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(54) **Title**: EMULSION SELECTION OF ANTIBODIES

(57) **Abstract**: Amplification of an antibody onto a solid support in an emulsion yields a reagent that provides an increased signal for use in detecting antibody/antigen interactions. The amplification proceeds by growing antibody-producing cells in emulsions in the presence of a solid support. The antibodies can bind to the solid support either by an antigen/antibody interaction or by another interaction, for example via a protein tag such as His6. The combination of emulsions and solid support permits the amplification. The use of antibody producing cells within the emulsions permits the phenotype to be linked to the genotype.

EMULSION SELECTION OF ANTIBODIES

TECHNICAL FIELD OF THE INVENTION

[01] This invention is related to the area of antibodies. In particular, it relates to methods for identifying and obtaining desirable antibodies.

BACKGROUND OF THE INVENTION

[02] Detailed knowledge of mRNA and protein expression, protein-protein interaction and genetic data is a key to elucidating gene regulatory networks, biological pathways and cellular responses to various perturbations, e.g. disease and response to treatment. The ability to analyze these data rapidly, effectively and comprehensively is a gateway for understanding cellular function. As a result, there is growing interest in high-throughput, high-content technologies that can be applied routinely to separate, identify and characterize these molecules on a global scale. Although such technologies are used for simultaneously characterizing the genome and genomic expression, analogous applications are not readily available for multiplexed analysis of the proteome and protein expression.

[03] For some characterizations, *e.g.,* analysis of polymorphisms and mutations, nucleic acid measurements provide useful information and these aspects have been comprehensively studied in efforts to better understand cellular events. However, because proteins, not nucleic acids, carry out key metabolic processes and since the relationship between the levels of an mRNA and the protein it encodes is inconsistent at best, there is a need to directly measure proteins accurately and comprehensively as well. In addition, a considerable proportion of proteins in the proteome undergo posttranslational

modification (post translational modification) and neither DNA nor RNA analysis provides any information regarding the extent of such modification. This creates a substantial gap in our effort to understand cellular behavior because post translational modification is known to be extensively involved in regulatory processes.

[04] Though the technical challenges for proteome analysis are far greater than those for genome analysis, new innovative technologies and tools are being developed to provide solutions for the post-genomic era. Affinity reagents, and in particular antibodies (Abs), are molecular tools that greatly expand our ability to characterize and study proteins, both qualitatively and quantitatively. Antibodies allow researchers to identify, track, capture, quantify, control and image individual proteins. To be useful in characterizing protein levels in a biological sample, antibodies should detect protein levels with a linear dose response, preferably at a range as low as 20–200 pg/ml, but also quantitatively at higher levels and with an extensive dynamic range. The antibodies employed should display strong and specific signals. Ideally, the antibodies would be recombinant to enable maximum molecular biological manipulation.

[05] Although assays (typically enzyme-linked) using antibodies and other affinity reagents (*e.g.,* receptors for measuring ligands and vice versa) have been developed for measurement of protein levels in biological samples and are broadly used, they typically have neither the desired sensitivity nor the dynamic range of methods that have been implemented for tracking levels of individual nucleic acids.

[06] While chip and bead arrays permit accurate measurement of nucleic acids in a convenient and highly parallel fashion, efforts to provide reagents for simultaneous quantitation of large numbers of different proteins have been disappointing. Analyzing protein arrays is a complex task: typically the antibodies used have differing, non-matched affinities for their antigen (Ag) targets, the sample proteins label unevenly with colorimetric or fluorimetric dyes and, for the latter, quenching or enhancing of neighboring fluorescent molecules can distort the data obtained. Not least, there is the issue of a limitation of suitable antibodies; to measure the proteome comprehensively, one would require tens of

thousands of different antibodies, and preferably monoclonal antibodies (mAbs), of high specificity for their targets as well as relatively similar affinities so that they could act better as a matched reagent set, as oligonucleotides do with respect to nucleic acid analyses.

[07] Limitations on generating a matched set of mAbs targeting the entire proteome are related to both technical issues and cost considerations. Unlike nucleic acid analysis, in which oligonucleotide reagents can be readily predicted and prepared based on knowledge of the sequences of the targets, affinity reagents such as antibodies must typically be individually selected for desired properties of affinity and specificity from among massive libraries. Not only is this process typically time consuming and expensive, but for most of the technologies employed, prior to selecting the desired antibodies it is necessary to synthesize or purify the antigens. This carries additional challenges, sometimes considerable, with respect to technology and cost. The situation is exacerbated in efforts to characterize the entire proteome because identification of antibodies and other affinity reagents that can reliably distinguish between pinpoint differences in protein sequence or structure, e.g., polymorphisms or the difference between a protein with and without post translational modification, is particularly difficult. As noted above, because nucleic acid analysis does not provide information regarding the state of post translational modification of their encoded proteins, determination of levels of post translational modification can only be made directly on the proteins per se and so there is a particular need for antibodies that can reliably distinguish between the same protein with and without post translational modification.

[08] IgG antibodies are comprised of two heavy chains and two light chains. Diversity in antibody-antigen interactions is achieved through the somewhat independent interaction of six short (3-25 amino acids) variable complement-defining regions (CDRs) in the Fab portion of the Ab. An Fab portion can be used as an abbreviated Ab structure, as can single chain Ab variable region fragments (scFvs), which contain the CDRs of heavy and light chains combined into a single peptide. scFvs are ideally suited for molecular screening because they can be cloned and manipulated as individual peptides while

retaining the ability to bind antigen with high affinity. Moreover, most scFvs can be converted to, and retain activity as, conventional two-chain antibodies, if desired.

[09] Several projects have been initiated to generate antibodies on a large scale. For example, Uhlen et al. over-produce short peptides in *E. coli* corresponding to unique amino acid sequences of individual proteins (Kampf 2004; Lindskog 2005; Uhlen, 2005, 2005a). They then inoculate rabbits with these proteins and purify the resultant antibodies from the sera. Several thousand new antibodies are being generated using this method. However, the antibodies are polyclonal, the amount of each Ab generated and purified is miniscule and thus the commercial opportunity of this method is limited. As such the project is primarily a research project.

[10] A number of technologies have been proposed and/or used for the rapid and cost-effective production of mAbs, including phage display, yeast display, yeast-two hybrid (Y2H) and microfluidics. Each of these techniques carries its own advantages and disadvantages for various applications in which mAbs are desired, but none of these satisfies all of the criteria necessary for providing a full complement of mAbs for characterizing the proteome.

[11] One of the most widely adopted techniques for selecting mAbs in vitro is phage display, wherein the mAbs are displayed on the surface of a filamentous bacteriophage (Lee 2004; Sheets 1998; Schofield 2007). Among bacteriophage that are desirable for use in mAb selection is M13. In these methods a library of scFv antibodies is displayed on the surface of M13 bacteriophage gpIII as genetic fusions to the gpIII protein. M13 is a male-specific single-stranded filamentous bacteriophage of E. coli, which infects its host via the F-pilus. Upon entry into the cell, the phage particle is stripped of coat proteins and its circular single stranded DNA molecule is converted into a double-stranded replicative form (RF). Replication of this form generates about 100 double-stranded copies, from which new single-stranded DNA and phage proteins are synthesized. The single-stranded DNA is packaged and phage particles are extruded from the cell in a non-lytic manner. Approximately 200-1000 mature phages are produced per cell per generation. M13 does

not cause cell lysis, but growth of infected cells is slowed, resulting in turbid plaques on a lawn of uninfected cells. Infection of E. coli with M13 requires a suitable F+ or F'episome-containing host strain, such as TG1 or XL1Blue.

[12] Phage displaying desired mAbs must be separated from those displaying irrelevant mAbs so that the former can be isolated and the genes encoding the desired mAbs obtained. This is typically achieved by "biopanning," an iterative procedure in which phage displaying appropriate mAbs on their surface bind to antigen attached to the surface of a vessel, typically wells of a microtiter dish. A number of washes occur in this iterative selection, and because each non-covalent binding interaction is characterized by a certain on-off rate, even a mAb that binds tightly to its target antigen will not always be bound at any point in time and if the mAb should happen not to be bound during a washing step that characterizes the iterative selection procedure, the phage displaying that mAb is thereafter lost from consideration for selection.

[13] Notwithstanding the foregoing limitations, phage display has been used successfully by a number of laboratories for isolating individual mAbs even though the process is time-consuming and tedious. However, there are other limitations to the conventional approach to phage display that make it less than desirable when the objective is to select mAbs to very large numbers of different proteins. The procedure can be relatively expensive and challenging because of the requirement for a considerable amount of pure Ag. Additionally the process, as it has been applied in the past, is not readily amenable to multiplexing. Furthermore, selection is typically based upon affinity between the mAb and its antigen and additional efforts have to be expended to optimize for selectivity.

[14] Similar limitations apply to yeast display (Boder et al., 2000). Additional disadvantages to yeast display include smaller mutagenic library sizes compared to alternative methods and differential glycosylation (and other post-translational modifications) in yeast compared to mammalian cells. It should be noted that these disadvantages have not limited the success of yeast display for a number of applications, including engineering

the highest monovalent ligand-binding affinity reported to date for an engineered protein (Boder, et al. 2000).

[15] Yeast two-hybrid (Y2H) assays overcome the requirement for pure antigen (since the yeast synthesize the antigen), the time-consuming iterative process used in phage display is unnecessary and selection can be multiplexed. However, because yeast do not recapitulate post translational modification as it occurs in higher mammalian cells it is not ideal for selection of mAbs that discriminate between proteins with and without post translational modifications.

[16] Microfluidic approaches have a potential for convenient and rapid selection of mAbs that will discriminate between proteins with and without post translational modifications (Weiner, PCT/US08/73839) but this technology is not readily amenable to multiplexing.

[17] Emulsions are heterogeneous systems of two immiscible liquid phases with one of the phases dispersed in the other as droplets of microscopic or colloidal size (Becher, 1957; Sherman, 1968; Lissant, 1974; Lissant, 1984). Emulsions may be produced from any suitable combination of immiscible liquids. For the purposes of the present invention, the emulsion is comprised of an aqueous phase (containing sources of antibody and antigen) present as finely divided droplets (the disperse, internal or discontinuous phase) and a hydrophobic, immiscible liquid (an "oil") as the matrix in which these droplets are suspended (the non-disperse, continuous or external phase). Such emulsions are termed "water-in-oil" (W/O); they have the advantage that the aqueous phase contains all of the biochemical and biological components whereas the external phase, being hydrophobic, generally contains none and hence is inert. The emulsion may be stabilized by addition of one or more surface-active agents (surfactants) which act at the water/oil interface to prevent (or at least delay) separation of the phases. Many oils and many emulsifiers can be used for the generation of W/O emulsions; one compilation lists more than 16,000 surfactants, many of which are used as emulsifying agents (Ash and Ash, 1993). Suitable oils include, but are not limited to, light white mineral oil and non-ionic surfactants (Schick, 1966) such as sorbitan monooleate (Span 80; ICI) and polyoxyethylenesorbitan

monooleate (Tween 80; ICI). The use of anionic surfactants such as sodium cholate and sodium taurocholate might also be beneficial at a concentration <0.5% w/v. Inclusion of such surfactants can in some cases increase the expression of the genetic elements and/or the activity of the gene products. Addition of some anionic surfactants to a non-emulsified reaction mixture completely abolishes translation. During emulsification, however, the surfactant is transferred from the aqueous phase into the interface and activity is restored. Addition of an anionic surfactant to the mixtures to be emulsified ensures that reactions proceed only after compartmentalization.

[18] W/O emulsions have been used by Tawfick and Griffiths for the in vitro compartmentalization (IVC) of biochemical reactions. They have previously shown how IVC can be used to select for catalysis by selecting DNA-methyltransferases [13] and for the directed evolution of Taq DNA polymerase [14]. IVT microbead display libraries can be selected by flow cytometry, which has a variety of practical advantages for the selection of ligand binding [26]. Ligand binding equilibria and dissociation kinetics can be determined and clones selected accordingly. Indeed, flow cytometry has been used to select an extremely high affinity anti-fluorescein scFv (Kd = 48 fM) from libraries displayed on yeast [27]. Throughput is relatively high (up to 70,000 sorted events per second), but flow cytometry does impose an upper limit of ~109 on the size of libraries that can be selected in an hour. The small size of the compartments (~5 fL) means that very large gene libraries could potentially be selected: a 100 µl reaction mix dispersed in 0.5 ml oil forms ~2x1010 aqueous compartments. However, to do so it is probably necessary to select the microbead display libraries by affinity purification.

[19] There is a continuing need in the art for methods to identify the components of the proteome and their post-translationally modified versions. There is a continuing need in the art for methods for quickly obtaining antibodies that have specified properties of affinity and specificity.

SUMMARY OF THE INVENTION

[20] According to one aspect of the invention a method separates antibody molecules one from another. An emulsion is formed comprising a first liquid and a second liquid which are immiscible. The first liquid is aqueous and comprises antibody-producing cells, and an antigen attached to a solid substrate which is suspendable in the first liquid. The emulsion is incubated under conditions and for a time such that the antibody-producing cells produce antibody molecules within the first liquid and the antibody molecules bind to antigen when the antibody molecules have appropriate specificity and affinity. The emulsion is broken and the first liquid is collected. The solid substrate attached to antigen which is bound to antibody molecules is separated from solid substrate attached to antigen which is not bound to antibody molecules and/or from antibody molecules which are not bound to solid substrate.

[21] According to another aspect of the invention a method makes a reagent useful for detecting an interaction with an antigen. An emulsion is formed comprising a first liquid and a second liquid which are immiscible. The first liquid is aqueous and comprises antibody-producing cells, and a solid substrate which is suspendable in the first liquid. The emulsion is incubated under conditions and for a time such that the antibody-producing cells produce antibody molecules within the first liquid and a complex is formed comprising the solid substrate and the antibody molecules.

[22] Another aspect of the invention is a composition comprising an emulsion. A first phase of the emulsion is aqueous and comprises a solid substrate and an antibody-producing cell. The antibody-producing cells express a protein which strongly interacts with the solid substrate.

[23] Yet another aspect of the invention is a method for making a cell which displays a secreted protein on its surface. An emulsion is formed comprising a first liquid and a second liquid which are immiscible. The first liquid is aqueous and comprises a cell which produces and secretes a desired protein, and a bifunctional reagent which binds to

the desired protein and to the cell. The emulsion is incubated under conditions and for a time such that the cell produces and secretes the desired protein within the first liquid and the bifunctional reagent binds to the desired protein and to the cell, whereby the desired protein is bound to the cell which produces it.

[24] Still another aspect of the invention is a composition comprising an emulsion. A first phase of the emulsion is aqueous and comprises a cell which produces and secretes a desired protein and a bifunctional reagent which binds to the desired protein and to the cell. When the cell expresses the desired protein, the bifunctional reagent binds the desired protein to the cell which produced it.

[25] Another aspect is a composition comprising a mixed population of cells which are each decorated with a plurality of molecules of a desired protein. A bifunctional reagent binds the desired protein to the surface of the cell. The cell contains a nucleic acid which encodes the desired protein. Different cells in the mixed population are decorated with different desired proteins.

[26] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with methods and reagents for obtaining antibodies of desired specificity and affinity.

BRIEF DESCRIPTION OF THE DRAWINGS

[27] Fig. 1A-1B. Cloning anti-phosphotyrosine mAb PY20 as an scFv. The cloning was performed by annealing and ligating oligos F1-10 (SEQ ID NO:9-18) with R1-11 (SEQ ID NO:19-29; all 80mers that overlap each other by 40 bp; Fig. 1A), then amplifying that product using F_X (SEQ ID NO:7) and R_X (SEQ ID NO:8) to add XhoI sites at each end. The product (which encodes a protein as shown in Fig. 1B; SEQ ID NO: 30) will be cloned into a vector containing superfolding GFP at a SalI site immediately 5' of the start codon. A clone with PY20 upstream of, and in frame with GFP will be identified. The

PY20 scFv was designed using VH and VL sequences from Ruff-Jamison and Glenney (J. Immunology 1993) with VH 5' of VL, separated by a (Gly4Ser)3 (SEQ ID NO: 3) linker.

[28] Fig. 2. Expression of scFv PY20 in *E. coli*. Overnight cultures of BL21(DE3) in LB+Ampicillin (60 mg/ml) were diluted and grown to mid-log phase (OD 600nm= ~0.6). The cells were induced with 0.1, 0.5, or 1 mM IPTG for 3 hours at 37°C. Western analysis using anti-GFP antibody and HRP was used to develop the Western. The protein was purified over a Cobalt column and eluted with imidazole.

[29] Fig. 3. pY20-GFP scFV binds to a phosphopeptide. Cell lysate from *E. coli*-expressing pY20-GFP was bound to magnetic beads coated with either Streptavidin (SA), Streptavidin + biotinylated Her2 Y1222 peptide (no phosphate; SA + Y1222), or Streptavidin + biotinylated Her2 pY1222 phosphopeptide (SA + pY1222) for 90 minutes. Beads were then washed 3 times with lysis buffer. The lysate-supernatent (S), first wash (W), and bead-eluate (E) were loaded directly onto a polyacrylamide gel, subjected to electrophoresis, electroblotted and a Western performed using anti-GFP Ab.

[30] Fig. 4. pY20-GFP binds to a phosphopeptide within an emulsion. Cell lysate from *E. coli* -expressing pY20-GFP was bound to magnetic beads coated with either Streptavidin (SA), Streptavidin + biotinylated Her2 Y1222 peptide (no phosphate; SA + Y1222), or Streptavidin + biotinylated Her2 pY1222 phosphopeptide (SA + pY1222). Bulk emulsions were generated with surfactant-containing oil (oil supplied by Raindance Technologies, Lexington, MA) either prior to binding reaction (right) or following the binding reaction (left), and binding reactions were carried out for 90 minutes. Emulsions were broken, and beads were then washed 3 times with lysis buffer. The lysate-

supernatent (S), first wash (W), and bead-eluate (E) were loaded directly onto a polyacrylamide gel, subjected to electrophoresis, electroblotted and a Western performed using anti-GFPAb.

[31] Fig. 5A-5F. Growth of bacteria and protein production in an emulsion. The obligate aerobe B. subtilis bacteria producing cytoplasmic-bound GFP protein were encapsulated in a 30 micron emulsion and incubated overnight at 37 C. (Top), Single bacteria (Fig. 5A) are able to replicate within the emulsion to a density of approximately 30 cells (Fig. 5B). In addition, they are able to secrete an extracellular protease (Fig. 5C). (Bottom) Overnight incubated droplets are examined for growth (Fig. 5D), protease production (Fig. 5E) and growth and protease production (Fig. 5F).

[32] Fig. 6. Emulsion size as a function of homogenizer speed. Size distribution of the aqueous droplets formed by emulsification of an in vitro transcription-translation reaction as determined by laser diffraction: the emulsions were either stirred or stirred and then homogenized for 3 min at the indicated speeds.

[33] Fig. 7. Bacterial secretion in an emulsion. In the example shown, bacteria secrete phage (e.g., bacteriophage M13 displaying scFv Ab fusion on gp3 protein). In some droplets, scFv will become bound to antigen-coated bead. In potentially the majority of droplets, the bacteriophage will not adsorb to the Ag-coated beads. As a further example, additional beads may also be included in the droplets to compete with the antigen-coated bead for non-specific M13 interactors. Other non-limiting examples could include lambda and T7 phage.

[34] Fig. 8A-8B. (Fig. 8A) Phage ESCape Steps: (A) M13K07Δ9 is transfected into *E. coli* (F', pGP9::his6, pGP3::scFv), (B) Progeny M13 are produced packaged with pGP3::scFv DNA and displaying his6 fused to gp9, and an scFv fused to gp3, (C) the progeny phage

will attach to a cobalt-coated bead via the strong interaction between the his6 and cobalt-chelate on the bead, (D) the emulsion is broken using a surfactant, (E) labeled antigen is added, and (F) the beads are washed under varying conditions and labeled beads are sorted away from the population of beads by flow cytometry or in bulk using a tyramide amplification assay. Phage and Plasmid constructs [their gene markers (and function in the phage ESCape system)] are as follows: 1) F' [F origin, Tet resistance, (the F pilus is needed for M13 phage infection)]; 2) pGP9::his6 [CloDF13 origin, spec resistance, (provides a gp9-his6 fusion complement in trans to M13K07Δ9)]; 3) M13K07Δ9 [p15A origin, oriF1-, gp9::lacZ, kan resistance (helper phage, requires gp9 in trans to form phage particles, will preferentially package oriF1+)], and; 4) pGP3-scFv [colE1 origin, oriF1+, amp resistance (provides a gp3-scFv fusion complement in trans to M13K07Δ9, preferentially-packaged by the M13K07Δ9 phage)]. (Fig. 8B) Protocol for selecting mAbs by the use of emulsions and flow cytometry. In the example shown, E. coli cells transformed with a phagemid library (encoding, for example, an scFv library and an antibiotic-resistant gene) are infected with helper phage (M13KO7) and the infected cells are emulsified in a W/O droplet. After incubation for a time period sufficient for bacteriophage production, the emulsion is collected and broken. The aqueous solution from the broken emulsion is (optionally) washed, and fluorescently-labeled anti-M13 Ab is added. The beads within the broken emulsion are sorted by flow cytometry to collect those beads having a high-fluorescence signal. The collected beads (which may be collected into one or more test-tube(s) or individually collected in, for example, one or more 96- or 384-well microtiter plates) are spread onto a lawn of E. coli such that it allows transduction of the attached phagemid into the cells, rendering them resistant to the appropriate antibiotic.

[35] Fig. 9A-9B. Phage growth in W/O emulsion. (Fig. 9A) Transduction of E. coli by filtered lysates of M13 after growth of M13KO7-infected, phagemid containing E. coli incubated overnight in bulk (blue) or in an emulsion (red). (Fig. 9B) ELISA performed with phage lysates produced in bulk (blue) or in an emulsion using anti-M13 antibody.

[36] Fig. 10A-10B. FACS analysis of coated beads. Flow analysis of Ag-coated beads pre-treated with positive-binding phage (B3) or negative-binding phage (P3) at a ratio of 90:10 and post-treated with fluorescently-labeled anti-M13 Ab.

[37] Fig. 11. Tyramide signal amplification. Peroxidase converts a fluorescein-labeled tyramide substrate to a reactive intermediate that forms a second reaction to tyrosine residues in any nearby protein.

[38] Fig. 12. Design of a self signal-amplifying M13 phage. A genetic fusion of an enzyme [in the example shown, horse radish peroxidase (HRP)] to the M13gp7 (or 9) protein is used to generate a bacteriophage with HRP activity. A scFv library can be fused to the gpIII protein. Phage attach to an Ag-coated bead (in solution, or on a solid substrate) and the signal is amplified through addition of a modified tyramide moiety.

[39] Fig. 13. Biotinylation of a substrate through tyramide signal amplification. Peroxidase converts a biotin-labeled tyramide substrate to a reactive intermediate that forms a second reaction to tyrosine residues in any nearby protein.

[40] Fig. 14. Affinity-sorting using tyramide signal amplification: generation of biotinylated beads. Beads, cells and HRP-modified, scFv-displaying phage are encapsulated in an emulsion. The droplets are incubated for a time-period sufficient to allow phage secretion and capture onto the antigen-coated bead(s). The emulsion is broken and the beads washed. The biotin-tyramide reagent is added to the beads and the biotinylation of phage-coated beads allowed to occur. Biotin-labeled tyramide substrate is turned over by peroxidase to form a reactive intermediate that forms a second reaction to tyrosine residues in any nearby protein.

[41] Fig. 15. Affinity-sorting using tyramide signal amplification: separation. Biotinylated beads are added to a separating means (shown is a Streptavidin-well, but one could also use, as a non-limiting example, a Streptavidin or avidin-coated magnetic bead). The

beads are washed to remove non- or under-biotinylated beads and the remaining beads are plated onto a lawn of, for example, E. coli cells.

[42] Fig. 16. Proposed work-flow for high-throughput mAb production using emulsions. Shown is an example using FACS sorting. The method would potentially be modified for use in another sorting means, for example, magnetic sorting.

[43] Fig. 17. Yeast surface display. As an example, the Aga2P gene of yeast can be genetically-modified to have fused to it a hemagglutination (HA) tag site and a means for capturing IgG molecules secreted into the droplet. In the non-limiting example shown the capturing molecule is a staphA protein. The tag site can be used to calibrate/quantify the concentration of the fusion protein to the yeast surface. Other examples could include an anti-human IgG protein.

[44] Fig. 18. Yeast secretion and capture within a droplet. A yeast is either coated or made to genetically expose a surface moiety capable of binding an IgG or Fab molecule. An IgG or Fab molecule library is transformed into yeast cells to create a yeast IgG or Fab library, respectively. Cells from this library, along with a fluorescently-labeled peptide, are emulsified and incubated in the droplet. After a sufficient period of time to allow the yeast to secrete and capture the either IgG or Fab molecule, the emulsion is broken and the aqueous phase is sorted using, as a non-limiting example, flow cytometry.

[45] Fig. 19A-19B. Non-yeast cell secretion and capture within a droplet. (Fig. 19A) A non-yeast cell is either coated with, or made genetically to expose a surface moiety capable of capturing an IgG or Fab molecule. An IgG or Fab molecule library is transformed into the non-yeast cells to create a IgG or Fab library. (Fig. 19B) Cells from this library, along with a fluorescently-labeled peptide, are emulsified and incubated in the droplet. After a sufficient period of time to allow the non-yeast to secrete and capture the either IgG or Fab molecule, the emulsion is broken and the aqueous phase is sorted using, as a non-limiting example, flow cytometry. As a non-limiting example, B-cells are enriched from a human tissue or blood sample. The B-cell is coated with a bispecific antibody

consisting of anti-CD19 antibody coupled to a goat anti-human IgG antibody. The coated B-cell is emulsified along with a labeled peptide in a droplet. The labeled-peptide/B-cell emulsion is incubated for a time-period sufficient to allow secretion and capture of the IgG antibody on the B-cell surface. The emulsion is broken, the aqueous phase washed, and the washed cells flow sorted.

[46] Fig. 20. Construction of a random peptide library. Triplet codons can be incorporated into a growing chain using synthetic codon linkers as shown. Step A: In the example shown, an anchor primer is attached to a solid substrate, either a bead or the surface of a solid substrate such as a 96-well microtiter plate. The substrate is used to facilitate washing of the growing chain between steps. In the example shown, the anchor primer is a hairpin loop that contains a upstream priming site and ends in a blunt-end. The anchor primer also contains a means for its removal from the substrate, for example a restriction enzyme site. Alternatives could also include a cleavable linker to the substrate, or the use of an organic solvent or physical condition (heat as an example) that would separate the anchor primer from the substrate. Alternatively the anchor could be left attached and PCR using the upstream primer site used to copy the final product. In step A, a codon linker is ligated to the anchor primer. The codon linker consists of three specific bases (denoted by NNN) that are placed upstream of a type II restriction enzyme such as MlyI. In the example shown the 5' end of the codon link is phosphorylated. In a non-limiting example the codon linker is constructed such that there is a hairpin-loop. Optionally in step A the number of codon linkers can be varied such that 1 to 64 possible triplet codons can be added to the growing chain. Alternatively, one could add a subset of the 64 triplet codons, such as just the 20 that code for the unique set of amino acids, or the preferred codons used for expression in a specific organism, or codons that do not make a MlyI site (see step C). As a further non-limiting example, one can add more than 3 bases at a time to the growing chain. As an extension of this idea, one could add two codons at a time, using 4096 different codon-linkers if using the complete set of 64-codons, or using 400 codon-linkers if one uses the set of 20 amino acids. Or as another non-limiting alternative, if a defined amino acid is known to be wanted at a particular position in the

chain, then a set of 20 codon linkers can be synthesized such that the first (or second) codon is "fixed". This would also be performed if more than one amino acid can be fixed within the sequence. For example, a set of 20 codon linkers could be made with the end sequence equivalent to 5'-ATGNNNATG-3' (SEQ ID NO: 1) wherein the middle base form one of 20 amino acid codons, and the flanking ATG sequences incorporate a methionine amino acid into the protein chain. An advantage of adding two or more codons at a time is that the number of steps gets reduced and there would be an overall increase in efficiency in time and accuracy, and decrease in cost. As a further example, the codon-linkers can be added at differing or identical ratios such that there either will or will not be a skewing of which codon linkers get ligated into the growing chain. It is known that T4 DNA ligase has preferred DNA sequences for ligation. This ratio can be based on the molar mass ratio of the various codon-linkers or could be based on the relative efficiency of a particular codon linker to be ligated into the growing chain. Step B: To increase ligation efficiency, a crowding agent(s) such as (10-25%) polyethylene glycol may be optionally added to the ligation mix. Optionally, if both the anchor and linker primers are 5' phosphorylated (either during oligonucleotide synthesis or either post-ligation or synthesis using T4 polynucleotide kinase and ATP), then exonuclease can be used to remove any unligated anchor or codon primer remaining in the reaction. In the non-limiting example shown, the ligated anchor-primer/codon-linker forms a molecules with both ends sealed (i.e., a single DNA strand without a free end). Such a covalently-sealed molecule would be resistant to an exonuclease. An alternative means of protecting the end of the codon linker would be to incorporate a thiol-group that would render the end of the molecule similarly resistant to exonuclease digestion. Step C: The restriction endonulcease MlyI makes a double-strand cut 5 bases upstream (on both strands of the DNA) of its recognition site. The restriction digested product can be washed away from the bound anchor-primer. Step D: the process is repeated (steps A-C) to add more codon linkers. Step E: When the chain has grown such that the end is reached, then a terminal linker can be attached. This terminal linker can optionally incorporate aNNN sequence at its 5' end. The terminal linker could also contain a downstream primer-binding site that could be used in conjunction with the anchor primer binding site to enable one to PCR or

amplify the grown chain. Step F: The completed chain can be optionally separated from the substrate using various means, including a restriction enzyme, chaotropic agent, change in pH, organic solvent, heat, chemical or enzymatic decoupling. The freed DNA fragment can be cloned directly into a vector, used for splice overlap extension, or amplified by one of many means, including PCR and rolling circle amplification.

[47] Fig. 21. Cryogenic storage of phage-coated bead emulsions. M13K07 helper phage and antigen-coupled beads were added to E. coli (F') containing ampicillin-resistant phagemid expressing an anti-interferon gamma scFv-gp3 fusion. An emulsion was generated by subsequent addition of perfluorocarbon oil. Infected cells were incubated at 30°C overnight for phage production. Emulsions were either frozen at -80°C or broken. Cells were pelleted, beads were washed and frozen in the absence or presence of a cryoprotectant. Stability of phage-containing beads after freezing was examined by ELISA using anti-M13-HRP antibody.

[48] Fig. 22. Schematic of Method of Phage ESCape. *E. coli* cells transformed with a phagemid library is infected with helper phage (M13KO7△9 helper phage. The infected bacterium is then compartmentalized along with a cobalt-chelate "capture-bead" in a water-in-oil [W/O] emulsion. Consequently, in each isolated compartment during overnight incubation, thousands of copies of the recombinant phage displaying both an scFv protein at one end (fused to the gp3 protein) and a his6 peptide at the opposite end (fused to the gp9 protein) are produced. The his6-end of the display phage binds to the cobalt-chelate capture-bead. The emulsion is broken and these beads, along with any phage bound to them, are isolated. In an initial application, the phage-bound beads are incubated with a fluorescein-labeled antigen and then washed to remove any excess ligand. The labeled beads are then sorted (together with the phage attached to them) from the population of beads by flow cytometry. Enriched beads are plated onto a lawn of *E. coli* and the phagemid-encoded scFv further tested for specificity and affinity.

DETAILED DESCRIPTION OF THE INVENTION

[49] The applicants have invented emulsion-based methods for selection of antibodies. The methods have applicability for multiplexing, require relatively small amounts of antigen, can be used to select for antibodies directed to post-translational modifications, and can be designed to select simultaneously on the basis of affinity and specificity. In addition, it provides a convenient approach for library construction that is amenable to long-term storage. The use of emulsions permits, for example, individual query of the ability of each antibody to bind to an antigen-coated solid substrate, yet the procedure is massively multiplexed, so that upwards of 10^9 individual queries can be carried out in approximately one hour. The methods can be applied to a number of different sources of antibody including bacteriophages, viruses, bacteria, yeast, or higher eukaryotic cells, including hybridomas and normal immune cells, such as B cells.

[50] Emulsions permit the production of solid substrates or cells which can be decorated with a single species of protein or antibody molecule. Yet the solid substrates or cells can be in a mixture such that the single species on one solid substrate or cell differs from the next. Thus a solid substrate or cell becomes an anchor for identical antibodies or proteins, thus the signal of each antibody or protein is amplified. The ratio of solid substrate to antibody producing cell to emulsion compartments can be readily adapted to maximize the number of compartments with a single antibody–producing cell and one or more solid substrates. At least 5 %, at least 10 %, at least 15 %, at least 20 %, at least 25 %, at least 30 %, at least 35 %, at least 40 %, at least 45 %, at least 50 % or more of the solid substrates may carry a single antibody or protein species.

[51] Antibody molecules in cells can be encoded by a natural antibody gene, such as in a B cell or in a hybridoma. Alternatively, the antibody molecules can be encoded by recombinant constructs. The recombinant constructs may make truncated molecules that retain the antibody binding moieties. For example single chain variable region molecules can be used. These can be made as fusion protein, optionally. Fusion proteins may comprise a desirable detectable moiety, such as green fluorescent protein or an enzyme,

or may comprise a desirable tag, such as a his tag, or may comprise a structural protein, such as an M13 structural protein.

[52] After a particular antibody is identified as having desirable binding properties, such as specificity and avidity, additional amounts of the antibody can be generated using the cell which made the antibody, or using the recombinant construct to transform additional cells, or by using the bacteriophage or virus to infect additional cells. The methods are particularly designed to facilitate this process by physically linking the desired antibodies to the nucleic acid sequences encoding them. This physical linkage need not be direct. In most cases there are one or more intermediaries in the linkage. For example in a cell that expresses a desired antibody or protein, the linkage to the desired antibody or protein may be made via a bifunctional reagent that binds both to the cell and to the antibody or protein. A non-limiting example of a bifunctional reagent is a bifunctional antibody. Similarly, a cell surface may be modified either chemically or genetically to display a moiety on its surface through which a cell may be attached to an antibody or other protein. Alternatively, a cell may express a protein on its cell surface or on the surface of a virus or bacteriophage that binds strongly to a moiety on the solid substrate.

[53] Labeling of antibodies may similarly be accomplished directly or indirectly. An example of indirect labeling is the binding of a labeled goat anti-human antibody to a human antibody. A non-limiting example of direct labeling is the use of a genetic fusion construct between an antibody molecule and a green fluorescent moiety.

[54] Separating, detecting, and analyzing solid supports or cells bound to antibodies can be accomplished by any means known in the art. Two useful means are flow cytometry and flow sorting. Separations can also be accomplished by means of centrifugation, by means of magnetic attraction of paramagnetic particles, and by affinity attraction. In some cases it may be useful that the solid substrate itself be detectably labeled. Once separated, a cell, virus, or bacteriophage encoding a desirable antibody molecule can be cultured to achieve a larger stock of the antibody, cell, virus, or bacteriophage. The culturing may be

used to achieve a sufficient amount of nucleic acids to conveniently sequence the antibody-encoding nucleic acid species.

[55] According to some configurations, a solid substrate is complexed with antibodies, wherein in others, the solid substrate is complexed with antigens. In either configuration, the reagents are useful for detecting binding between antibodies and antigens and for recovery of the nucleic acids encoding the antibodies. Either antigens or antibodies may be physically complexed with the solid substrate, usually through intermediate binding moieties. For example, a solid substrate can be coated with nickel or cobalt ions and these can be used to bind his-tagged proteins or antibodies to the solid support. Other binding pairs can be used to link a protein or antibody to the solid support, such as using avidin or streptavidin and biotin.

[56] Competing species may be introduced into binding reactions in order to increase the stringency of detected binders. Species which may be used, include without limitation, solid substrates with no attached proteins, soluble proteins which may be similar to an antigen of interest, and soluble proteins which are not similar to an antigen of interest.

[57] The technology is useful *inter alia* for detecting an interaction between an antigen and a mAb. The antigen may be a protein, a polypeptide, a peptide, a protein carrying a post-translational modification including but not limited to phosphorylation, glycosylation, sialylation and the like, or even a non-peptidic molecule. The mAb may be a conventional IgG, a Fab fragment or a scFv. Each of these antibody or antibody-like structures is referred to here generically as a mAb. The mAb may be a hybrid protein comprising, variously, a conventional IgG, a Fab fragment or a scFv fused to a polypeptide. The polypeptide fusion partner may have properties that influence the location of the mAb (*e.g.*, on the external surface of a cell or a virus or secreted into the milieu) and/or enhance the ability of the mAb to be detected and/or facilitate purification of the mAb. Non-limiting examples of such polypeptides are surface structural polypeptides of cells, bacteriophage or viruses; enzymes, including, without limitation, alkaline phosphatase, halogenase or horseradish peroxidase (HRP), ligands that bind

tightly to cell surface receptors or polypeptides that can readily be detected by fluorimetry or colorimetry, including, without limitation, green fluorescence protein (GFP). Also according to an alternative to this aspect of the invention, the hybrid protein comprises a multiplicity of polypeptides that influence mAb location and/or enhance the ability of the mAb to be detected and/or facilitate purification of the mAb.

[58] Each mAb can be synthesized within a host cell. The host cell may variously be a recombinant bacterial cell, a bacterial cell infected with a bacteriophage, a recombinant yeast cell, a recombinant higher eukaryotic cell, a eukaryotic higher cell infected with a virus, a hybridoma or a mammalian immune cell that is programmed to express one or more of its endogenous Ab genes. One or more Ab genes may be introduced recombinantly into the genome of a host cell. The host cell may be a bacterium and one or more Ab genes may be introduced into the bacterium recombinantly. The host cell may be a bacterium and one or more Ab genes may be introduced into the bacterium via a bacteriophage. The host cell may be a eukaryotic cell and one or more Ab genes may be introduced into the cell recombinantly. The host cell may be a eukaryotic cell and one or more Ab genes in the cell is introduced into the cell via a virus. The host cell may be an antibody-producing cell that had been obtained from or derived from the immune system of a mammal. The mammalian source of the immune cell may optionally be human. The host cell may also be a hybridoma of either human or non-human origin.

[59] Bacterial host cells can be recombinantly manipulated so that as a population they collectively express a library of mAbs. The bacteria may express mAbs on their surface. The bacteria may be infected with a population of bacteriophage that has been recombinantly manipulated so as to express on their surfaces a library of mAbs. The bacteriophage may be released from the host bacteria into the medium.

[60] Eukaryotic cells may be recombinantly manipulated so that as a population they collectively express a library of mAbs. The eukaryotic cells may express mAbs on their surface. Alternatively, the mAbs may be secreted into the medium and captured by a molecule located on the eukaryotic host cell surface. For example, the cells may be

genetically modified to produce Staph A protein on their surfaces, the mAbs produced by the cells may be IgG molecules which, following secretion, may be captured by the surface Staph A protein. Alternatively, the antibody-producing cells may be hybridoma cells.

[61] Eukaryotic cells can be infected with a virus recombinantly manipulated so that as a population they collectively express a library of mAbs. Optionally each mAb is part of a fusion protein that additionally contains an epitope that binds to a receptor on the surface of the host cell. Alternatively, the eukaryotic cells express mAbs on their surface. Optionally, virus expressing mAbs on their surface are released from the host cells into the medium.

[62] An antigen may be attached to a solid substrate that can optionally be suspended in an emulsion. The solid substrate may be a bead, microsphere, particle etc. that is relatively inert. One bead that is of suitable size for use in emulsions is a 5-micron bead. Another suitable solid substrate is a magnetic bead. The solid substrate may be suitably marked so that it can be tracked by a fluorimetric or colorimetric detector. The marker may be on the surface or internal to the bead, microsphere, or particle. The antigen may be modified so that when it is attached to the solid substrate, the solid substrate can be tracked by a fluorimetric or colorimetric detector. Both or either of the solid substrate and the antigen may be so marked, so that they can be differentially or additively tracked by a fluorimetric or colorimetric detector.

[63] Antibodies may be selected on the basis of specificity as well as affinity. Optionally, a non-target molecule can be added to the medium at a concentration such that if a mAb is non-specific it is sequestered in the medium by interaction with the non-target molecule such that binding sites on the mAb will not be available for interaction with the targeted Ag. Optionally, antigen can be selected on the basis of specificity by being bound to a first bead that can be distinguished from a second bead to which the desired target antigen is not bound.

[64] The source of mAb (whether cells, phage, or virus) can be mixed with antigen attached to an appropriate solid substrate (*e.g.*, a bead) in a suitable first liquid and an emulsion of that first liquid can be created in an appropriately immiscible second liquid. Optionally, the first liquid is an aqueous medium and the second liquid is a hydrophobic liquid, such as, an oil. Any two immiscible liquids known in the art may be used, so long as the first liquid can sustain cell growth and optionally virus or phage production. The emulsion can be created by manual mixing of the first and second liquids. The emulsion can be created by a device that generates an emulsion in which droplets are relatively uniform in size. Emulsions may alternatively be made in a device possessing a series of nozzles so that emulsions are effectively produced in a multiplexed fashion. Any means known in the art for making emulsions may be used.

[65] After an appropriate incubation period, in terms of time and temperature, of the emulsion to allow mAb to be produced by the cells and (if antigen is present) to interact, or not interact, with antigen within the same droplet within the emulsion, the emulsion can be broken by any means known in the art. Centrifugation and/or addition of a suitable agent or mixture of agents, such as a detergent or mixture of detergents, can be used to break the emulsions. The first liquid from the combined droplets within the emulsions are collected and pooled. After collection and pooling, the first liquid can be subjected to a separation procedure that stratifies phage or virus or cells that are bound to one or more beads containing antigen. One such stratification procedure which may be used is by flow cytometry. Fluorescence activated cell sorting may alternatively be used. Bacteriophage or virus or cell that is attached to one or more antigen-containing solid substrates may be recovered and used to produce more of the mAb which has been selected. The bacteriophage or virus or cell may be used as a source of genetic material for sequencing of the mAb-encoding gene(s).

[66] Optionally, the emulsions can be used for creating libraries of antibodies attached to a solid substrate, without the presence of antigen. For example, the solid substrate may be coated with a binding partner for antibodies, with a binding partner for a fusion partner of the antibodies (if made as a fusion protein), or for the bacteriophage producing the

antibodies on their surfaces or for the cells expressing the antibodies on their surfaces. The solid substrate from a single emulsion droplet would be homogeneously decorated with a single antibody member of the library. Different solid substrates within the population would be decorated with different library members. The libraries of antibodies attached to the solid substrates can be stored before further use, either as an emulsion or as the first liquid phase of the broken emulsion. Optionally they can be frozen for storage. If desired, additional cryoprotective substances can be added to enhance the shelf-life of the libraries. Suitable cryoprotective substances include those which increase the osmolality of the medium, such as glycerol, mannitol, sucrose.

[67] After a period of storage, target antigen can be added to aliquots of the library of antibodies on solid substrates. After a suitable period to allow appropriate antigen-antibody interaction to occur, the mixture can be subjected to a separation procedure, *e.g.,* that stratifies Ag-mAb-solid substrate complexes from complexes that do not contain antigen.

[68] The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

[69] Cloning and testing of synthetic pY20 scFv. HER2 (human) is a proto-oncogenic receptor tyrosine kinase of the EGFR family. A phosphorylated peptide of Her2_Y1222, has the sequence PAFDNLYpYWDQDPPE (SEQ ID NO: 2). This sequence is used as a target epitope in the Example. We constructed a synthetic anti-pY20 scFv based on the published sequence of the anti-phosphotyrosine IgG molecule. To test the ability of this scFv to bind to phosphotyrosine, the scFv was genetically fused to GFP and expressed in

E. coli under lacI control and with a bacteriophage T7 promoter. IPTG was used to induce protein production.

[70] The sequence of the PY20 peptide is shown in Fig. 3B. A his6 tag was fused to the construct for use in protein purification. A (Gly4Ser)3 (SEQ ID NO: 3) linker at the 3' end was also added to allow fusion to another protein. The amino acid sequence was reverse-translated and the codons were optimized for expression in *E. coli*. Splice overlap PCR was used to generate the full-length gene fragment. The synthetic gene was cloned into a pET vector under the control of a T7 promoter. DNA sequencing was performed to identify a clone with the correct sequence.

[71] Protein induction is shown in Fig. 4.

Example 2

[72] Testing of scFv PY20 for anti-pY activity in vitro and in emulsion oils. The PY20-GFP construct was tested for anti-phosphotyrosine binding activity in vitro and in the presence of oils typically that can be used for emulsion generation. The results are shown in the Figs. 5 and 6. These results demonstrate that the PY20 retained anti-PY activity as an scFv. Additionally, we have demonstrated that this binding activity was not affected by the perfluorocarbon oils being used to generate the emulsion.

Example 3

[73] Isolation of Interferon gamma scFvs with different Kd range. We have isolated recombinant scFvs against Interferon gamma (IFNγ) using M13 phage display against two separate epitopes of IFNγ. Biacore analysis is used to derive affinity-binding information for these antibodies.

Example 4

[74] Bacteria and bacteriophage can survive the emulsion process. Bacteria have survived compartmentalization for up to several days in the perfluorocarbon oils we are using for making our emulsions (*e.g.*, Fig. 7). We have also determined that *E. coli* produced M13KO7 helper phage when incubated overnight in oil droplets and survived for at least six days thereafter.

Example 5

[75] Model system for demonstration of mAb selection in an emulsion. Peptides Her2_Y1222 (PAFDNLYYWDQDPPE; SEQ ID NO:4), Her2_pY1222 (PAFDNLYpYWDQDPPE; SEQ ID NO: 2), IFNγ_3 peptide (biotin-GSLGCYCQDPYVKEAENLKK; SEQ ID NO:5), and IFNγ_4 (biotin-DNGTLFLGILKNWKEESDRK; SEQ ID NO:6) are synthesized (Sigma-Aldrich, St Louis, MO) and attached to a Luminex bead (Austin, TX). The method for attachment is EDC-based direct chemical coupling to derivatized 5.6 μm beads through an activated amine group on the peptide. Biotin-coupled peptides are attached to streptavidin-coated beads. The phosphorylated peptide (Her2_pY1222; Cell Signaling, Inc. (Beverly, MA) is attached to one set of beads; the same peptide absent the phosphotyrosine (Her2_Y1222) is attached to a fluorescently-distinguishable set of beads and used as a negative control. The commercially-available murine IgG anti-phosphotyrosine hybridoma CRL-1955 from ATCC (Manassas, VA) is used as a control antibody. Fluorescein is attached to the Luminex and streptavidin beads at several calibrated concentrations. These fluorescein-bound control beads are used to determine and optimize detection and differentiation of the background and fluorescein-generated signal.

[76] Recombinant M13 bacteriophage. We have obtained 10 different scFvs against two peptide epitopes of IFNγ by biopanning, 6 different scFv mAbs against the IFNγ_3 peptide that recognize both the peptide and full-length protein and 4 different scFv mAbs against the IFNγ_4 peptide that recognize both the peptide and full-length protein. A 3+3 phasmid system is used based on pBlueScript and which can rescue the phasmid with M13KO7 helper phage using published protocols. PY20 scFv is sub-cloned from the scFv-GFP fusion (see Example 1) into this plasmid system.

[77] scFv protein purification and measurement of binding kinetics. We have purified PY20 and anti-IFNγ scFvs to near homogeneity on the appropriate columns. The purified scFvs have been cloned as enzyme fusions (typically with alkaline phosphatase) for use in Western analysis. Our yields for scFv protein purification were in the range of approximately 100 μg to 1 mg per liter. The binding kinetics of purified scFvs are determined by BiaCore analysis. Instrumentation. A Luminex 100 IS (Austin, TX) xMap instrument is used to analyze and decode at least two different Luminex-type beads and fluorescein dye intensities on a bead as a model for flow cytometry studies. The Luminex machine uses a 532 nm green laser to excite a reporter molecule (phycoerythrin, Cy3, etc.) and a 635 nm diode laser to excite the red and near infrared fluorescence from the dyes used to color-code the beads. Fluorescein dye and antibodies are purchased from Invitrogen, Carlsbad, CA.

[78] A flow cytometer may be used to sort the beads. Among non-limiting alternatives for flow cytometry, any of the following high-speed sorters can be used: a BD FACSVantage SE, a BD Aria, and a Dako MoFlo. Cells are sorted at rates of up to 20K/sec. at 99+% purity, using a variety of commonly used fluorochromes, including but not limited to FITC, PE, PE-Cy5, APC, APC-CY7, PE-CY7, CY5, Alexa680 and PI. The MoFlo has 7-color, Vantage SE has 6-color and the Aria has 10-color capability. Cells are sorted into 5 or 15 ml tubes or into various plates as single or multiple cells/well or onto microscope slides for analysis. In addition, the sorters are capable of simultaneous 4-way sorting.

FACS Vantage SE and FACS Aria are also equipped with aerosol management systems making them suitable for sorting live human, primate or other potentially biohazardous cells.

[79] During flow cytometry, individual beads flow past a laser detector for fluorescence analysis. Based on this real-time analysis, a decision is made whether an individual bead has a fluorescent signal coincident with the bead above a chosen threshold (*e.g.,* 3x above background). As one alternative, droplets with signals above the threshold trigger voltage generation in the sorting electrode which causes the droplet to move into the "keep" channel. Beads with signals below the threshold flow into the waste channel.

[80] Flow-sorting technology can process nearly 1 billion beads per hour, in a total volume of less than 1 ml. We have determined that bacterial cells and bacteriophage remain alive and functional in our emulsion solutions for over 24 hours. This time is sufficient for the phage-screening proposed. We and others (Sepp 2002) have shown that we can detect increased fluorescence signal on a bead. Spiking experiments (i.e., adding a phage expressing an mAb that binds to a bead in the presence of an excess of non-binding phage) are carried out to optimize the experimental conditions for mAb screening. Emulsions are prepared using variations of the procedure described in Example 6 to vary the size of the emulsion droplets, phage and bacterial titers and concentration of beads.

Example 6

[81] Development of reproducible methods for generating emulsions of consistent size. Creation of an emulsion generally requires the application of mechanical energy to force the phases together. There are a variety of ways of generating emulsions which utilize a variety of mechanical devices, including stirrers, homogenizers, colloid mills, ultrasound and "membrane emulsification" devices (Becher, 1957; Dickinson, 1994). Aqueous microcapsules formed in W/O emulsions are generally stable with little if any exchange of genetic elements or gene products between microcapsules.

[82] Generation of droplets. The methods for generating the emulsions have been published in detail [Oliver J Miller, Kalia Bernath, Jeremy J Agresti, Gil Amitai, Bernard T Kelly, Enrico Mastrobattista, Valérie Taly1, Shlomo Magdassi, Dan S Tawfik & Andrew D Griffiths. Directed evolution by in vitro compartmentalization. *Nature methods.* 3 (2006) 561-570]. These conditions are used to generate our initial W/O emulsions. Briefly, to generate a emulsion, 950 µL of an oil-surfactant mixture [Span 80 2.25 ml 4.5% (wt/wt) Tween 80 250 µl 0.5% (wt/wt), Mineral oil to 50 ml) is cooled on ice along with a 3x8 mm stir bar. The mixture is stirred at 1,150 r.p.m. on a magnetic stirrer with a tube supported in an aluminum block pre-cooled on ice (or in a beaker containing ice water). The aqueous phase is added gradually to the oil-surfactant mixture as 5 aliquots of 10 µl over a period of 2 min. After the addition is complete, stirring is continued for 1 min to generate a W/O emulsion estimated to contain ~1010 droplets with a mean diameter of 2.6 microns. For a greater degree of mono-dispersity, we homogenize the emulsion in a CryoTube vial for 3 min with an Ultra Turrax T8 homogenizer. Note that greater homogenization speeds produce smaller droplets with narrower size distributions (Fig. 6).

[83] The reactions are quenched when the emulsion is broken by spinning at 3,000 xg for 5 minutes and removing the oil phase, leaving the concentrated emulsion at the bottom of the vial. The size distribution of the aqueous droplets in the emulsions can be determined by laser diffraction using a Coulter LS230 Particle Size Analyzer. An aliquot of emulsion, freshly diluted (1:10) in mineral oil is added to the micro-volume chamber containing stirred mineral oil. Results are analyzed with the instrument's built-in Mie optical model using refractive indices of 1.468 for mineral oil and 1.350 for the aqueous phase.

[84] Determination of optimal number of cells, beads and phage for selection in an emulsion droplet. Procedures for the use of bacteria to produce and secrete phage expressing mAbs in emulsions and the subsequent sorting of the mAb-producing phage are summarized in Figs. 7 and 8 and elaborated upon in Examples 10 and 12.

[85] As a non-limiting example, E. coli encoding an scFv phasmid are infected with M13KO7 helper phage at an MOI = 10 for 5 minutes at 37 °C and then washed with an excess of cold, sterile LB medium to remove unadsorbed helper phage. The infected cells are resuspended in Luria-Bertani (LB) medium containing a predetermined number of Ag-coated beads. The concentration (and number) of beads are estimated using a hemocytomer. The number of viable cells is estimated by absorbance at 600 nm (A600) and (prior) calibration of A600 to colony-forming units on agar plates. The ratio of cells:beads and total concentration of cells and beads are varied in the LB medium. The aqueous medium is emulsified as described above. In addition, the size of the microdroplets is varied. The emulsion is incubated for up to 24 hours, with portions removed at appropriate (*e.g.,* 2 hr) intervals. The phage multiply within the aqueous droplets within the emulsions (Fig. 9A and 9B). The emulsions are broken as described above and the number of transducing phage particles in the aqueous phase determined.

[86] In initial experiments, the antigen under investigation (*i.e.,* the phosphotyrosine-encoding peptide Y1222) is conjugated to one bead type whereas a second bead type is either 'naked' (i.e., nothing attached to the bead) or is conjugated with at least one of either non-phosphorylated peptide, or BSA. The PY20 antibody is used as a first test mAb. The beads are blocked with BSA or powdered milk prior to use. It is noted that the amount of time that encapsulated bacteria need to be incubated to express sufficient scFv-M13 for detection is an important aspect of this specific aim. It is assumed that 6-12 hours will be sufficient, but this number is determined empirically for the various emulsions.

[87] Correlating results with flow cytometry and validating that only one scFv-displaying phage-type is attached to one bead. Fig. 10 provides an example of how coated beads are sorted by flow cytometry. Aliquots from the above set of experiments are also used to determine the correlation between flow-cytometry sensitivity, plaque-forming units, ELISA results and the number of transducing phage particles. Flow cytometry is carried out as described in Example 5. This step provides guidance for calibration of results in

future experiments. Transducing phage are resuspended on single beads, collected and scFv-specific primers are used to validate the percent homogeneity of bound phage on each particle.

[88] Determine emulsion-breaking protocol and optimal wash conditions. The beads isolated from broken emulsions are washed using several protocols to determine which conditions best able differentiates between specific binding and non-specific binding. PY20 scFv is initially tested against separate Her2_pY1222 and Her2_Y1222 peptide-coated beads. Initial tests are performed in a strictly aqueous environment using a plate-reader, and then checked for reproducibility in an emulsion using a flow cytometer. Wash conditions are varied for reduction of non-specific binding. Specifically, the concentration of added detergents (e.g., Tween20, and NP40), blocking agents (powdered milk, BSA), NaCl concentration, pH and number of washes are all varied. Additionally, the washing temperature is varied: washes are performed either on ice or at room temperature. Conditions are defined that reduce or eliminate non-specific mAb binding within a sorted emulsion to less than 5%.

Example 7

[89] Test wash conditions as a function of Kd for IFNγ phage. To test if Kd can be calibrated by wash conditions, 109 different phage carrying mAbs against IFNγ are tested in separate compartments via formation of an emulsion. A 3+3 phagemid system, which is considered to generate 99% of the particles with 0 gpIII-scFv fusions and approximately 1% of phage having 1 gpIII-scFv fusion product on the phage particle is used for these experiments in order to reduce avidity effects. It is determined under conditions of saturation of the beads in each compartment with phage whether making the wash conditions progressively more stringent leads to isolation of phage having greater affinity for antigen on the beads. This potential correlation between wash stringency and mAb affinity is evaluated by determining whether there is a correlation between fluorescence signal on the bead with affinity. IFNγ scFvs selected by these procedures are analyzed by

Biacore analysis to determine their Kds and a curve of fluorescence vs Kd for each of the various wash conditions is generated.

Example 8

[90] Production of large (up to $>10^9$) synthetic human framework phage libraries expressing scFv with or without fusion to dehalogenase or HRP for tyramide analysis. The objective of this example is to prepare a large, quality-controlled naïve phage library that carries a scFv framework that is functional in an emulsion system in the presence of *E. coli*. Optionally, and in addition, the distal end of the phage is constructed to carry an enzyme system that will increase efficiency of selection of desired mAbs.

[91] The use of a naïve bacterial phage library for the emulsion screen has several advantages, including reduced costs, system simplification, less dependence on vertebrate animals, better cell recovery and increased ease of handling. A naïve yeast library of 108 separate clones, in a small compartment such as a yeast nucleus, can be used very effectively to isolate Ag-specific mAbs. Most phage libraries with sizes on the order of 109 have been successful in isolating high-affinity mAbs. By fusing an enzyme to the gp7 or gp9 proteins of our M13 library clones the system can potentially be simplified and a possible source of artifact eliminated by avoiding the use of anti-M13 Ab and replacing it by covalently-bound fluorescein. There are 5 copies each of gp7 and gp9 at the distal end (opposite of gp3) in M13. Fusions to these genes are possible. The halo-tag technology sold by Promega Corporation (Madison, WI) uses a mutant dehalogenase enzyme that forms a covalent intermediate to its normal substrate. By fusion of this mutant dehalogenase to one of the distal M13 gene products, five fluorescein molecules are covalently bound to the recombinant phage. As few as 1000 fluorescein molecules can be measured on a bead in a dilute solution of fluorescein.

[92] Tyramide is a phenolic compound that, when activated by the enzyme horseradish peroxidase (HRP), covalently binds to electron rich moieties on a surface (i.e.,

predominantly to tyrosine residues in proteins). Tyramide Signal Amplification (Fig. 11) is based upon derivatized tyramide. In the presence of small amounts of hydrogen peroxide, immobilized HRP converts the labeled substrate (tyramide) into a short-lived, extremely reactive intermediate. The activated substrate molecules then very rapidly react with and covalently bind to electron-rich regions of adjacent proteins. This binding of the activated tyramide molecules occurs only immediately adjacent to the sites at which the activating HRP enzyme is bound. Multiple deposition of the labeled tyramide occurs in a very short time (generally within 3-10 minutes). Subsequent detection of the label yields an effectively large amplification of signal. HRP on either gp7 or gp9 can be used with tyramide-fluorescein to significantly increase signal on the phage-bound bead. In addition, the approximately 2,700 copies of gp8 are modified to encode extra tyrosine residues on the phage surface. Figs. 12 and 13 illustrate examples of the use of tyramide substrate to amplify signal when phage carrying scFvs bind to bead coated with cognate Ag. Fig. 14 illustrates a non-limiting procedure for generating phage:bead complexes in which signal has been amplified with tyramide. Fig. 15 illustrates a non-limiting procedure for recovering phage:bead complexes in which signal has been amplified with tyramide.

[93] In the foregoing examples, the same library construction method used to synthesize a yeast scFv library can be used for *E. coli*. A scFv framework is chosen that enables efficient expression in the bacterium and functions with appropriate characteristics in an emulsion once secreted. In order to prevent the biased outgrowth of a limited number of clones, all growth of the transformants take place on a solid agar medium. M13 phage and host E coli can survive in emulsion droplets for at least 2 days and can secrete active phage particles into the medium. A framework is chosen that enables functionality in our emulsions.

Example 9

[94] Functional Subtraction of Libraries to remove mAbs with non-specific binding properties. mAb libraries are initially functionally subtracted to eliminate non-specific interactors by flow-sorting the entire library against beads carrying a non-target molecule and thereby removing any library members that either specifically bind to that molecule, or non-specifically bind to that molecule or anything else on the bead. Functional subtraction is carried out on libraries in bacteria, yeast, other eukaryotic cells, phage or viruses, provided that they surface display the mAb. The source of the mAb library, be they bacteria eukaryotic cells, phage or virus, are screened for binding to a one or more non-target molecule(s) covalently attached to a bead. Luminex beads are used in this procedure; they contain bound non-target molecule(s) and are also labeled with a fluorescent dye molecule. After incubation for sufficient time to allow binding to occur, the solution is injected into a flow cytometer. Beads (with attached mAbs) are sorted out of the mixture and discarded; non-binding library members are collected. This procedure is reiterated as necessary until few or no library members bind to beads. The remaining library members are expanded and become the source of the "functionally-subtracted" library.

Example 10

[95] Use of emulsions to select for mAbs. As outlined in Fig. 8B, *E. coli* infected with a library of phage M13 encoding scFv variants are compartmentalized with Ag-coated beads in a W/O emulsion to give, on average, ~1 bead and 1-10 infected bacteria per compartment. The library is prepared as described in Example 8 (as an option, the gp7- or gp9-modified library described in Example 8 is used). In each compartment, multiple copies of the recombinant phage are produced, some of which may bind to the Ag-coated bead. The emulsion is broken and the microbeads, along with bound phage, are isolated. In one alternative of this method, the beads are incubated with ligand or anti-M13 IgG Ab coupled to HRP (see Example 8), washed to remove unbound ligand or mAb, and incubated with hydrogen peroxide and fluorescein tyramide. Immobilized HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with

adjacent proteins. Hence, beads coated with M13 phage that bind to the Ag-coated beads become labeled with multiple fluorescein molecules. These beads are then enriched (together with the phage attached to them) by flow cytometry.

Example 11

[96] Use of emulsions to select for specific mAbs against a protein carrying a post translational modification. Initially, the entire library is flow-sorted to remove non-specific binders as described in Example 9. The functionally subtracted library is then re-sorted, using conditions established in Examples 6 and 7 in an emulsion together with Luminex beads to which the target antigen (e.g., Her2_pY1222 described in Example 5) is attached. The Luminex beads used in this assay are also labeled with a dye-molecule fluorescently discernible from fluorescein.

[97] The [bacteria + Ag-coated beads] emulsion is generated and collected for incubation in a syringe. After incubation at 37°C for sufficient time to allow phage production and binding of mAb to Ag-bead, the emulsion is broken, the beads separated and the washed beads are injected into a flow cytometer and subjected to sorting. Individual beads flow past a laser detector for fluorescence analysis. Based on this real-time analysis, a decision is made whether an individual bead has a fluorescent signal coincident with the bead above a chosen threshold (e.g., 3x above background). Droplets with signals above the threshold trigger voltage generation in the sorting electrode which causes the droplet to move into the "keep" channel. Beads with signals below the threshold flow into the waste channel.

[98] After sorting the beads, the sorted phage are recovered and plated on agar along with a bacterium capable of infection by the phage: the beads from the "keep" channel are spotted in the center of the plate, and mechanically dispersed using a sterile glass spreader. The resulting transduced bacteria, after overnight incubation, are grown in LB liquid medium and transduced using M13KO7 helper phage for scFv-M13 production.

These phage particles are subsequently tested by standard ELISA format for binding to both phosphorylated and non-phosphorylated Y1222 peptide.

[99] In a non-limiting version to this Example, non-phosphorylated Her2_pY1222 peptide is added to the medium prior to formation of the emulsion at a concentration such that phage and beads are incubated together in the presence of an excess of non-target peptide. Accordingly, the preponderance of phage that bind to beads have a higher affinity for the phosphorylated peptide (target) than for the non-phosphorylated (non-target) version of the peptide.

Example 12

[100] A high throughput approach for producing mAbs by the use of emulsions. This example outlines a procedure for high-throughput selection of mAbs in emulsions. Fig. 8B illustrates the procedure. scFv-displaying phage clones are emulsified in droplets with antigen-coated beads. After overnight growth in droplets, the emulsions are broken and the beads washed to remove unbound phage. The beads, which are labeled with a fluor if they are bound by phage, are analyzed by flow cytometry; beads labeled above a background threshold level are flow-sorted to individual wells of a 96-well microtiter plate. LB medium, F+ *E. coli* and helper phage are added to the wells and the plate incubated overnight. Clones are streaked for single-colony transductants and the colonies are expanded as a source of mAb fro purification and analysis. To confirm specificity, an aliquot of recombinant phage [displaying the encoded scFv] is added to wells containing a Luminex bead coated with the original antigen + 7 different Luminex beads, each coated with a different antigen. Labeled anti-M13 antibody is added to these wells and subjected to Luminex multiplex and the mAb gene is characterized by DNA sequencing (Fig. 16).

Example 13

[101] A high throughput approach for selecting mAbs by the use of emulsions and yeast display. In this non-limiting example yeast cells are genetically altered so as to express and secrete a library of IgG genes as well as to express and display on their surface a recombinant fusion protein containing the Aga2P gene of yeast-hemagglutinin (HA) site-staph A protein. The expressed protein is anchored to the surface of yeast cells via interaction of the Aga2P moiety to Aga1P (Fig. 17). Emulsions are formed as in the examples described above and mAbs secreted in the droplet bind to the surface of the mAb-secreting yeast. Also present in the droplets are beads carrying target antigen linked to a fluorescent moiety. Yeast with adherent mAb that binds to the antigen on the bead thus bind to the fluorescent beads. The emulsion is broken and yeast associated with fluorescent beads are sorted (Fig. 18).

Example 14

[102] This example outlines a procedure for high-throughput selection of mAbs produced by non-yeast eukaryotic cells in emulsions. Fig. 19A-19B illustrates the procedure. The cells alternatively express IgG endogenously (*e.g.,* hybridoma cells) or an IgG library is transformed into the cells. The cells are either coated with, or made genetically to expose, a surface moiety capable of capturing an IgG molecule. In the present non-limiting example, the cells are genetically manipulated to express Staph A protein on their surface. The cells, along with a fluorescently-labeled antigen, are emulsified and incubated in the droplet in the presence of medium such that if serum-containing medium is used, the serum has been depleted of exogenous immunoglobulin. After a sufficient period of time to allow the cells to secrete and capture the IgG molecule, the emulsion is broken and the aqueous phase is sorted using, as a non-limiting example, flow cytometry, to capture fluorescently-labeled cells.

Example 15

[103] In this example a mAb library, alternatively scFv or Fab or IgG, is transformed into non-yeast eukaryotic host cells. The mAbs are each part of a fusion protein together with a ligand that binds to a surface receptor on the host cell. Any of a large number of ligand:receptor pairs known in the art is used. The host cell is emulsified along with a labeled antigen in a droplet. The labeled Ag-host cell emulsion is incubated for a time-period sufficient to allow secretion and capture of the recombinant mAb-ligand fusion protein on the host cell surface. The emulsion is broken, the cells are washed, the washed cells flow sorted and fluorescent cells are collected.

Example 16

[104] The foregoing procedures with eukaryotic cells (yeast or higher eukaryotes) are alternatively carried out such that the target antigen is attached to a bead and such that the Ag-bead complex is fluorescent and cells binding such fluorescent beads are sorted on the basis of fluorescence.

Example 17

[105] The procedure in Example 10 is alternatively carried out such that the aqueous phase in the emulsion also contains one or more non-target molecules as described in Example 11, such that the mAb attached to the cells preferentially binds the target antigen as opposed to the non-target molecules in the aqueous solution.

Example 18

[106] This example provides an efficient and convenient procedure for constructing a random library, preferentially a mAb library. Triplet codons can be incorporated into a growing chain using DNA ligation and synthetic codon linkers. In one non-limiting example, an anchor primer is attached to a solid substrate, either a bead or the surface of a solid

substrate such as a 96-well microtiter plate. The substrate is used to facilitate washing of the growing chain between steps. In this example, the anchor primer is a hairpin loop that contains a upstream priming site and ends in a blunt-end. The anchor primer also contains a means for its removal from the substrate, for example a restriction enzyme site. Alternatives could also include a cleavable linker to the substrate, or the use of an organic solvent or physical condition (heat as an example) that would separate the anchor primer from the substrate. Alternatively the anchor could be left attached and PCR using the upstream primer site used to copy the final product. In a first step, a codon linker is ligated to the anchor primer. The codon linker consists of three specific bases (denoted by BBB) that are placed upstream of a type II restriction enzyme such as MlyI. In this example the 5' end of the codon linker is phosphorylated. In a non-limiting example the codon linker is constructed such that there is a hairpin-loop. Optionally in this first step the number of codon linkers can be varied such that 1 to 64 possible triplet codons can be added to the growing chain. Alternatively, one could add a subset of the 64 triplet codons, such as just the 20 that code for the unique set of amino acids, or the preferred codons used for expression in a specific organism, or codons that do not make a MlyI site (see step C). As a further non-limiting example, one can add more than 3 bases at a time to the growing chain. As an extension of this idea, one could add two codons at a time, using 4096 different codon-linkers if using the complete set of 64-codons, or using 400 codon-linkers if one uses the set of 20 amino acids. Or as another non-limiting alternative, if a defined amino acid is known to be wanted at a particular position in the chain, then a set of 20 codon linkers can be synthesized such that the first (or second) codon is "fixed". This would also be performed if more than one amino acid can be fixed within the sequence. For example, a set of 20 codon linkers could be made with the end sequence equivalent to 5'-ATGNNNATG-3' (SEQ ID NO: 1) wherein the middle bases form one of 20 amino acid codons, and the flanking ATG sequences incorporate a methionine amino acid into the protein chain. An advantage of adding two or more codons at a time is that the number of steps gets reduced and there would be an overall increase in efficiency in time and accuracy, and decrease in cost. As a further example, the codon-linkers can be added at differing or identical ratios such that there either will or

will not be a skewing of which codon linkers get ligated into the growing chain. It is known that T4 DNA ligase has preferred DNA sequences for intermolecular and intra-molecular DNA ligation. This ratio can be based on the molar mass ratio of the various codon-linkers or could be based on the relative efficiency of a particular codon linker to be ligated into the growing chain. This relative ratio can be empirically-determined by constructing a pilot library, and sequencing several clones to determine the cloning efficiency of each codon linker. To increase the ligation efficiency, a crowding agent(s) such as (10-25%) polyethylene glycol may be optionally added to the ligation mix. Optionally, if both the anchor and linker primers are 5' phosphorylated (either during oligonucleotide synthesis or either post-ligation or synthesis using T4 polynucleotide kinase and ATP), then exonuclease can be used to remove any unligated anchor or codon primer remaining in the reaction. In the non-limiting example shown, the ligated anchor-primer/codon-linker forms a molecules with both ends sealed (i.e., a single DNA strand without a free end). Such a covalently-sealed molecule would be resistant to an exonuclease. An example alternative means of protecting the end of the codon linker would be to incorporate a thiol-group that would render the end of the molecule similarly resistant to exonuclease digestion.

[107] A restriction enzyme is used to remove the codon-linker (except for the NNN sequence) from the ligated anchor-primer/codon linker product. The restriction endonulcease MlyI makes a double-strand cut 5 bases upstream (on both strands of the DNA) of its recognition site. The restriction digested product can be washed away from the bound anchor-primer. The previous process can be repeated to add more codon linkers. When the chain has grown such that the desired end is reached, then a terminal linker can be attached. This terminal linker can optionally incorporate NNN sequence at its 5' end. The terminal linker could also contain a downstream primer-binding site that could be used in conjunction with the anchor primer binding site to enable one to PCR or amplify the grown chain. The completed chain can be optionally separated from the substrate using various means, including a restriction enzyme, chaotropic agent, change in pH, organic solvent, heat, chemical or enzymatic decoupling. The freed DNA fragment can

be cloned directly into a vector, used for splice overlap extension, or amplified by one of many means, including PCR and rolling circle amplification.

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I CLAIM:

1. A method for separating antibody molecules, comprising the steps of:
   - forming an emulsion comprising a first liquid and a second liquid which are immiscible, wherein the first liquid is aqueous and comprises:
     - antibody-producing cells, and
     - an antigen attached to a solid substrate which is suspendable in the first liquid,
   - incubating the emulsion under conditions and for a time such that the antibody-producing cells produce antibody molecules within the first liquid and the antibody molecules bind to antigen when the antibody molecules have appropriate specificity and affinity;
   - breaking the emulsion and collecting the first liquid;
   - separating solid substrate attached to antigen which is bound to antibody molecules from solid substrate attached to antigen which is not bound to antibody molecules or from antibody molecules not bound to solid substrate.
2. The method of claim 1 wherein the antibody-producing cells produce a library of diverse antibody molecules, and at least 10 % of the solid substrate bound to antibody is bound to a homogeneous population of antibody molecules.
3. The method of claim 1 wherein the antibody molecules are encoded by a recombinant construct in the antibody-producing cells, or in a virus infecting the antibody-producing cells, or in a bacteriophage infecting the antibody-producing cells, wherein the antibodies are produced on the surface of said antibody-producing cells, or said virus, or said bacteriophage, and said method further comprises the step of:
   - culturing the antibody-producing cells, or virus, or bacteriophage to produce additional quantities of antibody molecules or additional quantities of nucleic acids encoding the antibody molecules.
4. The method of claim 1 wherein the first liquid further comprises competitor molecules which are not attached to the solid substrate and which can compete with the antigen attached to the solid substrate for antibody binding.

5. The method of claim 1 wherein the competitor molecules are soluble molecules of the antigen.
6. The method of claim 1 wherein the competitor molecules are distinct from the antigen.
7. The method of claim 1 wherein the first liquid further comprises a competitor solid substrate to which none of the antigen is attached.
8. The method of claim 1 wherein the solid substrate is detectably labeled.
9. The method of claim 1 wherein the antibody-producing cells express the antibody on the cell surface.
10. The method of claim 1 wherein the antibody-producing cells secrete the antibody into the first liquid and the antibody-producing cells capture the antibody on their surface.
11. The method of claim 1 wherein the antibody is produced by the antibody-producing cells as a fusion protein with an enzyme.
12. The method of claim 1 wherein the antibody is produced by the antibody-producing cells as a fusion protein with a structural protein expressed on an outer surface of an antibody-producing cell, a virus infecting the antibody-producing cell, or a bacteriophage infecting the antibody producing cell.
13. The method of claim 1 wherein the antibody is produced by the antibody-producing cells as a fusion protein with a ligand for a receptor on the surface of the antibody-producing cell.
14. The method of claim 1 wherein the antibody is produced by the antibody-producing cells as a fusion protein with a detectable protein.
15. The method of claim 1 wherein prior to the step of separating, the antibody molecules are fluorescently labeled and the separating step is by flow cytometry.
16. The method of claim 1 wherein prior to the step of separating, the antibody molecules are attracted to a paramagnetic particle and the separating step is by magnetic attraction.
17. The method of claim 1 wherein prior to the step of separating, the antibody molecules are attracted to an antibody affinity reagent and the separating step is by affinity attraction.
18. The method of claim 1 wherein prior to the step of separating, the antibody molecules are labeled with a detectable reagent.
19. The method of claim 1 wherein the antibody is a monoclonal antibody, a Fab fragment, or a single chain Fv molecule.

20. The method of claim 1 wherein the antibody-producing cells are selected from the group consisting of human cells, bacterial cells, yeast cells, hybridoma cells, virus-infected cells, and bacteriophage–infected cells.
21. The method of claim 1 wherein the antibody is detectably labeled.
22. The method of claim 1 wherein the solid substrate is a bead.
23. A method for making a reagent useful for detecting an interaction with an antigen, comprising the steps of:
    - forming an emulsion comprising a first liquid and a second liquid which are immiscible, wherein the first liquid is aqueous and comprises:
        - antibody-producing cells, and
        - a solid substrate which is suspendable in the first liquid; and
    - incubating the emulsion under conditions and for a time such that the antibody-producing cells produce antibody molecules within the first liquid and a complex is formed comprising the solid substrate and the antibody molecules.
24. The method of claim 23 wherein the antibody molecules are produced as fusion proteins comprising a first and a second polypeptide, wherein the first polypeptide is the antibody molecule and the second polypeptide binds to the solid substrate.
25. The method of claim 23 wherein the second polypeptide is a His6 tag.
26. The method of claim 23 further comprising the step of storing the emulsion.
27. The method of claim 23 further comprising the step of freezing the emulsion.
28. The method of claim 23 further comprising the steps of:
    - breaking all or a part of the emulsion and collecting the first liquid; and
    - adding a detectably labeled antigen to the complex comprising the antibody molecules and the solid substrate in the first liquid and incubating under conditions and for a time such that the antibody molecules bind to the antigen when the antibody molecules have appropriate specificity and affinity.
29. The method of claim 28 further comprising the step of separating the complex bound to antigen from antigen not bound to the complex.
30. The method of claim 28 further comprising the step of separating the complex bound to antigen from complex not bound to antigen.

31. The method of claim 28 further comprising the step of adding non-antigen molecules which can compete with the detectably labeled antigen for binding of relatively non-specific antibody.
32. The method of claim 28 wherein the first liquid further comprises a competitor substrate which can compete with the complex comprising the antibody molecules for relatively non-specific binding to antigen.
33. The method of claim 23 wherein the antibody molecules are encoded by a recombinant construct in the antibody-producing cells, or in a virus infecting the antibody-producing cells, or in a bacteriophage infecting the antibody-producing cells, and wherein the antibodies are produced on the surface of said antibody-producing cells, or said virus, or said bacteriophage.
34. The method of claim 33 wherein the antibody-producing cells express a protein which strongly interacts with the solid substrate, wherein the antibody and the protein are expressed on the surface of the antibody-producing cells, the virus, or the bacteriophage, whereby the antibody and the solid substrate are linked via the antibody-producing cells, virus, or bacteriophage.
35. The method of claim 33 further comprising the steps of:
    - separating the complex bound to antigen from antigen not bound to the complex;
    - culturing the antibody-producing cells, or virus, or bacteriophage to produce additional quantities of antibody molecules or additional quantities of recombinant construct encoding the antibody molecules.
36. The method of claim 33 wherein the antibody, the virus, or the bacteriophage is detectably labeled.
37. The method of claim 33 further comprising determining sequence of the recombinant construct encoding said antibody molecules by performing nucleic acid sequencing reactions.
38. The method of claim 23 wherein the solid substrate is detectably labeled.
39. The method of claim 23 wherein the antibody is a monoclonal antibody, a Fab fragment, or a single chain Fv molecule.
40. The method of claim 23 wherein the antibody-producing cells are selected from the group consisting of human cells, bacterial cells, yeast cells, hybridoma cells, virus-infected cells, and bacteriophage–infected cells.

41. The method of claim 23 wherein the antibody-producing cells produce a library of diverse antibody molecules, and at least 10 % of the solid substrate bound to antibody is bound to a homogeneous population of antibody molecules.
42. A composition comprising an emulsion, wherein a first phase of the emulsion is aqueous and comprises a solid substrate and an antibody-producing cell, wherein the antibody-producing cells express a protein which strongly interacts with the solid substrate.
43. The composition of claim 42 which is frozen.
44. The composition of claim 42 wherein the protein and the antibody are expressed as a fusion protein.
45. The composition of claim 42 wherein the protein and the antibody are expressed as separate molecules.
46. The composition of claim 42 wherein the antibody-producing cell expresses antibody molecules that are encoded by a recombinant construct in the antibody-producing cells, or by a virus infecting the antibody-producing cells, or by a bacteriophage infecting the antibody-producing cells, and wherein the antibody and the protein are expressed on the surface of the antibody producing cells, the virus, or the bacteriophage, whereby the antibody and the solid substrate are linked via the antibody-producing cells, virus, or bacteriophage.
47. The composition of claim 42 wherein the antibody is a monoclonal antibody, a Fab fragment, or a single chain Fv molecule.
48. The composition of claim 42 wherein the antibody-producing cells are selected from the group consisting of human cells, bacterial cells, yeast cells, hybridoma cells, virus-infected cells, and bacteriophage–infected cells.
49. The composition of claim 42 comprising a plurality of antibody-producing cells, wherein the antibody-producing cells produce a library of diverse antibody molecules, and wherein at least 10 % of the solid substrate bound to antibody is bound to a homogeneous population of antibody molecules.
50. A method for making a cell which displays a secreted protein on its surface, comprising:
    - forming an emulsion comprising a first liquid and a second liquid which are immiscible, wherein the first liquid is aqueous and comprises:
        - a cell which produces and secretes a desired protein, and

- a bifunctional reagent which binds to the desired protein and to the cell; and

♦ incubating the emulsion under conditions and for a time such that the cell produces and secretes the desired protein within the first liquid and the bifunctional reagent binds to the desired protein and to the cell, whereby the desired protein is linked to the cell which produces it.

51. The method of claim 50 wherein the cell is a B cell.
52. The method of claim 50 wherein the cell is a hybridoma.
53. The method of claim 50 wherein the desired protein is an antibody molecule.
54. The method of claim 50 wherein the desired protein is a growth factor.
55. The method of claim 53 wherein the bifunctional reagent comprises Staph A protein.
56. The method of claim 53 wherein the bifunctional reagent comprises an anti-Fc antibody.
57. The method of claim 50 wherein the bifunctional reagent comprises an antibody molecule which binds to a cellular protein displayed on the cell surface.
58. The method of claim 50 wherein the bifunctional reagent comprises a ligand for a cellular protein displayed on the cell surface.
59. The method of claim 50 further comprising the step of breaking the emulsion and contacting it with a labeled molecule which specifically binds to the desired protein.
60. The method of claim 59 further comprising the step of subjecting the broken emulsion to flow cytometry to detect and/or isolate cells which are bound to the labeled molecule.
61. The method of claim 50 wherein the emulsion comprises a population of mixed cells which produce a plurality of diverse proteins.
62. The method of claim 50 wherein the first liquid further comprises a cell surface modifying reagent, and wherein the bifunctional reagent binds to the cell via the modifying reagent.
63. The method of claim 62 wherein the cell surface modifying reagent biotinylates the cell surface.
64. A composition comprising an emulsion, wherein a first phase of the emulsion is aqueous and comprises:
    - a cell which produces and secretes a desired protein; and
    - a bifunctional reagent which binds to the desired protein and to the cell;

wherein when the cell expresses the desired protein, the bifunctional reagent binds the desired protein to the cell which produced it.

65. A composition comprising a mixed population of cells which are each decorated with a plurality of molecules of a desired protein, wherein a bifunctional reagent binds the desired protein to the surface of the cell, wherein the cell contains a nucleic acid which encodes the desired protein, wherein different cells in the mixed population are decorated with different desired proteins.
66. The composition of claim 64 or 65 wherein the cell is a B cell.
67. The composition of claim 64 or 65 wherein the cell is a hybridoma.
68. The composition of claim 64 or 65 wherein the desired protein is an antibody molecule.
69. The composition of claim 64 or 65 wherein the desired protein is a growth factor.
70. The composition of claim 68 wherein the bifunctional reagent comprises Staph A protein.
71. The composition of claim 68 wherein the bifunctional reagent comprises an anti-Fc antibody.
72. The composition of claim 64 or 65 wherein the bifunctional reagent comprises an antibody molecule which binds to a cellular protein displayed on the cell surface.
73. The composition of claim 64 or 65 wherein the bifunctional reagent comprises a ligand for a cellular protein displayed on the cell surface.

Fig. 1A-1B

F_X_PY20	ACTGTCCTCGAGGAATTCATGGGTGTTCAGCTGCAGCAGTCT
R_X-PY20	GACAGTCTCGAGAGAACCACCACCACCAGAACCA
PY20_F1	ATGGGTGTTCAGCTGCAGCAGTCTGGTCCGGAACTGGTTAAACCGGGTGCTTCTGTTAAAATCTCTTGCAAAACCTCTGG
PY20_F2	TTACACCTTCACCGAATACACCATGCACTGGATGAAACAGTCTCACGGTAAATCTCTGGAATGGATGGGTGGTATCAACC
PY20_F3	CGAACTCTGGTGGTACCCGTGACAACCAGCGTTTCAAAGGTCGTGCTACCCTGACCGTTGACAAATCTTCTTCTATCGCT
PY20_F4	TACATGGAACTGCGTTCTCTGACCTCTGAAGACTCTGCTGTTTACTACTGCGCTCGTCGTGGTCCGTACGGTAACTACTA
PY20_F5	CAACTCTTACTACTTCGACTACTGGGGTCAGGGTACCACCCTGACCGTTTCTTCTGCTCGTTCTGGTGGTGGTGGTTCTG
PY20_F6	GTGGTGGTGGTTCTGGTGGTGGTGGTTCTATGGACGTTCAGATGACCCAGACCACCTCTTCTCTGTCTGCTTCTCTGGGT
PY20_F7	GACCGTGTTACCATCTCTTGCTCTGCTTCTCAGGGTATCTCTAACTACCTGAACTGGTACCAGCAGAAACCGGACGGTAC
PY20_F8	CGTTAAACTGCTGATCTACTACACCTCTTCTCTGCACTCTGGTGTTCCGTCTCGTTTCTCTGGTTCTGGTTCTGGTACCG
PY20_F9	ACTACTCTCTGACCATCTCTAACCTGGAACCGGAAGACGTTGCTACCTACTACTGCCAGCAGTACTCTAAAGTTCCGTGG
PY20_F10	ACCTTCGGTGGTGGTACCAAACTGGAAATCAAATCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTC
PY20_R1	GAACCACCACCACCAGAACCACCACCACCAGAACCACCAC
PY20_R2	CACCAGATTTGATTTCCAGTTTGGTACCACCACCGAAGGTCCACGGAACTTTAGAGTACTGCTGGCAGTAGTAGGTAGCA
PY20_R3	ACGTCTTCCGGTTCCAGGTTAGAGATGGTCAGAGAGTAGTCGGTACCAGAACCAGAACCAGAGAAACGAGACGGAACACC
PY20_R4	AGAGTGCAGAGAAGAGGTGTAGTAGATCAGCAGTTTAACGGTACCGTCCGGTTTCTGCTGGTACCAGTTCAGGTAGTTAG
PY20_R5	AGATACCCTGAGAAGCAGAGCAAGAGATGGTAACACGGTCACCCAGAGAAGCAGACAGAGAAGAGGTGGTCTGGGTCATC
PY20_R6	TGAACGTCCATAGAACCACCACCACCAGAACCACCACCACCAGAACCACCACCACCAGAACGAGCAGAAGAAACGGTCAG
PY20_R7	GGTGGTACCCTGACCCCAGTAGTCGAAGTAGTAAGAGTTGTAGTAGTTACCGTACGGACCACGACGAGCGCAGTAGTAAA
PY20_R8	CAGCAGAGTCTTCAGAGGTCAGAGAACGCAGTTCCATGTAAGCGATAGAAGAAGATTTGTCAACGGTCAGGGTAGCACGA
PY20_R9	CCTTTGAAACGCTGGTTGTCACGGGTACCACCAGAGTTCGGGTTGATACCACCCATCCATTCCAGAGATTTACCGTGAGA
PY20_R10	CTGTTTCATCCAGTGCATGGTGTATTCGGTGAAGGTGTAACCAGAGGTTTTGCAAGAGATTTTAACAGAAGCACCCGGTT
PY20_R11	TAACCAGTTCCGGACCAGACTGCTGCAGCTGAACACCCAT

Fig. 1A

MGVQLQQSGPELVKPGASVKISCKTSGYTFTEYTMHWMKQSHGKSLEWMGGINPNSGGTRDNQRFKGRATLTVDKS
SSIAYMELRSLTSEDSAVYYCARRGPYGNYYNSYYFDYWGQGTTLTVSSARSGGGGSGGGGSGGGGSMDVQMTQTT
SSLSASLGDRVTISCSASQGISNYLNWYQQKPDGTVKLLIYYTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDVA
TYYCQQYSKVPWTFGGGTKLEIKSGGGGSGGGGSGGGG

Fig. 1B

Fig. 2

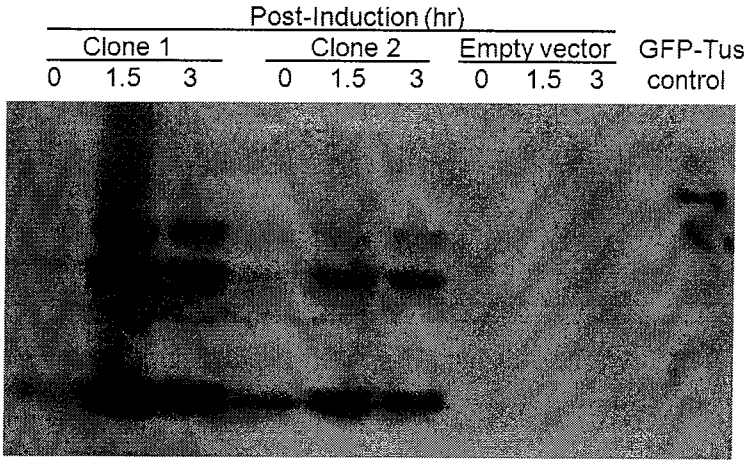

Post-Induction (hr)
Clone 1
0 1.5 3
Clone 2
0 1.5 3
Empty vector
0 1.5 3
GFP-Tus
control

Fig. 3

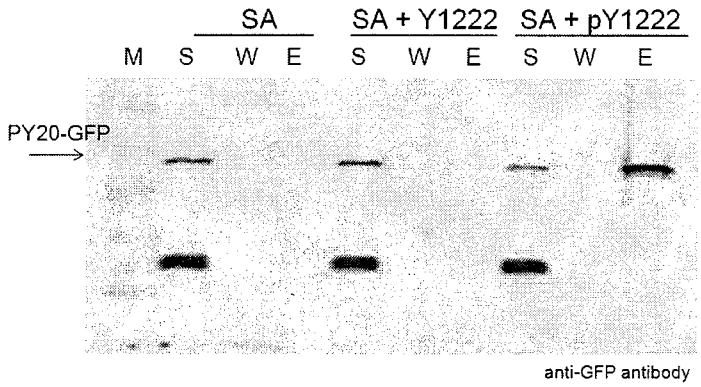


Fig. 4

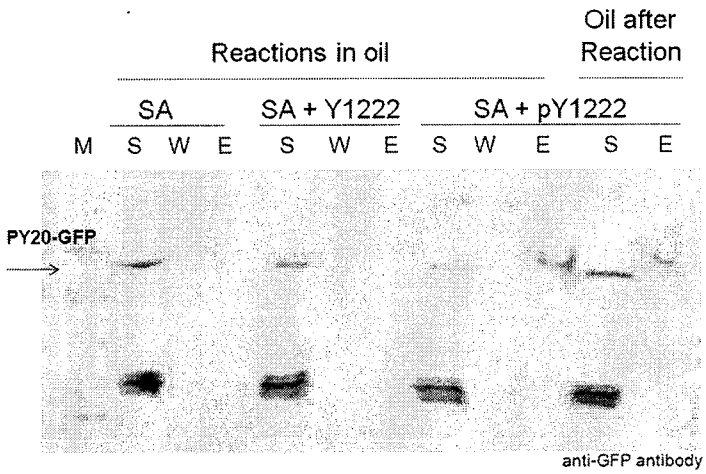
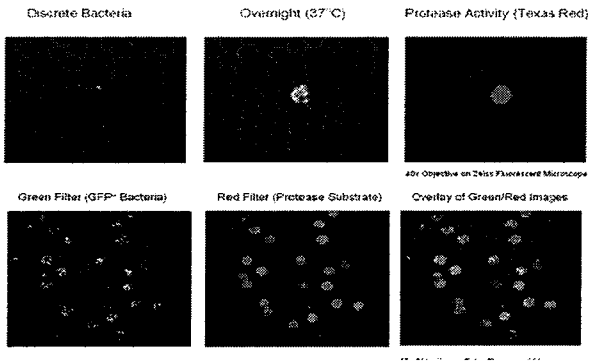

Reactions in oil
Oil after Reaction
SA
SA + Y1222
SA + pY1222
M S W E S W E S W E S E
PY20-GFP
anti-GFP antibody

Fig. 5A-5F



5A	5B	5C
5D	5E	5F

Fig. 6

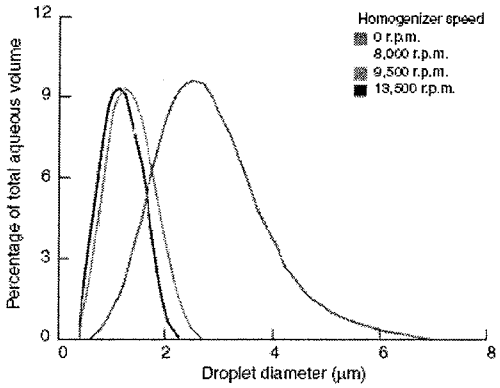

Homogenizer speed
0 r.p.m.
8,000 r.p.m.
9,500 r.p.m.
13,500 r.p.m.
Percentage of total aqueous volume
12
9
6
3
0
0
2
4
6
8
Droplet diameter (μm)

Fig. 7

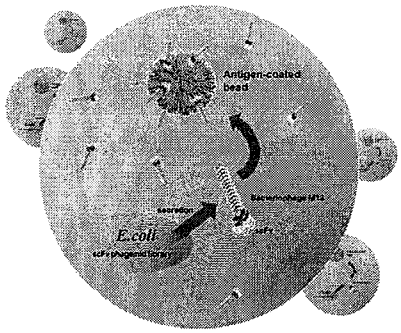
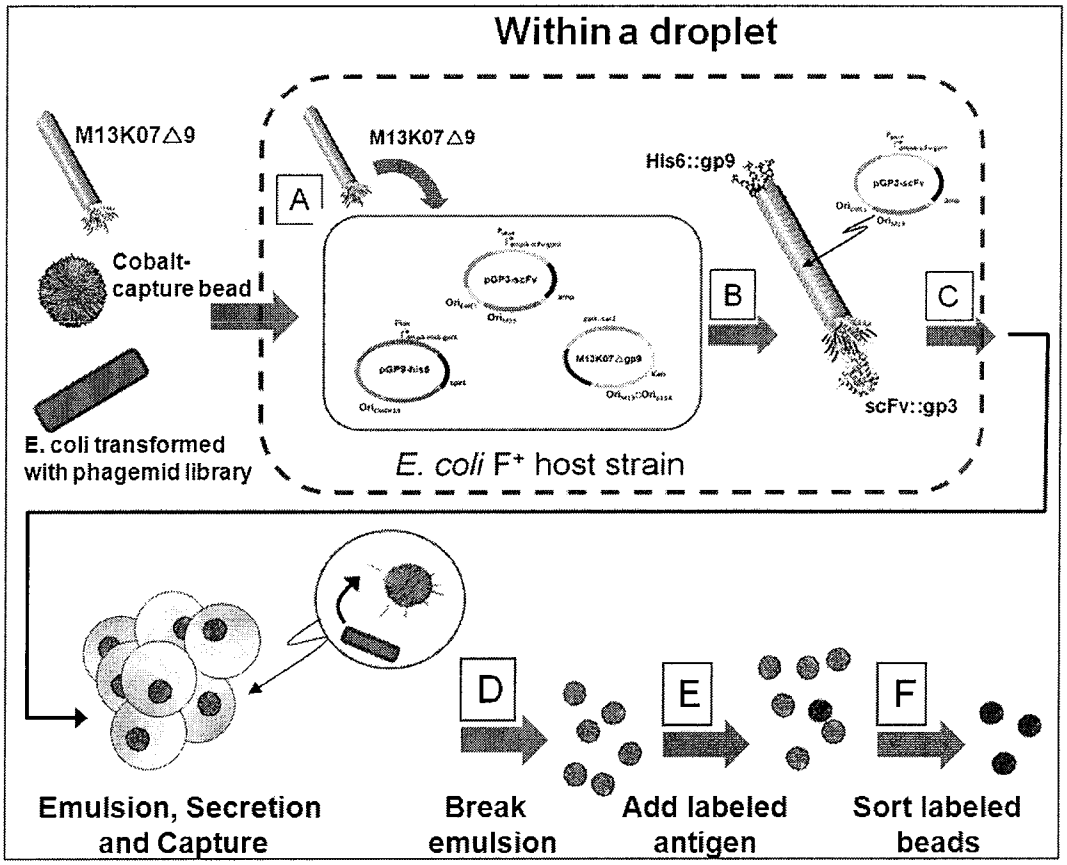


Fig. 8A


Within a droplet
M13K07△9
M13K07△9
His6::gp9
A
B
C
Cobalt-capture bead
E. coli transformed with phagemid library
E. coli F+ host strain
scFv::gp3
D
E
F
Emulsion, Secretion and Capture
Break emulsion
Add labeled antigen
Sort labeled beads

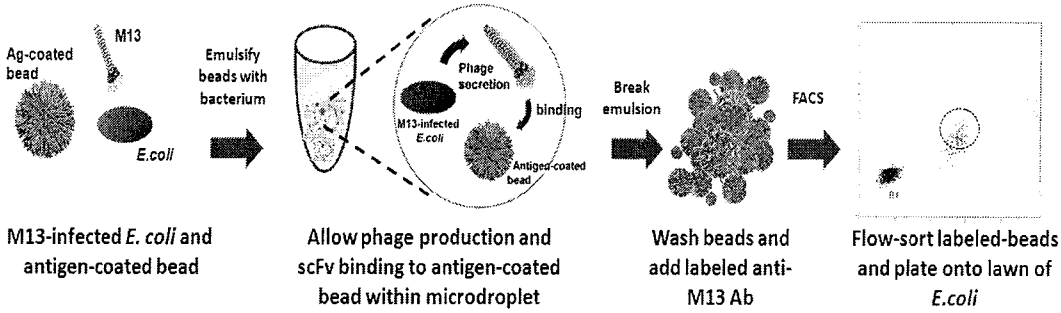

Ag-coated bead
M13
E.coli
Emulsify beads with bacterium
Phage secretion
binding
M13-infected E.coli
Antigen-coated bead
Break emulsion
FACS
M13-infected E. coli and antigen-coated bead
Allow phage production and scFv binding to antigen-coated bead within microdroplet
Wash beads and add labeled anti-M13 Ab
Flow-sort labeled-beads and plate onto lawn of E.coli

Fig. 8B

Fig. 9A-9B

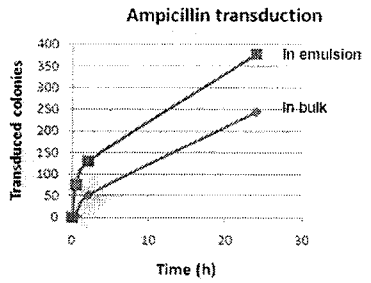


Fig. 9A

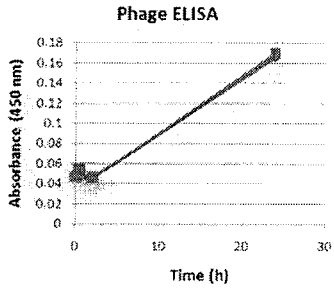


Fig. 9B

Fig. 10A-10B

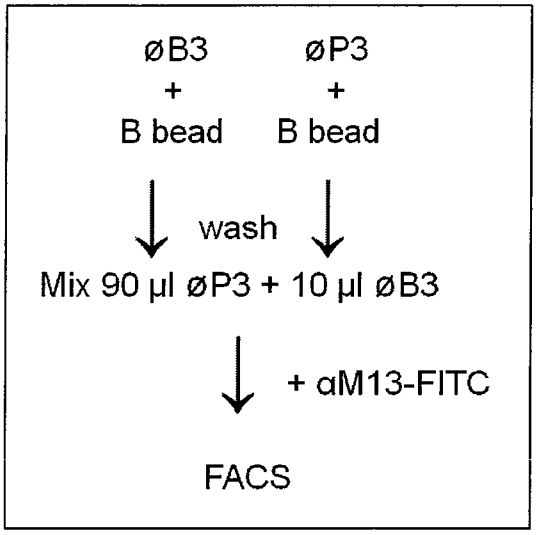


Fig. 10A

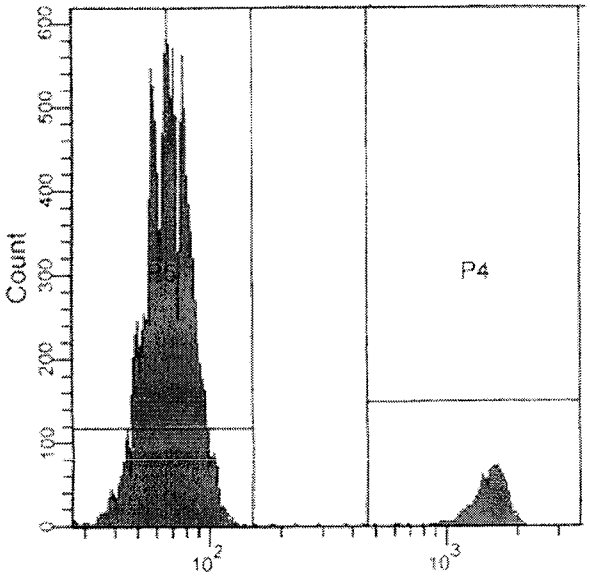


Fig. 10B

Fig.11

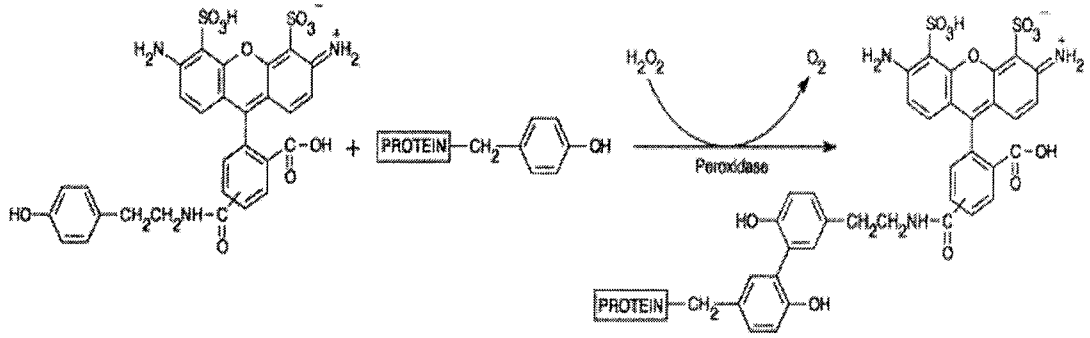


Fig. 12

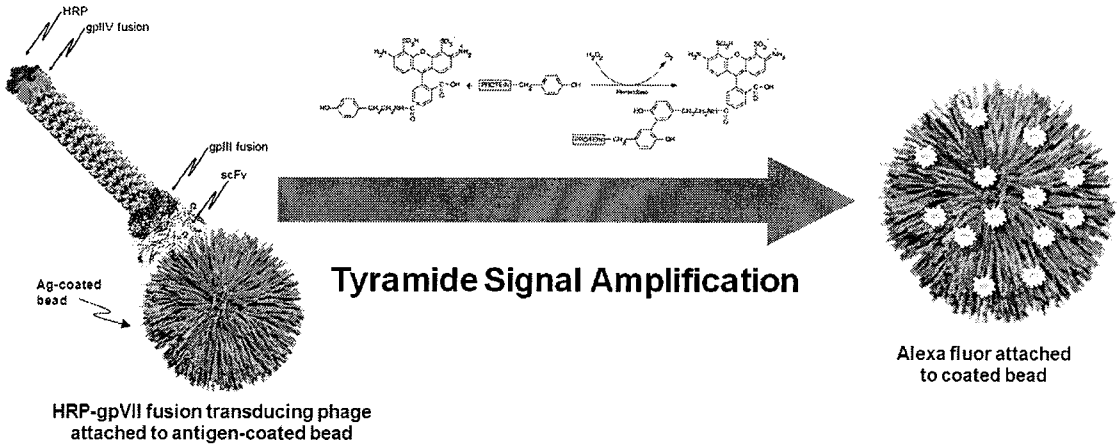

HRP
gpIIV fusion
gpIII fusion
scFv
Ag-coated bead
Tyramide Signal Amplification
HRP-gpVII fusion transducing phage attached to antigen-coated bead
Alexa fluor attached to coated bead

Fig. 13

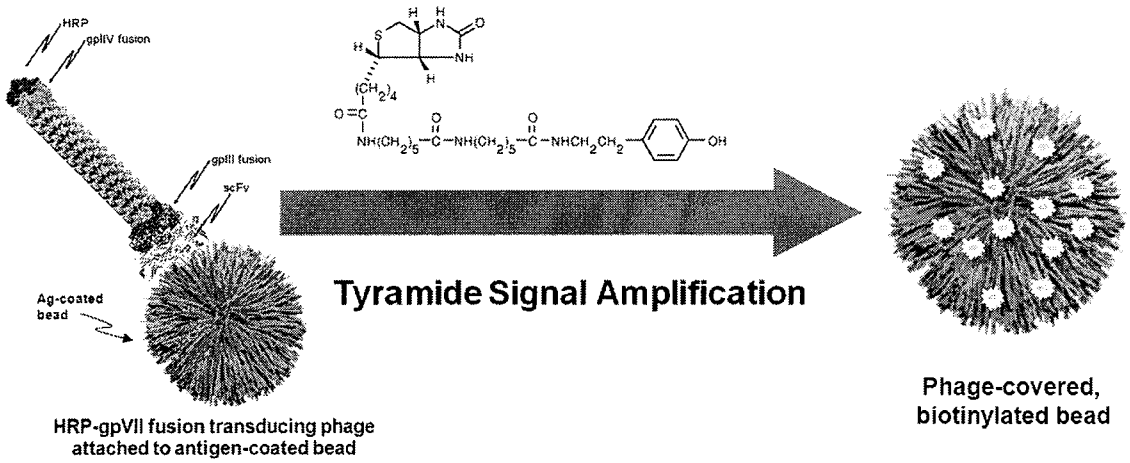
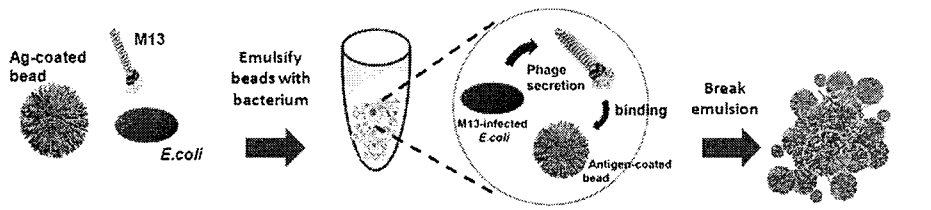

HRP
gpIIV fusion
gpIII fusion
scFv
Ag-coated bead
Tyramide Signal Amplification
HRP-gpVII fusion transducing phage attached to antigen-coated bead
Phage-covered, biotinylated bead

Fig. 14



E.coli is infected with a gp7 (or gp9) HRP-encoding M13KO7 derivative

Allow phage production and scFv binding to antigen-coated bead within microdroplet

Wash beads and add biotin-tyramide reagent

Fig. 15

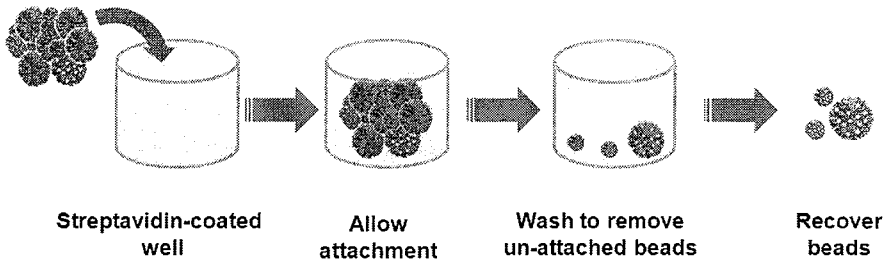

Streptavidin-coated well
Allow attachment
Wash to remove un-attached beads
Recover beads

Fig. 16

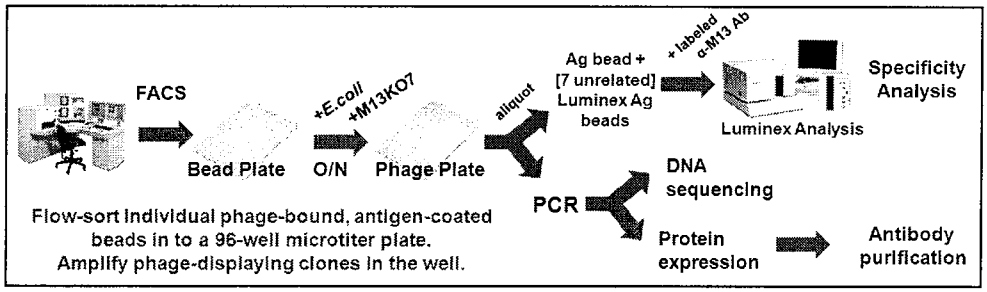

FACS
Bead Plate
+E.coli
+M13KO7
O/N
Phage Plate
aliquot
Ag bead +
[7 unrelated]
Luminex Ag
beads
+ labeled
α-M13 Ab
Luminex Analysis
Specificity
Analysis
PCR
DNA
sequencing
Protein
expression
Antibody
purification
Flow-sort individual phage-bound, antigen-coated
beads in to a 96-well microtiter plate.
Amplify phage-displaying clones in the well.

Fig. 18

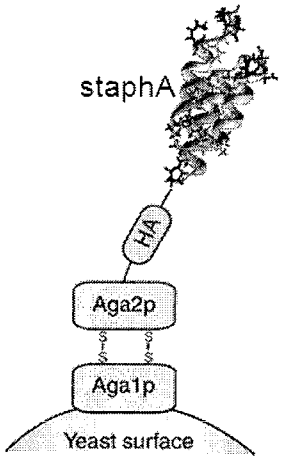


Fig. 18

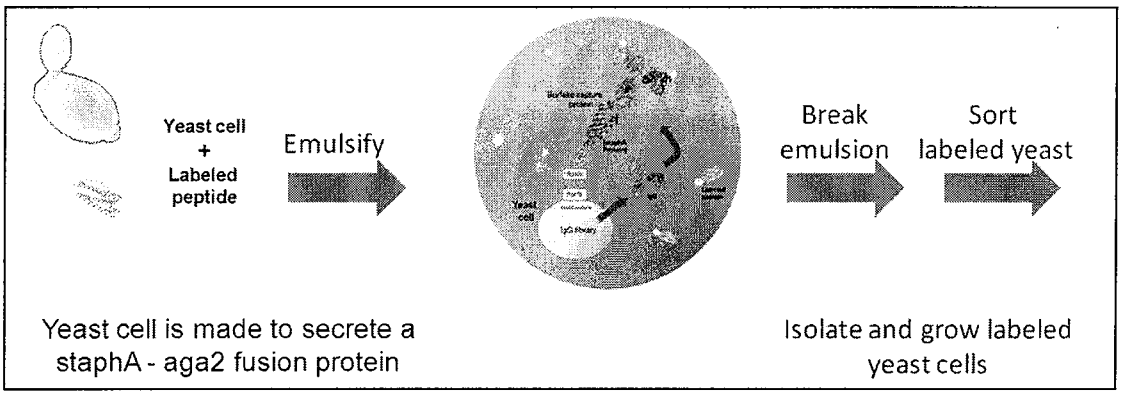

Yeast cell
+
Labeled
peptide
Emulsify
Break
emulsion
Sort
labeled yeast
Yeast cell is made to secrete a
staphA - aga2 fusion protein
Isolate and grow labeled
yeast cells

Fig. 19A

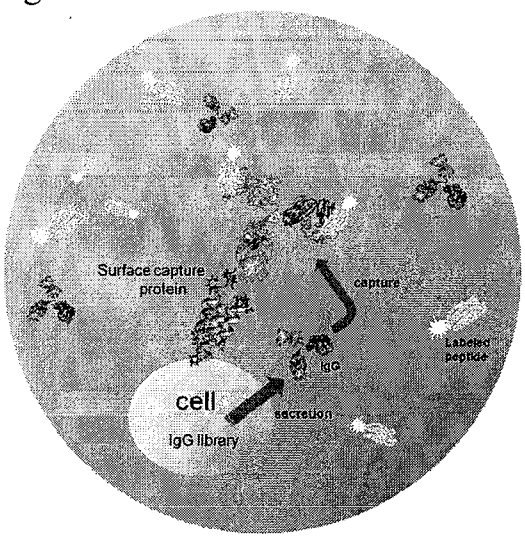


Fig. 19B

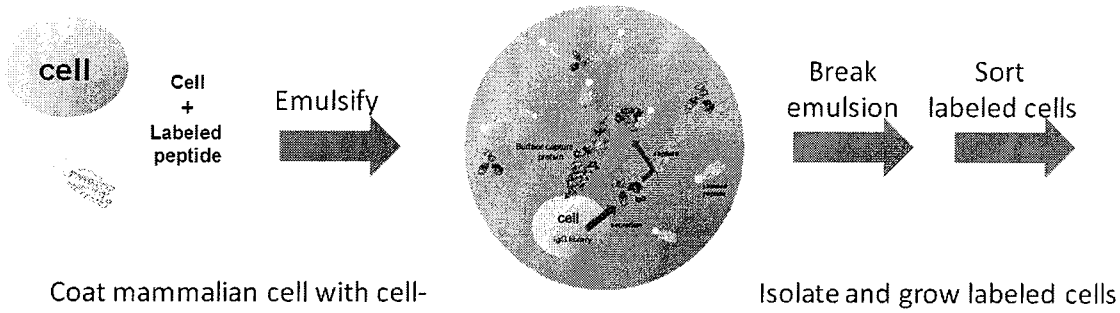
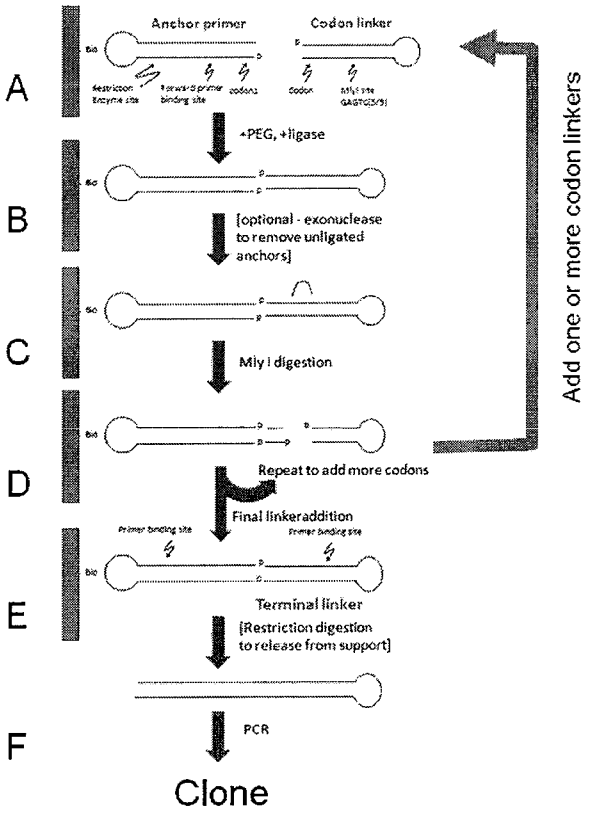


Fig. 20


Anchor primer
Codon linker
A
+PEG, +ligase
B
[optional - exonuclease to remove unligated anchors]
C
Mly I digestion
D
Repeat to add more codons
Final linker addition
E
Terminal linker
[Restriction digestion to release from support]
F
PCR
Clone
Add one or more codon linkers

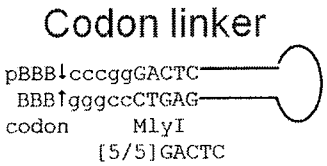

Codon linker
pBBB↓cccggGACTC
BBB↑gggccCTGAG
codon MlyI
[5/5]GACTC

Fig. 21

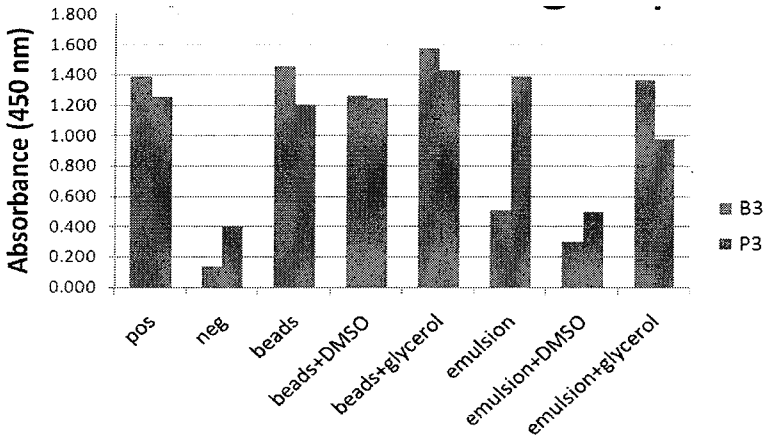
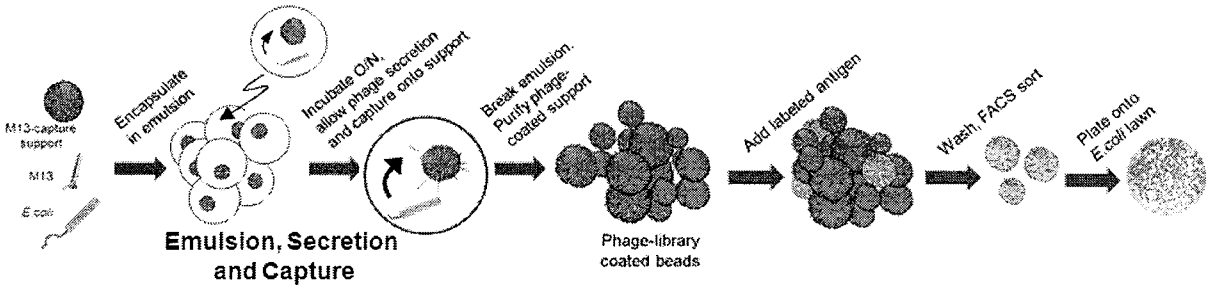

Absorbance (450 nm)
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
pos
neg
beads
beads+DMSO
beads+glycerol
emulsion
emulsion+DMSO
emulsion+glycerol
B3
P3

Fig. 22.


M13-capture support
M13
E.coli
Encapsulate in emulsion
Incubate O/N, allow phage secretion and capture onto support
Emulsion, Secretion and Capture
Break emulsion. Purify phage-coated support
Phage-library coated beads
Add labeled antigen
Wash, FACS sort
Plate onto E.coli lawn

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/33537

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53; C07K 16/00 (2010.01)
USPC - 435/7.1; 530/387.1
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)
USPC- 435/7.1; 530/387.1

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)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed: antibody, in vitro compartmentalization, screen, library, emulsion, bifunctional, cell surface, cell-based

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 2007/0077572 A1 (TAWFIK et al.) 5 April 2007 (05.04.2007) para [0023]-[0024], [0027], [0029], [0031], [0038]-[0039], [0042]-[0044], [0048], [0051], [0071], [0097], [0101], [0106], [0110], [0116], [0119], [0126], [0153], [0156]-[0162], [0164], [0171], [0174], [0191]-[0195], [0248], [0252], [0344]	23, 26-27, 33, 35, 37-41, 50-58, 64-73 --- 1-22, 24-25, 28-32, 34, 36, 42-49, 59-63
Y	ROTHE et al. In vitro display technologies reveal novel biopharmaceutics. FASEB J. 2006, 20(10):1599-1610; Fig 1; pg 1599, para 1-2; pg 1600, para 3	1-22
Y	ABI-GHANEM et al. Phage display selection and characterization of single-chain recombinant antibodies against Eimeria tenella sporozoites. Veterinary Immunology and Immunopathology 2008, 121(1-2):58-67; abstract; pg 61, para 1	14, 21, 24-25, 34, 36, 42-49
Y	US 2009/0005264 A1 (RAKESTRAW et al.) 1 January 2009 (01.01.2009) abstract; para [0004], [0008], [0063], [0076]-[0077]	10, 13, 18, 28-32, 59-63
Y	LEE et al. Bivalent antibody phage display mimics natural immunoglobulin. J. Immunol. Methods 2004, 284(1-2):119-132; abstract; pg 124, para 4-5	4-6, 31
Y	US 2007/0009959 A1 (LAWSON et al.) 11 January 2007 (11.01.2007) para [0049]	7, 32
A	BERNATH et al. In vitro compartmentalization (IVC): a high-throughput screening technology using emulsions and FACS. Discovery Med. 2004, 4(20):49-53	1-73

☒ 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
13 June 2010 (13.06.2010)	09 JUL 2010
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: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/33537

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YONEZAWA et al. DNA display for in vitro selection of diverse peptide libraries. Nucleic Acids Res. 2003, 31(19): article e118; pages 1-5	1-73
A	HOSSE et al. A new generation of protein display scaffolds for molecular recognition. Protein Sci. 2006, 15(1):14-27	1-73
A	US 2005/0042648 A1 (GRIFFITHS et al.) 24 February 2005 (24.02.2005)	1-73
A	US 2006/0153924 A1 (GRIFFITHS et al.) 13 July 2006 (13.07.2006)	1-73
A	US 2006/0154298 A1 (GRIFFITHS et al.) 13 July 2006 (13.07.2006)	1-73
A,P	SUMIDA et al. Bicistronic DNA display for in vitro selection of Fab fragments. Nucleic Acids Res. December 2009, 37(22):article e147, pages 1-9	1-73