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Methods for immuno-detection of epitopes on molecules and for detection of interactions of molecules via fluorescent dyes

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(71)   Applicant(s)
         The Trustees of the University of Pennsylvania

(72)   Inventor(s)
         Liu, Qingdu; Li, Bin; Greene, Mark I.; Murali, Ramachandran; Zhang, Hongtao

(74)   Agent/Attorney
         FB Rice & Co, Level 23 200 Queen Street, Melbourne, VIC, 3000

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(71) Applicant: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Center For Technology Transfer, Suite 300, 3700 Market Street, Philadelphia, PA 19107 (US).

(72) Inventors: GREENE, Mark, L.; 300 Righters Mill Road, Penn Valley, PA 19071 (US). ZHANG, Hong, Tao; 377 Poplar Avenue #N488, Devon, PA 19333 (US). LI, Bin; 4218 Baltimore Avenue, Philadelphia, PA 19104 (US). LIU, Qindu; 250 Beverly Boulevard, F 109, Upper Darby, PA 19082 (US). MURALI, Ramchandran; 41-6 Revere Road, Drexel Hill, PA 19026 (US).

(74) Agents: LICATA, Jane, Massey et al.; Licata & Tyrrell P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

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(54) Title: METHODS FOR IMMUNO-DETECTION OF EPITOPES ON MOLECULES AND FOR DETECTION OF INTERACTIONS OF MOLECULES VIA FLUORESCENT DYES

(57) Abstract: Methods, systems and kits are provided for detecting molecules expressing a selected epitope in a sample through use of an epitope detector containing a single chain Fv for the selected epitope or a constrained epitope specific CDR, CDR mimetic or engineered CDR structure attached to an oligonucleotide.

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METHODS FOR IMMUNO-DETECTION OF EPITOPES
ON MOLECULES AND FOR DETECTION OF
INTERACTIONS OF MOLECULES VIA FLUORESCENT DYES

Introduction

5   This application is a continuation-in-part of U.S. Patent 09/783,896, filed February 15, 2001, which is a continuation-in-part of U.S. Patent Application Serial No. 09/624,946, filed July 25, 2000.

Background of the Invention

10   Traditional methodologies for protein detection and quantification include 2-D gel electrophoresis, mass spectrometry and antibody binding. Each methodology has been used to quantify protein levels from relatively large amounts of tissue, yet each suffers from a lack of
15 sensitivity.

        Improvement of the ability to monitor proteins, lipids, sugars and metabolite levels and their modifications is needed for cell biology and medicine. A variety of technologies have been employed to improve the sensitivity
20 of detecting these molecules. Recent examples of detection methods include immuno-PCR, RCA and immuno-aRNA.

        Immuno-PCR (U.S. Patent 5,665,539) combines the polymerase chain reaction (PCR) technology with conventional detection methods to increase the sensitivity to detect
25 protein. However, a major limitation of immuno-PCR lies in the non-linear amplification ability of PCR reaction, which limits this technique as a quantitative detection method. Thus, this method provides no direct correlation between the amount of signal and the amount of protein present.

30   A relatively isothermal rolling circle DNA amplification technique (RCA; Schwietzer et al., Proc. Natl. Acad.Sci. USA 97, 10113, 2000)) provides an improvement over

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immuno-PCR as this technique overcomes some of the quantitation problems associated with immuno-PCR.

U.S. Patent 5,922,553 discloses a method for quantifying levels of a selected protein via a technique
5 referred to as immuno-aRNA. In this method, a first antibody targeted to a selected protein is immobilized to a solid support. The support is then contacted with the selected protein so that the selected protein is immobilized to the first antibody. The solid support is then contacted
10 with a RNA promoter-driven cDNA sequence covalently coupled to a second antibody targeted to the selected protein so that the second antibody binds to the bound selected protein. The amount of selected protein is determined by quantifying levels of the promoter driven cDNA sequence
15 covalently coupled to the bound second antibody via an amplified RNA technique. In a preferred embodiment, a T7 promoter driven cDNA sequence is covalently coupled to the second antibody.

It has now been found that single chain fragments as
20 well as exocyclic peptide based complementarity determining region (CDR) subunits can be used in this immuno-aRNA technique. Further, it has been found that PCR, as well as amplified RNA techniques, can be used to quantify the promoter driven cDNA sequence covalently coupled to the
25 bound single chain fragment or CDR subunit. The use of smaller antibody binding units and fragments coupled with the already existing large single chain or cyclic peptide libraries and the use of robotic assistance renders this method widely useful for both medicinal and research
30 purposes. Furthermore, a single third detector species can be coupled with double-stranded DNA and bound to either the single chain Fv or the CDRs, rendering detection uniform and simple.

Summary of the Invention

It is desirable that the present invention provide a method for detecting molecules expressing a selected epitope in a sample. In this method, an epitope anchor specific for a selected epitope is immobilized to a selected surface. The epitope anchor

5 may comprise a single chain Fv fragment, a CDR, CDR mimetic or engineered CDR structure, an antibody, or other ligand peptide or chemical or pharmaceutical that interacts with a selected epitope. The surface is then contacted with a sample suspected of containing molecules which express the selected epitope so that the molecules bind to the immobilized epitope anchor. An epitope detector comprising a single chain Fv

10 for the selected epitope or a constrained epitope specific CDR, CDR mimetic or engineered CDR structure attached to an oligonucleotide, preferably a double-stranded cDNA, is then used to detect any bound molecules. The oligonucleotide can be chemically conjugated or linked through biotin-streptavidin or biotin-avidin binding to the single chain Fv, CDR, CDR mimetic or engineered CDR structure. Alternatively,

15 the method of the present invention can be performed without an epitope anchor. In this embodiment, the epitope detector is employed to define molecules bound directly to a surface.

In a preferred embodiment of this method, detection is performed via readout using a standard fluorimetric device of a fluorescence derived numerical value. More

20 specifically, in this embodiment, following amplification of the oligonucleotide of the epitope detector, a fluorescent dye is incorporated into the nucleic acid sequence in a linear manner. Upon excitation at a selected wavelength, the dye yields a quanta of fluorescence signals directly proportional to the mass of sample loaded.

With a mixture of epitopes detectors comprising either monoclonal antibodies

25 for selected epitopes, single chain Fvs for selected epitopes or constrained epitope specific CDRs, CDR mimetics or engineered CDR structures, conjugated to cDNAs of different lengths, the method of the present invention can also be used to profile proteins in a cell lysate.

In one embodiment, CDRs, CDR mimetics or engineered CDR structures for use

30 in the epitope detector are identified via screening of a library of CDRs, CDR mimetics or engineered CDR structures to define a CDR, CDR mimetic or engineered CDR structure which binds a molecule with the epitope. CDRs or CDR mimetics identified via screening of these libraries can also be used as therapeutic agents targeted to a receptor or protein interacting with the receptor. When used as a therapeutic agent, it is

35 preferred that the CDR or CDR mimetic be reinserted into a humanized antibody or attached to an Fc.

The present invention provides a method for detecting molecules that express a selected epitope comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected surface;

5   (b) contacting the surface with an epitope detector so that the epitope detector binds to immobilized molecules on the surface, said epitope detector comprising

(i) an oligonucleotide comprising an RNA promoter; said oligonucleotide attached to

(ii) a monoclonal antibody that specifically binds to the selected epitope, a 10 single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected epitope;

(c) producing an amplified RNA product from the oligonucleotide by RNA 15 amplification; and

(d) detecting the presence of the amplified RNA product by a method selected from the group consisting of:

(i) contacting the amplified RNA product with a fluorescent dye which stains the RNA product and measuring a quanta of fluorescence signals emitted from 20 stained RNA, wherein fluorescence emitted from stained RNA which is directly proportional to epitope detector bound to the selected epitope on molecules immobilized on the surface, and

(ii) further amplifying the RNA product using reverse transcriptase or replicase and detecting a product of the further amplification by contacting said product 25 of further amplification with a fluorescent dye or probe which stains said product of further amplification and measuring fluorescence emitted from the stained product, wherein detection of a product of the further amplification is directly proportional to epitope detector bound to the selected epitope on molecules immobilized on the surface.

30   The present invention also provides a method for quantifying molecules that express a selected epitope comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected surface;

(b) contacting the surface with an epitope detector so that the epitope detector 35 binds to immobilized molecules on the surface, said epitope detector comprising

(i) an oligonucleotide comprising an RNA promoter; said oligonucleotide attached to

(ii) a monoclonal antibody that specifically binds to the selected epitope, or a single chain Fv that specifically binds to the selected epitope, a constrained CDR that
5 specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected epitope;

(c) producing an amplified RNA product from the oligonucleotide by RNA amplification;
10 (d) contacting the amplified RNA product with a fluorescent dye which stains the amplified RNA product; and

(e) quantifying a quanta of fluorescence signals emitted from the stained RNA product, wherein the quantity of fluorescence emitted is directly proportional to the number of molecules that express the epitope present in the sample.
15 The present invention also provides a method for monitoring interaction of molecules *in vitro* using a two-component system comprising:

(a) immobilizing a first molecule to a solid surface;

(b) adding to the solid surface a second molecule that interacts with the first molecule, wherein the second molecule comprises a universal epitope;
20 (c) adding to the solid surface a universal epitope detector conjugated to an oligonucleotide that comprises an RNA promoter;

(d) performing RNA amplification;

(e) contacting the amplified RNA product with a fluorescent dye which stains the amplified RNA product; and
25 (f) measuring a quanta of fluorescence signals emitted from stained RNA, wherein fluorescence emitted from stained RNA is directly proportional to binding of the first molecule to the second molecule.

The present invention also provides a method of monitoring interaction of molecules *in vitro* comprising:
30 (a) using a two-component system in accordance with the method of the invention;

(b) adding third molecule to the two-component interaction system; and

(c) monitoring effects of the third molecule on the binding and interaction of said first and second molecules of said two-component system by measuring changes in
35 fluorescence wherein a positive change in fluorescence is indicative of the third molecule facilitating binding of the first and second molecule and a negative change in

fluorescence is indicative of the third molecule inhibiting binding of the first and second molecule.

The present invention also provides a method for identifying a CDR structure that specifically binds to a specific epitope, a CDR mimetic structure that specifically
5 binds to a specific epitope or an engineered CDR structure that specifically binds to a specific epitope, the method comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected surface;

(b) contacting the surface with a library of CDRs, CDR mimetics or engineered
10 CDR structures wherein:

(i) the CDRs, CDR mimetics or engineered CDR structures of the library are each linked to an oligonucleotide comprising an RNA promoter; or

(ii) the CDRs, CDR mimetics or engineered CDR structures of the library comprise a universal epitope and an epitope detector that binds to the universal epitope
15 is further added, wherein the epitope detector comprises an oligonucleotide comprising an RNA promoter

(c) producing an amplified RNA product from the oligonucleotide by RNA amplification;

(d) detecting the presence of amplified RNA product by contacting the
20 amplified RNA product with a fluorescent dye which stains the amplified RNA product, and

(e) measuring a quanta of fluorescence signals emitted from the stained RNA product, wherein fluorescence emitted from the stained RNA product is directly proportional to identifying a CDR structure that specifically binds to a specific epitope
25 on molecules immobilized on the selected surface, a CDR mimetic structure that specifically binds to a specific epitope on molecules immobilized on the selected surface or an engineered CDR structure that specifically binds to a specific epitope on molecules immobilized on the selected surface.

The present invention also provides a method for profiling proteins in a cell
30 lysate comprising:

(a) contacting the cell lysate with a mixture of epitope detectors so that the epitope detectors specifically binds to selected epitopes on proteins present in the cell lysate, each of said epitope detectors comprising:

(i) cDNA comprising an RNA promoter; said cDNA conjugated to
35 (ii) a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that

specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or engineered CDR structure that specifically binds to the selected epitope wherein epitope detectors that specifically bind to the same specific epitope are conjugated to cDNAs that produce RNA amplification products of the same length, and

5 epitope detectors that specifically bind to different specific epitopes are conjugated to cDNAs that produce RNA amplification products of different lengths;

(b) performing RNA amplification;

(c) separating amplified RNA by size

(d) contacting the amplified RNA with a fluorescent dye that stains RNA,

10 (e) visualizing separated stained amplified RNA and identifying the presence of the different size amplified RNA so that the profile of proteins expressing different selected epitopes in the lysate can be determined.

It is also desirable that the present invention provide kits for the detection of molecules expressing a selected epitope which comprise an epitope detector comprising

15 a single chain Fv for the selected epitope or a constrained epitope specific CDR, CDR mimetic or engineered CDR structure attached to an oligonucleotide, preferably a double-stranded cDNA. In addition, the kits preferably comprise an RNA polymerase, an amplification buffer and a fluorescent dye for staining of amplified products. Kits of the present invention may further comprise an epitope anchor specific for a selected

20 epitope. In one embodiment, the single chain Fv or the constrained epitope specific CDR, CDR mimetic or engineered CDR structure is modified to allow for attachment of the oligonucleotide. In another embodiment, the kit comprises a mixture of epitope detectors for profiling of proteins in a cell lysate.

The present invention provides a kit when used for the detection or

25 quantification of molecules that express a selected epitope using fluorescence comprising:

(a) an epitope detector comprising

(i) an oligonucleotide comprising an RNA promoter; the oligonucleotide linked to

30 (ii) a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, or a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected epitope;

35 (b) an RNA polymerase;

(c) an amplification reaction buffer; and

(d) a fluorescent dye that binds to RNA.

The present invention also provides a kit when used for profiling proteins comprising:

(a) a mixture of epitope detectors that specifically bind to selected epitopes on
5 proteins present in the cell lysate, each of said epitope detectors comprising

(i) cDNA comprising an RNA promoter; said cDNA linked to

(ii) a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, a CDR mimetic that specifically binds to the
10 selected epitope, or an engineered CDR structure that specifically binds to the selected epitope; wherein epitope detectors that specifically bind to the same specific epitope are conjugated to cDNAs that produce RNA amplification products of the same length, and epitope detectors that specifically bind to different specific epitopes are conjugated to cDNAs that produce RNA amplification products of the different lengths;

15 (b) an RNA polymerase;

(c) an amplification reaction buffer; and

(d) a fluorescent dye that binds to RNA.

Methods and kits of the present invention can be formulated into a convenient 96- or 384-well plate platform for high-throughput handling and screening of samples.

20 Methods and kits of the present invention are particularly useful in analyzing the interactions of molecules, e.g. protein-protein interactions, ligand-induced protein-protein interactions and sugar-protein interactions, as well as for monitoring changes in these interactions which are induced by chemicals, pharmaceuticals and other molecular species including, but not limited to, proteins, lipids and sugars.

25 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Detailed Description of the Invention

30 The present invention relates to improved methods for quantifying levels of selected molecules and systems and kits for performing these improved methods. In one embodiment, the method comprises binding an epitope anchor specific for a selected epitope of the molecule to a selected surface. The epitope anchor may comprise a single chain Fv fragment, a CDR, CDR mimetic or engineered CDR

structure, an antibody, or other ligand peptide or chemical specific for a selected epitope. In a preferred embodiment, the epitope anchor is bound to a designated spot on the surface. For example, the surface may comprise a chip and the epitope anchor is bound to a defined spot on the chip. In one embodiment, the epitope anchor is

5   deposited onto a surface or plate with the aid of a pipettor or similar device which permits application at a single site. The surface with the bound epitope anchor is then contacted with a sample suspected of containing molecules expressing the selected epitope so that the molecule binds to the epitope anchor. In another embodiment, the molecule is attached to a surface directly, without the use of an epitope anchor.

10   Examples of samples which can be assayed via the methods of the present invention include, but are not limited to, individual cells and solutions including biological fluids such as serum.

An epitope detector which can bind to any bound molecule on the surface is then used to detect and quantify

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the amount of bound molecule. The epitope detector
comprises a monoclonal antibody, a single chain Fv for the
selected epitope or a constrained epitope specific CDR, CDR
mimetic or engineered CDR structure or which have been
5   modified to allow for attachment of oligonucleotides,
preferably double-stranded DNA at a single site.
Alternatively, fragments of antibodies with the binding
activity, scFv or CDR peptides can be used to replace
antibodies in this technology.

10   Fv fragments for selected epitopes can be produced in
cells or on microorganisms by use of recombinant DNA
technology. For example, Skerra and Pluckthun (Science 1988
240:1038-1044) describe an expression system for production
of functional Fv fragments in *E. coli*.

15   A method for producing Fv fragments in eukaryotic
host cells with a eukaryotic expression vector which has an
operon having a DNA sequence which encodes the variable
domain only of an antibody light or heavy chain has also
been described (J. Mol. Biol. 1988 203:825-828). Chains of
20 the Fv fragment are secreted and correctly assembled by the
host cell such that fully functional Fv fragments are
produced in the culture supernatant. In addition, the DNA
coding sequence may be altered toward its 5' end so that the
amino terminal end expresses a residue or residues with a
25 surface suitable for covalent coupling of an
oligonucleotide. In addition, the 3' terminal end may be
varied so that cysteine residues are produced towards the
C-terminal end of each variable domain permitting the
variable domains in the dimer to become linked together by
30 disulphide bonding. This may also promote assembly of the
Fv fragment. Alternatively, the Fv fragment may be
stabilized by use of a vector having a first DNA sequence
encoding a first variable domain and a second DNA sequence
encoding a second variable domain, the first and second
35 sequences being linked by a third DNA sequence which encodes

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a joining peptide sequence. In this case, the joining peptide sequence is sufficiently long and flexible to allow folding of the two polypeptides into a functional single chain Fv. Preferably, the host cell is a myeloma cell line

5 which, prior to transformation, does not secrete whole antibody or light chains. Such cells lines are well known and widely available (Reichmann et al. J. Mol. Biol. 1988 203:825-828).

It is believed that random phage technology to any
10 hapten or chemical compound can also be used to select Fvs. (Harrison et al. United States Biochemical Pharma Ltd. (Europe), Watford, United Kingdom)

The CDR technology is well known and has been described in U.S. Patent 5,334,702, U.S. Patent 5,663,144,
15 and U.S. Patent 5,919,764. Antibody molecules bind to their antigens via six variable loops called Complementary Determining Regions (CDRs). The CDR3 from heavy chain often mediates that specificity. A general methodology for design of constrained cyclic CDR mimetics is described by Williams
20 et al. (Proc. Natl Acad. Sci. USA 1989 86:5537-5541), Sargovi et al. (Science 1991 253:792-795) and Murali et al. (Immunol. Res. 1998 17:163-169). In general, CDRs comprise a 6 to 15 mer peptide constrained to be cyclic and modified by aromatic residues. CDR mimetics are small molecules
25 (about 1 kDa) which are capable of mimicking their parent antibodies in terms of specificity, affinity and biological activity. Cyclic forms of CDR mimetics contain approximately 5 to 13 constrained amino acids.

For purposes of the present invention, an epitope
30 detector or epitope anchor comprising a CDR may consist essentially of the CDR or CDR mimetic. Alternatively, the epitope detector or epitope anchor may comprise a CDR or CDR mimetic defined as binding which is reinserted into a humanized antibody framework or attached to an Fc. CDRs or
35 CDR mimetics reinserted into a humanized antibody or

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attached to an Fc are also referred to herein as engineered CDR structures. However, as will be understood by those of skill in the art upon reading this disclosure, the term "engineered CDR structure" is inclusive of any protein into 5 which a CDR or CDR mimetic is inserted. Examples of such proteins include, but are not limited to, members of the immunoglobulin gene family, minibodies or small antibody-like molecules, or any other framework which allows the CDR to be functional as an antigen binding surface.

10   An important step in the design of CDRs, CDR mimetics and engineered CDR structures for use in the present invention is the delineation of the residues that are important for activity. This is generally accomplished by first synthesizing a set of analogs from the bioactive 15 domain of the original antibody or receptor or ligand of different lengths and establishing the minimal chain lengths for the complete and partial activities. Once the minimal chain length has been established, each side chain can be systematically varied to determine the importance of charge, 20 steric bulk, hydrophobicity, aromaticity, and chirality at each position. After evaluation of the properties of a large set of analogs, it is possible to identify the functional groups and conformation features involved in binding. Different conformationally constrained analogs can 25 then be developed. Various means for constraining peptides have been developed.

One means involves introducing a conformationally constrained amino acid. Hruby (Life Sci. 1982 31:189-199) describes the synthesis of a large number of amino acid and 30 dipeptide derivatives with built-in conformational constraints, as well as their incorporation into biologically active peptides. Prasad et al. (Biopolymers 1995 35:11-20) also describes a method of constraining the conformation of an amino acid unit by replacing the hydrogen 35 atom at the α-carbon with a methyl group to produce a

dialkylamino acid. U.S. Patent 6,022,523 describes a method that restricts the conformational freedom of amino acids by introducing a double-bond at the C-α and C-β atoms.

5   Another means for constraining peptides involves introduction of covalent cross-links. Constraining the peptide backbone by introduction of covalent cross-links provides more dramatic effects than incorporating unusual amino acids. Macrocyclization is often accomplished by forming an amide bond between the peptide N- and C-termini,

10 between a side chain and the N or C terminus, or between two side chains. A head-to-tail cyclization of side protected peptides synthesized by Fmoc/t-butyl solid phase procedures on polysterine resin derivatized with 4-hydroxymethyl-3-methoxyphenoxyacetic acid, the first generation dialkoxy-

15 benzyl linkage agent, has been described by Sheppard, R.C. (Int. J. Peptide Res. 1982 20:451-454). In addition, the analogous   linkage   agent,   4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HAMA), was recently employed in fragment condensation and solid phase synthesis of

20 peptides with these highly acid sensitive linkers (In Peptides, E. Giralt and D. Andreu eds, ESCOM, Leiden, The Netherlands 1991, 131-133). The enkephalin analogs described by Schiller provide an example of side-chain to backbone covalent cyclization in which covalent attachment

25 of the e-amino group of the D-lys residue to the C terminal backbone carboxylate group of Leu produces a cyclic 16-membered ring analog with high potency and significant μ receptor selectivity (Schiller et al. Int. J. Pep. Prot. Res. 1985; 25:171-177). BOP-reagent and carboimide/1-

30 hydroxy-benzotriazole combinations have also been reported to be useful in the formation of cyclic peptides (Felix, A.M. Int. J. Pep. Prot. Res. 1988 31:231-238). Degrado et al. have also developed a biologically active cyclized peptide analog of the GP IIb/IIIa complex using m-

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aminomethylbenzoic acid as the linker (U.S. Patent
6,022,523).

　　　Disulphides can also be formed by oxidation via
introduction of cysteine at certain positions. For example,
5　Romani, S. (Int. J. Pep. Prot. Res. 1987 29:107-117)
demonstrated that non-symmetrical disulphides can be built
with the help of the di-tertbutyl aster of azodicarboxylic
acid. Ploux, O. (Int. J. Pep. Prot. Res. 1987 29:162-169)
also describes a method for formation of non-symmetrical
10　disulphides via thiol displacement of the 3S-3-nitro-2-
pyridinesulfenyl group.

　　　In a preferred embodiment, the oligonucleotide used
in the present invention is double-stranded and comprises
a T7 promoter driven cDNA sequence so that it can be
15　amplified using T7 RNA polymerase. In this embodiment,
double-stranded cDNA is synthesized for use as a template
for T7 RNA polymerase transcription. T7 RNA polymerase
requires its promoter site to be double-stranded.

　　　In one embodiment, the site on the Fv or CDR, CDR
20　mimetic or engineered CDR structure to which the
oligonucleotides are attached comprises a series of residues
which allow the attachment of linkers consisting of
chemicals such as heterodimeric coupling reagents or other
linkers. These residues provide a uniform binding site for
25　the linker attachment. The linkers attach to this site and
also link oligonucleotides to the Fv or CDR, CDR mimetic or
engineered CDR structures. Oligonucleotides may be
unmodified or modified. For example, the presence of the
amplified oligonucleotide can be enhanced by incorporating
30　a beacon or fluorescent labeled oligonucleotide into the
mixture allowing for rapid semi quantitative assessment of
the epitope expressing molecules (Tan et al. Chemistry 2000
6:1107-1111; Leone et al. Nucleic Acids Res. 1998
26(9):2150-2155).

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In another embodiment, the oligonucleotide of the epitope detector is coupled to biotin and the monoclonal antibody, single chain Fv or constrained epitope specific CDR, CDR mimetic or engineered CDR structure is coupled to
5 streptavidin and attachment of the oligonucleotide to the monoclonal antibody, single chain Fv or constrained epitope specific CDR, CDR mimetic or engineered CDR structures occurs via complexing of the biotin to the streptavidin.

Bound epitope detectors may be quantified by methods
10 such as amplification by conventional PCR or aRNA techniques. If the detection method used is immuno aRNA, double-stranded cDNA are preferably used in the epitope detector. In this embodiment, aRNA is transcribed on the solid support using a polymerase, unlabeled ribonucleotides,
15 and fluorescently labeled ribonucleotides. By "polymerase" for purposes of the present invention, it is meant a polymerase which recognizes a specific promoter. Examples of polymerases useful in the present invention include, but are not limited to, T7 RNA polymerase, T3 RNA polymerase,
20 SP6 RNA polymerase, φ29 polymerase, and Taq polymerase. In another embodiment, the amplified products can serve as templates for further amplification with reverse transcriptase or replicases to increase the sensitivity.

A variety of means are available for detection of
25 amplified products of the epitope detector. In one embodiment, the nucleic acid sequence is detectably labeled such as with a radioactive label or a fluorescent label. In a preferred embodiment, the nucleic acid sequence is not labeled but rather is stained by fluorescent dye. Other
30 methods such as gel electrophoresis, high performance liquid chromatography, hybridization assays, immunohistochemical assays and/or specific binding protein assays can also be used for detection.

Use of Fvs and CDRs, CDR mimetics or engineered CDR
35 structures as the epitope detector renders the methods of

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the present invention useful in identifying larger
polypeptides than can be identified by the immuno-aRNA
technique of U.S. Patent 5,922,553, as well as in
identifying motifs in supernatants, fluids, extracts of
5 cells or bacteria or any other eukaryotic organism.
Accordingly, the method of the present invention has
widespread applicability in both medicinal and research
purposes. Further, the method of the present invention is
more sensitive than currently available methods and provides
10 quantitative results.

The ability of Fvs and CDRs, CDR mimetics or
engineered CDR structures to serve as epitope detectors of
selected molecules in the method of the present invention
was demonstrated for the p185 receptor. For these
15 experiments, a single chain Fv (ScFv) construct of 7.16.4
(designated as 7.16.4 ScFv) was produced in accordance with
the procedure outlined by Peterson & Greene (DNA and Cell
Biology 1998 17(12):1031-1040) wherein the Fv region of the
heavy chain and light chain region was joined by a
20 (gly4-Ser)5 linker. Since ScFv7.16.4 contained a
poly-histidine tag, it was purified over Ni-NTA resin.
After purification, the binding of 7.16.4ScFv was confirmed
by FACS analysis on B104-1-1 cells, in both direct binding
and competitive binding against the monoclonal antibody
25 7.16.4.

AHNP, a constrained exocyclic peptide designed from
the CDR3.H region of the anti-human p185 antibody 4D5 was
also used. AHNP binds to p185 and mimics the growth-
inhibitory effects of 4D5 (Park et al. Nature Biotechnology
30 2000 18:194-198).

Both ScFv7.16.4 and AHNP were coupled to a double-
stranded oligonucleotide (ds-oligo) to form epitope
detectors. Both ds-oligo coupled 7.16.4ScFv and AHNP were
able to detect their antigens, rat p185neu from B104-1-1
35 cells and human p185her2/neu from T6-17 cells, respectively.

Further, conjugation with the ds-oligo to form the epitope detector did not change the binding affinity of the CDR detection molecules with their antigens as determined by plasmon resonance analysis. Since 7.16.4ScFv and mAb 7.16.4 have comparable binding affinity against the p185 receptor, they were used at comparable molar concentration in this assay. However, AHNP was used at a higher concentration since its affinity is lower against p185Her2/neu than 4D5.

AHNP was also expressed as a streptavidin fusion protein using the M13 phage system. The cDNA for the core streptavidin sequence from *Streptomyces avidinii* was cloned by PCR using a 5'PCR primer which also contains the sequence for a (Gly4Ser)2 linker. Complementary oligonucleotide encoding AHNP and an additional Gly4Ser linker were synthesized and annealed to form the double strand DNA. After ligation, the fusion consists of AHNP at the N-terminus of a (Gly4Ser)3 linker followed by the core streptavidin (SA) sequence. The linker region is introduced to produce flexibility between the tetrameric AHNP mimetic and SA which normally resembles a tetramer. Both long linkers comprising 3 or more Gly4Ser linkers and short linkers comprising 2 or 1 linkers be used in these fusion proteins. However, longer linkers of 3 Gly4Ser linkers are preferred as they provide for greater flexibility.

The fusion construct was cloned into the SalI-ApaI site in the phage expression vector phage3.22 (Maxim Biotechnology, CA). The fusion was expressed first in the pIII phage protein reading frame of the TG1 bacterial strain. AHNP-SA fusion can also be expressed in another bacterial host strain (HP2151) free of the pIII component. A chimeric protein consisting of the extracellular domain of p185Her2/neu and the immunoglobulin Fc region (Her2-Fc) was used to test the binding affinity of the expressed AHNP-SA forms. Her2-Fc was coated onto a 96-well immunoassay plate and the phage supernatant was added to the plate.

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Biotin-conjugated peroxidase was then used to detect binding
activity of AHNP-SA molecule to purified Her2-Fc.

Thus, since, as shown herein, the AHNP-SA species is
amenable to polymerase-based amplification when attached to
5   a biotinylated oligonucleotide, the AHNP-SA species provides
a vehicle to develop a rapid and diverse proteomics
approach.

A CDR mimetic of anti-EGFr molecules, AERP, which a
different number of constrained amino acids has also been
10   developed and shown to have nM affinity for EGFr.

A preferred means for detection in the present
invention comprises staining with a fluorescent dye. In
this embodiment, after RNA amplification with a polymerase
such as T7 RNA polymerase, T3 RNA polymerase, SP6 RNA
15   polymerase, φ29 polymerase or Taq polymerase, a portion of
the reaction mixture can be mixed with a fluorescent dye
such as RiboGreen reagent (Molecular Probes, Inc) (U.S.
Patent 5,436,134), a unsymmetrical cyanine dye that binds
to RNA directly in the solution and then releases
20   fluorescence signals. Examples of other fluorescent dyes
with similar properties useful in this method include, but
are not limited to, PicoGreen, TOTO-1 or YOYO-1. The
reactions are preferably performed in microplates and the
fluorescence is read using a fluorimeter such as the
25   Spectra Fluora 5 to 15 minutes after mixing RNA solutions
with RiboGreen dye.

In the method of the present invention, a mixture of
epitope detectors comprising either monoclonal antibodies
to selected epitopes, single chain Fvs for selected
30   epitopes, or constrained epitope specific CDRs, CDR mimetics
or engineered CDR structures, conjugated with cDNAs of
different lengths can be used in a single reaction to probe
the cell lysate to provide a profile of proteins in the cell
lysate via automatic sequencing. In this method, after
35   amplification and staining of the amplified products with

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a fluorescent dye, the reaction mixture is separated by electrophoresis and the size of the RNA products are visualized by fluorescent dyes or probes. Moreover, specific probes can hybridize with the reaction mixture and
5 reveal the presence and abundance of the corresponding antigens.

The sensitivity of the fluorescence detection method of the present invention was demonstrated for the p185 receptor. In these experiments, a chimeric p185-Fc
10 polypeptide was first coated directly to a 96-well plate. The 4D5 monoclonal antibody conjugated with a 1 kb cDNA was then used to detected the chimeric p185-Fc receptor. Since the cDNA contained a T7 promoter, it was amplified by the T7 RNA polymerase. After amplification, the RNA product was
15 mixed with the RiboGreen reagent in a 96-well plate and excited at 485 nm after 5 to 10 minutes. The fluorescent reading was collected at 535 nm. Using this method, concentrations as low as 0.177 pg/ml were detected.

Coupling of a ds-oligo directly to the CDR or single
20 chain Fv can be a time-consuming procedure, particularly if the purpose is to detect hundreds or thousands of antigens in a mass screening proteomic assay. In addition, variation in the coupling efficiency can complicate the interpretation of the amplification results. Accordingly,
25 in a preferred embodiment of the present invention, the Fv or CDR contains a general or universal epitope such as an hemagglutinin HA tag or polyhistidine tag. An example of a general or universal epitope is the poly-His-tag in the 7.16.4ScFv initially designed for the purification of the
30 protein. A single monoclonal antibody or single chain Fv coupled with ds-DNA is then used to bind to the general epitope to create a universal epitope detector. The efficacy of a universal epitope detector in the method of the present invention was demonstrated with the poly-His-tag
35 in the 7.16.4ScFv. In these experiments, p185 receptors

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were captured by A11 coated on the plate, free 7.16.4ScFv
was added, followed by extensive washing and then incubating
with ds-oligo conjugated anti-His antibody.   After T7
polymerase amplification, specific bands from lysates of
5 10^{-6} dilution of the cells were detected. Accordingly, this
sensitivity was even higher than that seen with the basic
protocol without a universal epitope detector.

The method of the present invention is also useful
in the detection of post-translation modifications. PCR and
10 aRNA techniques were originally developed to detect the
activity of target genes at the DNA level. These methods
have been adopted exclusively in the application of genomics
research, sometimes combined with hybridization. Regardless
of sensitivity, these methods are not able to detect the
15 post-translation   modification   at   the   protein   level.
Monitoring of such events, however, is very critical since
many   modifications   including,   but   not   limited   to,
phosphorylation   and   glycosylation   are   related   to   the
functional status of the protein. Thus, experiments were
20 performed to demonstrate the ability of the method of the
present invention to detect the phosphorylation of the p185
receptor induced by EGF treatment. A signaling model was
established   in   which,   upon   EGF   stimulation,   EGFR
heterodimerizes with and trans-activates p185, resulting in
25 the   phosphorylation of tyrosine   residues   on   the   p185
receptor (Qian et al. Proc. Natl Acad. Sci. 1994 15:1500-
1504). The A431 cell line, which overexpresses EGFR as well
as p185 erbB2, was used in these experiments. After EGF
stimulation,   the   p185   receptor   in   the   cell   lysate   was
30 captured by 1E1, a monoclonal antibody developed against
p185erB2/neu. PY99, an IgG2b type of anti-phosphorylated
Tyr antibody, was used to detect phosphorylated receptors.
A second antibody, anti-IgG2b, coupled with ds-oligo, was
used to probe the antigen-antibody sandwich complex. A431
35 cells stimulated with EGF produced a positive band, which

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was not observed in cells without EGF treatment. T6-17 cells, however, also showed a positive band, indicating constitutive phospho-tyrosine on the p185 receptor. These data indicate that this method is capable of detecting the
5 functional status of a protein by analyzing its modification. Epitope detectors comprising an Fv or CDR coupled to the ds-oligo can also be used to detect the functional status of the protein.

Thus, the present invention provides a sensitive
10 detection method which eliminates concerns about the non-quantitative nature of immuno-PCR techniques and which offers vast potential in the field of proteomics. By using a polymerase which recognizes a specific promoter such as T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase,
15 φ29 polymerase or Taq polymerase as well as the specific promoter in the amplification step, assays performed in accordance with this method possess linear amplification and precise quantification which are relevant to biological and medical assays. The number of factors that affect the
20 sensitivity of detection have also been reduced. The specific binding between antigens and their Fv or monovalent CDR, CDR mimetic or engineered CDR structure is the only critical parameter of this method.

The ability to provide universal epitope detectors
25 provides the method of the present invention with multiple additional advantages. First, any cellular antigens can be detected without having been first coupled to a monoclonal antibody with ds-oligo. Without the universal probe, the method would only be useful in looking at one or several
30 particular antigens at a time. The universal probe, on the other hand, allows for the detection of any cellular or fluid residing antigen with available Fvs or CDRs, CDR mimetics or engineered CDR structures. In addition, with slight modification in the protocol, different proteins can
35 be detected simultaneously in a single electrophoresis lane

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when oligonucleotides of different sizes are attached to the Fv or CDR, CDR mimetic or engineered CDR structure of the epitope detector. Thus, as demonstrated herein, the method of the present invention provides a versatile technique that

5   is applicable in the identification of protein antigens as well as post-translational modification of polypeptides and other structures such as sugars or lipids at the single cell level of detection.

The method of the present invention is also useful in

10   the analysis of interactions of molecules and the detection of small molecules. For example, ligand peptides can be used as epitope detectors on tissue samples to identify the expression of specific receptors, or verse visa. With available Fvs, CDRs, CDR mimetics or engineered CDR

15   structures, or binding proteins, small molecules such as toxins or drug metabolites, can be detected in any solution including, but not limited to, water, foods, and body fluids.

To study the interactions of molecules, a two-

20   component (molecule A/molecule B) interaction system is developed. The two components, molecule A and molecule B may comprise proteins, sugars, or other types of chemical entities including, but not limited to, carbohydrates, DNA or RNA, or peptides with structural conformations such as

25   alpha helices or beta-sheets. To develop this two-component system, an epitope detector such as an antibody for a first molecule, referred to hereafter as molecule A, is placed in proximity with a sample comprising molecule A so that molecule A binds to the epitope detector. A solution

30   containing products of an expression library constructed so that each expressed protein also contains a HA tag or similar tag can then be added to identify molecules which interact with molecule A. For purposes of the present invention, these molecules are referred to herein as

35   molecule B or a second molecule. Alternatively, normal

cellular extracts or lysates or any fluid containing potential molecule B can be used.

If the second molecule, molecule B, in the library product or cellular extract or lysate binds to the first
5   molecule A bound by the epitope detector, the new molecules can be detected with either a universal detector that binds to the tag or marker or by the use of a CDR library or a scFv library specific against molecule B. In a preferred embodiment, monitoring of the interaction between molecule
10 A and molecule B is performed by the fluorescence based quantifiable assay which detects the amplified nucleic acid sequence from the oligonucleotide conjugated to the universal detector.

When one of the molecules of the two-component
15 (molecule A/molecule B) interaction system binds a ligand or pharmaceutical drug, the two-component system can be used to investigate the effects of the ligand or drug on the binding of molecule A to molecule B. Accordingly, the present invention also provides an *in vitro* system for monitoring
20 drug affects on interactions of molecules. The ligand or drug can be added at any step in the assay to determine how the ligand or pharmaceutical drug alters the binding of molecule A to molecule B. In addition, more than one drug can be added to the two-component interaction system. For
25 example, a second drug such as an antagonist of the first drug can be added and the level of binding of A to B as well as to the complex of A and B can be determined. Further, instead of known ligands or drugs, a third solution containing the products of another library of molecule C
30 marked with other tags could be added to the complex of A and B and the effects of this third solution on binding can be determined.

Thus, the present invention provides a rapid *in vitro* screening assay with a biological readout, namely the
35 formation of a complex interaction. Further, using this type

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of system it is possible to build screening systems that act like organic analogue computers whose output is dependent on the number of events developed with each progressive addition. These progressive events are disturbed upon

5   addition of a third molecule such as a pharmaceutical drug or ligand that interferes with this assembly. The quantification of signals before and after addition of the third molecule defines the change in output. A positive change means that the pharmaceutical drug or ligand

10 facilitates the binding of molecule A to molecule B, while a negative change means that the pharmaceutical drug or ligand inhibits the binding of molecule A to molecule B.

Active CDRs, CDR mimetics or engineered CDR structures can also be used as starting template molecules to derive a

15 spectrum of CDRs, CDR mimetics or engineered CDR structures to identify and study via the present invention families of proteins associated with a particular disease state, i.e the erbB family and it association with origin and early detection of malignancy. For example, a library has been

20 developed containing a large and diverse population of CDR mimetics ($>10^6$) of a single ring size that develop into constrained turns and also possess aromatically modified termini. This particular library was produced using the M13 phage system and contains a large repertoire of CDR-

25 streptavidin binding moieties diversified from a 6 membered AHNP peptide through randomization. For diversification of the library, the synthesized oligonucleotide library encoding randomized CDR regions, but with a fixed constrained framework region, are inserted into the phage construct that

30 leads to the expression of the highly varied CDR streptavidin fusion proteins.

CDR-SA libraries can be used in the present invention, for example, to screen transformed cell lines and tumor cells for tumor surface markers. In this embodiment, initial

35 screening preferably employs fluorescence based

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microfluorimetry for binding to cells followed by an ELISA
type assay for binding to captured proteins derived from cell
lysates. For example, a monoclonal antibody can be attached
to PI3-kinase or ras and any bound CDR-SA forms can be
5 detected. The sequence of any unknown targets of tumor
specific selected CDR-SA molecules can then be determined.
In one embodiment, purified CDR-SA is used to screen a cDNA
expression library constructed in cells such as COS7 cells
and in expression libraries prepared from freshly isolated
10 tumor tissues from various stages of malignancy.

Libraries of CDRs, CDR mimetics or engineered CDR
structures can also be used to generate a spectrum of probes
to detect receptors, proteins associated upon activation of
the receptors and proteins involved in pathways associated
15 with the receptors. For example, a CDR mimetic library can
be used to generate a spectrum of probes to erbB receptors,
proteins associated upon activation of erbB receptors and
proteins involved in pathways associated with erbB receptors
such as the PI-3 kinase pathway.

20 CDRs or CDR mimetics of libraries defined as binding
to a receptor, proteins associated upon activation of a
receptor or proteins involved in pathways associated with the
receptor are can also be used therapeutically to target the
receptor or protein.   In a preferred embodiment for
25 therapeutic use, the CDR or CDR mimetic is reinserted into
a humanized antibody framework or attached to an Fc. In this
embodiment, the engineered CDR structure may be administered
alone or may further comprise a radiolabel or cytotoxin
attached thereto.

30 Methods for preparing scFv libraries with some
randomly changed CDRs are described by Winter et al. in U.S.
Patent 6,291,650, U.S. Patent 6,225,447, U.S. Patent
6,172,197 and U.S. Patent 6,140,471. However, production of
CDR-SA libraries are not described by Winter et al.

Also provided in the present invention are kits for performing the methods of the present invention. In one embodiment, a kit is provided for the detection of molecules expressing a selected epitope. In a preferred embodiment, detection of the molecule is performed via a fluorescent dye which stains nucleic acid sequences. Thus, this kit preferably comprises an epitope detector comprising an oligonucleotide attached to a monoclonal antibody for the selected epitope, a single chain Fv for the epitope or a constrained epitope specific CDR, CDR mimetic or engineered CDR structure, as well as an RNA polymerase, an amplification reaction buffer, and a fluorescent dye. In another embodiment, kits are provided for profiling proteins in a mixture such as a cell lysate. In this embodiment, the kit preferably comprises a mixture of epitope detectors comprising monoclonal antibodies for selected epitopes, single chain Fvs for selected epitopes or constrained epitope specific CDRs conjugated with cDNAs of different lengths, as well as an RNA polymerase, an amplification reaction buffer, and a fluorescent dye.

The original immuno-PCR used pure antigens in the assay. Later iterations of immuno-PCR examined mixed antigens (Hendrickson et al. Nucleic Acids Research 1999 23(3):522-529) but only showed sensitivity of two to three orders of magnitude higher than ELISA. In a real-world assay with the background comprising a huge variety of non-specific antigens, sensitivity is always limited by the specificity of the assay. Epitopes bound by the Fvs or CDR fragments are expected to identify larger polypeptides and can be used to identify motifs in supernatants, fluids, extracts of cells or bacteria or any other eukaryotic organism. Further, actual identity of the polypeptides, organic molecules or sugar structures can be determined by computer aided analysis of data bases using the binding of several epitopes by Fvs as a guide. For example, binding by Fv a, d, e, and f would

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identify a sugar molecule as having side chains a, d, e, and
f, and hence belonging to a family of sugars having these
same side chains. In this way the present invention allows
definition and identification of many, if not all molecules
5 in a cell at any one particular time. Moreover this approach
can be used to identify alternative transcriptional forms
translated in an active cell or cellular supernatant. This
procedure is easily amenable to 1) use with nonradioactive
detection methods, most preferably fluorescent dyes 2)
10 microtized liquid handling procedures, 3) low sample volume
detection such as "protein chip" analysis and 4)
robotization. For example, a chip can be developed which
contains multiple binding elements or units and a single
universal epitope detector.   A binding element that
15 recognizes a common surface on the monoclonal antibody, the
single chain Fv, or the CDR, CDR mimetic or engineered CDR
structure, such as a universal antibody can be used.
Alternatively, avidin can by built into each antibody, single
chain Fv or CDR, CDR mimetic or engineered CDR structure and
20 biotin can be coupled to the oligonucleotide. These chips
provide the advantage of more rapid and higher affinity
thereby multiplying the signal.

The following nonlimiting examples are provided to
further illustrate the present invention.

25 **EXAMPLES**

Example 1: Materials

Antibodies used include mAbs 7.16.4 and A11, each of
which is reactive with a different epitope in the
extracellular domain of p185neu.   1E1 is an IgG1 monoclonal
30 antibody generated against the ectodomain of human
p185her2/neu.   rhuMAb 4D5 (Herceptin) was obtained from
Genentech. The anti-phosphotyrosine monoclonal antibody PY99
was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).
The cell line B104-1-1 was derived from NIH3T3 cell by
35 expressing rat oncogenic p185neu. The expression level of

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p185neu in B104-1-1 cells was determined using an ^{125}I-labeled anti-neu mAb binding assay. NR6, negative for both EGFR and p185neu, was a subclone from NIH3T3. T6-17 was derived from NIH3T3 by over-expressing the human p185her2/neu receptor.

5   These cell lines were all cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone) at 37°C in a 5% CO_2 atmosphere.

Example 2: Expression and Purification of His-tagged 7.16.4

LB media (150 ml) containing 50 μg/ml ampicillin was
10 inoculated with a 15 ml overnight culture of *E. coli* DH5 α and maintained at 30°C until an optical density of 0.5 (600 nm) was obtained. Isopropyl-β-(-D-thiogalactopyranoside) (IPTG) was added at a final concentration of 1 mM to induce His-tagged 7.16.4ScFv. After 3 hours, cells were harvested
15 and lysed by freezing and thawing and resuspended in 10 ml of urea lysis buffer (10 mM Tris-Cl, pH 8.0, 0.1 M NaH_2PO_4, 1 M NaCl, 8 M urea) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 2 μM leupeptin. The insoluble cellular debris was removed by
20 centrifugation (12,000 x g for 15 minutes, followed by 12,000 x g for 30 minutes). The clarified supernatant was mixed with 2 ml of Ni-NTA agarose followed by gentle shaking on ice for 1 hour. The mixture was loaded into an empty column and unbound protein was washed with urea lysis buffer (pH 6.3)
25 at 4°C. His-tagged protein was eluted with urea lysis buffer (pH 4.5), and eluate fractions were examined by SDS-PAGE and FACS analysis as described in Example 3. Proteins in the peak fractions were pooled and dialyzed against TKC buffer (50 mM Tris-Cl, pH 8.0, 100 mM KCl, 10 mM $CaCl_2$, 1 mM EDTA,
30 0.1 mM PMSF).

Example 3: Confirmation of 7.16.4ScFv binding by FACS Analysis

Cell-surface antigens were detected by Fluorescence-activated cell sorting (FACS). B104-1-1 cells
35 (about 5x10^{5}) were incubated (30 minutes at 4°C) in 200 μl of

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FACS buffer (PBS containing 0.5% bovine serum albumin and 0.02% sodium azide) containing the purified 7.16.4ScFv. Cells were then washed in FACS buffer and incubated (30 minutes, 4°C) with anti-His antibody (Invitrogen). After

5 this second incubation, cells were washed again in FACS buffer and further incubated with FITC-labeled IgG against mouse immunoglobulins (Sigma Chemical Co., St. Louis, MO). After washing with FACS buffer, the cell pellet was suspended in PBS buffer and processed for analysis by a FACS scan flow

10 cytometer (Becton Dickinson). For each sample, 10,000 viable cells were gated following size (forward scatter, FSC) and granularity (side scatter, SSC) parameters and analyzed with CellQuest Software (Becton Dickinson). For competitive binding, B104-1-1 cells were first incubated with the

15 monoclonal antibody 7.16.4 in the presence of different concentrations of 7.16.4ScFv. Cells were then washed in FACS buffer and further incubated with FITC-labeled IgG against mouse immunoglobulins (Sigma Chemical Co.) before analysis on the flow cytometer.

20 **Example 4: Synthesis of biotinylated ds-cDNA to and streptavidin conjugates**

An oligonucleotide of the following sequence (GGCTAACTAGAGAACCCACT (SEQ ID NO:1)) was synthesized with a biotin at the 5' end to allow for binding to streptavidin or

25 avidin. Another oligonucleotide primer (GCTGGGATCTGTCTCTACAA (SEQ ID NO:2))was derived from an internal sequence from the VCP cDNA. Using these two primers, a cDNA fragment of ~ 0.6 kb was amplified from the plasmid pcDNA-VCP. This fragment contains a biotin at the 5' end and a T7 promotor site

30 (TAATACGACTCACTATAGGG (SEQ ID NO:3)) used to direct the synthesis of RNA from the cDNA template through the enzymatic activity of T7 RNA polymerase. For attachment of 2.5 μg of antibody to 10 μg of streptavidin, a half volume of 0.8% glutaraldehyde was added to 4 μl aliquots. The solution was

35 mixed on a rotation device for 3 hours at room temperature.

Ethanolamine (1 M; 1/20 volume; pH 7.5) was then added. The solution was mixed for an additional 2 hours at room temperature. The antibody-streptavidin complex was stored at 4°C.

Example 5: RNA Amplification Procedure

5   Ninety-six well microtiter plates (Nunc-Immuno Plate, MaxiSorpTM) were coated with different concentrations of chimeric p185 extra-cellular domain-Fc polypeptide by incubating plates overnight at 4°C with 100 µl of coating buffer (antibody concentration: 5 µg/ml). Plates were then washed three times with PBS containing 0.2% TWEEN 20 (PBS-T; 200 µl/well), blocked with PBS containing 0.5%

10 FBS and 0.2% TWEEN 20 (200 µl/well) for 1 hour at room temperature, and washed again three times with PBS-T (200 µl/well). After this incubation step, plates were washed with PBS-T (six times, 200 µl/well) and incubated with humanized anti-p185Her2 antibody 4D5-streptavidin conjugate (150 ng/ml, 50 µl/well) for 2 hours at room temperature. Plates were washed with PBS-T (six times) and incubated with

15 biotinylated cDNA (800 ng/ml, 0.05 ml/well) for 1.5 hours. Subsequently, plates were washed six times and RNA amplification was performed. The following reagents were added: 1X RNA amplification buffer (50 mM Tris (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM Spermidine; 10 mM DTT; 750 µM ATP, UTP, GTP and CTP; 0.5 µl RNAsin; 200 U T7 RNA. The solution was then incubated for 4 hours at 37°C. The

20 RNA product was removed from the wells and 25 µl was diluted with 75 µl TE buffer and then mixed with 100 µl of 2000-fold diluted RiboGreen reagent. Ten minutes later, samples in the 96-well plate were excited at 485 nm, and the fluorescence emission was collected at 535 nm using a Spectra Fluora reader (Tecan, Austria).

Any discussion of documents, acts, materials, devices, articles or the like which

25 has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   A method for detecting molecules that express a selected epitope comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected
5 surface;

(b) contacting the surface with an epitope detector so that the epitope detector
binds to immobilized molecules on the surface, said epitope detector comprising

(i) an oligonucleotide comprising an RNA promoter; said oligonucleotide
attached to

10   (ii) a monoclonal antibody that specifically binds to the selected epitope, a
single chain Fv that specifically binds to the selected epitope, a constrained CDR that
specifically binds to the selected epitope, a CDR mimetic that specifically binds to the
selected epitope, or an engineered CDR structure that specifically binds to the selected
epitope;

15   (c) producing an amplified RNA product from the oligonucleotide by RNA
amplification; and

(d) detecting the presence of the amplified RNA product by a method selected
from the group consisting of:

(i) contacting the amplified RNA product with a fluorescent dye which
20 stains the RNA product and measuring a quanta of fluorescence signals emitted from
stained RNA, wherein fluorescence emitted from stained RNA which is directly
proportional to epitope detector bound to the selected epitope on molecules
immobilized on the surface, and

(ii) further amplifying the RNA product using reverse transcriptase or
25 replicase and detecting a product of the further amplification by contacting said product
of further amplification with a fluorescent dye or probe which stains said product of
further amplification and measuring fluorescence emitted from the stained product,
wherein detection of a product of the further amplification is directly proportional to
epitope detector bound to the selected epitope on molecules immobilized on the
30 surface.

2.   A method for quantifying molecules that express a selected epitope comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected
surface;

35   (b) contacting the surface with an epitope detector so that the epitope detector
binds to immobilized molecules on the surface, said epitope detector comprising

    (i) an oligonucleotide comprising an RNA promoter; said oligonucleotide attached to

    (ii) a monoclonal antibody that specifically binds to the selected epitope, or a single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected epitope;

    (c) producing an amplified RNA product from the oligonucleotide by RNA amplification;

    (d) contacting the amplified RNA product with a fluorescent dye which stains the amplified RNA product; and

    (e) quantifying a quanta of fluorescence signals emitted from the stained RNA product, wherein the quantity of fluorescence emitted is directly proportional to the number of molecules that express the epitope present in the sample.

3. A method for monitoring interaction of molecules *in vitro* using a two-component system comprising:

    (a) immobilizing a first molecule to a solid surface;

    (b) adding to the solid surface a second molecule that interacts with the first molecule, wherein the second molecule comprises a universal epitope;

    (c) adding to the solid surface a universal epitope detector conjugated to an oligonucleotide that comprises an RNA promoter;

    (d) performing RNA amplification;

    (e) contacting the amplified RNA product with a fluorescent dye which stains the amplified RNA product; and

    (f) measuring a quanta of fluorescence signals emitted from stained RNA, wherein fluorescence emitted from stained RNA is directly proportional to binding of the first molecule to the second molecule.

4. The method of claim 3 wherein the first and second molecules are proteins, sugars, carbohydrates, DNA, RNA, or peptides with structural conformations.

5. A method of monitoring interaction of molecules *in vitro* comprising:

    (a) using a two-component system in accordance with the method of claim 3 or 4;

    (b) adding third molecule to the two-component interaction system; and

(c) monitoring effects of the third molecule on the binding and interaction of said first and second molecules of said two-component system by measuring changes in fluorescence wherein a positive change in fluorescence is indicative of the third molecule facilitating binding of the first and second molecule and a negative change in fluorescence is indicative of the third molecule inhibiting binding of the first and second molecule.

6.   The method of claim 5 wherein the third molecule comprises a ligand or a pharmaceutical drug.

7.   A method for identifying a CDR structure that specifically binds to a specific epitope, a CDR mimetic structure that specifically binds to a specific epitope or an engineered CDR structure that specifically binds to a specific epitope, the method comprising:

(a) immobilizing a molecule that expresses a selected epitope to a selected surface;

(b) contacting the surface with a library of CDRs, CDR mimetics or engineered CDR structures wherein:

(i) the CDRs, CDR mimetics or engineered CDR structures of the library are each linked to an oligonucleotide comprising an RNA promoter; or

(ii) the CDRs, CDR mimetics or engineered CDR structures of the library comprise a universal epitope and an epitope detector that binds to the universal epitope is further added, wherein the epitope detector comprises an oligonucleotide comprising an RNA promoter

(c) producing an amplified RNA product from the oligonucleotide by RNA amplification;

(d) detecting the presence of amplified RNA product by contacting the amplified RNA product with a fluorescent dye which stains the amplified RNA product, and

(e) measuring a quanta of fluorescence signals emitted from the stained RNA product, wherein fluorescence emitted from the stained RNA product is directly proportional to identifying a CDR structure that specifically binds to a specific epitope on molecules immobilized on the selected surface, a CDR mimetic structure that specifically binds to a specific epitope on molecules immobilized on the selected surface or an engineered CDR structure that specifically binds to a specific epitope on molecules immobilized on the selected surface.

8.   The method of any one of claims 1 to 7 wherein the selected surface is selected from the group consisting of: a chip, a 96-well plate and 384 well-plate.

9.   The method of any one of claims 1 to 8 wherein the oligonucleotide is linked to the monoclonal antibody, a single chain Fv, a constrained CDR, a CDR mimetic or engineered CDR structure either:
        (a) by biotin-streptavidin linkers; or
        (b) the oligonucleotide being conjugated to a molecule that specifically binds to an epitope on the monoclonal antibody, a single chain Fv, a constrained CDR, a CDR mimetic or an engineered CDR structure.

10.   The method of any one of claims 1 to 9 wherein the oligonucleotide is a double stranded cDNA molecule.

11.   A method for profiling proteins in a cell lysate comprising:
        (a) contacting the cell lysate with a mixture of epitope detectors so that the epitope detectors specifically binds to selected epitopes on proteins present in the cell lysate, each of said epitope detectors comprising:
            (i)   cDNA comprising an RNA promoter; said cDNA conjugated to
            (ii)   a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or engineered CDR structure that specifically binds to the selected epitope wherein epitope detectors that specifically bind to the same specific epitope are conjugated to cDNAs that produce RNA amplification products of the same length, and epitope detectors that specifically bind to different specific epitopes are conjugated to cDNAs that produce RNA amplification products of different lengths;
        (b) performing RNA amplification;
        (c) separating amplified RNA by size
        (d) contacting the amplified RNA with a fluorescent dye that stains RNA,
        (e) visualizing separated stained amplified RNA and identifying the presence of the different size amplified RNA so that the profile of proteins expressing different selected epitopes in the lysate can be determined.

12.   The method of claim 11 wherein the amplified RNA is separated by size by electrophoresis.

13.   The method of any one of claims 1 to 12 wherein the RNA promoter is selected from the group consisting of: a T7 RNA promoter, a T3 RNA promoter and an SP6 RNA promoter.

5   14.   The method of any one of claims 1 to 13 wherein the fluorescent dyes is selected from the group consisting of: an unsymmetrical cyanine dye.

15.   A kit when used for the detection or quantification of molecules that express a selected epitope using fluorescence comprising:

10   (a) an epitope detector comprising

            (i) an oligonucleotide comprising an RNA promoter; the oligonucleotide linked to

            (ii) a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that

15   specifically binds to the selected epitope, or a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected epitope;

        (b) an RNA polymerase;

        (c) an amplification reaction buffer; and

20   (d) a fluorescent dye that binds to RNA.

16.   A kit when used for profiling proteins comprising:

        (a) a mixture of epitope detectors that specifically bind to selected epitopes on proteins present in the cell lysate, each of said epitope detectors comprising

25   (i) cDNA comprising an RNA promoter; said cDNA linked to

            (ii) a monoclonal antibody that specifically binds to the selected epitope, a single chain Fv that specifically binds to the selected epitope, a constrained CDR that specifically binds to the selected epitope, a CDR mimetic that specifically binds to the selected epitope, or an engineered CDR structure that specifically binds to the selected

30   epitope; wherein epitope detectors that specifically bind to the same specific epitope are conjugated to cDNAs that produce RNA amplification products of the same length, and epitope detectors that specifically bind to different specific epitopes are conjugated to cDNAs that produce RNA amplification products of the different lengths;

        (b) an RNA polymerase;

35   (c) an amplification reaction buffer; and

        (d) a fluorescent dye that binds to RNA.

17.   The kit of claim 15 or 16 wherein the oligonucleotide is linked to the monoclonal antibody, a single chain Fv, a constrained CDR, a CDR mimetic or an engineered CDR structure either:

     (a) by biotin-streptavidin linkers; or

5   (b) the oligonucleotide is conjugated to a molecule that specifically binds to an epitope on the monoclonal antibody, a single chain Fv, a constrained CDR, a CDR mimetic or an engineered CDR structure.

18.   The kit of any one of claims 15 to 17 wherein the oligonucleotide is a double
10   stranded cDNA molecule.

19.   The kit of claim 15 to 18 wherein: the RNA promoter is T7 RNA promoter and the RNA polymerase is T7 RNA polymerase, the RNA promoter is a T3 RNA promoter and the RNA polymerase is T3 RNA polymerase, or the RNA promoter is a SP6 RNA
15   promoter and the RNA polymerase is SP6 RNA polymerase.

20.   The kit of any one of claims 15 to 19 wherein the fluorescent dye is an unsymmetrical cyanine dye.

20   21.   A method for detecting molecules that express a selected epitope substantially as hereinbefore described with reference to the examples, excluding the comparative examples.