including any time within that range. As used herein, the term "maintained" is used to refer to the preservation or retention of the physical characteristics of the bioparticle while in the preserved state, including the structural integrity of the bioparticle and retention of the functional heterologous antigen-binding molecule(s) displayed on the outer surface.

The present invention also provides bioprobe compositions wherein the lyophilized bioparticle is capable of being maintained in the preserved state under a wide range of storage temperatures ranging between below freezing and above 50° Celsius, such as between about 4°C and about 40 C, or any range therein. Preferably, to maximize stability (i.e., retention of desired binding characteristics), the lyophilized bioparticle is maintained within about 10°C of room temperature, which is typically considered to be

between 20°C and 25°C. More preferably, the lyophilized bioparticle is maintained within about 9°C, 8°C, 7°C, 6°C, 5°C, 4°C, and 3°C of room temperature.

As used herein, the term "reconstitution" refers to the conversion of the bioparticle from a preserved state to an active state wherein the bioparticle is capable of interacting with components of a biological sample and specifically binding to its cognate antigen. In some embodiments, reconstitution of the bioparticle involves contacting the lyophilized bioparticle with an appropriate buffer according to protocols known in the art. In some embodiments, buffers useful for reconstitution comprise PBS and/or BSA. For example, in an illustrative embodiment, the reconstitution buffer for a small aliquot of lyophilized yeast bioprobes is 100 µL of PBS + 0.5% BSA (PBSB). In other embodiments, the reconstitution can be accomplished primarily by rehydration effected by the biological sample itself, if the biological sample contains sufficient liquid. In some embodiments, the buffer contains additional components, such as protease inhibitors, to assist preservation of the antigen of interest and the heterologous antigen-binding molecule.

As used herein, the term "antigen of interest" refers to any antigen that serves as a cognate antigen to the heterologous antigen-binding molecule. It will be apparent to persons of ordinary skill in the art that there is no limitation on the character, type, or source of antigen in the practice of the present invention. For example, embodiments of antigens can include toxins, biomarkers for microbial or parasitic infection, biomarkers for the presence of transformed cells, such as cancer cells, biomarkers for a biological state, such as a disease state, and endogenous biomarkers indicating a particular genotype or phenotype. Antigens can be peptides, polypeptides, and non-protein antigens. In some embodiments, the antigens are non-protein small molecule compounds, such as drugs, metabolites, environmental pollutants, and the like.

The bioprobe compositions of the present invention, such as lyophilized yeast-scFv, provide several advantages over the affinity reagents currently available. By virtue of having the heterologous antigen-binding protein displayed on the surface of the lyophilized bioparticles, there is no need for further induction or assembly cycles after reconstitution. No additional culture or growth media (and related equipment) is required, adding to the efficiency and cost savings. Thus, in contrast to the labor and time intensive procedures required to generate and store monoclonal antibodies or soluble antibody fragments, the generation of bioprobes of the present invention permits

production of large quantities (e.g., hundreds to thousands) of aliquots in a short time period at a cost of cents per aliquot. Furthermore, the lyophilization of the bioparticles permits easy and long-term storage of the bioprobes of the present invention. Therefore, shipping and storage do not require expensive maintenance of low temperatures or use of additional chemicals. Finally, the bioprobes are amenable for use as affinity reagents in locations that lack growing or storage facilities in simple, yet sensitive assays that do not require sophisticated equipment.

In another aspect, the present invention provides a bioprobe composition for selectively binding an antigen of interest in a sample, which comprises a lyophilized particle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the bioparticle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

As used herein, the term "particle" refers to the same elements as a bio-particle. However, in addition to any particle derived from a biological system, the term particle also refers to particles that are not derived from a biological system, but rather are commonly used in the art to interact with biological systems. Such particles include inorganic microspheres, microparticles, nanoparticles, beads, and the like, to which antigen-binding molecules can be attached. The antigen-binding molecules can include the same embodiments described above. However, instead of utilizing the expression of fusion protein incorporating trans-surface anchoring domains, embodiments provided by this aspect are attached according to routine protocols known in the art. Illustrative examples include, but are not limited to, homo- and hetero-bifunctional crosslinking chemistries (e.g. carbodiimide, *N*-hydroxysuccinimide ester, maleimide, etc.), and biotin/streptavidin coupling.

In another aspect, the described bioprobe compositions can be employed in methods for detecting and/or selectively binding an antigen of interest in a sample. In some embodiments, the bioprobe compositions can be used as a capture reagent that selectively binds to the antigen of interest (i.e., molecules comprising the cognate antigen for the displayed heterologous antigen-binding protein) and does not bind the other non-cognate antigens present in the sample. Thus, any antigen of interest that is bound to the bioprobe can be selectively removed from the other components of the sample. Detection of binding can be accomplished according to various assay formats known in the art.

Illustrative examples are described below. Accordingly, in one embodiment of this method, the method comprises contacting a sample with a bioprobe composition as described herein under conditions sufficient to permit the binding of the bioprobe (via the heterologous antigen-binding protein) to the antigen of interest, and detecting the binding of the bioprobe to the antigen of interest.

In some embodiments, the method further comprises contacting the sample with a detection reagent that also binds to the antigen of interest. While it is preferred that the detection reagent specifically binds to the antigen of interest, it is not critical that it demonstrates a high degree of specificity because the specificity is provided in the assay by the bioprobe composition. However, it will be apparent that the detection reagent should not compete with the binding of the bioprobe composition to the antigen of interest. Nonlimiting examples of the detection reagent include polyclonal antibodies (e.g., as included in a polyclonal antiserum), monoclonal antibodies, TCRs, lectin, and any known natural binding partner to the antigen.

In a further embodiment, the detection reagent is detectably labeled. Detectable labels can include, but are not limited to, functional enzymes, chemiluminescent molecules, fluorescent molecules, phosphorescent molecules, radioactive labels, spin labels, redox labels, and the like, specific examples of which are well known in the art.

In some embodiments, the sample is also contacted with a reporter reagent. The reporter reagent performs as a secondary detection reagent, wherein the reporter reagent binds to a component common to the (primary) detection reagents. For example, as described below, a goat anti-rabbit antibody was used to bind to and detect the polyclonal rabbit antibodies (in serum), which was bound to the antigen of interest, which in turn was bound to the yeast bioprobes. In these embodiments, the detection reagent is not detectably labeled, whereas the reporter reagent that binds to the detection agent is detectably labeled.

In preferred embodiments, the method contains a step wherein the bioprobe/antigen/detection reagent complex is separated from any unbound detectable label. In a standard ELISA, the complex is typically immobilized on a solid substrate, such as the bottom of a well. The unbound reagent with a detectable label is removed by one or more rinse procedures. In some methods of the present invention, no immobilization is required by virtue of the size of the bioprobe, which facilitates the separation of the labeled bioprobe/antigen complex from unbound detectably-labeled

reagent and/or unbound antigen. In other methods, the bioprobe can be immobilized to solid surfaces such as a microwell plates. Simple wash protocols can be used. In some embodiments, centrifugation can be employed to generate pellets followed by resuspension in a wash buffer. This can be performed once or in a series to selectively isolate the labeled bioprobe/antigen complex from unbound detectably label reagent and/or unbound antigen.

In some embodiments, the labeled bioprobe/antigen complex is separated from unbound detection reagent, followed by detection and/or quantification by use of various assays known in the art. As described below, such as in Examples 3 and 5, an antibody sandwich flow cytometric assay (ASFC) is used to quantify antigen binding by the bioprobe. In another embodiment, the labeled bioprobe/antigen complex is detected and/or quantified by use of an ELISA-like enzymatic assay, which does not necessarily require immobilization of the antigen/capture reagent complex. The labeled bioprobe/antigen complex is quantified by use of a detection reagent bound to an enzyme with detectable activity, such as horseradish peroxidase (HRP). Upon separation of the labeled bioprobe/antigen complex from unbound detection reagent, the activity of the enzyme is quantified by virtue of a characteristic of the product of enzyme activity, such as a color change, or light absorbance at a specific wavelength. A representative ELISA-like assay using HRP is described in more detail in Example 8. In another embodiment, an immunofluorescence microscopy assay is used to detect and quantify the presence of labeled bioprobe/antigen complex, such as described in more detail in Example 7.

In yet another embodiment, binding of the bioprobe to the antigen complex is detected and quantified without the use of a labeled reporter, but rather is detected and quantified using a competitive inhibition assay. In a representative example, the assay comprises further contacting the sample with a detectably-labeled antigen of interest, wherein binding of the bioprobe to any unlabeled antigen of interest from the sample blocks binding of the bioprobe to the detectably-labeled antigen of interest. By virtue of the competitive binding with the bioprobe, binding of the antigen of interest results in a detectable reduction of labeled antigen of interest bound to the bioprobe compared to a control sample not containing any antigen of interest. See, e.g., Gray, S.A., et al., *Biotechnol Bioeng 105*:973–981 (2010).

The foregoing presentation of the methods of the present invention is generally in the context of the bioprobe serving as a capture reagent. However, the invention is not

limited to this aspect. Persons of ordinary skill in the art will recognize that alternative capture reagents can be used that incorporate antigen-specific binding molecules. Non-limiting examples include magnetic beads conjugated to polyclonal antibodies (antiserum), immobilized antibodies, antibody-like molecules, or fragments thereof, lectins, TCRs, or any naturally occurring binding partner. In these embodiments, the bioprobe compositions of the present invention can thus be used as a detection reagent. Detection/quantification of binding of the bioprobe composition to the captured antibody can be performed by incorporating any well-known detectable label, or by use of a detectably-labeled secondary detection reagent specific for a component of the bioprobe composition.

In some embodiments, the sample is a biological sample. Non-limiting examples of biological samples include blood, urine, sputum, mucus, saliva, cerebral spinal fluid, tissues, stool, or processed derivatives thereof. Use of such samples can facilitate the use of the methods of the present invention for purposes of detecting particular biological states, the presence of pathogens, parasites, or to determine particular phenotypes, depending on the antigen of interest. In additional embodiments, the sample is an environmental sample, such as a water sample. In additional embodiments, the sample is from a nutrient source, such as from the food or potable water supply, for example, milk, juice, meat, crop samples, animal feed, and the like, or any processed derivatives thereof. The methods of the present invention can be applied to the monitoring of these samples to monitor for toxins and contaminants, or, for example, in efforts to monitor against agents used in acts of bioterrorism.

Biomarker discovery is the search for novel molecular indicators of biological state. For example, proteomic analysis using tandem mass spectrometry can be used to identify proteins that are found in the sera of people with a specific disease, but not in the sera of healthy people. In this process, analysis of a small set of diseased and healthy samples can identify scores or hundreds of candidate markers. These must be validated by analysis of much larger sample sets. As described herein, validation steps typically begin with the generation of antibodies in animals immunized with recombinant candidate biomarkers as antigens. The antibodies are then used in formats such as dot blot or ELISA to test large sample sets for antigen occurrence. Raising antibodies in animals is costly and requires months to complete. As a result, candidate markers must be carefully prioritized for validation, and many promising candidates are never pursued.

Overall, validation is widely considered to be the most significant bottleneck in biomarker discovery. The present invention streamlines this step by providing for very rapid generation of specific binding reagents. In so doing, it also makes it possible to screen larger numbers of candidate markers, thereby increasing the probability of a successful biomarker search.

Accordingly, the present invention provides a method for validating a candidate biomarker as a biomarker for a biological state. The method comprises contacting a library comprising a plurality of bioparticles displaying heterologous antigen-binding molecules with a detectably-labeled candidate biomarker of a biological state under conditions sufficient to permit the binding of a displayed heterologous antigen-binding molecule with the labeled candidate biomarker. In preferred embodiments, each bioparticle in the library displays a clonal population of the same antigen-binding molecule. Thus, each bioparticle specifically binds to a single cognate antigen, although the antigen-binding molecules preferably vary from bioparticle to bioparticle such that the library can be used for a wide variety of candidate antigens/biomarkers.

In additional steps, the bioparticles displaying heterologous antigen-binding molecules bound to the candidate biomarker are detected, isolated, and propagated. In some embodiments, the now clonal (or near clonal) population of bioparticles can be induced to express and/or display the heterologous antigen-binding molecule on its surface. The bioparticles displaying the heterologous antigen-binding molecule are then preserved, preferably by lyophilization. The preserved bioparticles can be stored or distributed for use in validation studies from a plurality of sources to statistically ascertain whether the candidate biomarker is associated with the biological state.

As will be apparent, the bioparticle embodiments of this aspect must be capable of propagation. Illustrative examples are cells and viruses. However, as described above, once the bioparticles are isolated, propagated, and induced to display the heterologous antigen-binding molecule, they can be preserved without regard to their viability after reconstitution. As explained above, the necessary feature is that the preserved bioparticles must retain structural integrity, including the display of functional heterologous antigen-binding molecules.

Any biological state can be assessed for biomarkers, such as the presence or absence of disease, pathogen, macroparasite, or toxin, the stage of disease or infection,

the receptivity to particular treatments, or susceptibility/sensitivity to a disease, and the like.

An example of a library useful in the practice of this method is a yeast library displaying human-derived scFv, as described below (see, e.g., Examples 2 and 3). Other well-known examples include *E. coli*, bacteriophage, and ribosome display libraries, as described herein.

In another aspect, the present invention provides a kit for selectively binding an antigen of interest in a sample to permit the use of the compositions and methods described above. In one embodiment, the kit comprises the bioprobe compositions as described above and a reconstitution buffer. The kit can be modified to also include detection reagents that specifically bind to the same antigen of interest as the bioprobe composition, albeit non-competitively. As described, the detection reagent can contain a detectable label. Alternatively, the kit can also include a reporter reagent that serves as a secondary detection reagent. In some embodiments, the kit contains detectably-labeled antigen of interest for use as control. In some embodiments, the kit contains printed instructions for use.

In one illustrative embodiment, the kit of the present invention comprises at least one aliquot of the bioprobe in a tube-like device. In a further embodiment, the tube-like device is separated into at least two chambers by a filter or permeable membrane. One chamber contains the lyophilized bioprobe, whereas a second chamber contains reconstitution buffer. The reconstitution buffer can optionally comprise additional reagents, such as protease inhibitors. Upon use, the reconstitution buffer is mixed or contacted with the sample (such as feces from a fecal swab). The buffer and sample are mixed and allowed to contact the lyophilized bioprobe. This can be performed by centrifugation, which in this embodiment would permit the flow of antigen and reconstitution buffer to the chamber with the bioprobe composition, but would prevent the flow of macro particulates.

The following is a description of a representative method to generate bioprobe compositions capable of easy storage and extended shelf life, and methods of their use in selectively detecting cognate antigens, also described in Gray, S.A., "Toward low-cost affinity reagents: lyophilized yeast-scFv probes specific for pathogen antigens," *PLoS ONE 7(2)*:e32042, 2012, hereby expressly incorporated by reference in its entirety.

The generation of affinity reagents, usually monoclonal antibodies, remains a critical bottleneck in biomedical research and diagnostic test development. Recombinant antibody-like proteins such as scFv have yet to replace traditional monoclonal antibodies in antigen detection applications, in large part because of poor performance of scFv in solution.

The present study sought to overcome the various limitations inherent in antibody and antibody fragment production to yield practical and direct yeast-scFv bioprobe assay formats. Induced yeast-scFv bioprobes were lyophilized to yield stable reagents that did not require cultivation or special storage conditions, and were immediately usable following rehydration. In addition, simple direct assay formats were developed that used yeast-scFv reagents in combination with basic laboratory resources.

To evaluate these methods, yeast-scFv probes were isolated that bound specifically to putative cyst proteins of the parasite *Entamoeba histolytica*, the causative agent of intestinal amoebiasis in developing countries (Haque, R., et al., "Epidemiologic and Clinical Characteristics of Acute Diarrhea With Emphasis on *Entamoeba histolytica* Infections in Preschool Children in an Urban Slum of Dhaka, Bangladesh," *Am J Trop Med Hyg 69*:398–405, 2003; Petri, W.A., Jr., et al., "Estimating the Impact of Amebiasis on Health," *Parasitol Today 16*:320–321, 2000). These proteins were previously shown by gene expression profiling analyses to be up-regulated in *E. histolytica* cysts (Ehrenkaufer, G.M., et al., "Identification of Developmentally Regulated Genes in *Entamoeba histolytica*: Insights Into Mechanisms of Stage Conversion in a Protozoan Parasite," *Cell Microbiol 9*:1426–1444, 2007), thereby making them candidate stool biomarkers of *E. histolytica* infection. The study demonstrates the utility of a new type of renewable affinity reagent that can be selected from nonimmune yeast display libraries in 2–3 weeks, produced in vast quantities by yeast culture, and packaged in lyophilized form for extended shelf life and ease of distribution and use.

Library Selections

Four *E. histolytica* proteins were selected for this study on the basis of their elevated mRNA expression in the cyst stage relative to the trophozoite stage of the parasite (Ehrenkaufer, G.M., et al., "Identification of Developmentally Regulated Genes in *Entamoeba histolytica*: Insights Into Mechanisms of Stage Conversion in a Protozoan Parasite," *Cell Microbiol 9*:1426–1444, 2007). Fragments of each protein were selected

and cloned to minimize sequence overlap with homologs from the commensal species *E. dispar* and *E. moshkovskii*. These species are closely related to *E. histolytica* and are often found in stool samples, but are not pathogenic to humans (Haque, R., et al., "Rapid Diagnosis of *Entamoeba* Infection by Using *Entamoeba* and *Entamoeba histolytica* Stool Antigen Detection Kits," *J Clin Microbiol 33*:2558–2561, 1995; Haque, R., and W.A. Petri, Jr., "Diagnosis of amebiasis in Bangladesh," *Arch Med Res 37*:273–276, 2006; Haque, R., et al., "Diagnosis of Amebic Liver Abscess and Amebic Colitis by Detection of *Entamoeba histolytica* DNA in Blood, Urine, and Saliva by a Real-Time PCR Assay," *J Clin Microbiol 48*:2798–2801, 2010). The protein fragments, which ranged in size from 12 to 34 kDa, were cloned for expression in *E. coli* (Table 1). One of the proteins, *Eh*Jacob1, is expressed at approximately 1290 fold higher mRNA level in the cyst form relative to the trophozoite form of the parasite (Ehrenkaufer, G.M., et al., "Identification of Developmentally Regulated Genes in *Entamoeba histolytica*: Insights Into Mechanisms of Stage Conversion in a Protozoan Parasite," *Cell Microbiol 9*:1426–1444, 2007). In early encysting *E. invadens* cells (a model parasite for studying encystation in *E. histolytica*), Jacob proteins appear on the surface of encysting parasite and are abundant in the walls of mature cysts (Chatterjee, A., et al., "Evidence For a "Wattle and Daub" Model of the Cyst Wall of *Entamoeba*," *PLoS Pathog* 5:e1000498, 2009; Ghosh, S.K., et al., "The Jacob2 Lectin of the *Entamoeba histolytica* Cyst Wall Binds Chitin and Is Polymorphic," *PLoS Negl Trop Dis 4*:e750, 2010). Three chromodomain-containing proteins (EHI_142000, EHI_115350 and EHI_000780; hereafter termed 142, 350, and 780, respectively) were also selected because chromodomain proteins fulfill stage-specific functions in other systems (Perez-Toledo, K., et al., "*Plasmodium falciparum* Heterochromatin Protein 1 Binds to Tri-Methylated Histone 3 Lysine 9 and Is Linked to Mutually Exclusive Expression of Var Genes," *Nucleic Acids Res 37*:2596–2606, 2009; Smith, T.J., et al., "Sequence Variation Within *Botulinum* Neurotoxin Serotypes Impacts Antibody Binding and Neutralization," *Infect Immun 73*:5450–5457, 2005). Additionally, 780 was detected in 2 out of 5 *E. histolytica* cyst samples that were subjected to shotgun proteomic analysis.

Table 1. *E. histolytica* proteins used in this study.

Protein	EHI #	NCBI Accession	Total AA[1]	AA Expressed	MW (kDa)[2]	Annotation	Yeast-scFv probe
Jacob	EHI_044500	XP_657246	574	323	34	Cyst wall-specific glycoprotein Jacob[1]	*Jacob-A11*
780	EHI_000780	XP_653178	1641	148	19	Chromodomain-helicase-DNA-binding protein	*780-23*
350	EHI_115350	XP_649984	1247	138	17	Chromodomain-helicase-DNA-binding protein	*350-E2*, *350-12*
142	EHI_142000	XP_654629	414	117	12	Histone acetyltransferase	none

[1]AA, number of amino acids.

[2]Calculated molecular weight of expressed protein fragments.

The Wittrup library of human nonimmune scFv antibodies displayed on the surface of yeast was used to isolate scFv that bind to *E. histolytica* proteins. Following magnetic and FACS selections, sorted clones were tested for binding to cognate antigen as well as for specificity. To determine specificity, antigen-binding clones were tested for non-binding to a minimum of four additional *E. histolytica*-derived non-cognate antigens.

A total of 48 selected clones were tested to confirm binding to a biotinylated 350 fragment (350-biotin). Of these, 30 clones specifically bound the antigen. However, BstN1 fingerprint analysis followed by DNA sequencing revealed that only two clones, designated *350-12* and *350-E2*, were isolated. Clone *350-E2* was isolated more than 20 times from multiple sorts with this antigen. Further characterization of these clones revealed that *350-12* bound only biotinylated-350, and failed to bind unlabeled 350, while clone *350-E2* bound both labeled and unlabeled 350.

For the 780 antigen, eight unique clones were isolated from 48 selected clones. All eight of these clones bound biotinylated-780, and none of them bound unlabeled 780 antigen. Biotinylation may have altered antigen structure such that unlabeled antigen was no longer recognized. One of the unique clones, designated *780-23*, bound the 780-biotin antigen at a moderately high affinity of ~1 nM as determined by using a flow cytometric assay described previously (Gray, S.A., et al., "Synergistic Capture of *Clostridium botulinum* type A Neurotoxin by scFv Antibodies to Novel Epitopes," *Biotechnol Bioeng*, 2011; Gray, S.A., et al., "Flow Cytometry-Based Methods for Assessing Soluble scFv

Activities and Detecting Antigens in Solution," *Biotechnol Bioeng 105*:973–981, 2010). This clone was chosen for lyophilization studies.

Selections with the Jacob antigen generated several unique clones. One clone, designated *Jacob-A11*, bound to both biotinylated and unlabeled Jacob antigen. The affinity was determined to be 43 nM and this clone was selected for further lyophilization studies.

The 142 fragment generated a panel of yeast scFv that exhibited good binding. However, all of these scFv exhibited non-specific binding to at least one of the four non-cognate control proteins employed for the specificity analysis. No clones were isolated that bound specifically to 142.

In summary, yeast-scFv clones were isolated that bound specifically to 3 out of 4 protein fragments examined. The following 4 yeast-scFv clones that bound specifically to putative *E. histolytica* cyst proteins were chosen for assay development: clones *350-E2* and *350-12* which bound the 350 antigen; clone *780-23* which bound the biotinylated 780 antigen, and clone *Jacob-A11* which bound the Jacob antigen.

Yeast-scFv bioprobe lyophilization and flow cytometric assay formats

The selected yeast clones were induced to express scFv and antigen binding was confirmed by flow cytometry. The yeast cells were then lyophilized as described below in the EXAMPLES section. Yeast that were lyophilized and rehydrated in assay wash buffer appeared morphologically similar to fresh, pre-lyophilized yeast, as determined microscopically. Specifically, 10 mL of fresh induced or lyophilized and rehydrated yeast-scFv clone *350-E2* were analyzed by light microscopy at 40× magnification. Both pre-lyophilized yeast and reconstituted yeast appeared rounded and uniform with approximately half of the yeast in the process of budding (not shown). By flow cytometry, forward scatter (FSC) versus side scatter (SSC) plots indicated slight differences between fresh and lyophilized yeast. Specifically, yeast that had been lyophilized and rehydrated shifted to the left on SSC histograms relative to fresh yeast, while remaining largely unchanged on FSC histograms (not shown). This suggests that lyophilization and rehydration reduces the complexity and granularity of yeast-scFv cells without significantly changing their size.

Lyophilized yeast-scFv probes were used in three antigen detection assay formats. In one format, biotinylated antigens were captured by yeast-scFv and bound antigen was then detected by staining with streptavidin conjugated to phycoerythrin (SA-PE) followed

by flow cytometric analysis (results illustrated in FIGURE 1A). In a second, more broadly useful format, unlabeled antigens were incubated with yeast-scFv and their capture was detected by incubating the complexes with rabbit polyclonal antiserum raised against the antigen. FIGURE 1B shows the results of rehydrated yeast-*350-E2* binding to unlabeled 350 antigen. The antigen-rabbit polyclonal antibody complex was further detected using a phycoerythrin-conjugated goat-anti-rabbit MAb (GaR-PE) and analyzed by flow cytometry. The rabbit polyclonal antiserum was not affinity purified and therefore was unlikely to be fully specific to the cognate antigens. However, when such antibodies were used in sandwich assays in combination with monoclonal yeast-scFv probes, the combined assays were expected to be specific. In a third assay format, a sandwich assay incorporating yeast-scFv capture probes, polyclonal rabbit antibody signal probes and GaR-FITC detection probes were combined with an immunofluorescence (IFA) microscopy readout rather than flow cytometry (results illustrated in FIGURE 3).

In the flow cytometry-based assays, binding of antigen was quantified as the percent of PE-positive yeast relative to the no-antigen control. In most such assays, positive binding resulted in PE-fluorescence of approximately 45–65% of yeast cells.

Lyophilization and reconstitution had little effect on binding of the yeast-scFv bioprobes to antigens. For example, freshly grown yeast-scFv *350-E2* exhibited approximately 53% binding to both unlabeled (FIGURE 1B) and biotinylated 350 antigen (FIGURE 1A) prior to lyophilization, and approximately 64% binding after lyophilization and reconstitution. The same probe exhibited only <6% binding to three non-cognate antigens (142, Jacob, and 780). This specificity did not change with lyophilization and rehydration, although slightly greater background binding to biotinylated noncognate antigens was observed in lyophilized yeast. Specifically, fresh yeast exhibited 0.16–0.27% binding to biotinylated noncognate antigens while lyophilized yeast exhibited 3.27–5.15% binding to these antigens. When unlabeled antigens were detected using rabbit polyclonal antibody followed by GaR-PE in place of SA-PE, both fresh and lyophilized yeast exhibited similar background binding levels. Therefore, it is likely that the SA-PE reagent is responsible for the non-specific binding seen in FIGURE 1A. It is noted that in addition to clone *350-E2* (FIGURE 1), full activity following lyophilization was observed in eight additional, distinct yeast-scFv clones (i.e., *350-12*, *350-10*, *Jacob-cl.12*, *Jacob-cl.5*, *Jacob-cl.15*, *780-23*, *780-18*, and *780-17*) specific to three

different antigens (350, Jacob, and 780). Briefly, 100 μL of either fresh or lyophilized/rehydrated yeast were stained first for c-*myc*-FITC expression followed by binding to cognate antigen and to a non-cognate control (100 nM antigen concentration). Binding to antigen was indicated by an increase in PE fluorescence. Clones *350-10* and *780-18* are myc-negative due to premature stop codons following the scFv gene. However, these clones appeared to bind antigen before and after lyophilization. Thus, all examined clones bound their cognate antigen following lyophilization (not shown). Therefore, antigen-specific binding after lyophilization is demonstrated to be a consistent property of yeast-scFv bioprobe reagents.

While appearing healthy microscopically and retaining scFv display/expression and antigen binding, the lyophilized yeast did not retain viability under the lyophilization conditions used. Attempts to culture rehydrated cells demonstrated complete loss of viability. This demonstrates, therefore, that the bioprobes of the present invention are not required to maintain viability upon preservation and/or reconstitution, but rather only require that the preservation process (e.g., lyophilization) leave the bioprobes intact with the surface display of the antigen-binding compounds.

Lower limit of detection (LLD) of lyophilized yeast-scFv bioprobes

To quantify the sensitivity of antigen detection by yeast-scFv reagents, the *350-E2* probe were tested against 10 concentrations of 350 antigen ranging from 20 nM to 0.04 nM in twofold serial dilutions, each in a volume of 2.5 mL. Bound 350 antigen was detected by incubation with rabbit anti-350 antiserum followed by GaR-PE and analyzed by flow cytometry. As shown in FIGURE 2, the LLD was approximately 310 picomolar, comparable to an ELISA assay using a typical monoclonal antibody. The LLD was determined as the concentration of antigen resulting in binding was two standard deviations above the "no antigen" negative control.

Immunofluorescence microscopy assay using yeast-scFv bioprobes

The flow cytometry-based assays are sensitive and specific; however, flow cytometry capabilities are not available to all potential users of the bioprobes (e.g., yeast-scFv bioprobes) of the present invention. Therefore, an alternative immunofluorescence microscopy assay (IFA) was developed using the lyophilized yeast-scFv bioprobes. To evaluate the IFA, the reconstituted yeast-scFv (*350-E2* in FIGURE 3) were incubated with antigen in a concentration series ranging from 500 nM to 0.69 nM in threefold dilutions. Antigen binding was detected by staining with rabbit

anti-350 antiserum followed by GaR-FITC, and quantified by fluorescence microscopy and computer-assisted image analysis as described in the EXAMPLES section below. FITC fluorescence in the presence of the cognate 350 antigen was clearly visible relative to a non-cognate control antigen (antigen 142). Under increased magnification, as illustrated in FIGURE 3A, the majority of the fluorescent staining appeared localized to the perimeter of the yeast-scFv particles, consistent with staining of the cell wall associated scFv. By eye, the LLD of the IFA format was 6.17 nM (not shown). To better quantify the LLD, three separate, randomly chosen fields were imaged first by visible light and then with FITC, to determine the percentage of total yeast-scFv particles that exhibited antigen binding above a threshold value (FIGURE 3B). By this approach, a LLD of 5.0-2.5 nM was observed. Although somewhat less sensitive than the flow cytometry assays, these results demonstrate that the immunofluorescence microscopy assay delivers useful sensitivity without requiring flow cytometry.

ELISA-like HRP assay using yeast-scFv bioprobes

An additional alternative assay was developed using an ELISA-like assay with the sandwich assay format. Aliquots of 1.5E6 reconstituted yeast-scFv bioprobes (clone *350-E2*) were incubated with cognate antigen 350 in a series of eight concentrations from 100 nM to 0.78125 nM. Additional yeast-scFv bioprobes (clone *350-E2*) were incubated in reactions with non-cognate antigens 142, 780, or Jacob (or no antigen for control). Antigen binding was detected by staining with rabbit anti-350 polyclonal antiserum (1/1000) followed by GaR conjugated to horseradish peroxidase (GaR-HRP, 1/50). Washes were performed with PBS + 0.5% BSA (YWB) and centrifugation after each reagent incubation to remove unbound reagents. 650 µL Thermo 1-step Turbo TMB-ELISA substrate was added, and the reaction was allowed to persist for three minutes before being quenched with an equal volume of 1N sulfuric acid. Antigen binding was quantified by measuring the absorbance spectrophotometrically at 450 nm. Assays were performed in triplicate and standard deviations were generated. As shown in FIGURE 4, absorbance was clearly detected in the presence of the cognate 350 antigen in a manner proportional to the concentration of the cognate antigen. In contrast, reactions incorporating non-cognate antigens or no antigen exhibited no or negligible absorbance. By this approach, binding of cognate antigen was detectable over no antigen or non-cognate antigen at a concentration as low as 0.78125 nM. Similar to the IFA, this assay

may be less sensitive than flow cytometry-based assays, but nevertheless delivers useful sensitivity without requiring sophisticated equipment.

Stability of lyophilized yeast-scFv bioprobes over time

The stability of lyophilized yeast-scFv over time was assessed by using clone *780-23*, which is specific for 780-biotin antigen (FIGURE 5). For each time point, an aliquot of lyophilized *780-23* yeast was rehydrated, stained for scFv expression via the anti-c-myc tag, and then split into smaller aliquots. One aliquot was incubated with 100 nM 780-biotin (the cognate antigen) and other aliquots were incubated with 100 nM of three non-cognate antigens (Jacob-biotin, 142-biotin, and 350-biotin). Jacob-biotin was used throughout the experiment, while the other non-cognate antigens were used only at later time points. After washing, the bound antigen was detected by the addition of SA-PE. An aliquot of unstained yeast was also analyzed as a negative control. As seen in FIGURES 1 and 2, antigen binding was quantified using flow cytometry to measure the percentage of yeast cells that expressed scFv (FITC on the x-axis) and bound to biotinylated antigen (PE on the y-axis). Binding activity was stable for at least 30 days, with decay in the PE fluorescence starting around day 35. Binding to non-cognate antigens was low throughout the experiment (FIGURE 5).

Similar time course assays were carried out using clone *350-12*, and both resulted in very similar trends. In this assay, binding to cognate antigen (350-biotin) remained constant (between 40% and 60%) for ≥ 30 days post lyophilization, while binding to a noncognate control antigen (780-biotin) was low throughout the experiment (FIGURE 6).

Discussion

Affinity reagents are critical limiting factors in biomedical research and diagnostics. The goal of the present efforts is to design faster, cheaper, and more versatile methods for generating new affinity reagents. Yeast display libraries of human scFv have been in use for more than 10 years and are well established for rapid *in vitro* antibody isolation. However, the use of scFv culled from these libraries has been limited by the slow and inefficient conversion of yeast-displayed scFv to soluble, secreted antibodies that can be incorporated into diagnostic tests. As a result, yeast display and related methods have not supplanted traditional methods for generating monoclonal antibodies.

To address these limitations, an alternative model for yeast-scFv generation and application was pursued. The question of whether scFv probes culled from yeast-display

libraries would function best if maintained in the environment in which they were selected, namely tethered by Aga1-Aga2 linkages to yeast cell walls, was investigated. Accordingly, methods were developed to use yeast-scFv directly for antigen detection, without requiring secretion of scFv in soluble form. The resulting end-use detection reagents can be generated in two to three weeks, much faster than MAb generation. Furthermore, the bioprobe reagents can be generated efficiently in a cost-effective manner in large batches and be stored at room temperature for long periods before use.

ScFv that bound to cyst proteins derived from the human pathogen *E. histolytica* were selected for initial study. Four proteins were selected for this study based on microarray analysis that indicated elevated mRNA levels in cysts relative to trophozoites. The proteins were produced recombinantly in *E. coli* and then biotinylated to perform selections for yeast-displayed scFv Abs. Only one of the four proteins (142 antigen) failed to yield specific yeast clones. While multiple clones were selected to each of the other three proteins, the majority of clones bound only to the biotinylated form of the proteins. The selected yeast-scFv clones that bound the biotinylated form of the protein did not bind to the biotin entity itself, as indicated by the fact that they did not bind to biotinylated non-cognate control antigens. Therefore, one explanation is that biotinylation may alter protein folding, exposing new epitopes not seen in the native protein. One clone, *Jacob-A11*, bound both unlabeled and Jacob-biotin protein, however, it bound much better to Jacob-biotin. Only two unique clones were isolated that bound 350 antigen. This may be because the 350 protein fragment used for selections was small (~18 kDa) and, therefore, may have presented a small number of epitopes. One of these clones, *350-E2*, bound labeled and unlabeled 350 antigen equally well and was isolated more than 20 times from multiple independent selections. This clone was used for most of the subsequent work.

A major goal of the study was to determine whether whole (i.e., intact) yeast displaying scFv could be preserved by lyophilization. Yeast clones were induced to express scFv on their surfaces and then lyophilized in single use aliquots. Microscopically, lyophilized/reconstituted yeast were indistinguishable from freshly induced yeast. By flow cytometry, the rehydrated yeast exhibited much lower side scatter fluorescence, suggesting that they lacked the complexity/granularity of fresh yeast. The lyophilized yeast did not form colonies when plated on SDCAA medium (synthetic dextrose medium with casamino acids deficient in histidine, uracil, and tryptophan). The

loss of viability and shift in side scatter fluorescence may be a result of the simple lyophilization protocol, which did not use cryoprotectants. However, the goal was not to maintain cell viability, but rather to maintain the specific antigen-binding activities of the surface-displayed scFv after lyophilization and subsequent reconstitution.

In flow cytometric assays, bioprobe clones maintained binding to cognate antigen that was similar to that of freshly induced yeast. Lyophilization did not increase non-specific binding to noncognate antigens. Lyophilized yeast-scFv bioprobe *350-E2* bound to its cognate antigen with a lower limit of detection (LLD) of less than 1 nM. This LLD approaches that of a typical ELISA assay using a monoclonal IgG antibody. In addition to flow cytometry, diverse alternative read-outs can be used including the IFA described here which detected 350 antigen with an LLD in the range of 2.5–6.17 nM.

The lyophilized yeast were stored at room temperature, reconstituted in simple buffers (typically PBS or PBS+5% BSA), and were immediately usable following rehydration. Although stabilizers such as lyophilization buffers, nitrogen gas, oxygen absorbers, and desiccants were not used, all three yeast-scFv bioprobe clones examined maintained stable binding for at least 30 days. Longer shelf lives may be possible when yeast-scFv are stored with cooling or desiccants.

Lyophilization of yeast-scFv facilitates storage and shipment. Moreover, it abrogates the need for overnight growth of yeast in SDCAA medium, followed by overnight induction of scFv expression in SG/RCAA media, as well as the need to perform quality control (QC) analysis of each induction. With lyophilized yeast cultures, the QC is performed just once immediately prior to lyophilization. A typical flow cytometry assay, such as illustrated in FIGURE 1, requires approximately 1e7 yeast. With lyophilization, liter-scale batches of induced yeast (at a concentration of 2-4e7 yeast per mL) can be stored in single-use aliquots of 1e7 yeast. This means that a single 1-liter culture of induced yeast-scFv can yield enough material for up to 4000 tests at a cost of a few pennies per test.

Most of the assays describe herein utilized rabbit polyclonal antisera for secondary detection of unlabeled antigens. Additionally, polyclonal mouse and chicken antisera have been successfully used for this purpose. The use of polyclonal detection antibodies extends the time required to generate functional yeast-scFv-based antigen detection assays. However, polyclonal detection antibodies do not need to be affinity-purified, because assay specificity is conferred by the yeast-scFv bioprobes,

which are monoclonal. The only requirements for detection reagents (e.g. antibodies) are: 1) that they bind to antigens of interest, and 2) that they don't cross-react with yeast-scFv bioprobes in the absence of antigen. Thus, polyclonal detection antibodies are inexpensive and easy reagents to prepare.

In summary, whole-cell yeast-scFv affinity reagents can be rapidly selected and prepared in lyophilized form. With its fast turnaround time (3 weeks from expressed antigen to lyophilized probe) and low cost, the process has utility to produce affinity reagent compositions that can routinely overcome a significant bottleneck in diagnostics and research.

The following examples are provided for the purpose of illustrating, not limiting, the invention.

EXAMPLES

Example 1

Reagents

In this Example, materials used to generate illustrative reagents and assays of the present are described.

Except where indicated, all chemicals were from Sigma-Aldrich (St. Louis, MO). All secondary reagents for immuno-cytometry were acquired through Molecular Probes (Invitrogen, Carlsbad, CA). Nickel-NTA sepharose for purification of expressed antigens was obtained from Qiagen (Qiagen, Germantown, MD).

The polyclonal rabbit anti-350 and anti-780 antisera were both generated by Cocalico Biologicals, Inc. (Reamstown, PA).

Proteins 780, 350, 142, and Jacob were biotinylated using either the Pierce EZ-Link Peg-Biotin or the Sulfo-NHS-LC-Biotin kit (Thermo Scientific, Rockford, IL) per manufacturer's instructions. The degree of biotinylation was quantified using the Pierce Biotin Quantitation (HABA) Assay (Thermo Scientific, Rockford, IL).

Example 2

Protein purification

In this Example, a method for production of antigens of interest for later screening against yeast-scFv libraries is described.

The divergent region of *E. histolytica* Jacob protein (compared to *E. dispar* and *E. moshkovskii* proteins) was codon optimized (GeneArt, Invitrogen) and topo-cloned

into the pET SUMO vector. The correct orientation of insert in pET SUMO vector was verified by DNA sequencing.

For the chromodomain proteins (Genbank nos.: EHI_000780, EHI_115350, and EHI_142000), DNA segments corresponding to the selected region of proteins were PCR amplified from the *E. histolytica* genomic DNA, digested with *Nde*I and *Bam*H1 restriction enzymes, gel-purified, and ligated to pET11a vector using the T4 DNA ligase. Top10 cells were then transformed using the pET11a vector containing the insert. Both pET SUMO vector and pET11a vectors contain sequences for 6 histidine residues N-terminal to the gene of interest, allowing each protein to be purified using the Ni-NTA column according to the manufacturer's instructions (Qiagen). The SUMO fusion protein was cleaved from the *Eh*Jacob with the treatment of SUMO protease (LifeSensors, Malvern, PA).

Example 3

Library selections

In this Example, a method and reagents for screening of libraries of human non-immune scFv displayed on yeast with antigens of interest to produce yeast bioprobes are described.

Selections were performed on the Wittrup library of human nonimmune scFv antibodies displayed on the surface of yeast, according to protocols previously described (Feldhaus, M.J., et al., "Flow-Cytometric Isolation of Human Antibodies From a Nonimmune *Saccharomyces cerevisiae* Surface Display Library," *Nat Biotechnol 21*:163–170, 2003; Gray, S.A., et al., "Synergistic Capture of Clostridium Botulinum Type a Neurotoxin by scFv Antibodies to Novel Epitopes," *Biotechnol Bioeng*, 2011; Gray, S.A., et al., "Flow Cytometry-Based Methods for Assessing Soluble scFv Activities and Detecting Antigens in Solution," *Biotechnol Bioeng 105*:973–981, 2010; Chao, G., et al., "Isolating and Engineering Human Antibodies Using Yeast Surface Display," *Nat Protoc 1*:755–768, 2006). Yeast were routinely grown in SDCAA broth. To induce expression of scFv on the yeast surface, yeast were grown in selective synthetic galactose/raffinose plus casamino acids deficient in histidine, uracil, and tryptophan (SG/RCAA) broth. For round 1 (R1) and round 2 (R2) of selection, 10^{10} yeast were incubated in 10 mL yeast wash buffer (YWB; PBS 0.5% BSA) containing 100 nM of each of the four biotinylated proteins separately. Yeast that bound protein were then labeled by incubation with 200 µL of either streptavidin-magnetic particles (R1) or anti-biotin magnetic particles (R2).

Following the second magnetic sort, the eluted yeast (R2 output) were incubated with 1 μg mL^{-1} SA-PE. Yeast were then sorted by FACS for the top 10% of PE-positive cells and expanded into 5 mL of SDCAA broth (sorted yeast comprised the R3 output). For round 4 (R4) of the selection, the R3a outputs were incubated with anti-c-myc MAb and goat-antimouse-fluorescein isothiocyanate (GaM-FITC) to confirm expression of scFv before again being incubated with 100 nM of their specific biotinylated antigen followed by SA-PE. Yeast were then sorted by FACS for FITC-positive and the top 10% of PE-positive signal. Antigen-binding yeast were grown on synthetic dextrose casamino acids minus His, Ura, Trp plate (-HUT media) for single clone analysis. For each antigen, 24–48 clones were picked and grown in 96 deep well plates in 1 mL of SDCAA broth. Following induction in 1 mL SGRCAA, each clone was then tested by flow cytometry for binding to cognate antigen and for non-binding to all non-cognate antigens or the secondary reagents (SA-PE, FITC) themselves. Antigen-specific clones were then amplified by PCR, and the PCR amplicon was subjected to *Bst*N1 fingerprint analysis as described previously (Gray, S.A., et al., "Synergistic Capture of *Clostridium botulinum* Type A Neurotoxin by scFv Antibodies to Novel Epitopes," *Biotechnol Bioeng*, 2011; Gray, S.A., et al., "Flow Cytometry-Based Methods for Assessing Soluble scFv Activities and Detecting Antigens in Solution," *Biotechnol Bioeng 105*:973–981, 2010). In this method, the 850-1000 nucleotide PCR amplicons are digested with the restriction endonuclease *Bst*N1, usually resulting in 3–8 smaller fragments. The resulting fragment pattern, or fingerprint, after resolving the fragments by DNA gel electrophoresis was used to determine uniqueness. All apparently unique clones were then DNA sequenced to confirm uniqueness and confirmed clones were used in the study.

To determine affinities, a previously described flow cytometry assay which utilizes whole yeast was used (Chao, G., et al., "Isolating and Engineering Human Antibodies Using Yeast Surface Display," *Nat Protoc 1*:755–768, 2006; Van Antwerp, J.J., and K.D. Wittrup, "Fine Affinity Discrimination by Yeast Surface Display and Flow Cytometry," *Biotechnol Prog 16*:31–37, 2000; Siegel, R.W., et al., "High Efficiency Recovery and Epitope-Specific Sorting of an scFv Yeast Display Library," *J Immunol Methods 286*:141–153, 2004; Feldhaus, M., and R. Siegel, "Flow Cytometric Screening of Yeast Surface Display Libraries," *Methods Mol Biol 263*:311–332, 2004). In this assay, yeast-displaying scFv were incubated with twofold serial dilutions of biotinylated antigens spanning 3.125–250 nM in concentration and antigen binding was determined by

further staining with SA-PE. Samples were analyzed by flow cytometry, results graphed as a function of antigen-biotin concentration versus mean PE fluorescence. Affinities were then determined by using a nonlinear least squares fit of the curves as previously described (Feldhaus, M.J., et al., "Flow-Cytometric Isolation of Human Antibodies From a Nonimmune *Saccharomyces cerevisiae* Surface Display Library," *Nat Biotechnol 21*:163–170, 2003; Van Antwerp, J.J., and K.D. Wittrup, "Fine Affinity Discrimination by Yeast Surface Display and Flow Cytometry," *Biotechnol Prog 16*:31–37, 2000; Kemmer, G., and S. Keller, "Nonlinear Least-Squares Data Fitting in Excel Spreadsheets," *Nat Protoc 5*:267–281, 2010). This assay was used to measure the affinities of clones *780-23*, *Jacob-A11*, *350-12*, and *350-E2* (binding to 350-biotin). The affinity of clone *350-E2* to unlabeled 350 was measured by using a modification of this assay, in which binding was detected by staining the yeast-antigen complexes with a rabbit anti-350 polyclonal serum and then further stained with goat-anti rabbit PE. The affinity of clone *Jacob-A11* to unlabeled Jacob protein was not tested.

Example 4

Yeast lyophilization

In this Example, a method and reagents for lyophilization of yeast bioprobes are described.

Selected yeast clones were induced to express scFv and active antigen binding was confirmed as described above. Confirmed antigen-binding cells were lyophilized by harvesting 100 mL of SG/RCAA culture at an OD_{600} = 1.5–2 (1 $OD_{600} \approx 2\times10^7$ yeast/mL). An aliquot of culture with ~1×10^7 total yeast was transferred to 1.5 mL freezer tubes and centrifuged for 1 min at 13000 g. The supernatant was decanted, and the pellets were flash-frozen in liquid nitrogen for approximately 15 seconds. Without allowing the yeast to thaw, the tubes were loosely capped and transferred to a lyophilization beaker that had been previously cooled in a dry-ice/ethanol bath. The beaker was immediately capped, fit to the lyophilizer, and vacuum applied. Yeast were lyophilized overnight using a Millrock Technology Model BT48A lyophilizer (Kingston, NY). Following lyophilization, tubes were tightly capped and stored at room temperature without desiccants or stabilizers.

Example 5

Specificity analysis

In this Example, a method and reagents for determining the specificity of cytometry-based assays using yeast bioprobe reagents are described.

One aliquot of fresh, non-lyophilized *350-E2* yeast and one aliquot of lyophilized, rehydrated yeast (~1×10^7 yeast/aliquot) were incubated with anti-c-*myc* MAb followed by goat-anti-mouse FITC detection MAb to confirm expression of scFv. Following c-*myc* staining, the yeast were split into two sets of five equal aliquots. One set was incubated with 100 nM of biotinylated cognate antigen and three biotinylated non-cognate antigens (plus the no-antigen control) for 1 hr at RT. Binding of biotinylated antigens was detected by addition of 100 µL of a 1/800 dilution of SA-PE for 30 minutes. The second set of aliquots received 100 nM of unlabeled 350, 142, Jacob, and 780 (plus the no-antigen control). To detect binding of unlabeled antigens, the yeast were incubated with a 1/1000 dilution of rabbit anti-350 antiserum for 45 min at RT followed by a 1/800 dilution of goat-anti-rabbit-PE for 45 min at RT. Yeast were then analyzed by flow cytometry for scFv expression (x-axis; FITC fluorescence) and antigen binding (y-axis; PE fluorescence). The assay was performed three times, and each assay utilized an independent induction and lyophilization of yeast *350-E2*. The percent PE fluorescent yeast were determined by gating the percent PE-positive relative to the "no antigen" controls. All other yeast clones isolated in this study were analyzed for specificity in a similar fashion.

Example 6

Lower limit of detection

In this Example, a method and reagents for determining the sensitivity (i.e. the lower limit of detection characteristic) of cytometry-based assays using yeast bioprobe reagents are described.

One aliquot of lyophilized *350-E2* yeast clone was resuspended in 1 mL YWB and split into 11 equal aliquots. Each aliquot was incubated with 2.5 mL of 350 antigen ranging from 20 nM to 0.04 nM in twofold serial dilutions (plus the 0 nM control). Following a 1 hour antigen incubation, yeast were washed to remove unbound antigen, and the bound 350 antigen was detected by incubating with a 1/2500 dilution of rabbit anti-350 antiserum. Final detection of bound 350 was achieved by incubation with 1/2500 dilution of goat-anti-rabbit-PE (GaR-PE) detection MAb for 1 hr. Samples were analyzed by flow cytometry and the percent of PE-positive yeast, relative to a no-antigen control, were plotted versus the concentration of 350 antigen. The experiment was

repeated three independent times, and each data point reflects the average percent PE-positive yeast plus and minus the standard deviation.

Example 7

Immunofluorescence microscopy assay

In this Example, a method and reagents for performing an immunofluorescence microscopy assay to determine specific binding of the bioprobe reagents with antigen of interest are described.

One aliquot of lyophilized *350-E2* yeast clone was reconstituted in 1 mL YWB and split into 8 equal aliquots. Each aliquot received 100 µL of 7 concentrations of cognate 350 or non-cognate 142 ranging from 500 nM to 0.69 nM in threefold serial dilutions (plus the 0 nM control). Bound antigens were detected by incubating with a 1/2500 dilution of rabbit anti-350 antiserum followed by 1/800 dilution of goat-anti-rabbit-FITC (GaR-FITC) detection MAb for 1 hr. Following three washes to remove unbound antigen, yeast pellets were resuspended in 100 µL, and 10 µL of yeast were spotted onto microscope slides and a coverslip applied. The yeast were imaged at 40× magnification using a Nikon TE2000U inverted microscope (Nikon Corporation, Tokyo, Japan) outfitted with phase contrast and DIC optics for visible imaging, a FITC filter, a high-speed Cool Snap CCD camera, and a motorized stage for automated scanning. Image capture and analysis was performed using Metamorph software (Research Precision Instruments, Natick, MA).

To confirm staining of the perimeter of yeast, suggestive of scFv binding, the sample incubated with 500 nM antigen was examined at 90× magnification. For purposes of clarity, the image provided in FIGURE 3A was digitally zoomed approximately 3-fold to better discern the fluorescent staining pattern.

A second experiment was similarly performed to enumerate the approximate number of FITC-positive yeast relative to a concentration of cognate antigen. In this experiment, yeast were incubated with 11 concentrations of unlabeled 350 antigen ranging from 160 nM down to 0.16 nM in twofold serial dilutions. For each antigen concentration, a minimum of 3 independent fields were captured in visible mode followed immediately by the same three fields in FITC fluorescence mode. Total and FITC-fluorescent yeast were counted in each field using the Image J Software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, imagej.nih.gov/ij/, 1997–2011). Visible fields were background-subtracted with a

50-pixel rolling ball radius while sliding paraboloid was enabled and automated smoothing disabled. Automatic thresholding was performed in grayscale with the default settings. Particles (cells) were analyzed from 64-∞ without circularity compensation, with edge exclusion and hole inclusion. Fluorescent (FITC) fields were background-subtracted in the same fashion. Each FITC image then underwent a binary conversion allowing the watershed filter to be applied. After automatic triangle thresholding in grayscale using a dark background, the fluorescent particles were enumerated from 64-∞ without circularity compensation. In each field, percent fluorescence was calculated from the ratio of the number of fluorescent yeast (FITC) to the total number of yeast in the visible spectrum. The percentages were averaged and standard deviations calculated from at least three fields at a given antigen concentration.

Example 8

ELISA-like HRP assay

In this Example, a method and reagents for performing an ELISA-like HRP to determine specific binding of the bioprobe reagents with antigen of interest are described.

Aliquots of 1.5E6 reconstituted yeast-scFv bioprobes (clone *350-E2*) were incubated separately in YWB (PBS + 0.5% BSA) either with cognate antigen 350 in a series of eight concentrations from 100 nM to 0.78125 nM, or with non-cognate antigens 142, 780, or Jacob (or no antigen for control), in triplicate. Antigen binding was detected by staining with rabbit anti-350 polyclonal antiserum (1/1000) followed by GaR conjugated to horseradish peroxidase (GaR-HRP, 1/50). One wash was performed (with the addition of 1 mL YWB followed by centrifugation and the aspiration of the supernatant) after each reagent incubation to remove unbound reagents; three washes were performed after the final GaR-HRP incubation. Each reagent incubation was allowed to equilibrate for 45 minutes before washing. After unbound GaR-HRP was removed by washing, 650 µL Thermo 1-step Turbo TMB-ELISA substrate was added, and the reactions were allowed to persist for three minutes before being quenched with an equal volume of 1N sulfuric acid. Antigen binding was quantified by measuring the absorbance of supernatants spectrophotometrically at 450 nm. Assays were performed in triplicate and standard deviations were generated.

Example 9

Lyophilization time course assay

In this Example, a method and reagents for confirming the stability of lyophilized yeast bioprobe reagents are described.

To assess the stability of yeast-scFv following lyophilization, a fresh culture of yeast clone *780-23* was induced for expression of scFv and lyophilized in individual aliquots containing ~1×10^7 yeast. For the first two weeks, time points were taken twice weekly, and from then on time points were taken approximately once per week until little binding to cognate antigen was observed.

For each time point, one aliquot of lyophilized yeast was reconstituted in 1 mL YWB, stained for c-*myc* expression as described previously, and then split into 5 equal aliquots. The aliquots received 100 µL of 100 nM of one of either cognate 780-biotin or non-cognate Jacob-biotin antigen (plus a no-antigen control). Following 45 min of antigen binding, yeast were washed 2× and bound antigen detected by incubation with a 1/800 dilution of SA-PE. Yeast were washed 3× and analyzed for FITC (x-axis) and PE (y-axis) fluorescence by flow cytometry. A gate was set to measure the total percent of PE-positive yeast relative to the "no antigen" control. The percent of antigen binding (percent PE-positive) was then plotted versus the day post lyophilization. The cognate antigen throughout this assay was 780-biotin, and the non-cognate antigen was Jacob-biotin. Starting at day 30, two other non-cognate antigens, 350-biotin and 142-biotin, were included to better assess specificity. The assay was terminated on day 86 when binding to cognate antigen had dropped below 15%.

The stability of the yeast bioprobe clone *350-12* was similarly assessed. Briefly, three individual colonies of clone *350-12* were lyophilized separately on different days. Every 3 to 5 days, one aliquot of each was tested for binding to cognate antigen (100 nM 350-biotin) and to a non-cognate control antigen (100 nM 780-biotin) as described above for yeast bioprobe clone *780-23*. Because of the staggered lyophilization dates, data were grouped into groups of 2 to 3 days, each point representing the average and standard deviation for the day groupings.

While illustrative embodiments have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A bioprobe composition for selectively binding an antigen of interest in a sample, comprising a lyophilized bioparticle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the bioparticle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

2. The composition of Claim 1, wherein the bioparticle is selected from the group consisting of a cell, a cellular organelle, or a virus.

3. The composition of Claim 2, wherein the cell is selected from the group consisting of a yeast, bacterium, plant, or animal cell.

4. The composition of Claim 3, wherein the yeast is from the genera *Saccharomyces* or *Pichia*.

5. The composition of Claim 2, wherein the cellular organelle is a ribosome.

6. The composition of Claim 2, wherein the virus is a bacteriophage.

7. The composition of Claim 1, wherein the heterologous antigen-binding molecule is selected from the group consisting of an antibody-like molecule and a T cell receptor (TCR).

8. The composition of Claim 7, wherein the antibody-like molecule comprises an antigen-binding fragment of an antibody or T cell receptor.

9. The composition of Claim 7, wherein the antibody-like molecule is a single-chain antibody, a bispecific antibody, an Fab fragment, or an $F(ab)_2$ fragment.

10. The composition of Claim 9, wherein the single-chain antibody is a single-chain variable fragment (scFv), single-chain Fab fragment (scFab), V_HH fragment, V_{NAR}, or nanobody.

11. The composition of Claim 1, wherein the lyophilized bioparticle is capable of being maintained between about 4 and 40 degrees Celsius before reconstitution of the bioparticle.

12. The composition of Claim 1, wherein the lyophilized bioparticle is capable of being maintained for at least about 90 days before reconstitution of the bioparticle.

13. The composition of Claim 1, wherein the at least one heterologous antigen-binding molecule is displayed on the bioparticle surface prior to lyophilization by inducing expression and translocation of the molecule to, or assembly of the molecule on, the bioparticle surface.

14. A bioprobe composition for selectively binding an antigen of interest in a sample, comprising a lyophilized particle displaying on its outer surface at least one heterologous antigen-binding molecule, wherein upon reconstitution of the particle from the preserved state, the at least one heterologous antigen-binding molecule is capable of specifically binding the antigen of interest.

15. A method of detecting the presence of an antigen of interest in a biological sample, comprising:

A) contacting a biological sample with the bioprobe composition of any one of Claims 1-14 under conditions sufficient to permit the binding of the bioprobe with an antigen of interest; and

B) detecting the binding of the bioprobe to the antigen of interest.

16. The method of Claim 15, further comprising contacting the biological sample with a detection reagent that binds to the antigen of interest.

17. The method of Claim 16, further comprising contacting the biological sample with a detectably-labeled reporter reagent and separating the bioprobe from unbound reporter.

18. The method of Claim 15, wherein binding of the antigen of interest to the bioprobe is detected using an antibody sandwich flow cytometric assay, cell bioprobe immunofluorescence microscopy, an ELISA-like assay, or a competitive inhibition assay.

19. The method of Claim 15, wherein the biological sample is selected from the group consisting of blood, urine, sputum, mucus, saliva, cerebral spinal fluid, tissues, stool, nutrient sources, or processed derivatives thereof.

20. A method of detecting the presence of an antigen of interest in a biological sample, comprising:

A) contacting a biological sample with a capture reagent that binds to the antigen of interest; and

B) contacting the biological sample with a detection reagent under conditions sufficient to permit the binding of the detection reagent with an antigen of interest, wherein the detection reagent comprises the bioprobe composition of any one of Claims 1-14.

21. The method of Claim 20, wherein the detection reagent further comprises a detectable label.

22. The method of Claim 20, further comprising contacting the biological sample with a detectably-labeled reporter agent that specifically binds to the bioprobe composition, and separating the capture reagent from unbound reporter agent.

23. A method for validating a candidate biomarker as a biomarker of a biological state, comprising:

A) contacting a library comprising a plurality of bioparticles displaying heterologous antigen-binding molecules with a detectably-labeled candidate biomarker of a biological state under conditions sufficient to permit the binding of a displayed heterologous antigen-binding molecule with the labeled candidate biomarker;

B) isolating the bioparticles displaying heterologous antigen-binding molecules bound to the candidate biomarker;

C) propagating the bioparticles isolated in step (B);

D) inducing expression and surface display of the heterologous antigen-binding molecules in the bioparticles propagated in step (C);

E) preserving the bioparticles displaying the heterologous antigen-binding molecules induced in step (D); and

F) validating the candidate biomarker as a biomarker for the biological state by contacting biological samples obtained from a plurality of individuals exhibiting and not exhibiting the biological state with reconstituted bioparticles displaying the heterologous antigen-binding molecules, the plurality being sufficiently large to statistically associate the candidate biomarker with the biological state.

24. A kit for selectively binding an antigen of interest in a sample, comprising:

A) the bioprobe composition in any one of Claims 1-14; and

B) reconstitution buffer.

25. The kit of Claim 24, further comprising detection reagent.

26. The kit of Claim 24, further comprising detectably-labeled reporter reagent.

27. The kit of Claim 24, further comprising instructions for use.

28. The kit of Claim 24, further comprising at least one detectably-labeled antigen of interest as a control.

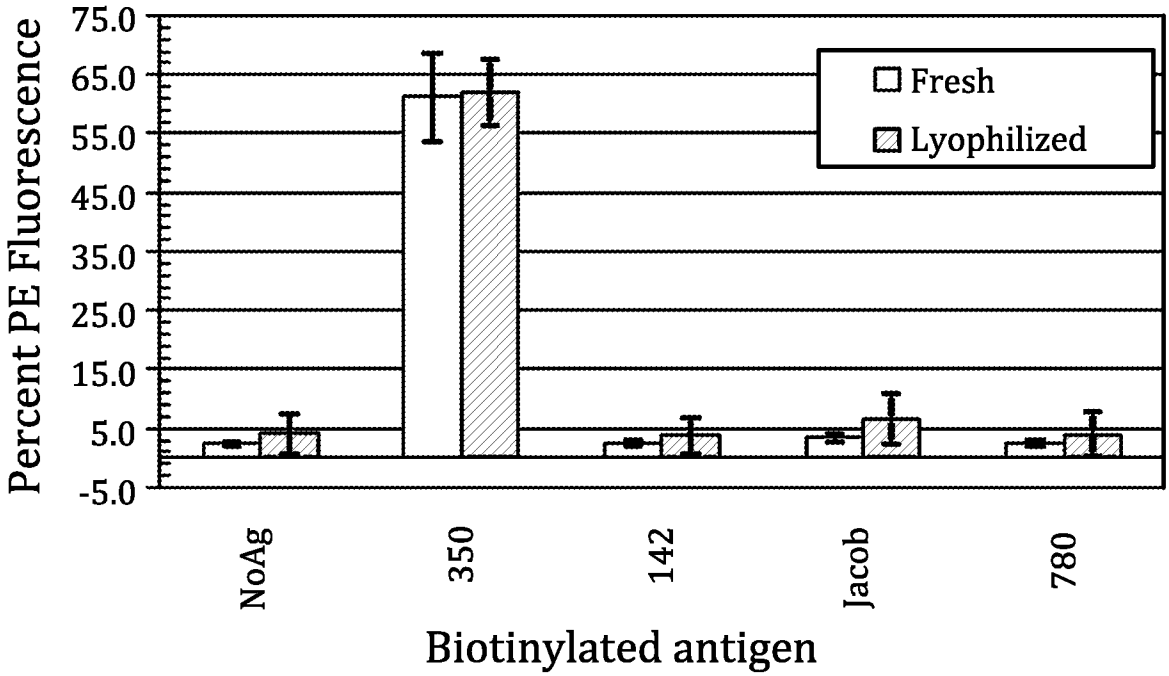

75.0
65.0
55.0
45.0
35.0
25.0
15.0
5.0
-5.0
Percent PE Fluorescence
Fresh
Lyophilized
NoAg
350
142
Jacob
780
Biotinylated antigen

Fig.1A.

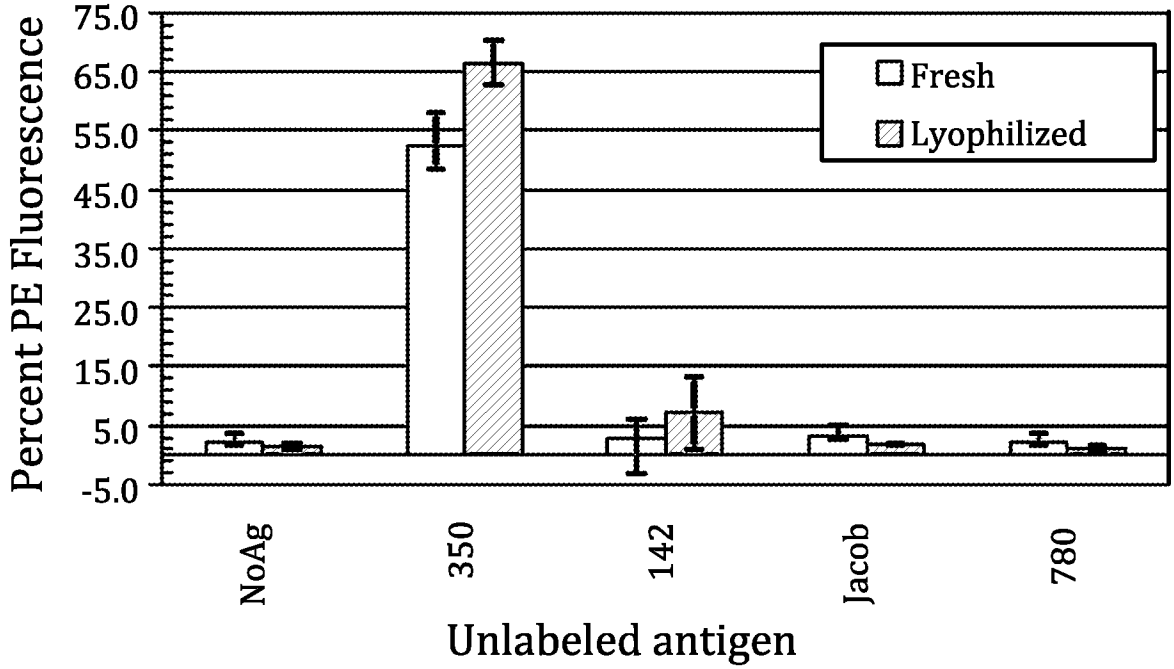

75.0
65.0
55.0
45.0
35.0
25.0
15.0
5.0
-5.0
Percent PE Fluorescence
Fresh
Lyophilized
NoAg
350
142
Jacob
780
Unlabeled antigen

Fig.1B.

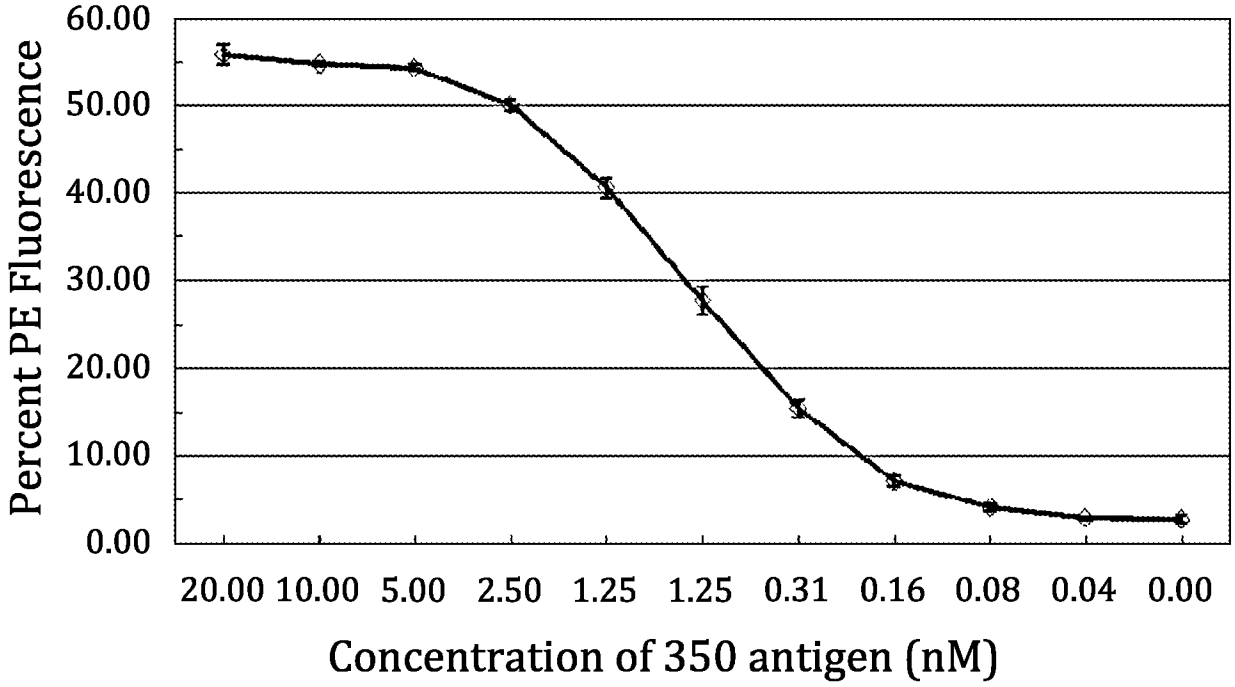

Percent PE Fluorescence
60.00
50.00
40.00
30.00
20.00
10.00
0.00
20.00 10.00 5.00 2.50 1.25 1.25 0.31 0.16 0.08 0.04 0.00
Concentration of 350 antigen (nM)

Fig.2.

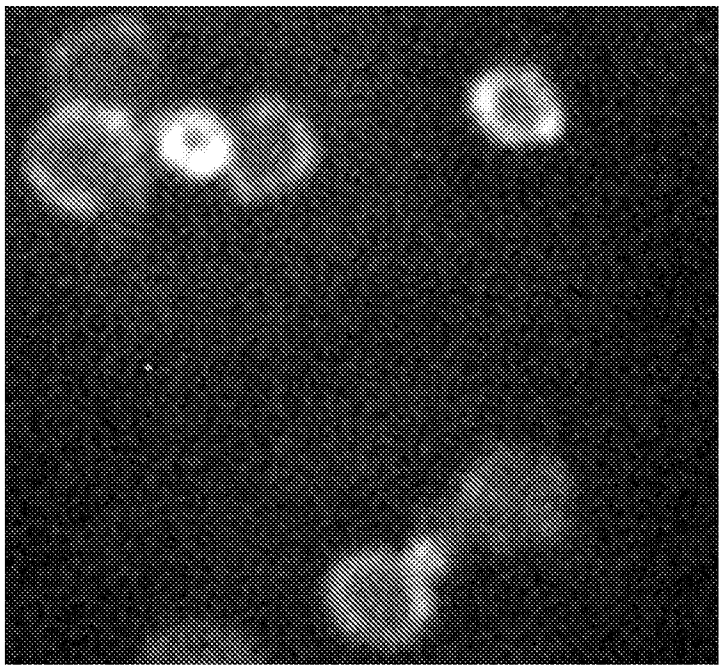


Fig.3A.

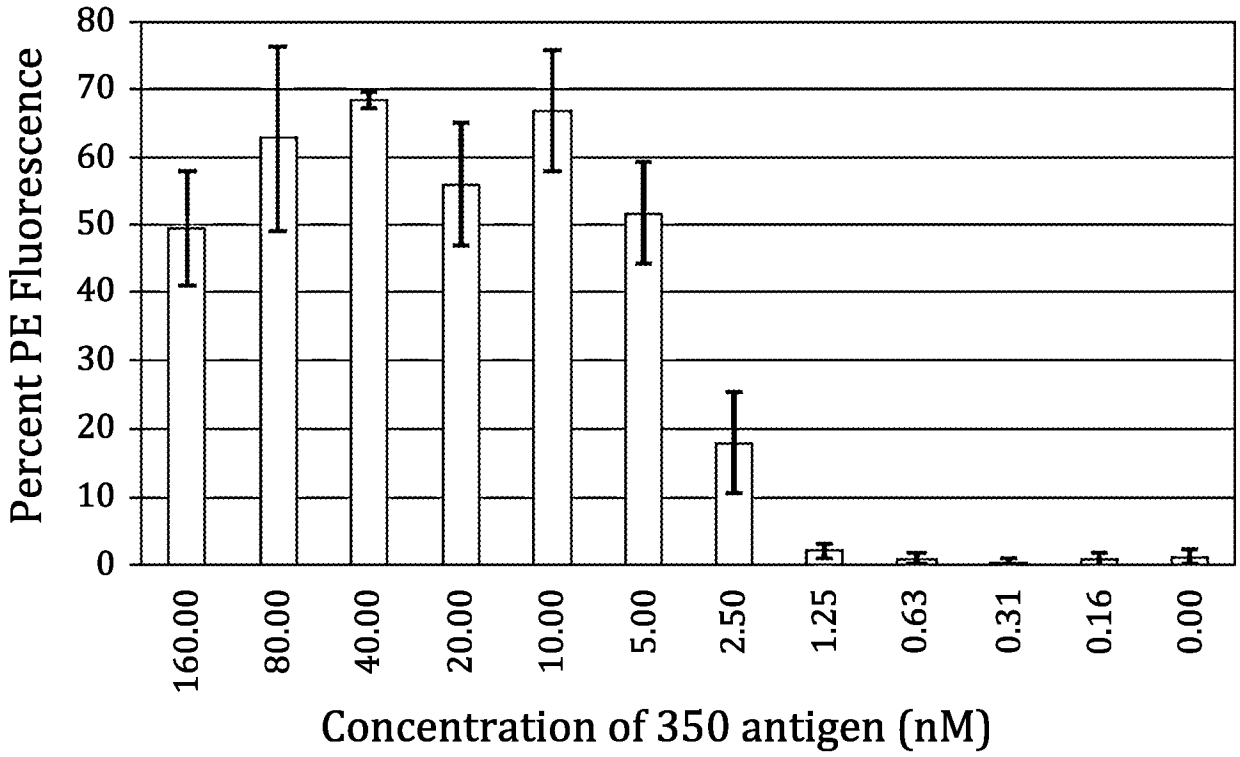
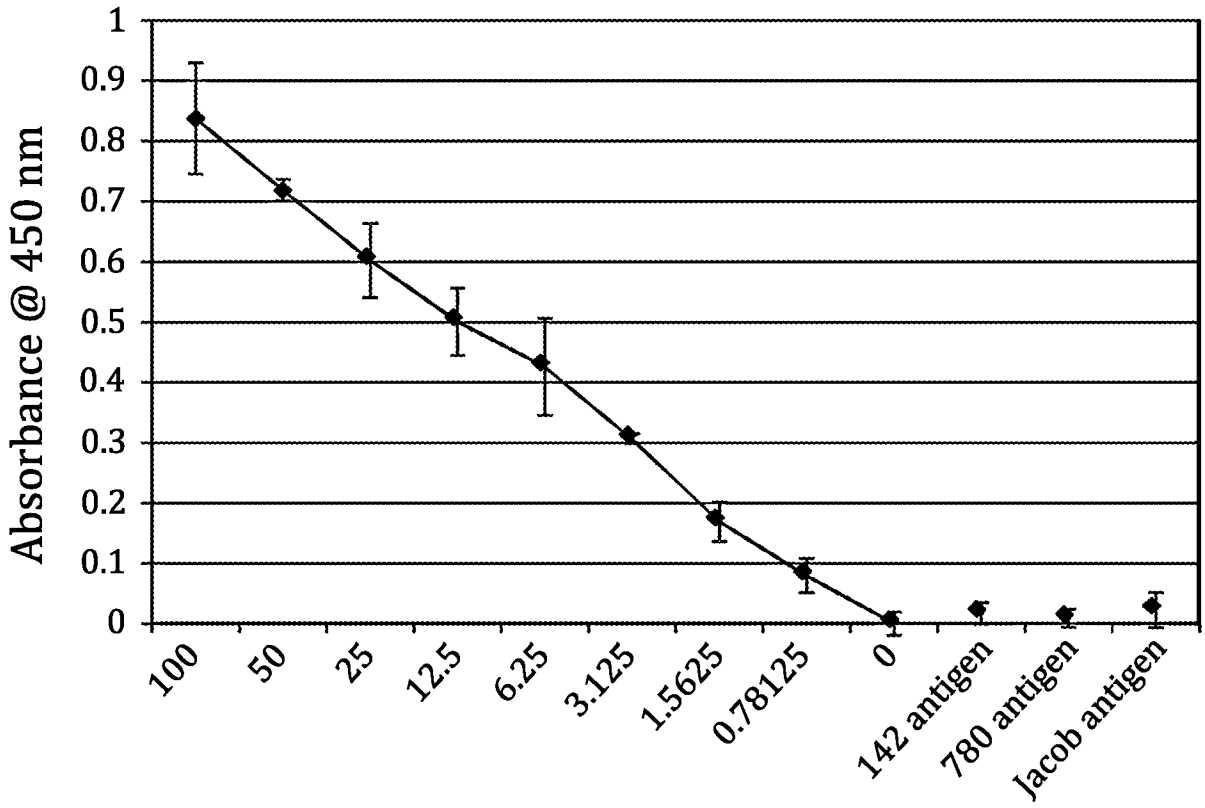

Percent PE Fluorescence
80
70
60
50
40
30
20
10
0
160.00
80.00
40.00
20.00
10.00
5.00
2.50
1.25
0.63
0.31
0.16
0.00
Concentration of 350 antigen (nM)

Fig.3B.



[nM 350], or non-cognate antigen @ 100 nM

Fig.4.

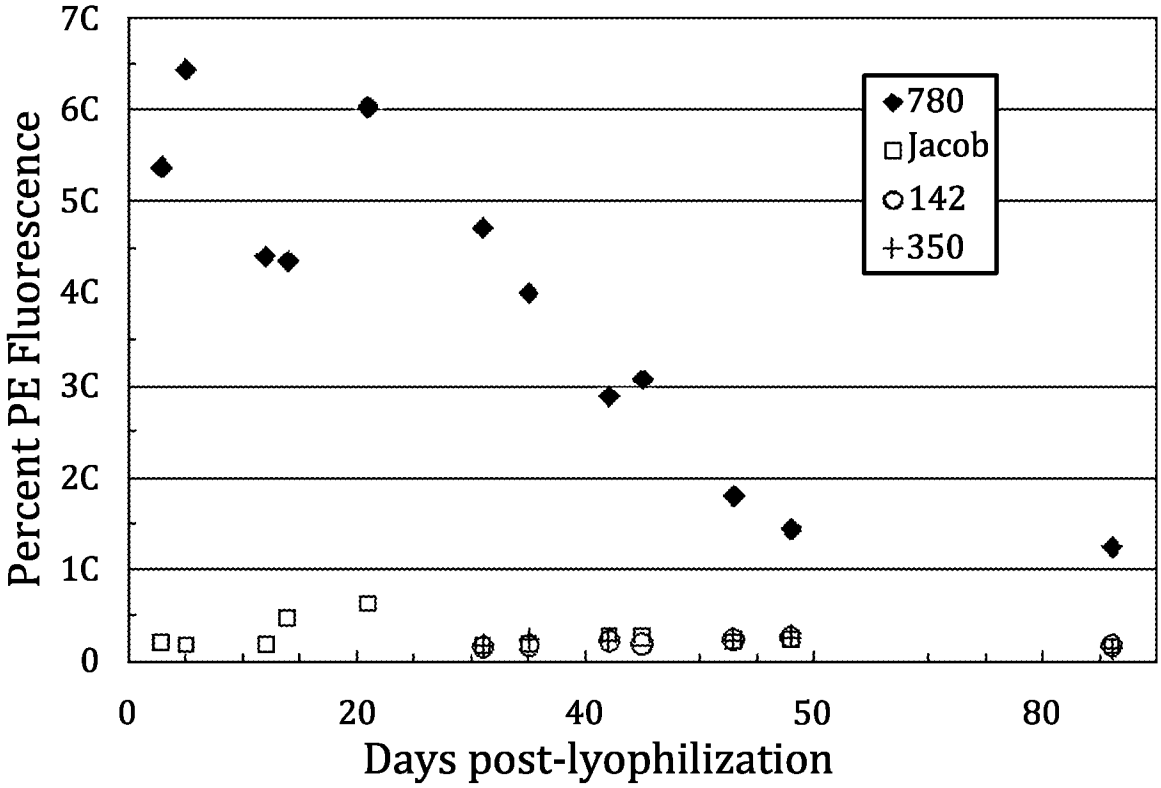

7C
6C
5C
4C
3C
2C
1C
0
Percent PE Fluorescence
780
Jacob
142
+350
0
20
40
50
80
Days post-lyophilization

Fig. 5.

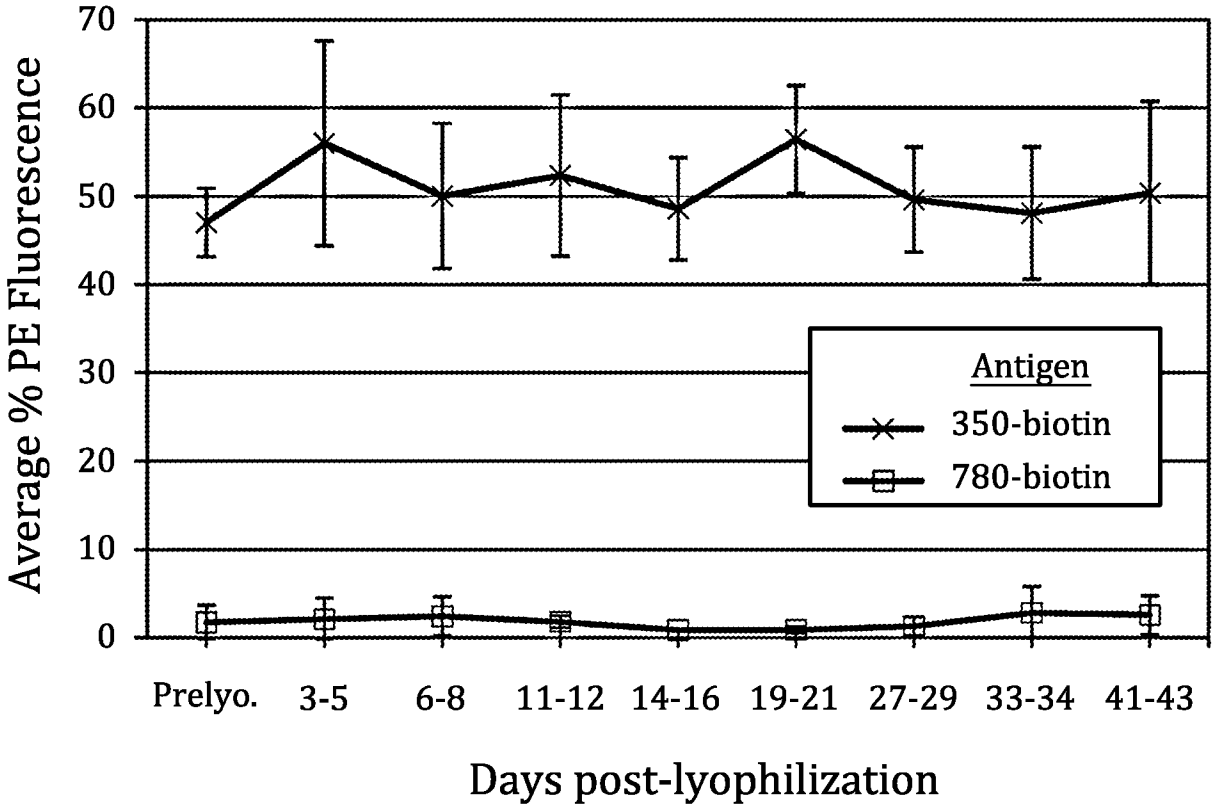

70
60
50
40
30
20
10
0
Average % PE Fluorescence
Antigen
350-biotin
780-biotin
Prelyo.
3-5
6-8
11-12
14-16
19-21
27-29
33-34
41-43
Days post-lyophilization

Fig. 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US12/38684

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/00, 1/70; G01N 33/53 (2012.01)
USPC - 435/4, 5, 7.1, 287.2, 287.1, 283.1; 436/86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/00, 1/70; G01N 33/53 (2012.01)
USPC: 435/4, 5, 7.1, 287.2, 287.1, 283.1; 436/86

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE_U, GB-A, FR-A); DialogPRO; Google; Google Scholar; bio*probe, bio*sensor, lyophilize*, free*dry*, heterologous*, antigen*, bind*, reconstitute*, bacteriophage, saccharomyces, pica

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2010/0303827 A1 (SHARMA, SR., MS et al.), December 2, 2010; abstract; paragraphs [0100], [0299], [0406], [0557], [0562]-[0565], [0692], [0833]	1-4, 6-11, 13-16, 18, 19, 24-28 ---- 5, 12, 15, 16, 18, 19, 23-28
Y	WO 2009/058564 A2 (KARRER, EE et al.), May 7, 2009; page 166, lines 15-18; page 163, lines 27-30; page 165, lines 25-26; page 233, lines 10-16 This document can be viewed by entering the doc number at the following url: http://worldwide.espacenet.com/numberSearch?locale=en_EP	5, 12, 15-22, 24-28
Y	WO 2003/031591 A2 (SHEN, L et al.), April 17, 2003; paragraphs [0014], [0022]-[0029], [0184]	17, 20-22
Y	WO 2007/047796 A2 (ZHANG, H et al.), April 26, 2007; page 7, lines 22-25; page 15, line 10; page 20, lines 12-14, page 30, line 16; page 193, lines 23-26	23

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2012 (23.07.2012)	03 OCT 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)