Selection of human antibody fragments by phage display

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Here, we describe a protocol for the selection of human antibody fragments using repertoires displayed on filamentous bacteriophage. Antigen-specific clones are enriched by binding to immobilized antigen, followed by elution and repropagation of phage. After multiple rounds of binding selection, specific clones are identified by ELISA. This article provides an overview of phage display and antibody technology, as well as detailed protocols for the immobilization of antigen, the selection of repertoires on purified or complex antigens and the identification of binders.

INTRODUCTION

Phage display technology is a powerful method for the selection of monoclonal antibodies against a given antigen. In addition to antibodies, phage display has also been used for the selection of other polypeptides, including zinc fingers[1], peptides[2], SH3 domains[3] and hormones[4]. More recently, the methodology has been expanded to allow for the selection of enzymatic function[5,6] and for the detection of protein domains[7].

Monoclonal antibodies

The development of monoclonal antibodies and hybridoma technology was pioneered by Georges Köhler and Cesar Milstein at the MRC Laboratory of Molecular Biology in Cambridge. Published in a landmark paper in 1975[8], their method was based on the immunization of mice, followed by fusion of antibody-secreting B cells with an immortal myeloma cancer cell line. This allowed the generation of hybrid cells (hybridomas) that could be propagated indefinitely in tissue culture. Hybridoma technology allowed the generation of defined affinity reagents and continues to provide the backbone of clinical diagnostics, as well as a wide range of antibodies for academic research. The initial impact of the technology on therapeutic applications, however, was limited, mainly due to unwanted immune responses elicited by the murine monoclonals[9]. Other limitations of the technique relate to the restriction to the set of natural antibody isotypes, poor immunogenicity of some targets[10–12] and general issues associated with the expression of full-length antibodies, such as cost, glycosylation patterns and a lack of purification tags[13].

Phage display technology

The generation of fully human monoclonals was closely linked to the development of phage display technology. Phage display was first described by George Smith[2] in 1985, but initially the technique was limited to the selection of peptides. However, it was later shown that antibody fragments could be successfully displayed on phage[14]. Phage display is based on genetic engineering of coat proteins of filamentous bacteriophages of *Escherichia coli* such as M13 or Fd (**Fig. 1**). During phage propagation, elongated phage particles assemble through polymerization of the major geneVIII coat protein, before being capped by the terminal geneIII protein. Genetic fusions with both coat proteins are possible, leading to either high-level (geneVIII, >1,000 copies)[15,16] or low-level display (geneIII, 1–5 copies) (ref. 2). Unlike hybridoma technology, phage display allows the selection of antibodies against an almost unlimited array of biological[14] and nonbiological[17] targets, including self-antigens[18], while completely bypassing the use of animals. A prime example of a phage-selected monoclonal antibody is Adalimumab (Humira), an anti-TNF monoclonal selected by Cambridge Antibody Technology and developed and marketed by Abbott[19]. Used for the treatment of moderate to severe rheumatoid arthritis, Humira is the first fully human monoclonal to obtain regulatory approval.

Display formats

Although various display formats have been reported, by far the most common format for the display of antibody fragments on phage is the genetic fusion of variable chains to the terminal phage geneIII protein (**Fig. 1**). Such fusion can be achieved either by direct engineering of the phage genome (phage format)[2,14,20] or by using a geneIII-expressing plasmid in combination with a helper phage system (phagemid format)[4,21,22]. In the phage format, all geneIII molecules are expressed as antibody fusions and 3–5 copies are displayed on the tip of bacteriophage (multivalent display). By contrast, in most phagemid systems, the majority of copies are derived from the helper phage. Only a small proportion originates from the phagemid (less than one copy per phage on average), leading to mostly monovalent display[23,24]. Consequently, the phage format has the added potential of allowing the selection of binders

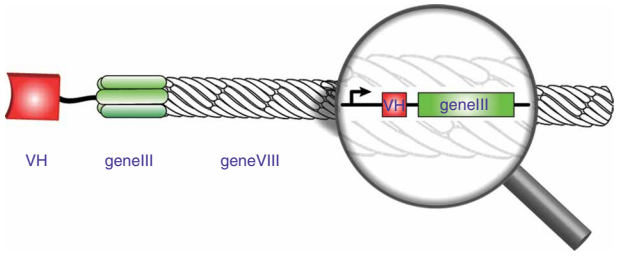

Figure 1 | Phage display of antibody fragments. The antibody fragment (such as variable heavy chain, VH, in red) is displayed as a fusion with the terminal phage geneIII protein (green). Both proteins are encoded by the phagmid DNA (magnified) and expressed from a common promoter. The major gene VIII coat protein is shown in white (other phage proteins are not shown).

with low to medium affinities due to tight binding to multivalent antigen, whereas higher affinities are required for selection in the phagemid format. This difference can be utilized for the affinity maturation of antibody fragments by switching from phage to a phagemid format during the selection process, thereby promoting the enrichment of high-affinity binders[25].

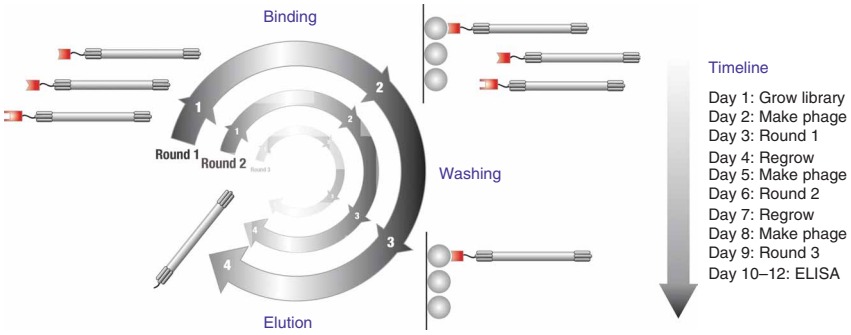

Figure 2 | Selection of antibody repertoire. Specific clones are captured by binding to antigen-affinity column and nonspecific clones are removed by washing the column, followed by elution through protease cleavage of linker region. Phages are amplified by infection and regrowth of phage-producing cells between selection rounds.

Antibody repertoires: from antibody fragments to domain antibodies

Although the expression levels of full-length antibodies in bacteria are generally poor, antibody fragments can be expressed at medium to high levels in the periplasm of *E. coli*[26]. This has allowed the selection of Fab fragments and, in particular, single chain variable fragments (scFv) by phage display. Such fragments do not rely on mammalian tissue culture for expression and can be promising candidates for therapeutic applications. Several antibody fragments are currently in clinical trials or have recently obtained regulatory approval, including certolizumab, an engineered Fab fragment for the treatment of Crohn's disease[29].

In 1989, Ward, Winter and co-workers[30] reported the selection of an even smaller antibody fragment on the basis of a single VH (variable heavy chain) domain. Despite its small size (14 kDa), this domain antibody was capable of binding the model antigen hen-egg lysozyme with high affinity and specificity. Although heavy and light chains are usually paired in the human immune system, VH and VL (variable light chain) can in some circumstances exist in isolation, for instance as Bence-Jones proteins in multiple myeloma[31]. Single domains have also been observed in other species such as camels[32] and sharks[33,34]. The small size and single domain nature of human domain antibodies allow them to be readily expressed in bacteria and yeast[35] and make them particularly amenable to engineering approaches[20]. The compact size of the domains is also expected to improve tissue penetration in therapeutic applications and the recognition of cryptic epitopes in enzyme active sites and membrane proteins.

The protocols presented in this article describe a general approach for the selection of human antibody fragments from phagemid repertoires. As an example, we describe the use of a human domain antibody repertoire recently developed in our laboratory[20], but the protocols can be readily adapted for other phage display selections. The antibody repertoire is based on the VH3-23 heavy-chain germline segment with synthetic diversity introduced by PCR mutagenesis into all three complementarity determining regions (CDR1-3). The antibody domains have been engineered to withstand heat-induced aggregation on phage and have been shown to refold without major loss of antigen-binding activity upon cooling[20]. This library is available to the research community on a nonprofit basis (contact details as below). A closely related library in which the VH3-23 heavy chain is paired with a Vκ repertoire (Tomlinson I and J) is available in an scFv format and can be selected according to this protocol.

Selection procedure

An overview of the phage display selection process can be seen in **Figure 2**. As a first step of the protocol, the frozen glycerol stock of the repertoire phagemid in TG1 bacteria is thawed and phage is produced in liquid culture after infection with KM13 helper phage[37] (Steps 1–3). Phage is then purified and concentrated by precipitation with polyethylene glycol (PEG) (Steps 4–5). After phage production, 5×10^{12} phages are incubated with antigen, thereby oversampling the antibody repertoires in excess of 1,000-fold (Step 6). For the immobilization of antigen, chemical coupling (by biotinylation) is used in this protocol, although nonspecific adsorption to plastic can also be utilized (**Box 1**). After incubation with antigen and stringent washes, phages are eluted by incubation with trypsin protease (Steps 6–7). This step will elute phage by cutting the c-Myc tag between antibody fragment and the phage geneIII protein. It will also remove background infectivity originating from geneIII protein of the trypsin-sensitive KM13 helper phage (geneIII protein originating from the repertoire phagemid is trypsin-resistant). The eluted phage is then used to infect TG1 bacteria and titers are determined by plating of dilution series (Steps 8–9). For subsequent rounds of selections, colonies from the first round are scraped from agar plates, and phages are produced in liquid culture, PEG-purified and selected by binding to antigen (Step 10). After three rounds of selection, individual clones are isolated, grown overnight (Step 11) and phage is produced in a 96-well format (Steps 12–13). Finally, antigen-specific phage clones are identified by ELISA using an anti-phage conjugate in combination with a colorimetric assay system (Step 14) (**Figs. 3** and **4**).

MATERIALS

REAGENTS

- Domain antibody library or Tomlinson I and J scFv libraries, *E. coli* TG1 TR strain, positive control clone (β-galactosidase-specific), negative control clone (phagemid) and KM13 helper phage (contact d.christ@garvan.org.au; see **Box 2**). Requests from European laboratories can be directly addressed to Geneservice Ltd. (info@geneservice.co.uk)
- TYE (tryptone yeast extract) agar plates, M9 minimal medium plates and 2×TY medium (see REAGENT SETUP)
- Ampicillin solution (Sigma, cat. no. A9518; see REAGENT SETUP)
- Kanamycin solution (Sigma, cat. no. K1377; see REAGENT SETUP)
- Glycine solution (see REAGENT SETUP)
- Trypsin solution (Sigma, cat. no. T1426; see REAGENT SETUP)
- Glucose solution (see REAGENT SETUP)
- PBS, PBST, MT and TBSC buffers (see REAGENT SETUP)
- PEG solution (Sigma, cat. no. 81260; see REAGENT SETUP)
- Tween-20

BOX 1 | IMMOBILIZATION OF ANTIGEN BY BIOTINYLATION

1. Use 500 µg of protein at 1 mg ml^{-1} in PBS, pH 7.4.
2. Add 5 µl of 10 mg ml^{-1} of NHS-biotin solution and mix well. **? TROUBLESHOOTING**
3. Incubate at room temperature for 30 min.
4. Add 50 µl of glycine solution to stop the reaction and mix well.
5. Incubate at room temperature for 10 min. Check molecular weight by mass spectrometry. Use 10 µg of nonbiotinylated and biotinylated protein for analysis. **? TROUBLESHOOTING**
6. Dialyze against 1 liter of PBS at 4 °C overnight and change PBS at least three times. A small-scale dialysis chamber can be fabricated as follows: prewet dialysis membrane in PBS, cut hole in lid of Eppendorf tube, completely cover tube-opening with membrane, close lid and fix lid by wrapping Parafilm around it. Dialyze by inverting tube in PBS and stir buffer overnight. Ensure that no bubbles are trapped by the membrane.
7. After dialysis, determine protein concentration in a UV-visible spectrophotometer at 280 nm.
8. Wash 100 µl of streptavidin agarose resin twice with PBS: centrifuge at 376*g* for 5 minutes at 4 °C in Eppendorf tube and carefully remove PBS. Streptavidin agarose should only be used in the first round of selection, whereas neutravidin agarose is used in all subsequent rounds. This prevents the selection of streptavidin binders.
9. Add 50 µg of protein in PBS to prewashed resin.
10. Incubate for 30 min.
11. Wash 2× with PBS (as in step 8).
12. Add additional resin (2× PBS-washed) up to 2 ml to increase resin volume to manageable level. For this purpose, streptavidin agarose (or neutravidin agarose in subsequent selection rounds), G25 sepharose or 0.2 mm glass beads can be used.
13. Add 2 ml of MT blocking buffer.
14. Incubate for 30 min.

Note: Alternatively, protein antigens can be coated onto a plastic surface by adsorption. Although a simple and straightforward approach, unfortunately, adsorption to plastic often denatures proteins. In addition, protein adsorption is highly dependent on the specific protein, its concentration, buffers and temperature. As a starting point, 0.1 mg ml^{-1} of protein in PBS at 4 °C overnight can be used, although less protein may be required in some cases. For this purpose, immunotubes or 96-well plates (Nunc MaxiSorb) can be utilized. After the coating step, tubes and plates should be carefully washed with PBS and blocked overnight at 4 °C with PBS supplemented with 2% (wt/vol) BSA or 5% (wt/vol) marvel milk powder.

- Marvel milk powder
- Anti-M13 horseradish peroxidase (HRP) conjugate (Amersham, cat. no. 27-9421-01)
- TMB (3.3′,5.5′-tetramethylbenzidine) solution (Becton Dickinson Biosciences, cat. no. 555214)
- *N*-hydroxysuccinimido (NHS)-biotin solution (Sigma, cat. no. H1759; see REAGENT SETUP)
- Sulfuric acid (see REAGENT SETUP)
- Streptavidin agarose (Sigma, cat. no. 85881)
- Neutravidin agarose (Pierce, cat. no. 29200)
- Streptavidin plates (High Bind Streptawell; Roche, cat. no. 11 989 685 001)
- Control antigen (β-galactosidase biotinylated; Sigma, cat. no. G5025)

EQUIPMENT

- Incubator 37 °C
- Shaker 37 °C, 250 r.p.m.
- Shaker 25 °C, 250 r.p.m.
- Falcon centrifuge, plate centrifuge and (preferably) Beckman centrifuge
- 2-liter glass flasks (nonbaffled)
- Plastic boxes
- 96-well round-bottomed plates (Corning, cat. no. 3799)
- Disposable plastic columns (Bio-Rad, cat. no. 731-1550)
- Silicone tubing
- 0.45 µM filters (Millipore, cat. no. SLHV 033RS)
- 0.2 µM filters (Millipore, cat. no. SLGP 033RS)
- UV-visible spectrophotometer
- Plate reader

REAGENT SETUP

TYE ampicillin glucose agar plates Dissolve 15 g of agar, 8 g of NaCl, 10 g of bacto-tryptone and 5 g of yeast extract in 800 ml of deionized water. Autoclave. Cool down to 50 °C and add 1 ml of ampicillin solution and 200 ml of glucose solution. Pour plates. Plates can be stored at 4 °C for up to 4 weeks. Plates should be dried in flow bench before use.

M9 minimal medium glucose plates Dissolve 15 g of agar in 800 ml of deionized water. Autoclave. Cool down to 50 °C and add 200 ml of 5×M9 salts, 10 ml of 20% (wt/vol) glucose, 1 ml of 1 M $MgSO_4$, 100 µl of 1 M $CaCl_2$ and 1 ml of 1 mg ml^{-1} VitB1. For M9 salts (5× solution), add 64 g of Na_2HPO_4, 15 g of KH_2PO_4, 5 g of NH_4Cl and 2.5 g of NaCl to 1 liter of deionized water and autoclave. Pour plates. Plates can be stored at 4 °C for several months.

2×TY medium Dissolve 16 g of bacto-tryptone, 10 g of yeast extract and 5 g of NaCl in 1 liter of deionized water. Autoclave. Cool to room temperature (25 °C) and add antibiotic solutions and glucose solution as required. Can be stored at 4 °C for up to 4 weeks.

Kanamycin solution Dissolve kanamycin powder at 50 mg ml^{-1} in deionized water. Filter through 0.2 µM filter. Aliquot in 1 ml portions. Can be stored at −20 °C indefinitely. Thawed aliquots should be freshly diluted 1,000-fold into medium or agar.

Ampicillin solution Dissolve ampicillin powder at 100 mg ml^{-1} in deionized water. Filter through 0.2 µM filter. Aliquot in 1 ml portions. Can be stored at

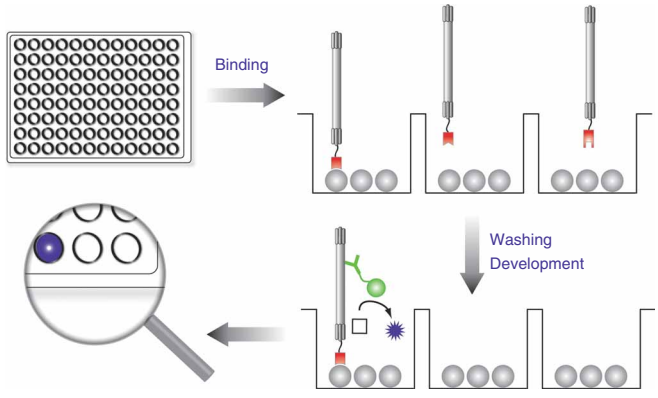

Figure 3 | Monoclonal phage ELISA. Individual phage clones are produced in a 96-well format and bound to antigen-coated wells. Binders are detected using an antiphage conjugate in combination with a colorimetric substrate.

−20 °C indefinitely. Thawed aliquots should be freshly diluted 1,000-fold into medium or agar.

NHS-biotin solution Dissolve 10 mg of NHS-biotin in 1 ml of DMSO. Use directly for reaction and discard remaining solution. Do not store.

Glycine solution 100 mM glycine, pH 7.4. Dissolve 7.5 g of glycine in 1 liter of deionized water. Adjust pH to 7.4. Filter through 0.2 μM filter. Can be stored at 4 °C for several months.

PBS buffer 1× phosphate buffer, pH 7.4. Dissolve 3.6 g of Na_2HPO_4, 0.2 g of KCl, 0.24 g of KH_2PO_4 and 8 g of NaCl in 1 liter of deionized water. Adjust pH to 7.4 and autoclave. Can be stored at 4 °C for several months.

PBST buffer Add 0.1% Tween-20 to PBS buffer.

MT buffer PBS buffer supplemented with 5% (wt/vol) marvel milk powder and 2% (vol/vol) Tween-20. Use directly and discard remaining buffer. Do not store.

TBSC buffer 10 mM Tris pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$. Dissolve 1.5 g of Trizma base, 8 g of NaCl and 0.15 g $CaCl_2$ in 1 liter of deionized water. Adjust pH to 7.4 and autoclave. Can be stored at 4 °C for several months.

PEG solution 20% PEG, 2.5 M NaCl. Dissolve 100 g of PEG-6000 and 73 g of NaCl in 500 ml of deionized water. Filter through 0.2 μM filter. Can be stored at room temperature for up to a year.

Trypsin solution Dissolve trypsin powder at 10 mg ml^{-1} in TBSC (trypsin stock). Freeze in 20 μl aliquots in liquid nitrogen. Can be stored at −20 °C for several months. For the experiment, dissolve 100 μl of trypsin stock in 10 ml of TBSC (trypsin solution).

Glucose solution 20% glucose solution. Dissolve 200 g of glucose in 1 liter of deionized water. Filter through 0.2 μM filter. Can be stored at 4 °C for several months.

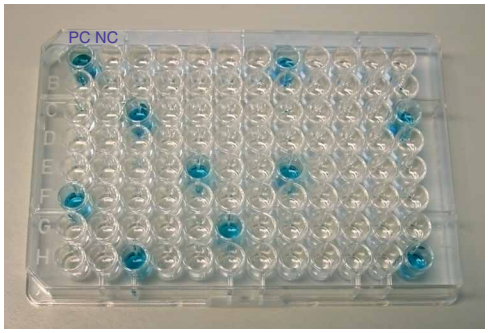

Figure 4 | Anticipated results for monoclonal phage ELISA. Plate was developed using TMB substrate solution and anti-M13 HRP conjugate. Positive controls (PC) and negative controls (NC) were included in the plate. The color of TMB substrate will turn yellow after quenching the reaction with sulfuric acid.

Sulfuric acid 1 M sulfuric acid. Add 10 ml of concentrated sulfuric acid to 187 ml of deionized water. **! CAUTION** Add acid to water, not water to acid; mix in fume hood, stir with teflon stir bar, wear nitrile gloves, goggles and lab coat.

PROCEDURE

Growth of phage antibody repertoire

1| Thaw aliquot of frozen antibody library on ice. Dilute with 500 ml 2×TY medium supplemented with 4% (wt/vol) glucose and 100 μg ml^{-1} of ampicillin. The presence of 4% glucose allows the effective suppression of antibody expression during bacterial growth. Measure OD600 in a UV-visible spectrophotometer. It should be ∼0.1. Grow culture at 37 °C and 250 r.p.m. in a 2-liter glass flask until OD600 = 0.5 (∼1.5–2 h).

? TROUBLESHOOTING

2| Add 2 × 10^{12} at 4 °C KM13 helper phages (from **Box 2**) and incubate in a water bath at 37 °C for 30–60 min. Spin culture at 3,200*g* for 10 min at 4 °C in ten Falcon tubes (50 ml each). Discard supernatant. Resuspend pellets in 500 ml of 2×TY medium supplemented with 0.1% (wt/vol) glucose, 100 μg ml^{-1} of ampicillin and 50 μg ml^{-1} of kanamycin. Grow for 16–20 h at 25 °C and 250 r.p.m. in a 2-liter glass flask.

3| Spin culture at 17,700*g* in a Beckman centrifuge at 4 °C for 10 min. Alternatively, centrifugation can be performed in a Falcon centrifuge at 3,200*g*, but may require multiple runs to properly remove cells and debris. Filter supernatant through a 0.45 μM filter (do not use a 0.2 μM filter for this step). The use of a 500 ml disposable vacuum filter unit is recommended. Discard pellet.

? TROUBLESHOOTING

BOX 2 | PREPARATION OF HELPER PHAGE STOCK

1. Streak TG1 bacteria from glycerol stock on an M9 minimal medium plate and incubate for 36 h at 37 °C.
2. Grow overnight culture in 5 ml of 2×TY medium from single colony at 37 °C and 250 r.p.m.
3. Dilute 100-fold into 5 ml of 2×TY medium. Grow at 37 °C and 250 r.p.m. until OD600 = 0.5.
4. Prepare a dilution series of KM13 helper phage in PBS (10^{12} ml^{-1} to 10^4 ml^{-1}).
5. Add 10 μl of diluted KM13 helper phage to 200 μl culture each.
6. Incubate in water bath at 37 °C for 30 min.
7. Add 3 ml of melted H-top agar, mix gently with culture and pour onto prewarmed TYE agar plates. Top agar should be completely melted in microwave and cooled to 42 °C in a water bath before use. Allow plates to solidify at room temperature and incubate overnight at 37 °C.
8. Pick a small plaque with a sterile toothpick and place into 5 ml of TG1 culture at OD600 = 0.5 (grown as above). Grow for 2 h at 37 °C and 250 r.p.m.
9. Dilute 100-fold into 500 ml of 2×TY medium. Shake in a 2-liter flask for 2 h at 250 r.p.m. at 37 °C. Add kanamycin to a concentration of 50 μg ml^{-1} and grow overnight at 250 r.p.m. at 30 °C.
10. Clear supernatant by centrifugation and filtration; purify phage by PEG precipitation (as described above). Phage can be frozen in liquid nitrogen (add 20% glycerol) and stored at −80 °C for many years.
11. Test trypsin cleavage: incubate 10^{10} phages in 1 ml of trypsin solution for 30 min at room temperature. Prepare a dilution series in PBS (10^{10}–10^2 phages) and infect 200 μl each of TG bacteria (OD600 = 0.5, grown as described above) with 10 μl of dilution series. Plate on TYE plates supplemented with kanamycin 50 μg ml^{-1} and grow at 37 °C overnight. The number of colonies obtained from trypsin-treated phage should be at least 10^6-fold lower than from nontreated phage. If not, discard helper phage preparation and start from different plaque.

BOX 3 | SELECTION ON CELLS

Phage display can be used to select binders against complex antigens, including antigens expressed on cells. However, a related cell preparation or cell line not expressing the antigen (background cell line) should be available for negative selection. This allows the reduction of nondesired binding specificities that otherwise frequently dominate the selection. High-level expression of functional antigen is crucial for the success of such selections and should be checked by FACS or binding of radiolabeled ligand. The protocol below is optimized for cells growing in suspension, but can be adapted for adherent cells. All centrifugation steps are carried out at 4 °C and 1,120*g* for 5 min in a Falcon centrifuge.

Negative selection on background cells

1. Incubate 5×10^{12} phages in 2 ml of tissue culture media supplemented with 2.5% BSA for 1 h at room temperature.
2. Spin down 4×10^{6} cells, and resuspend pellet in 2 ml of fresh tissue culture medium; repeat twice.
3. Pool cells with phage and incubate for 1 h at room temperature with gentle agitation.
4. Spin down cells, carefully remove supernatant, and discard the pellet.
5. Repeat steps 2–4 twice using supernatant of previous step and fresh cells.

Binding selection on antigen-expressing cells

6. Repeat steps 2–4 using antigen-expressing cells and depleted supernatant from negative selection.
7. Resuspend pellet in PBS, spin down cells; repeat twice; do not discard pellet.
8. Resuspend pellet in 1 ml of trypsin solution and incubate for 30 min at room temperature.
9. Spin down cells and carefully remove and store supernatant; discard the pellet.

Further steps

10. Infect TG1 bacteria by adding supernatant, plate, regrow library and prepare phage. Repeat negative and binding selections for another 2–4 rounds.
11. Test for positives by phage ELISA: spin down 2×10^{5} cells in a V-bottomed plate, resuspend pellet in PBS, repeat twice, add 1:5,000 anti-M13–HRP conjugate in PBS 2.5% (wt/vol) BSA, spin down cells, resuspend pellet in PBS, spin down cells, transfer cells in 100 µl of TMB solution to a flat-bottomed ELISA plate, develop, stop with 1M H_2SO_4 and read at 450–650 nM.

Purification of phage repertoire by PEG precipitation

4| Precipitate phage by adding 100 ml of PEG solution to 400 ml of filtered supernatant. Incubate on ice for 1 h. Then spin at 3,200*g* for 30 min in ten Falcon tubes at 4 °C. Discard supernatant. Resuspend pellets in a total of 5 ml of PBS buffer and pool in a 15-ml Falcon tube. Add 1 ml of PEG solution. Incubate on ice for 10 min. Spin for 30 min at 3,200*g* at 4 °C. Discard supernatant. Resuspend pellet in 1 ml of PBS.

5| Estimate phage titers by measuring absorption at 260 nm: dilute the phage preparation 100-fold in PBS. Titers can be estimated according to the following empirical formula: phage $ml^{-1} = OD260 \times 100 \times 22.14 \times 10^{10}$.

■ **PAUSE POINT** Phage can be stored at 4 °C for up to 2 weeks. Add 1% (wt/vol) EDTA and 0.1 mg ml^{-1} of BSA to reduce proteolysis.

? TROUBLESHOOTING.

First round of selection: binding and elution of phage

6| Add 5×10^{12} phages to 6 ml of MT buffer and incubate at room temperature for 30 min. Mix phage and antigen bound to streptavidin agarose resin (**Box 1**) and rotate at 4 °C overnight in a 15-ml Falcon tube. Pour resin into disposable column. Wash column 10× with 10 ml of PBST buffer and 2× with 10 ml of PBS buffer.

▲ **CRITICAL STEP** This is the enrichment of antigen-specific phage by binding to immobilized antigen. The first round of selection is the most important one, as any bias or loss of diversity will be amplified in the subsequent rounds.

? TROUBLESHOOTING

7| Close the column with plug. Add 2 ml of trypsin solution and incubate for 1 h at room temperature. Remove the plug and collect flow-through. Add an additional 1 ml of trypsin solution, collect flow-though and combine.

First round of selection: infection of TG1 bacteria with eluted phage

8| Streak TG1 bacteria from glycerol stock on an M9 minimal medium plate and incubate for 36 h at 37 °C. Grow overnight culture in 5 ml of 2×TY medium from a single colony at 37 °C and 250 r.p.m. Dilute culture 100-fold into 2×TY medium. Grow at 37 °C and 250 r.p.m. until OD600 = 0.5. Add 30 ml of TG1 culture to eluted phage (prepared above) and incubate at 37 °C for 1 h in a water bath. Centrifuge at 3,200*g* for 5 min at 4 °C in a Falcon centrifuge.

9| Resuspend pellet in 1 ml of 2×TY medium. Plate 166 µl each on six TYE plates supplemented with 100 µg ml^{-1} of ampicillin and 4% (wt/vol) glucose. Incubate at 37 °C overnight. Plate dilution series on TYE plates supplemented with 100 µg ml^{-1} of ampicillin and 4% (wt/vol) glucose. Tenfold dilution steps should be used. To reduce the number of plates, 10 µl aliquots can be spotted on a single agar plate.

? TROUBLESHOOTING

Subsequent rounds of selection

10| Scrape cells from agar plates using 5 ml of 2×TY medium per plate and a glass spreader. Mix cells thoroughly by vortexing in a 50-ml Falcon tube. Dilute with 500 ml of 2×TY medium supplemented with 4% (wt/vol) glucose and 100 μg ml^{-1} of ampicillin to an OD600 of 0.1. Grow to OD600 = 0.5 at 37 °C and 250 r.p.m. Then infect with helper phage, grow overnight and purify phage by PEG purification as described above (follow protocol from Step 2 onward). Repeat binding, elution and infection steps. Neutravidin agarose (rather than streptavidin agarose) should be used in subsequent rounds to prevent the enrichment of streptavidin binders.

Screening of clones by monoclonal phage ELISA

11| After three rounds of selection, individual colonies from the dilution series can be tested for antigen binding by phage ELISA (**Fig. 3**). Pick colonies using sterile toothpicks or pipette tips into a 96-well round-bottomed plate containing 200 μl of 2×TY medium supplemented with 100 μg ml^{-1} of ampicillin and 4% (wt/vol) glucose. Pick positive and negative control clones into A1/A2 wells from freshly streaked TYE plates supplemented with 100 μg ml^{-1} of ampicillin and 4% (wt/vol) glucose. Grow overnight at 37 °C and 250 r.p.m. in a plastic box. The 96-well plate can be secured inside the box with pieces of foam or with paper towels. The plate lid should be removed; however, the plastic box should be closed. Carefully keep box horizontal to avoid spills and cross-contamination.

12| The next day, a fresh 96-well round-bottomed plate containing 200 μl of 2×TY medium supplemented with 100 μg ml^{-1} of ampicillin and 4% (wt/vol) glucose should be inoculated with 5 μl of the overnight culture. Glycerol stocks of the original 96-well overnight cultures should be prepared by adding glycerol to the plate (20% final concentration) and placing it in a −80 °C freezer. The freshly inoculated plate should be shaken at 37 °C and 250 r.p.m. for 3 h (in a plastic box, as described above).

13| After 3 h, add 50 μl of 2×TY medium supplemented with 4×10^8 KM13 helper phages to each well (add 4×10^{10} KM13 phages to 5 ml of 2×TY medium). Mix by gentle agitation. Incubate plate at 37 °C without shaking for 1 h. Spin at 3,200*g* in a plate centrifuge for 10 min at room temperature. Discard supernatant by quickly inverting the plate. Resuspend pellets in 200 μl of 2×TY medium supplemented with 100 μg ml^{-1} of ampicillin, 50 μg ml^{-1} of kanamycin and 0.1% (wt/vol) glucose by gentle agitation. Grow overnight at 25 °C and 250 r.p.m. in a plastic box for 16–24 h.

14| The next day, spin plate at 3,200*g* for 10 min at room temperature in a plate centrifuge and transfer the supernatant to a new 96-well plate and store at 4 °C. The phage clones can now be tested by ELISA: add 100 μl of 1 μg ml^{-1} biotinylated antigen in PBS to each well of a streptavidin plate. Add control antigen to A1 and A2 wells. Incubate at room temperature with gentle agitation for 1 h. Wash wells with 2× PBS and block with 300 μl per well of MT at 4 °C overnight. Then wash wells with 2× PBST. Using a separate 96-well round-bottomed plate, dilute 25 μl of phage supernatant each in 75 μl of MT with a multichannel pipette.
? TROUBLESHOOTING

15| Transfer diluted phage (100 μl) to streptavidin ELISA plate and incubate at room temperature for 1 h with gentle agitation. Wash wells with 5× PBST and add 100 μl per well of 1:5,000 HRP–anti-M13 conjugate in MT buffer. Incubate at room temperature for 1 h with gentle agitation. Wash wells with 3× PBST and 1× PBS, then add 100 μl of TMB solution at room temperature to each well. Wait for blue color to develop (1–30 min). Stop with 50 μl of 1 M sulfuric acid. Color will turn to yellow. Read at 450–650 nm in a UV-visible plate reader.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
1	Limited glycerol stocks of original repertoire	Additional stocks of the repertoire can be produced by plating half of the original glycerol stock on eight large square (30 cm) TYE plates supplemented with 4% glucose and 100 μg ml^{-1} of ampicillin. After growing overnight at 37 °C, colonies should be scraped off plates with a glass spreader in 20 ml of 2×TY medium per plate. Carefully mix cells by vortexing and freeze in 2 ml aliquots in liquid nitrogen (add 20% glycerol, store at −80 °C). Only regrow once. Do not regrow in liquid culture. Use at least 2.5 ml of OD600 = 20 of cells for each selection
3	Filter clogs upon phage purification	Spinning at 17,700*g* or multiple spins at 3,200*g* may be required to clear supernatant before filtration
5	No or few phages produced	Carefully check conditions, in particular, glucose (0.1%) and kanamycin (50 μg ml^{-1}) concentrations during phage production. Increase production time to 20–24 h. Use 0.45 μM and not 0.2 μM filters for phage purification. Check calculation: ~0.1–1.0 of OD260 are expected after 100-fold dilution into PBS

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Solution
6	Column flow too fast or too slow	Flow should be several milliliters per minute. Resin volume should be around 2 ml using a 0.8 cm column. Volume can be increased by mixing with additional streptavidin agarose or with cheaper column materials such as G25 sepharose or 0.2 mm glass beads to decrease flow rate. Silicone tubing can be attached to column to increase flow rate (10–30 cm)
9	Phage titers too high or too low after binding step	Carefully check the number of phages before and after the binding step by infecting TG1 bacteria and plating dilution series. As one milliliter of OD600 = 0.5 cells corresponds to only about 4×10^8 cells, phages may have to be diluted before infection. Do not add more that 4×10^7 phages to each milliliter of cells
14	High background in ELISA	Insufficient blocking: block wells with MT buffer overnight at 4 °C. Completely fill wells. Insufficient washing: plates can be washed by hand in a small Tupperware box filled with buffer. All liquid has to be removed by inverting the plates and hitting forcefully onto absorbent material (such as paper towels). Change buffer and absorbent material frequently
	No positives obtained in phage ELISA	Develop ELISA for up to 30 min (**Box 3**). Continue with next round of selection and repeat phage ELISA. Some antigens may require more rounds of selection. This is particularly the case for complex antigens (cells, lysates) and those with multiple conformations
Box 1	No biotinylation detected by mass spectrometry	Presence of primary amines (such as Tris buffer) will inhibit the reaction of NHS-biotin with proteins. Reaction also requires accessible lysine or arginine residues in the protein. Biotinylation reagents other than NHS-biotin can also be utilized: NHS-SS-biotin (succinimidyl 2-(biotinamido)-ethyl-1-3′ dithiopropionate; Pierce) can be used for the specific elution by reducing agents (such as DTT), whereas variants with longer linkers (such as NHS-PEO_{12}-biotin (Pierce)) can reduce steric hindrance
	Excess biotinylation detected by mass spectrometry	Proteins should contain as few biotins as possible with a mean of 1–5 for small globular proteins and 1–10 for larger proteins and complexes. Excess biotinylation can alter epitopes and induce aggregation. Reduce concentration of NHS-biotin and repeat labeling

ANTICIPATED RESULTS

Step 5

Approximately 10^{13} phages should be obtained from 400 ml of supernatant. Phage titers can be estimated by measuring absorption at 260 nm. Alternatively, a more accurate way is by dilution of phage in PBS and infection of bacteria, followed by plating on TYE glucose ampicillin plates.

Step 9

It is essential to carefully monitor the titers after each round of selection. With an input of 5×10^{12} phages, one would expect to obtain approximately 10^5–10^7 bacterial colonies after the first and second round of selection. If the figure is outside this range, blocking, washing and elution conditions should be carefully checked. After 3–4 rounds of selections, titers will often rise to 10^7–10^9. Such an increase frequently indicates the selection of binders. However, the opposite is not necessarily true, and individual clones should always be checked by phage ELISA after three rounds of selection, even if no increase is observed. The outcome of each selection will be strongly dependant on the antigen, immobilization conditions and stringency of washing and blocking. Some antigens may yield positive clones after as few as two rounds of selection, whereas others require additional rounds.

Step 14

Figure 4 shows an example of a monoclonal phage ELISA. Positive and negative controls should be included in the assay. The signal obtained for the negative control should be at least 10- to 20-fold lower than that for the positive control. Otherwise, blocking and washing conditions should be optimized.

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