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Protein and Antibody Engineering by Phage Display

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Abstract

Phage display is an in vitro selection technique that allows for the rapid isolation of proteins with desired properties including increased affinity, specificity, stability, and new enzymatic activity. The power of phage display relies on the phenotype-to-genotype linkage of the protein of interest displayed on the phage surface with the encoding DNA packaged within the phage particle, which allows for selective enrichment of library pools and high-throughput screening of resulting clones. As an in vitro method, the conditions of the binding selection can be tightly controlled. Due to the high-throughput nature, rapidity, and ease of use, phage display is an excellent technological platform for engineering antibody or proteins with enhanced properties. Here, we describe methods for synthesis, selection, and screening of phage libraries with particular emphasis on designing humanizing antibody libraries and combinatorial scanning mutagenesis libraries. We conclude with a brief section on troubleshooting for all stages of the phage display process.

1. INTRODUCTION

Phage display is an in vitro selection technique for the isolation of binding peptides or proteins from diverse mutagenic libraries. The method was first described in Smith (1985) using peptides as fusions to the pIII minor coat protein. Since then, a range of different peptide/protein sizes, scaffolds, and expression formats have been described (elaborated later); we will focus our discussion here on phage display of proteins or antibodies. Displayed elements are expressed on the surface of filamentous M13 bacteriophage typically as a fusion to either the major (pVIII) or minor (pIII) phage coat protein; the DNA of each library member is packaged within the phage particle, thus linking its phenotype with its genotype (Fig. 1). This critical phenotype-to-genotype linkage allows for the high-throughput selection of variants and rapid identification of selectants with desirable characteristics such as high affinity and increased specificity. Proteins up to 100 kDa, such as bivalent Fabs, have been successfully displayed on the surface of phage, and therefore this technique is amenable to a diverse size range of protein scaffolds (Lee, Sidhu, & Fuh, 2004).

Diversity can be incorporated into phage libraries via both random and targeted mutagenesis methods. In this chapter, we focus on the use of degenerate oligonucleotides and Kunkel mutagenesis for the targeted incorporation of diversity during library production. After a phage library is produced, biopanning can be used to selectively enrich for clones with desired properties such as affinity, specificity, stability, or even new or improved enzymatic

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properties (Forrer, Jung, & Plückthun, 1999). For binding selections, the target is immobilized onto a solid support, such as the surface of a 96-well plate or magnetic beads, then the library phage added, nonbinders washed away, and binders eluted and amplified for subsequent rounds of selection (Fig. 1). Various strategies can be employed to enrich for binders with very specific preferences such as negative selection, and/or competitive selection to drive recognition of one target or binding region over another (Clackson & Lowman, 2004).

The utility of phage display is illustrated by its extensive use for the engineering of widely different protein scaffolds for a variety of applications. Some examples include: antibodies that target specific fusion-relevant intermediates of virus glycoproteins (Koellhoffer et al., 2012), humanized antibodies that retain high affinity for their original antigens (Baca, Presta, O'Connor, & Wells, 1997; Chen et al., 2014), human growth hormone variants with increased affinity for the growth hormone receptor (Lowman & Wells, 1993), cytochrome b562 variants that selectively bind BSA conjugated to *N*-methyl-*p*-nitrobenzylamine derivative 1 (Ku & Schultz, 1995), fibronectin type III domain variants that bind ubiquitin (Koide, Bailey, Huang, & Koide, 1998), Zinc finger domains that bind novel DNA sequences (Choo & Klug, 1994; Jamieson, Kim, & Wells, 1994; Rebar & Pabo, 1994), knottins that bind glycosylated alkaline phosphatase with micromolar affinity (Smith et al., 1998), cytotoxic T lymphocyte-associated antigen 4 variants that bind specifically to human $\alpha\beta 3$ integrin (Hufton et al., 2000), tendamistat, a β -sheet protein, that binds to the monoclonal antibody A8, which recognizes endothelin (McConnell & Hoess, 1995), and bilin binding protein, a lipocalin, that specifically binds to fluorescein (Beste, Schmidt, Stibora, & Skerra, 1999).

Phage display can also be used to rapidly dissect the energetics of protein interaction surfaces using combinatorial scanning mutagenesis, a technique in which libraries are designed to allow interface residues of one protein to vary between wild type and alanine (combinatorial alanine scanning) or other variations such as homolog scanning. This technique readily identifies “hotspot residues,” or residues that contribute substantial binding energy to the interaction. Combinatorial alanine scanning with phage display has been used to probe the energetics of the growth hormone and growth hormone receptor interface, as well as, to characterize residues that contribute substantially to the binding of a Dengue virus (DENV) broadly neutralizing antibody to its antigens (Frei, Kielian, & Lai, 2015; Pal, Kossiakoff, & Sidhu, 2003; Weiss, Watanabe, Zhong, Goddard, & Sidhu, 2000). We describe here how to design, synthesize, and screen phage libraries, including a list of troubleshooting steps for common problems that arise during the phage display process.

2. EQUIPMENT

- Pipettes
- Pipette tips with filters (phage manipulations should be done with filter tips)
- Multichannel pipettes—10 and 300 μ L volumes
- Reagent reservoirs (USA Scientific, Ocala, FL, catalog #: 1346-2510)

- Pipette gun
- Serological pipettes
- pH indicator strips (Sigma-Aldrich; Whatman, St. Louis, MO, catalog #: Z134147)
- 96-Well plates (USA Scientific, Ocala, FL; CytoOne, catalog #: CC7682-7596)
- 96-Well high-binding plates (Corning Incorporated; Costar, Corning, NY, catalog #: 3590)
- Plastic or foil plate covers (USA Scientific, Ocala, FL, catalog #: 2921-0000 or 2923-0100)
- 96-Well 2 mL deep-well plate (USA Scientific, Ocala, FL; PlateOne, catalog #: 1896-2000)
- Gas-permeable plate covers (Thermo Scientific, Waltham, MA, catalog #: AB-0718)
- Repeater pipette and tips
- 250 mL, 500 mL, 1 L, 2 L, and 4 L baffled glass flasks
- Electroporator (BioRad; GenePulser Xcell™, Hercules, CA, catalog #: 1652660)
- 2 mm- and 1 mm-gap cuvettes (Fisher Scientific, Hampton, NH; Fisherbrand™ Electroporation Cuvettes Plus™, catalog #: FB102 and FB101)
- Shaking incubators
- Incubator
- 1 L Bottle Top Filters (Corning, Corning, NY, catalog #: 431174)
- Plate shaker (USA Scientific, Ocala, FL, catalog #: 7400-4000)
- Plate washer
- Plate reader (Molecular Devices, Sunnyvale, CA, SpectraMax M5 Multi-Mode plate reader)

Other disposables

- Plates for agar plates
- ColiRollers Plating Beads (Novagen, Darmstadt, Germany, catalog #: 71013)
- Amicon Ultra 4 Centrifugal Filters (Millipore, Darmstadt, Germany, catalog #s: UFC800396 (3 k MWCO), UFC801096 (10 k MWCO), and UFC903096 (30 k MWCO))
- SnakeSkin Dialysis Tubing (Thermo Scientific, Waltham, MA, catalog #s: 68035 (3500 MWCO) and 88245 (10 k MWCO))
- PD-10 Desalting Columns (GE Healthcare, Piscataway, NJ, catalog #: 17-0851-01)

3. MATERIALS

3.1 Cell Lines

SS320 cells—The success of any phage display project relies on the ability to electroporate a large amount of library DNA into *Escherichia coli* with high efficiency. The *E. coli* strain SS320 was developed by crossing XL1-Blue, which contains the F' pilus that is required for phage entry on a tet-selectable episome, and MC1061, which tolerates high cell densities and thus can be prepared in high electroporation competency (Sidhu, Lowman, Cunningham, & Wells, 2000). The SS320 strain cell line can be propagated in-house as detailed in Protocol 1, or can be purchased from Lucigen (Middleton, WI, catalog #s: 60512-1 or 60512-2).

If using cells from Lucigen, a critical step of library production is the addition of M13KO7 helper phage after electroporation of library DNA into SS320 cells. If produced in-house following Protocol 1, SS320 cells will already be infected with M13KO7 phage, so that addition of exogenous helper phage is unnecessary.

3.1.1 Protocol 1: Production of SS320 Cells (Modified from Tonikian, Zhang, Boone, & Sidhu, 2007)

1. Day 1:
 - a. Streak a fresh LB/Tet plate with SS320 cells
 - b. Incubate overnight at 37°C
2. Day 2:
 - a. Inoculate SS320 colony into 25 mL 2xYT/25 µL Tet and grow to OD₆₀₀=0.6 at 37°C, 220 RPM
 - b. Serially dilute M13KO7 phage 1:10 in PBS, pH 7.4 (add 30 µL phage to 270 µL PBS)
 - c. Mix 500 µL SS320 cells with 200 µL of each M13KO7 dilution
 - d. Add to 4 mL Top Agar and pour onto LB/Tet plates
 - e. Incubate overnight at 37°C
3. Day 3:
 - a. Pick a plaque and inoculate into 1 mL 2xYT media/0.5 µL kanamycin (final kanamycin concentration=25 µg/mL)
 - b. Incubate at 37°C, 220 RPM, for 6–8 h
 - c. Transfer into 100 mL 2xYT/50 µL kanamycin (final kanamycin concentration=25 µg/mL)
 - d. Incubate overnight at 37°C and 220 RPM
4. Day 4:

- a. To twelve 2 L baffled flasks add the following: 500 mL SB media with 250 μ L kanamycin (final kanamycin concentration=25 μ g/mL)
- b. Add 5 mL of the overnight culture to each flask
- c. Incubate at 37°C, 220 RPM until culture reaches OD₆₀₀=0.5–0.8
- d. *Note:* Monitor the OD₆₀₀ closely
- e. Immediately chill cells on ice and transfer to preautoclaved and prechilled 1 L centrifuge bottles containing stir bars
- f. Spin down cells at 5000 RPM for 7 min at 4°C—discard supernatant
- g. Wash cells with 4 L of cold HEPES buffer (gently swirl centrifuge bottles to detach cells from bottle)—consolidate cells to end with highly concentrated cells
- h. Spin down as in step 4e—discard supernatant
- i. Wash cells with 2 L of cold HEPES buffer and consolidate cells as in step 4f
- j. Spin as in step 4e—discard supernatant
- k. Wash cells with cold 10% glycerol (in water, filter sterilized) and consolidate into 1 spin bottle
- l. Spin as in step 4e—discard supernatant
- m. Aliquot cells into preautoclaved ependorf tubes (350 μ L aliquots) and flash freeze with liquid nitrogen—store at –80°C

XL1-Blue cells: *E. coli* XL1-Blue cells harbor an episome that contains the F' (pilus) that is required for M13 bacteriophage infection, which allows for amplification of phage libraries. This episome also contains the tetracycline resistance gene, so cells should be grown in the presence of tetracycline to maintain the F' pilus. An original stock can be purchased from Agilent Technologies (Santa Clara, CA, catalog #: 200249) and propagated in-house following Protocol 2. A fresh XL1-Blue plate should be streaked out from a –80°C glycerol stock each week for all phage-based experiments.

3.1.2 Protocol 2: Production of XL1-Blue Cells

1. Day 1:
 - a. Streak out XL1-Blue cells from glycerol stock onto LB/Tet plate
 - b. Incubate overnight at 37°C
2. Day 2:
 - a. Pick single colony into 10 mL LB media/10 μ L tetracycline
 - b. Incubate overnight at 37°C and 220 RPM
3. Day 3:

- a. Inoculate 1 L LB/1 mL tetracycline with 5 mL of overnight culture (dilution of 1:2000)
- b. Incubate at 37°C and 220 RPM until cell density reaches OD₆₀₀=0.3–0.6
 - i. *Note:* OD₆₀₀=0.4 seems to work the best
- c. During cell incubation: prechill centrifuge bottles, autoclaved Eppendorf tubes, 10% glycerol, and sterile water (the rest of the protocol should be carried out either on ice or in a cold room)
- d. Once cells reach OD₆₀₀ of 0.3–0.6, immediately chill on ice for 10–15 min
- e. Transfer cells to centrifuge bottles and spin at 4000 RPM, 4°C for 20 min—discard supernatant
- f. Rinse cells with 1/10th original volume of ice cold sterile water (do not resuspend the cells at this time)
- g. Gently pour off the water
- h. Resuspend the cells in 1/10th original volume of ice cold sterile water
- i. Centrifuge at 4000 RPM, 4°C for 20 min—gently discard the supernatant
- j. Resuspend the cells with 1/50th volume of ice cold 10% glycerol and aliquot (200–300 µL) into autoclaved, prechilled Eppendorf tubes
- k. Flash freeze in liquid nitrogen and store at –80°C

Other cell lines needed

1. CJ236 (Lucigen, Middleton, WI, catalog #: 60701-1 or 60701-2)
2. One Shot TOP10 Chemically Competent cells (Invitrogen, Carlsbad, CA, catalog #: C4040-03)
3. One Shot BL21(DE3) Chemically Competent cells (Invitrogen, Carlsbad, CA, catalog #: C6000-03)
4. HEK239F (Invitrogen, Carlsbad, CA, catalog #: R790-07)

3.2 M13KO7 Helper Phage

Our group utilizes M13KO7 (NEB, Ipswich, MA, catalog #: N0315), although other helper phage strains are available (for example, R408 and VCSM13). Helper phage provide all of the necessary wild-type phage proteins required for the production of progeny phage particles; the phagemid (discussed later) contains only the engineered protein-pIII or protein-pVIII fusion and thus does not result in a replicative particle in the absence of the helper phage (Bass, Greene, & Wells, 1990). The helper phage genome contains mutations that reduce its packaging ability, so that the genome of the phagemid is preferentially packaged into progeny phage, preserving the phenotype-to-genotype linkage. The M13KO7

phage contain a kanamycin resistance gene; therefore, *E. coli* that are infected with M13KO7 can readily be selected by the addition of kanamycin to the media. For the production of M13KO7 phage from a glycerol stock, see Protocol 3.

3.2.1 Protocol 3: Production of M13KO7 Helper Phage (Modified from Tonikian et al., 2007)

1. Day 1:
 - a. Streak out a new XL1-Blue plate from glycerol stock
 - b. Incubate overnight at 37°C
2. Day 2:
 - a. Pick a single colony into 2 mL 2xYT/2 µL Tet media
 - b. Incubate at 37°C and 220 RPM for 6–8 h
 - c. Add M13KO7 helper phage from glycerol stock to a final concentration of 10^9 – 10^{10} phage per mL
 - d. Incubate at 37°C and 220 RPM for 30 min
 - e. Transfer to 1 L of 2xYT/1 mL Kan media in a 4 L baffled flask
 - f. Incubate overnight at 37°C and 220 RPM
3. Day 3:
 - a. Centrifuge cells at 4000 RPM for 15 min at 4°C—phage are in supernatant
 - b. Transfer supernatant to a flask containing 1/5th volume of 5 × PEG/NaCl and incubate with stirring for 1 h at 4°C
 - c. Centrifuge at 12,000 RPM for 20 min at 4°C—discard supernatant
 - d. Resuspend phage in 10 mL PBS, pH 7.4
 - e. Spin down in microcentrifuge at 7600 RPM for 10 min at 4°C
 - f. Transfer supernatant to fresh tubes and either store at 4°C or add 10% glycerol and store at –80°C
 - i. *Note:* Helper phage stored at 4°C is stable for 6 months

3.3 Phagemid Considerations

Phagemids are plasmids that contain only one of the phage structural proteins (pIII or pVIII), which is fused with the desired display protein. During phage production, the remaining phage proteins are supplied by coinfection of helper phage (see Section 3.2). Phagemids contain a bacterial origin of replication for propagation of the plasmid in *E. coli* and an F1 phage origin for replication and packaging of single-stranded DNA (ssDNA) (Qi, Lu, Qiu, Petrenko, & Liu, 2012).

Several phagemids are available for the production of phage libraries; the reader is directed to tables 1 and 2 of Qi et al., 2012 for a comprehensive list (Qi et al., 2012). Our lab has had the most success with phagemid HP153 (Persson et al., 2013). HP153 drives expression of the protein of interest fused to the C-terminal domain of the phage minor coat protein-pIII via the weak phosphatase A (phoA) promoter. This promoter is activated under low phosphate conditions. Thus, in phosphate-rich media the promoter is only minimally active, allowing for slow and steady expression of the pIII- or pVIII-fused protein (Lowman, Bass, Simpson, & Wells, 1991). HP153 contains a StII signal sequence N-terminal to the displayed protein, which delivers the protein to the periplasmic space, where folding and formation of disulfide bonds occur before it is incorporated into nascent phage particles. Additionally, this phagemid contains a dimerization domain allowing for the bivalent expression of the protein at the surface of phage. Cloning into HP153 using NsiI and FseI restriction sites results in bivalent display. Selection for *E. coli* containing HP153 plasmid is achieved by the addition of carbenicillin, as HP153 contains a β -lactamase gene to confer ampicillin resistance.

3.4 Reagents

3.4.1 Media

1. 2xYT media (Fisher Scientific, Hampton, NH, catalog #: BP97432)
2. Super Broth (SB) media
 - a. Per liter: 10 g MOPS, 30 g tryptone, 20 g yeast extract, pH 7.0
3. SOC media:
 - a. Per liter: 20 g tryptone, 5.0 g yeast extract, 0.5 g NaCl, 0.186 g KCl
 - b. Optional: add 10 mL 1 *MMgCl*₂ (filtered) and 20 mL 1 *MGlucose*
4. Low phosphate media:
 - a. Per liter: 3.57 g ammonium sulfate, 0.517 g sodium citrate monobasic anhydrous, 1.07 g potassium chloride, 5.36 g Hy-Case SF casein acid hydrolysate from bovine milk, 5.36 g yeast extract, pH 7.3 with KOH
 - b. After autoclaving, add 7 mL 1 *MMgSO*₄ and 14 mL 1 *MGlucose* (filtered) before use
 - c. Optional: add 1 *MMOPS* (filtered) to buffer media for optimal cell growth
5. LB Agar, Miller (Fisher Scientific, Hampton, NH, catalog #: BP9724-2):
 - a. Add either carbenicillin, tetracycline, kanamycin, carbenicillin and tetracycline, or carbenicillin and chloramphenicol at 1:1000 dilution
6. 2xYT Top Agar:
 - a. Per liter: 16 g tryptone, 10 g yeast extract, 5 g NaCl, 7.5 g granulated agar, pH 7.0 with NaOH

3.4.2 Buffers (Filter Sterilize)

1. Phosphate-buffered saline (PBS): 20 mM Na₃PO₄, 150 mM NaCl, pH 7.4 and 8.0 (pH 8.0 used as coating buffer)
2. PB-T: 20 mM Na₃PO₄, 150 mM NaCl, 1% BSA (w/v), 0.5% Tween-20 (v/v)
3. PBS-T: 20 mM Na₃PO₄, 150 mM NaCl, 0.5% Tween-20 (v/v)
4. Phage elution buffer: 100 mM Glycine, pH 2.0
5. Phage neutralization buffer: 2 M Tris, pH 7.5
6. Tris buffered saline (TBS): 20 mM Tris-HCl, 150–500 mM NaCl, pH 7.8
7. MOPS (1 M): 209.27 g in 1 L H₂O, pH 7.3
8. HEPES: 1 mM, pH 7
9. 8 M urea in TBS
10. 0.2% SDS, 8 M urea in TBS
11. MLB buffer: 1 M Sodium Perchlorate, 30% Isopropanol in water
12. MP buffer: 3.3 g citric acid monohydrate in 3 mL sterile H₂O; incubate with stirring for 5 min at room temperature, filter sterilize through 0.2 µm filter
13. Gentle Ag/Ab Binding Buffer, pH 8.0 (Pierce, Thermo Fisher Scientific, Waltham, MA, catalog #: 21020)
14. Gentle Ag/Ab Elution Buffer, pH 6.6 (Pierce, Thermo Fisher Scientific, Waltham, MA, catalog #: 21027)
15. 2% SDS (w/v) in H₂O

3.4.3 Solutions

1. 5× PEG/NaCl—filter sterilize
 - a. Per liter: 200 g PEG-8000 (20% w/v), 150 g NaCl (15% w/v)
2. 1–3% BSA (w/v) in PBS, pH 7.4
3. 2–5% Nonfat dry milk (NFDM) (w/v) in PBS, pH 7.4

3.4.4 Antibiotics (Filter Sterilize)

1. Carbenicillin: stock concentration of 100 mg/mL
2. Tetracycline: stock concentration of 5 mg/mL (made up in 70% ethanol)
3. Kanamycin: stock concentration of 50 mg/mL
4. Chloramphenicol: stock concentration of 34 mg/mL (made up in 100% ethanol)

3.4.5 Other Reagents

1. 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, catalog #: T0440)

2. 0.5 M H₂SO₄
3. Anti-M13-HRP conjugated antibody (GE Healthcare, Marlborough, MA, catalog #: GE27-9421-01)
4. T7 DNA Polymerase (NEB, Ipswich, MA, catalog #: M0274L)
5. T4 DNA Ligase (Invitrogen, Carlsbad, CA, catalog #: 15224-017)
6. 25 mM dNTPs (Fisher, Hampton, NH, catalog #: FERR1121)
7. 25 mg/mL Uridine (Sigma, St. Louis, MO, catalog #: U3750-100G)
8. BugBuster 10X Protein Extraction Reagent (Millipore, Billerica, MA, catalog #: 70921)
9. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany, catalog #: 28104)
10. QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany, catalog #: 28704)
11. QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany, catalog #: 27104)
12. NucleoBond Xtra Maxi Plus (Macherey-Nagel, Düren, Germany, catalog #: 740416.50)
13. Ni-NTA Agarose (Qiagen, Hilden, Germany, catalog #: 30210)
14. DNaseI (Invitrogen, Carlsbad, CA, catalog #: 18047-019)
15. SIGMAFast EDTA-free protease cocktail inhibitor (Sigma, St. Louis, MO, catalog #: S8830)
16. Precision Plus Protein Dual Color Standards (BioRad, Hercules, CA, catalog #: 161-03741)
17. Anti-His (C-term)-HRP antibody (Invitrogen, Carlsbad, CA, catalog #: 46-0707)
18. Protein A Agarose (Pierce, Thermo Fisher Scientific, Waltham, MA, catalog #: 15918-014)
19. Phusion High Fidelity PCR Master Mix with HF Buffer (NEB, Ipswich, MA, catalog #: M0531S)
20. Restriction enzymes: BssHII, BsiWI, NheI, NsiI, FseI (NEB, Ipswich, MA, catalog #:s: R0199S, R0553S, R0131S, R0127S, R0558S, respectively)
21. Alkaline Phosphatase, Calf Intestinal (NEB, Ipswich, MA, catalog #: M0290S)
22. Quick Ligation Kit (NEB, Ipswich, MA, catalog #: M2200S)
23. Polyethylenimine (PEI) Linear, 25,000 MW (Polysciences, Warrington, PA, catalog #: 23966)
24. FreeStyle™ 293 Expression Medium (ThermoFisher; Invitrogen, Carlsbad, CA, catalog #: 12338-018)
25. M2 antibody (Sigma-Aldrich, St. Louis, MO, catalog #: F1804)
26. Protein A-HRP conjugate (Invitrogen, Carlsbad, CA, catalog #: 101023)

4. PHAGE DISPLAY AND LIBRARY DESIGN

The first step in any phage display project is to clone the protein of interest into a suitable phagemid and verify that it is functionally expressed on the surface of phage. In our lab, phagemid HP153 has been shown to readily display many different proteins including domain III (DIII) of the glycoprotein E of DENV (Frei et al., 2015), Sudan virus (SUDV) Fabs (Chen et al., 2014), Dengue Fab E106 (unpublished results), HIV-1 Fab 2G12 (Stewart, Liu, & Lai, 2012), and protein G domain B1 from *Streptococcus* (unpublished results). To detect the protein of interest on phage and to monitor expression levels, we include a FLAG epitope (DYKDDDDK) either at the N-terminus (Dengue DIII, protein G) or C-terminus (light chain of Fabs), and probe for it using the anti-FLAG antibody M2. Protocol 4 details cloning into HP153. The protein insert should be designed with 5'-NsiI and 3'-FseI cut sites, 5'-"cc" (two cytosines) to keep the protein sequence in frame with the StII secretion sequence, and an N- or C-terminal FLAG-tag, or any tag of choice for which there is a well-characterized detection antibody.

Once cloned into HP153, expression of the protein at the surface of phage is confirmed by phage ELISA, in which the anti-FLAG antibody M2 is immobilized on a well, phage expressing the fusion protein are added, and bound phage are detected by the addition of an anti-M13-HRP conjugated antibody. ELISAs are developed using TMB and quenched with sulfuric acid, with absorbance measured at 450 nm (Abs. 450 nm) serving as a readout for phage binding. Although a positive ELISA result with M2 indicates expression of the protein, it does not necessarily confirm proper folding/function; function of the displayed protein can be determined by performing an ELISA with a known binding agent or antibody, preferably one that requires a conformational epitope for recognition. See Protocol 5 for phage amplification and titrating steps and Protocol 6 for phage ELISA. We typically observe Abs. 450 nm values of 2.5 or higher for well-expressing constructs with 1–20 min developing time (Fig. 2).

After confirming functional expression of a protein of interest on phage, library design can then ensue. The type of randomization scheme employed and the library format is highly dependent on the end goal of the project. If the goal is to optimize or alter selectivity of a given protein, then soft randomization libraries (discussed below) may be most appropriate. However, if the goal is to impart a protein scaffold with new function, then either hard randomization or limited diversity mutagenesis may be required. Furthermore, designing a library is easiest when high-resolution structures of the displayed protein or related homologues are available, however, this is not a requirement. In projects where the objective is to improve or broaden the specificity of an existing scaffold, the known contact residues can be targeted for mutagenesis. However, in other cases where one seeks to engineer new function into a scaffold or engineer an alternative binding interface, several sublibraries or a semiempirical step-wise approach may be required to identify which residues should be varied (Koide, Wojcik, Gilbreth, Hoey, & Koide, 2012; Mandal et al., 2012). Our lab has successfully used phage display to humanize an anti-SUDV mouse antibody (Chen et al., 2014), isolate antibodies specific to different proteolytic intermediates of the Ebola glycoprotein (Koellhoffer et al., 2012), isolate Fabs against proapoptosis protein BAX (Uchime et al., 2016), and map the hotspot residues in antibody–antigen interactions on both

the antigen (DENV DIII) or the antibody (Da Silva, Harrison, & Lai, 2010; Frei et al., 2015; Liu, Regula, Stewart, & Lai, 2011; Stewart, Harrison, Regula, & Lai, 2013; Stewart et al., 2012).

The targeted introduction of diversity into any protein is accomplished through the use of degenerate oligonucleotides. Many randomization schemes have been developed including hard randomization (allowing all 20 amino acids), soft randomization (high bias toward WT residue identity), and tailored/restricted diversity (Clackson & Lowman, 2004). It is important to keep in mind that the actual size of the library must surpass the theoretical diversity (all possible variants) of a phage library by 100-fold in order for all variants to be plausibly surveyed. This consideration will thus limit the number of residues that are randomized based on the diversification scheme selected. However, in cases where the library randomization scheme is highly biased, as in soft randomization or tailored/restricted diversity, there is often not a strict requirement that library size surpass the theoretical complexity of the library. Our lab typically achieves electroporation efficiencies of 10^8 – 10^9 transformants per reaction, and thus library sizes of 10^9 – 10^{10} can be achieved by pooling cultures from 10 to 20 electroporations.

In hard randomization, a position is allowed to vary among all 20 amino acids using the degenerate codon “NNS,” in which N codes for any of the four nucleotides and S codes for guanosine or cytosine. This randomization scheme encodes 32 of the 64 possible codons, including all 20 amino acids (some in duplicate) and one stop codon. When using a hard randomization strategy the number of residues that can be varied is limited to approximately 6, as the theoretical diversity becomes much too large past this (theoretical diversity = 32^6 at the genetic level = 1.1×10^9). In soft randomization, a position is allowed to vary among all 20 amino acids, but is biased toward the wild-type sequence by doping the four bases at each codon position at nonequivalent levels. To achieve approximately 50% of the wild-type sequence, each base of each codon is 70% wild type and 10% each of the other three nucleotides. With soft randomization, additional positions can be varied because, although the theoretical diversity is still 32^N (where N is the number of randomized residues), the library contains strong biases toward wild-type residue identity at any particular position. Soft randomization is best instituted in cases where one seeks to optimize, broaden, or even switch the specificity of a binding protein using the same contact residues. Lastly in tailored diversity strategies, degenerate codons are chosen that code for amino acids with desirable physicochemical properties, or in cases where only a subset of the 20 amino acids is to be considered. The reader is directed to table 1 of chapter 2 in Clackson and Lowman (2004) for a list of such degenerate codons.

In this section, we will focus the discussion of library design on the humanization of antibodies and mapping of hotspot residues in protein–protein interfaces.

4.1 Humanization of a Murine SUDV Antibody (Chen et al., 2014)

Full humanization of murine antibodies can be achieved rapidly by phage display if there is significant sequence and structural homology between the murine antibody and a human or humanized antibody. For the humanization of SUDV murine antibody 16F6, the structural alignment with a humanized template (YADS1, based on Herceptin[®]) was 1.7 Å in the non-

complementarity determining region (CDR) portions of the variable domains. The CDR loops of 16F6 and YADS1 began and ended at approximately the same locations within the Fv scaffolds, but with diverse structural configurations in some cases. A “grafted” chimeric clone, in which the CDR segments of 16F6 were grafted on YADS1 was found to be inactive. In our experience, a simple CDR graft between a murine antibody and a closely related human scaffold often results in significant diminishment or complete loss of binding activity.

For humanization of 16F6, the variable heavy (VH) and variable light (VL) domains of the grafted antibody were cloned into HP153 for display on phage protein-pIII using the restriction sites NsiI and FseI. The synthetic gene was ordered to contain the following 5′ to 3′: NsiI cut site, two cytosines, VL sequence, CGGS (amino acids), FLAG-tag, TAA stop codon, DNA region containing an internal ribosome binding site and StII sequence (5′-ttaactcgaggctgagcaaacgagactactaataacataaaagtctacgccggacgcacatcgtggccctagtacgcaagtccacgtaaaaagggttaactagaggttgaggtgattttatgaaaagaatatcgcatttcttctgca-tctatgttcgtttttctattgctacaaacgcgtacgtgagatctcc-3′), VH sequence, cysteine, gacaaaactcacatgc (DNA sequence 5′ to 3′), and FseI cut site. This format drives the bicistronic expression of both VH and VL and will result in bivalent display of a LC C-terminally FLAG-tagged Fab. Expression can be checked via phage ELISA against the anti-FLAG antibody M2 (see Protocol 6).

If a structure of the antibody–antigen complex is available, all paratope residues (residues of the antibody that contact the antigen) can be determined by visual inspection or calculation of buried surface area. In the case of 16F6, the humanization library maintained most of these contact residues as the native murine residue identity (ie, the residue present in the starting antibody). Non-contact residues that differed between 16F6 and YADS1 were varied among the human antibody identity and the starting antibody identity, as well as, residues with similar physicochemical properties (Chen et al., 2014). Two positions that differed between 16F6 and YADS1, whose contributions to direct binding or productive loop conformation were unclear, were varied among all 20 amino acids using the hard randomization “NNS” codon.

This randomization scheme minimizes the diversity of the library so that a phage library large enough to cover the theoretical diversity can be synthesized. Maintaining only the contacting residues as the starting antibody identity minimizes the nonhuman regions of the antibody and the potential for a human–antimouse–antibody response. Other methods for humanization of antibodies using phage display have been previously described and can serve as complementary or alternative approaches (Baca et al., 1997; Rader, Cheresh, & Barbas, 1998). In the case of 16F6 humanization, the strong sequence and structural agreement between the murine antibody 16F6 and a humanized scaffold YADS1 allowed precise prediction of sites that would require randomization. However, other cases for which the alignment of murine and human/humanized scaffolds is not as clear may necessitate randomization in positions of the framework that are distal to the CDRs.

4.2 Mapping Hotspot Residues of Protein–Protein Interfaces (Frei et al., 2015; Stewart et al., 2013)

While surfaces involved in protein–protein interfaces can be quite large and encompass many residues from both proteins, it has been well established that only a small subset of the contact residues contribute significantly to the energetics of binding and form the “functional” epitope (Bogan & Thorn, 1998; Clackson & Wells, 1995). Combinatorial alanine scanning mutagenesis by phage display is a method for rapidly mapping protein–protein interfaces to statistically determine hotspot residues, or residues that contribute significantly (>1 kcal/mole) to the energetics of binding. We have successfully applied this strategy to mapping the hotspot residues of the DENV domain III (DIII) protein binding to the broadly neutralizing antibody 4E11, and others have applied it to the human growth hormone binding to its receptor (Frei et al., 2015; Pal et al., 2003; Weiss et al., 2000). Here we describe the library design for mapping the functional epitopes of DENV DIII binding to antibody 4E11; however, this strategy can be generalized to other protein–protein interactions.

N-terminally FLAG-tagged DENV DIIs from all four serotypes (DENV1–4) were cloned into phagemid HP153 using NsiI and FseI restriction sites for bivalent display (see Protocol 4). For each serotype, the antibody-contacting residues (structural epitope) were determined from the crystal structure of the antibody–antigen complex (Cockburn et al., 2012). These residues were allowed to vary among the wild-type identity and alanine using the degenerate codons listed in table 1 of Weiss et al. (2000). Due to the degenerate nature of the genetic code, some residues varied among wild type, alanine and two other residues. For example if the wild-type residue is lysine, then at that position the library encodes for lysine, alanine, glutamate, or threonine. This strategy was applied to all four DIIs and resulted in the production of four phage libraries (Frei et al., 2015).

This approach can be generalized to any protein–protein interaction. A solved crystal structure of the complex, while useful, is not required for this analysis as multiple alanine scanning libraries can be synthesized and screened in parallel to map the complete binding interface. The reader is directed to Weiss et al. for a detailed explanation of the combinatorial shotgun scanning analysis that results in estimates of the ΔG of binding upon mutation to alanine (Weiss et al., 2000).

4.2.1 Protocol 4: Cloning into HP153

1. Day 1: Digestions (dilute vector and insert to 100 ng/mL in sterile H₂O)
 - a. Add the following:

HP153		Insert	
15 μ L	DNA	15 μ L	DNA
5 μ L	NEBuffer2.1	5 μ L	NEBuffer2.1
1 μ L	NsiI	1 μ L	NsiI

HP153		Insert	
1 μ L	FseI	1 μ L	FseI
28 μ L	H ₂ O	28 μ L	H ₂ O
50 μ L	Total vol.	50 μ L	Total vol.
Incubate at 37°C for 2.5 h			
Add 1 μ L CIP to vector			
Incubate at 37°C for 0.5 h			
Run a 1% agarose gel on digestion products			

- b.** Gel purify the appropriately sized bands (digested HP153 runs between 3000 and 4000 bp)
 - i.** Gel purify using Qiagen QIAquick Gel Purification Kit as per manufacturer's instructions
 - ii.** Elute with 30 μ L H₂O
 - iii.** Measure concentrations

2. Day 1: Ligation (NEB Quick Ligation Kit—*Note:* Modified manufacturer's protocol)

- a.** Add the following:

Ligation	
7.5 μ L	2 $\times$ Quick Ligase Buffer
2 μ L	Digested vector
5.5 μ L	Digested insert
1 μ L	Quick ligase
Incubate at room temperature for 5 min	
Incubate on ice for 5 min	

- i.** *Note:* May need to change the molar ratio of vector:insert for best results

3. Day 1: Transformation

- a.** Add 5 μ L ligation product to 25 μ L One Shot TOP10 Chemically Competent cells
- b.** Incubate on ice 10 min
- c.** Heat shock at 42°C for 45 s
- d.** Incubate on ice 2 min
- e.** Add 200 μ L SOC media and recover 45–60 min at 37°C, 220 RPM
- f.** Plate out 10 and 200 μ L onto LB/Carb plates
- g.** Incubate overnight at 37°C

4. Day 2: Verify sequences

- a. *Note:* We send several colonies to Genewiz for sequencing by making grid plates
- b. Day 3: Send for sequencing
 - i. Send 1 plate along with 400 μ L of 5 μ M primer for sequencing; store second plate at 4°C
 - ii. Primer sequences for sequencing (5' to 3'):
 - VH-For (VH domain):
TGTAACGACGGCCAGTGGACGCATCGTGG
CCCTA
 - VH-Rev (anneals in CH domain):
CAGGAAACAGCTATGACCCCTTGGTG
 - VL-For (VL domain):
TGTAACGACGGCCAGTCTGTCATAAAGTTG
TCACGG
 - VL-Rev:
CAGGAAACAGCTATGACCCCTTGGTACCCTGT
CCG
 - HP153-For (other proteins):
CTGTCATAAAGTTGTCACGG

4.2.2 Protocol 5: Amplifying and Titering Phage

1. Day 1: Electroporate phagemid into XL1-Blue cells
 - a. Thaw XL1-Blue cells on ice and place 2 mm-cuvette on ice
 - b. To 1 tube of XL1-Blue cells add 2–10 μ L of phagemid DNA
 - c. Transfer cells and DNA to cuvette on ice—tap to get rid of air bubbles
 - d. Electroporate using the following protocol:
 - i. *E. coli*—2 mm, 2.5 kV
 - ii. Voltage: 2500 V
 - iii. Capacitance: 25 μ F
 - iv. Resistance: 200 Ω
 - v. Cuvette: 2 mm
 - e. Resuspend cells in 2 $\times$ 200 μ L SOC media
 - f. Recover at 37°C, 220 RPM, 45–60 min
 - g. Plate various dilutions onto LB/Carb/Tet plates

b.

$$\text{Titer} = (\# \text{colonies}) \left(\frac{1}{5} \right) (1000) (10^{\text{dilution}+1})$$

- i. *Note:* The dilution factor contains “+1” to account for the 1:10 dilution from media to cells

4.2.3 Protocol 6: Phage ELISA

1. Day 1: Amplify phage (Protocol 5)
2. Day 2: Titer phage (Protocol 5) and coat plates
 - a. Coat antigen or antibody at 0.1–2 µg per well in coating buffer (100 µL total volume per well)
 - i. *Note:* Empirically determine which amount is necessary for each protein pair
 - b. Coating buffer: PBS, pH 8.0, serves as negative control
 - c. If coating with M2 (anti-FLAG antibody): dilute 1:500 in PBS, pH 8.0
 - i. *Note:* Can either coat overnight at 4°C or for 2 h at room temperature
3. Day 3: Phage ELISA
 - a. Make serial 1:10 dilutions of amplified phage stock in PB-T—switch tips between dilutions
 - b. Block plate with 1–3% BSA (w/v) or 2–5% NFDM (w/v) for 1.5–2 h at room temperature with shaking
 - c. Wash plate 5× with PBS-T
 - d. Add 100 µL of phage dilutions per well—incubate for 1 h at room temperature with shaking
 - e. Repeat step c
 - f. Add 100 µL per well of 1:1000 diluted anti-M13-HRP (dilute in PB-T)—incubate for 45–60 min at room temperature with shaking
 - g. Repeat step c
 - h. Develop with 100 µL per well TMB and quench with 100 µL per well 0.5 M H₂SO₄
 - i. Read absorbance at 450 nm

5. LIBRARY PRODUCTION

There are several methods to produce phage libraries; we favor the use of degenerate oligonucleotides and Kunkel mutagenesis to introduce targeted diversity as originally described by Kunkel, Roberts, and Zakourts, and Zakour (1987). Therefore, we chose to

focus on this strategy for this section. The reader is encouraged to review “[Phage display: A practical approach](#)” for other methods of library synthesis (Clackson & Lowman, 2004).

5.1 Kunkel Mutagenesis

The process of Kunkel mutagenesis is summarized in Fig. 3. Kunkel mutagenesis relies on the synthesis of a ssDNA template enriched in uracil (dU-ssDNA) by isolating the ssDNA from phage produced in an *E. coli* strain that lacks dUTPase and uracil-N-glycosylase (*dut⁻/ung⁻*) such as CJ236 cells. Deficiencies in these two enzymes result in production of DNA that has a high incorporation rate of uracil (dU-ssDNA). CJ236 cells are chloramphenicol (cmp) resistant and should be plated on LB/cmp plates, or on LB/cmp/carb plates after electroporating in the phagemid.

Once dU-ssDNA has been produced, 5'-phosphorylated degenerate oligonucleotides are annealed to the dU-ssDNA. These primers are designed so that the 5' and 3' ends are exactly complementary to the 5' and 3' DNA surrounding varied positions to allow for annealing. The mismatched region between the complementary segments contains the degenerate codons that encode for diversity according to the library design. Degenerate primers are specifically incorporated during the annealing step of library synthesis—thus allowing for the targeted incorporation of diversity. After the primers are annealed, the complementary strand is synthesized based on the dU-ssDNA template by the addition of all four dNTPs (dATP, dCTP, dGTP, and dTTP), T7 polymerase, and T4 ligase, which results in a hybrid double-stranded DNA (dsDNA) molecule. One strand contains the uracil-containing template while the newly synthesized strand contains thymine. At this point, there is a heterogeneous mixture of hybrid covalently closed circular double-stranded DNA (CCC-dsDNA). Hybrid CCC-dsDNA is electroporated into a high efficiency electroporatable cell line that is *dut⁺/ung⁺*, such as SS320. In SS320 cells the uracil-containing DNA strand is inactivated by cleavage of the uracil, and the newly synthesized (mutated) strand is preferentially replicated. During replication, the mutations encoded by the degenerate oligonucleotides are incorporated. Addition of M13KO7 helper phage after electroporation results in the production of library phage; each phage displays a different version of the mutated protein.

While Kunkel mutagenesis is a highly efficient mutagenesis technique, with an overall efficiency approaching 80% when a single primer is used, this still results in 20% of clones maintaining the wild-type sequence. If three to four primers are used, corresponding to three or four distal regions of sequence space that are randomized, then mutagenesis efficiency decreases to approximately 50% (Clackson & Lowman, 2004). One must consider that the wild-type population will dominate the selection, as 20% or more of the sequences will be wild type depending on the efficiency of primer incorporation. To circumvent the wild-type phenotype dominating selections, an inactive template may be synthesized in which residues that are to be varied in the library are replaced with stop codons (TAA) or rare arginine codons (AGA or AGG). Incorporation of stop codons or rare arginine codons results in the decreased expression of the protein at the surface of phage (Sidhu & Geyer, 2015). Therefore even though 20% of the clones will maintain the wild-type genotype, they are not represented at the phenotypic level, as they are not expressed.

An inactive template is synthesized using Kunkel mutagenesis as described above; however, because only one clone containing the inactivating mutations is required, the hybrid DNA can be electroporated into XL1-Blue cells instead of SS320. The inactivated template will match the wild-type protein sequence but will contain either stop or rare arginine codons at positions of diversity. Once the inactive dU-ssDNA template is synthesized, library synthesis continues with annealing of library primers to the inactive template as described earlier.

After library synthesis is completed, library phage should be titered and a number of clones should be sequenced to determine the incorporation rate of library primers and library diversity. While the efficiency for incorporation of mutations with one primer can reach 80%, this efficiency realistically decreases to 20–30% upon increasing the number of primers to be incorporated. Sequencing data from the naïve library allow one to determine whether or not to begin the next step of phage display: selection.

5.1.1 Protocol 7: dU-ssDNA Synthesis (Modified from Tonikian et al., 2007)

1. Electroporate into CJ236 cells
 - a. Electroporate 1 μ L phagemid DNA into 25 μ L CJ236 cells using 1 mm-gap cuvette
 - b. Recover in 3×200 μ L recovery medium (provided with cells)
 - c. Incubate at 37°C, 220 RPM for 1 h
 - d. Plate out 0.5, 1, 10, and 200 μ L onto LB/Carb/cmp plates—want individual colonies
 - e. Incubate overnight at 37°C
2. Grow CJ236 cells
 - a. Add 1 colony to 1 mL 2xYT/2 μ L cmp/5 μ L Carb
 - i. *Note:* Cells will grow slowly
 - b. Incubate at 37°C, 220 RPM until slightly dense
 - i. *Note:* Can require overnight incubation, therefore include a negative control
 - ii. Negative control: media+antibiotics but no colony
 - c. Add M13KO7 helper phage to a final concentration of 10^9 – 10^{10} CFU/mL
 - d. Incubate at 37°C, 220 RPM for 2 h
 - e. Add 1 mL culture to 25 mL 2xYT/12.5 μ L Carb/12.5 μ L Kan
 - f. Incubate at 37°C, 220 RPM for 3 h
 - g. Expand to 1 L 2xYT/500 μ L Carb/500 μ L Kan/10 μ L of 25 mg/mL Uridine
 - h. Incubate at 37°C, 220 RPM until dense—can require 24–36 h

3. Precipitate phage
 - a. Centrifuge at 4000 RPM, 4°C for 15 min to pellet cells
 - b. Pour supernatant into 250 mL 5× PEG/NaCl
 - c. Incubate at 4°C for 1 h with stirring
 - d. Centrifuge at 12,000 RPM and 4°C for 20 min to precipitate phage
 - e. Resuspend phage in 10 mL PBS, pH 7.4
 - f. Microcentrifuge at 7600 RPM and 4°C for 10 min
 - g. Transfer supernatant to clean 15 mL Falcon tube
4. Isolate dU-ssDNA (modified from Qiagen's QIAprep Spin M13 Kit protocol)
 - a. Add 7 µL Buffer MP (see Section 3.4.2) per 500 µL phage, vortex, and incubate at room temperature for 10 min
 - b. Apply 0.7 mL of phage to spin columns
 - i. *Note:* For 10 mL of phage, use four spin columns
 - c. Microcentrifuge at 8000 RPM for 30 s—discard the flow through
 - i. Continue loading and centrifuging spin columns until all phage has been loaded
 - d. Add 0.7 mL Buffer MLB (see Section 3.4.2) and centrifuge at 8000 RPM for 30 s
 - e. Add 0.7 mL Buffer MLB and incubate for 1 min at room temperature
 - f. Centrifuge at 8000 RPM for 1 min
 - g. Add 0.7 mL Buffer PE and centrifuge at 8000 RPM for 1 min—discard flow through
 - h. Repeat step g
 - i. Centrifuge at 8000 RPM for an additional 3 min
 - j. Add 50–100 µL sterile H₂O directly to membrane and incubate at room temperature for 10 min
 - k. Centrifuge at 8000 RPM for 1 min; combine into 1 tube

5.1.2 Protocol 8: Kunkel Mutagenesis (Modified from Kunkel et al., 1987; Tonikian et al., 2007)

1. Synthesis of hybrid covalently closed circular DNA (CCC-DNA)
 - a. Determine concentration of dU-ssDNA
 - i. Per reaction, use 10 µg dU-ssDNA
 - ii. For library synthesis start with four reactions

- iii. For template synthesis use one reaction with 5 µg dU-ssDNA
- b. Resuspend primers to 1 mg/mL in sterile H₂O and dilute 1:10 in sterile H₂O
- c. Determine number of base pairs (bp) in each primer
- d. Calculate dU-ssDNA:Primer ratio
 - i. Start with a dU-ssDNA:Primer ratio of 1:3
 - ii.

$$\text{Sample calculation: } = \frac{1}{3} = \frac{\frac{10 \mu\text{g (DNA)}}{\text{\#bp (phagemid)}}}{\frac{x}{\text{\#bp (primer)}}}$$
 - iii. May need to change the DNA:Primer ratio for best incorporation results
- e. Set up reactions as follows:

Per Kunkel reaction	
10 µg	dU-ssDNA
X ₁ µL	Primer 1
X ₂ µL	Primer 2
X ₃ µL	Primer 3
25 µL	5X T4 DNA Ligase Buffer (Invitrogen)
12.5 µL	10X T7 DNA Polymerase Buffer (NEB)
X ₄ µL	Sterile H ₂ O
250 µL	Total vol.

- f. For each reaction: mix and distribute to three PCR tubes (83 µL per tube)
 - g. Run the following PCR cycle:
 - h. 90°C (5 min), 75°C (45 s), 70°C (1 min), 65°C (1 min), 60°C (1 min), 55°C (1 min), 52°C (3 min), 45°C (30 s), 35°C (30 s), 30°C (45 s), 25°C (45 s), 22°C (1.5 min), 20°C (indefinitely)
 - i. Combine each starting reaction back into one Eppendorf tube (four tubes total)
 - j. Add 10 µL 25 mM dNTPs, 2 µL T4 DNA Ligase (Invitrogen), and 3 µL T7 DNA polymerase (NEB) to each
 - k. Incubate at room temperature for 3 h
 - l. PCR purify using four spin columns using Qiagen QIAquick PCR purification kit, but wash 2× with buffer PE
 - i. Elute with 50 µL sterile H₂O and combine into one tube

- m. Measure DNA concentration
2. Electroporate into SS320 cells
 - a. If using Lucigen SS320 cells, note that each tube contains two reactions worth of cells (50 μ L cells per tube)
 - b. Per 25 μ L SS320 cells add 15–20 μ L of DNA and electroporate using a 1 mm-gap cuvette (*E. coli*—1 mm, 1.8 kV)
 - i. *Note:* Time constants of 5.0 ms are desirable
 - ii. We start with 2–6 electroporations
 - c. Resuspend immediately in 2 $\times$ 500 mL SOC media for each electroporation
 - d. Recover in a total volume of 100 mL SOC media with 10^9 – 10^{10} CFU/mL M13KO7 helper phage at 37°C, 220 RPM for 1 h
 - e. Titer recovered transformants:
 - i. Serially dilute 10 μ L of the recovered phage in 90 μ L 2xYT (1:10 dilutions)
 - ii. Plate out 80 μ L of each dilution (10^{-1} through 10^{-8}) onto LB/Carb plates
 - iii. Incubate overnight at 37°C
 - iv.
$$\text{Transformants} = (\# \text{colonies}) \left(\frac{1}{80} \right) (1000) (100) (10^{\text{dilution}})$$
 - *Note:* Library size should exceed theoretical diversity by 100-fold
 - f. Add 100 mL recovery culture to 500 mL 2xYT/250 μ L Carb/250 μ L Kan
 - g. Incubate overnight at 37°C and 220 RPM
 3. Precipitating and storing library phage
 - a. Centrifuge at 4500 RPM, 4°C for 15 min
 - b. Add supernatant to 125 mL 5 $\times$ PEG/NaCl for 1 h at 4°C with stirring
 - c. Centrifuge at 12,000 RPM, 4°C for 20 min
 - d. Resuspend phage in 10 mL PBS, pH 7.4
 - e. Microcentrifuge at 7600 RPM, 4°C, for 10 min
 - f. Collect supernatant in clean 15 mL Falcon tube
 - i. *Note:* Use this library phage immediately in the first round of selection
 - g. Add final 50% (v/v) of pure glycerol to phage and thoroughly combine

- h. Aliquot and store phage library at -80°C

5.2 Primer Design

Degenerate oligonucleotides should be designed so that there are at least 15 complementary bases upstream and downstream of the targeted mutated sequence to ensure targeted and proper annealing during Kunkel mutagenesis. Primer length is limited to approximately 100 bp due to decreasing oligonucleotide synthesis efficiency past this size. The regions of randomization can be longer or shorter than the corresponding region of the native template protein, to either add or remove residues. For CDR loop segments of antibodies, the size range can be especially beneficial. All nonmutated bases covered by the primer are exactly complementary to the wild-type DNA sequence. Up to four primers can be incorporated in a library design; however, the sequences they cover cannot overlap, as annealing will be mutually exclusive in those cases.

In order for the ligation reaction during complementary strand synthesis to occur, the degenerate primers must be 5'-phosphorylated. We order our degenerate primers with a 5'-phosphorylation modification from commercial suppliers. However, primers can be ordered without the modification and then 5'-phosphorylated following the protocol found in Tonikian et al. (2007). We dilute our primers to 1 mg/mL in sterile H_2O and store them at -20°C . Before library synthesis, dilute primers 1:10 in sterile H_2O .

5.3 Library Quality Control

To determine the quality of the library, it is recommended that the titer plates (naïve library) be sequenced in large scale ($n > 100$). The primer incorporation rate should be determined for each of the primers individually and the total incorporation rate if more than one primer was used for mutagenesis. Although initial incorporation rates can be low (20–30%) with many clones containing the template sequence, the first round of selection against target often results in enrichment of library-encoded sequences. However, in cases where the selection is difficult, such as when there is little or no initial affinity for the target of interest, enrichment for library members may not be observed until later rounds. In this case, it is often best to optimize the synthesis of the library by empirically determining the best primer to dU-ssDNA ratio and annealing conditions that provide the highest incorporation levels of the primers. From the naïve library sequences, one should also verify the diversity of the sequences. At this stage no selective pressure has been applied, so a single sequence should not dominate. Additionally, the entire sequence of the protein should be checked to ensure no deletions or truncations have occurred.

6. LIBRARY SELECTIONS AND SCREENING

Libraries that are produced in sufficient quality can be subjected to selection to enrich for phage clones that demonstrate increased affinity or specificity for the target. Typically, two to four rounds of library selections are performed; however, it is important to avoid overselection since the “fittest” clones are frequently those that harbor some activity toward the target but also a distinct growth advantage and are not necessarily the highest affinity

binders. Protocols for phage selection and screening can be found below (Protocols 9 and 10).

6.1 Selection

Library selection is accomplished through the immobilization of target in wells, followed by blocking of nonspecific sites. The phage library is added in a binding buffer, which contains an irrelevant protein (BSA, NFDM, etc.) and/or a nonionic detergent (Tween-20), the wells are washed to remove phage that do not bind to the target, and bound phage are eluted by addition of an acidic solution. The phage solution is neutralized and amplified by infection of an XL1-Blue culture for subsequent rounds of selection. During each round of selection, a negative control well coated with an irrelevant protein, such as BSA or NFDM, should be included to determine enrichment for binding to target (see later).

Over the course of selection, the stringency should be increased to select for phage clones that demonstrate increased affinity or specificity for the target. This can be accomplished by decreasing the amount of target coated (ie, decrease the number of wells), increasing the number of washes between phage binding and elution, increasing the incubation time of each wash step, or increasing the phage binding time to select for clones with larger k_{off} values and thus smaller K_{D} s. If increased binding to the negative control becomes evident in later rounds of selection, it may be useful to switch binding and blocking buffers and repeat that round of selection.

During selection, it is important to monitor enrichment. This is done by titering the input, output, and control phage onto LB/carb and LB/kan plates. The carb plate provides information on the enrichment, the ratio of phage recovered from wells coated with target vs phage recovered from negative control wells. Often very little enrichment is seen in the first round of selection. Ideally enrichment ratios of 10-fold or more will be observed, but often this is not observed until later rounds. Once background enrichment is no longer observed, continuing with selection may result in overselection. The kan plate provides information on the specific enrichment of displaying phage vs nondisplaying helper phage. Over rounds of selection, the output population should decrease on the kan plate, as the control (nondisplaying) helper phage should not be binding to the target of interest.

After each round of selection, we send clones for sequencing to monitor the enrichment process. If a selection is proceeding efficiently, we see higher percentages of library members emerge as rounds of selection progress. After several rounds of selection, the sequences may converge to a consensus sequence. In this case, screening should begin from an earlier round, as convergence to a single sequence may indicate overselection. It is best to screen populations that have been selectively enriched for functional binders but that still retain some degree of sequence variation. It is also important to monitor if the selection is selecting clones with truncation mutations.

6.1.1 Protocol 9: Selection

1. Day 1: Coat plate with antigen

- a. Coat seven wells of a high-binding plate with 0.2–10 μg per well of antigen (or antibody if antigen is on phage) in coating buffer (PBS, pH 8.0)
 - i. Add 100 μL per well
 - b. Coat one well with 100 μL PBS, pH 8.0 as negative control
 - c. Incubate overnight at 4°C; alternatively, incubate for 2 h at room temperature with shaking
 2. Day 2: Selection
 - a. Discard coating solution and block with either 1–3% BSA (w/v) or 2–5% NFDM (w/v)
 - b. Incubate for 1.5–2 h at room temperature with shaking
 - c. Wash wells 3–5 $\times$ with PBS-T
 - d. Add 100 μL phage per well
 - e. Incubate for 1 h at room temperature with shaking
 - f. Repeat step c
 - g. Elute phage with 100 mM glycine, pH 2.0
 - i. Add 100 μL per well including the negative control
 - ii. Incubate for 5 min with shaking
 - iii. Remove phage from each well by vigorously pipetting—keep the negative control phage separate
 - iv. Per 100 μL phage add 30 μL 2 M Tris, pH 7.5—invert to mix
 - v. Confirm neutral pH with pH indicator strips
 3. Day 2: Amplification of output phage population
 - a. Grow XL1-Blue cells in 2xYT/Tet media in a baffled flask at 37°C, 220 RPM until $\text{OD}_{600}=0.6$
 - b. To 5 mL XL1-Blue cells add half of the volume of the output phage from the previous round of selection
 - c. Incubate at 37°C, 220 RPM for 30–60 min
 - d. Add a final concentration of $10^9\text{--}10^{10}$ CFU/mL M13KO7 helper phage
 - e. Incubate at 37°C, 220 RPM for 1 h
 - f. Expand to 25–50 mL 2xYT/25–50 μL Carb/25–50 μL Kan
 - g. Incubate at 37°C, 220 RPM overnight
 4. Day 2: Phage titrating
 - a. See Protocol 5 step 3b—titer the input, output, and control phage

- b. Using a multichannel pipette, plate out 5 μ L of each dilution of the input, output, and control phage onto LB/Carb, LB/Tet, and LB/Kan plates, respectively
 - i. *Note:* LB/Tet is a control for XL1-Blue cells
- c. Incubate at 37°C overnight
- d. Calculate phage titer as in Protocol 5 step 4b

6.2 Screening

A reliable and rapid way to interrogate a selected population of phage for binding to the target of interest is to use monoclonal phage ELISAs. In monoclonal phage ELISA, individual phage are amplified in small scale in a 96-well format. These phage are then bound at a single high-point concentration to the target, which is immobilized on a well of a 96-well high-binding plate. The phage are incubated separately in parallel with a negative control protein (BSA or NFDM) and a positive control (anti-M2 antibody for detection of the FLAG-tag). Unbound phage are removed by washing, and binding to all three targets (target protein, negative control, and positive control) is probed by the addition of an antibody that recognizes the major coat protein-pVIII of the M13 bacteriophage and is conjugated to horse radish peroxidase (HRP). Substrate (TMB) is added and the reaction is quenched with sulfuric acid. The readout for binding is the absorbance measured at 450 nm (Abs(450 nm)) for each well for each plate.

Monoclonal phage ELISA data is analyzed by comparing the absorbance of the target protein wells to the negative control wells. Generally, we prefer to carry forward only those clones that have a strong Abs(450 nm) (2.5 or higher after 1–20 min development) and with a high preference over background. The most robust clones show 10- or more fold preference for the target over negative control wells, but we have in the past characterized clones with more moderate preferences (2- or 3-fold) on phage and found them in some cases to be well behaved as soluble proteins. Furthermore, one should consider the absorbance values for binding to both the target and the positive expression control, M2. If the values for binding to target and to M2 are low, it is likely that the clone does not have high affinity for the target and is not expressed well at the surface of the phage. If the absorbance value for M2 is high and the absorbance value for target is low, it is likely that this phage clone expresses very well but does not have high affinity for the target. If both absorbance values are high it is likely that this clone is a true hit, and one should proceed with validating it (see Section 6.3 characterization of selected clones).

The protocol for monoclonal phage ELISAs can be found below in Protocol 10 and protocols for characterizing hits can be found in Section 6.3.

6.2.1 Protocol 10: Monoclonal ELISA

1. Day 1: Titer and plate out phage
 - a. Grow XL1-Blue cells in 2xYT/Tet media until $OD_{600}=0.6$

- b. Titer output phage (for different rounds of selection) as described in Protocol 5 step 3b
 - c. Plate out 50 µL of phage on LB/Carb plates from different dilutions—want individual colonies
 - d. Incubate at 37°C overnight
2. Day 2: Coat plates
 - a. For each screen, you will need a 96-well high-binding plate for the target(s), negative control (NFDM or BSA), and positive control (anti-FLAG M2)
 - b. Target plate: per well add 100 µL of 0.2–2 µg of target in PBS, pH 8.0
 - c. Negative control plate: per well add 100 µL of PBS, pH 8.0
 - d. Positive control plate: per well add 100 µL of 1:500 diluted M2 antibody in PBS, pH 8.0
 - e. Incubate overnight at 4°C, or alternatively incubate at room temperature for 2 h with shaking
3. Day 2: Make 96-deep-well plate and grid plates
 - a. To each well of a 96-deep-well plate add 1 mL 2xYT, 1 µL Carb, and final concentration of 10^{9-10} CFU/mL M13KO7 helper phage
 - i. *Note:* Make up 100 mL 2xYT/Carb/M13KO7 and distribute to deep-well plate using repeater pipette
 - b. Prewarm two LB/Carb plates and draw a 96-spot grid on each
 - c. Using sterile pipette tips, pick colonies from phage plates (made on Day 1) and spot onto each grid plate and then place in the deep-well plate
 - i. *Note:* Match up the grid plate numbers with the deep-well plate
 - d. Place a gas-permeable membrane cover over the deep-well plate
 - e. Incubate deep-well plate at 37°C, 220 RPM overnight
 - f. Incubate grid plates at 37°C overnight
4. Day 3: Monoclonal ELISA
 - a. Discard coating solutions and block with 2–5% NFDM (w/v) or 1–3% BSA (w/v) at room temperature with shaking for 1.5–2 h
 - b. Wash plates 5× with PBS-T; pat out excess solution onto paper towels
 - c. Spin down the deep-well plate at 4000 RPM, 4°C for 20 min
 - d. Using a multichannel pipette, add 100 µL phage from deep-well plate to each high-binding plate

- i. *Note:* Match up the rows of the deep-well plate with those of the high-binding plates
 - ii. *Note:* If high background binding is encountered, try adding binding buffer to wells before adding the phage
- e. Incubate at room temperature with shaking for 1 h
- f. Repeat step b
- g. Add 100 μ L of 1:1000 α -M13-HRP in PB-T to each well
- h. Incubate at room temperature with shaking for 1 h
- i. Repeat step b
- j. Develop by adding 100 μ L TMB to each well and shaking at room temperature for 1–10 min, or until a dark blue color appears
- k. Quench the reaction by adding 100 μ L of 0.5 M H_2SO_4 to each well
- l. Read the absorbance at a wavelength of 450 nm (A_{450}) for each plate
- m. Phage clones are considered “hits” if the (A_{450} target)/(A_{450} negative control) >2
 - i. *Note:* In some cases the cutoff value needs to be increased; this should be determined empirically for each selection
- n. Send colonies corresponding to hits for sequencing using appropriate primers (see Protocol 4)

6.3 Characterization of Selected Clones

Monoclonal phage ELISA measures binding to target only at a single high concentration of phage. It is therefore important to determine the binding reactivity over a range of phage concentrations. This can be accomplished by electroporating the phagemid DNA into XL1-Blue cells, amplifying phage from a single colony as described in Protocol 5, and probing binding to target at multiple serial dilutions of phage. Plotting absorbance vs log (phage titer) determines the half-maximal binding titer, which often provides a relative readout for the relative affinity of the interaction between the protein expressed on phage and the target (Fig. 4).

While the first stages of phage display are relatively rapid, the downstream steps often require much more time and optimization and are therefore the bottleneck to many phage-based projects. Even if a displayed protein demonstrates desirable properties on phage, it may not behave the same expressed as a soluble protein. Therefore, we include protocols for expressing phage-displayed proteins as soluble proteins (Protocols 11 and 12). Once the protein has been expressed off phage, biochemical characterization of the soluble protein can be done.

6.3.1 Protocol 11: Expressing Soluble Fabs (Or Other Proteins) in *E. coli*—If the phagemid was designed to include an amber stop codon (TAG) between the end of the

VH domain (or protein of interest) and the beginning of the pIII phage protein, then expressing soluble Fab is achieved by transforming the phagemid into a nonsuppressor strain of *E. coli* such as BL21(DE3). In XL1-Blue cells, an amber suppressor cell line, the amber stop codon will be read through and the protein will be expressed as a fusion to pIII. In the nonsuppressor cell line, a release factor will be recruited to the ribosome at the amber stop codon and the soluble protein will be expressed. If an amber stop codon was included in the construct, proceed to step 2 of Protocol 11. If no stop codon was included between the protein of interest and the phage pIII coat protein, see below.

1. Insertion of stop codon between protein of interest and phage pIII
 - a. Prepare dU-ssDNA as described in Protocol 7 for the phagemid
 - b. Order 5'-phosphorylated primer that is complementary to the last five C-terminal residues of the protein of interest and to the first five residues of the pIII gene with a histidine tag and stop codon (TAA) placed between the complementary bases.
 - i. *Note:* The primer can include a protease cut site for removal of His-tag
 - ii. *Note:* If the protein of interest is cloned into HP153 with NsiI and FseI, then annealing the primer to the last five C-terminal residues of the protein will result in the expression of monovalent soluble protein
 - iii. *Note:* If the protein of interest has an N-terminal FLAG-tag, this can be removed by annealing a primer complementary to the last five residues of the StII sequence followed by the first 5 N-terminal residues of the protein
 - c. Perform Kunkel mutagenesis as described in Protocol 8 and electroporate into XL1-Blue cells
 - i. *Note:* Only one clone containing the mutation needs to be isolated; therefore decrease the amount of dU-ssDNA to 5 µg and use one reaction
 - d. Screen for clones containing the correct mutations by sequencing
 - e. Isolate DNA using a Qiagen QIAquick Miniprep Spin Kit
 - f. Transform into BL21(DE3) cells for protein expression
2. Expression
 - a. Pick a single colony from a freshly transformed plate into 25–50 mL 2xYT/25–50 µL Carb.
 - b. Incubate at 37°C, 220 RPM overnight
 - c. Expand culture to Low Phosphate Media/Carb
 - d. Grow at 30°C and 220 RPM for 24 h

- e. Harvest cells by centrifugation and determine weight of cell pellet—freeze cells
3. Purification
- a. Per gram of cell pellet, add 5 mL of 1X BugBuster
 - b. Resuspend cell pellet in PBS, pH 7.4/EDTA-free protease cocktail inhibitor/DNaseI
 - c. Add enough 10X BugBuster to dilute to 1X
 - d. Incubate at room temperature with gentle rocking for 20 min
 - e. Centrifuge at 12,000 RPM (SS-34 rotor), 4°C for 30 min
 - f. Keep the supernatant and the pellet
 - i. *Note:* Fab should express periplasmically
 - ii. Western blot the supernatant (periplasm/cytosol) and pellet (inclusion body), dissolved in 0.2% SDS/8 *M* Urea/PBS buffer, with an anti-His (C-term)-HRP antibody to confirm expression location
 - g. Wash Ni-NTA agarose beads with TBS, pH 7.4
 - h. Apply clarified periplasmic fraction to beads and collect flow through
 - i. Wash with 10 column volumes (CV) of TBS+20–25 *mM* Imidazole and collect wash fractions
 - j. Elute with 5 mL TBS+250 *mM* Imidazole—collect individual fractions
 - k. Elute with 5 mL TBS+500 *mM* Imidazole—collect
 - l. Run SDS-PAGE on all fractions, pool relevant fractions, and concentrate using a 10 k MWCO Amicon Ultra 4
 - m. Optional secondary purification step with Protein A agarose:
 - i. Dialyze into TBS, pH 8.0
 - ii. Wash 1 mL Protein A agarose beads with Gentle Ag/Ab Binding Buffer, pH 8.0
 - iii. Batch bind Fab with beads at 4°C for 1–2 h
 - iv. Collect flow through
 - v. Wash with 2 × 10 CV of Gentle Ag/Ab Binding Buffer, pH 8.0—collect wash fractions
 - vi. Elute with 5 × 2.5 mL Gentle Ag/Ab Elution Buffer, pH 6.6—collect elution fractions

- vii. Desalt using disposable PD-10 desalting columns into TBS, pH 7.4
- viii. Run SDS-PAGE, pool relevant fractions, and concentrate using 10 k MWCO Amicon Ultra 4

6.3.2 Protocol 12: Expressing Fab as IgG in HEK293F Cells

1. PCR out VH and VL domains

a. PCR primer sequences:

- i. IgG-VL-For (Bold=BssHII cut site, underlined=annealing site): **CAGGCGCGC**ACTCCGATATCCAGATGACCCAG
- ii. IgG-VL-Rev (Bold = BsiWI cut site, underlined = annealing site): **CACCGTACG**TTTGATCTCCACCTTGGTAC
- iii. IgG-VH-For (Bold = BssHII cut site, underlined = annealing site):
CAGGCGCGCACTCCGAGGTTCAGCTGGTGGAGTC
- iv. IgG-VH-Rev (Bold = NheI cut site, underlined = annealing site): **GGTGCTAG**CCGAGGAGACGGTGACC

b. Setup PCR reactions for VH and VL by adding the following:

	Vol. (μL):
Phusion Master Mix	10
For Primer (diluted to 32 μM in sterile H ₂ O)	1
Rev Primer (diluted to 32 μM in sterile H ₂ O)	1
Phagemid DNA (want 10–30 ng DNA)	1
Sterile H ₂ O	7
Total vol.	20

c. Run the following PCR cycle:

98°C	0.5 min	×30
98°C	0.5 min	
53°C	0.5 min	
72°C	1.0 min	
72°C	10–15 min	
4°C	Indefinitely	

d. PCR purify using QIAquick PCR purification kit following manufacturer's protocol—elute with 30 μL sterile H₂O

- e. Measure concentration
2. Digestion and ligation into pMAZ vectors (Mazor, Barnea, Keydar, & Benhar, 2007)
 - a. The reader is referred to Mazor et al. for a detailed description of the pMAZ vectors. In brief, pMAZ-IgH contains human IgG1 constant domain and pMAZ-IgL contains human kappa constant domain. To clone VH and VL into their respective plasmids, digest both insert and vector with BssHII and BsiWI for VL or with BssHII and NheI for VH.
 - b. Digestion (dilute vector DNA to 100 ng/μL in sterile H₂O):

pMAZ-IgH		VH (Insert)		pMAZ-IgL		VL (Insert)	
15 μL	DNA	23 μL	DNA	15 μL	DNA	23 μL	DNA
5 μL	Buffer2.1	5 μL	Buffer2.1	5 μL	Buffer3.1	5 μL	Buffer 3.1
1 μL	BssHII	1 μL	BssHII	1 μL	BssHII	1 μL	BssHII
1 μL	NheI	1 μL	NheI	1 μL	BsiWI	1 μL	BsiWI
28 μL	H ₂ O	20 μL	H ₂ O	28 μL	H ₂ O	20 μL	H ₂ O
50 μL	Total	50 μL	Total	50 μL	Total	50 μL	Total
Digest for 2.5 h at 37°C							
Add 1 μL CIP to vectors only							
Incubate at 37°C for 0.5 h							
PCR purify VH and VL inserts following Qiagen's QIAquick PCR purification kit—elute with 30 μL sterile H ₂ O							
Run a 1% agarose gel on the vector digestion products and excise the upper band							
Gel extract the DNA following Qiagen's QIAquick Gel Extraction Kit protocol— elute with 30 μL H ₂ O							
Measure the concentrations of digested inserts and vectors							

- c. Ligations:
 - i. Ligate VH into pMAZ-IgH and VL into pMAZ-IgL as described in Protocol 7
- d. Transformations:
 - i. Transform 5 μL ligation products into 25 μL One Shot TOP10 Chemically Competent cells as described in Protocol 7
3. Maxiprep IgH and IgL DNA
 - a. Transform purified DNA into One Shot TOP10 Chemically Competent cells and plate onto LB/Carb
 - b. Pick colony into 500 mL 2xYT/500 μL Carb for IgH and IgL
 - c. Incubate at 37°C, 220 RPM, overnight
 - d. Centrifuge cells at 4000 RPM, 4°C for 15 min

- e. Isolate DNA following manufacturer's protocol using Macherey–Nagel maxiprep kit
 - f. Measure concentrations
 4. Transfection: 600 mL scale; using sterile disposable 2 L baffled flask
 - a. Seed 3×10^7 HEK293F cells in 600 mL FreeStyle™ Media
 - b. To 50 mL sterile PBS add 201 µg IgH, 201 µg IgL DNA, and 1.2 mL PEI at a stock concentration of 1 mg/mL (3:1 ratio of PEI:DNA)
 - c. Vortex 15 s
 - d. Incubate at room temperature 15 min
 - e. Exchange media
 - f. Add PBS/DNA/PEI mixture to cells while swirling flask
 - g. Incubate at 37°C, 120 RPM, 8% CO₂, and 85% humidity for 5 days
 5. Antibody purification
 - a. Centrifuge culture at 4000 RPM, 4°C for 15 min
 - b. pH supernatant to 8.0 with diluted NaOH
 - c. Wash 1 mL of Protein A agarose beads with 10 CV of Gentle Ag/Ab Binding Buffer
 - d. Incubate beads with supernatant for 1–2 h at 4°C with stirring
 - e. Apply to column—collect flow through
 - f. Wash with 2× 10 CV Gentle Ag/Ab Binding Buffer—collect washes
 - g. Elute with 5× 2.5 mL Gentle Ag/Ab Elution Buffer—collect elutes
 - h. Desalt into TBS, pH 7.4 using PD-10 Desalting columns
 - i. Run SDS-PAGE, pool relevant fractions, concentrate using a 30 k MWCO Amicon Ultra 4
 - j. Measure concentration by measuring the absorbance at wavelength 280 nm

6.4 Troubleshooting

1. If expression of the protein of interest on phage results in no appreciable affinity for the target, increase the linker length between the protein of interest and the pIII minor coat protein.
2. dU-ssDNA synthesis:
 - a. Decrease the amount of antibiotics used by approximately half.

- b. If large-scale production of dU-ssDNA results in low yields, try multiple small-scale preps (30 mL and using 1 spin column) and pooling preps together.
 - c. Run 1% agarose gel and verify that dU-ssDNA runs as predominantly 1 band—heat the DNA at 65°C prior to running the gel to reduce secondary structure.
3. Library synthesis:
 - a. Allow synthesis reaction to run overnight at 20°C.
 - b. Add additional ATP (10 mM) to assist ligation reaction.
 - c. Phosphorylate primers enzymatically instead of chemically modifying them—chemical phosphorylation is often incomplete.
 - d. Try concentrating ccc-dsDNA to greater than 100 ng/mL for more efficient electroporation.
 - e. Run 1% agarose gel on CCC-dsDNA next to dU-ssDNA. The majority of the CCC-dsDNA should run at the expected molecular weight, however, higher molecular bands corresponding to strand displaced DNA and nicked DNA may be visible. These higher molecular weight bands transform *E. coli* less efficiently and do not have a high rate of mutation incorporation.
 - f. If there is a low incorporation rate of primers during library synthesis, try different DNA:Primer ratios during synthesis and verify incorporation by sequencing the naïve library.
4. Library selection:
 - a. Change binding and blocking buffers to decrease background phage binding.
 - b. Change concentration of coated target to enrich for specifically bound phage vs nonspecific binding.
 - c. After elution of phage, check for complete elution by blocking the wells and running an ELISA. Make sure to include a blocked well that has no phage added. If needed, increase elution time to 10–30 min.
 - d. If output population of phage does not amplify, check that the output phage titer is greater than 10⁴. If so, streak out a fresh XL1-Blue plate and grow a fresh batch of XL1-Blue cells for infection. Check infectivity of M13KO7 helper phage by infecting XL1-Blue cells and titering.
5. Library screening:
 - a. If 300–400 clones have been screened with no hits observed, check the titer of the phage in the deep-well plate. If titers are low, try growing

phage at higher shaker speed and with a lower concentration of carbenicillin to increase titers.

- b. If high background (binding to negative control) is seen, before adding the phage, add binding buffer to the high-binding plates to decrease background binding.
- c. Check sequences of clones from different rounds of selection to verify that there are diverse clones available, and that frameshift or deletion mutants did not dominate the selection. Sequencing should also be done in parallel with selections to confirm this.

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Fig. 1.

Phage display selection. An initial population of phage, expressing different variants of the protein of interest (indicated by *different colors* (*different gray shades* in the print version)), are selected against the target. The target is first immobilized, the phage library is added with nonbinders washed away, followed by the elution of bound phage. The output phage population can then be amplified by infecting *E. coli* cells. After several rounds of selection, clones demonstrating desirable properties can be sequenced.

Fig. 2.

Phage ELISA at single high-point concentration of phage for Dengue DIII-displaying phage (A) and protein G-displaying phage (B). (A) DIII displayed on phage binds specifically to target antibodies 4E11 and 3H5-1 but not negative control BSA. The N-terminal FLAG-tag can also be probed by M2 binding. (B) Protein G displayed as a fusion to the pIII coat protein also shows specific binding to its target IgG over negative control and displays the N-terminal FLAG-tag as probed by M2 binding.

Fig. 3. Kunkel mutagenesis. Single-stranded DNA with uracil incorporated (dU-ssDNA; *green* (*gray* in the print version) above) is isolated from phage produced in CJ236 cells. Degenerate primers (*red* (*black* in the print version)) are annealed (mutagenesis regions indicated by *asterices*) to the dU-ssDNA. T7 DNA polymerase and T4 DNA ligase and dNTPs are added to synthesize the complementary strand based on the dU-ssDNA template. This covalently closed circular double-stranded DNA (CCC-dsDNA) is then electroporated into *E. coli* SS320 cells, in which the uracil-containing template is deactivated and the new, mutagenic strand is preferentially replicated.

Fig. 4.

Full binding curve phage ELISA. (A) Serial dilutions of DENV-2 DIII-expressing phage were incubated with DIII-binding antibody 4E11 (orange (gray in the print version) circles) or negative control, 1% BSA (green (dark gray in the print version) squares). The absorbance measured at 450 nm is a readout for phage binding. (B) As a negative control, dilutions of DENV-2 DIII-expressing phage were incubated with nonbinding antibody (purple (dark gray in the print version) circles), M2 (blue (dark gray in the print version) triangles) or 1% BSA (green (gray in the print version) squares). No binding to the negative control antibody was observed (cf. panel A), but DIII is expressed on the surface of phage as indicated by the M2 signal.