

## Ph.D.™ Phage Display Peptide Library Kits v2

NEB #E8210S, E8211S, E8111L, E8212S

10 panning experiments

Version 1.0\_3/22

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### Ph.D. Phage Display Peptide Library Kit v2 Components

*Kit components should be stored at -20°C except where noted:*

#### Ph.D. Phage Display Peptide Library

Infectious M13 particles displaying random peptide library on pIII coat protein,  $\sim 1 \times 10^{13}$  pfu/ml. Ph.D.-7, Ph.D.-12 or Ph.D.-C7C (pfu = plaque forming unit i.e., virions), 0.1 ml.

#### *E. coli* K12 ER2738 host strain

*F'* *proA*<sup>+</sup> *B*<sup>+</sup> *lacI*<sup>q</sup>  $\Delta$ (*lacZ*)M15 *zzf::Tn10*(*Tet*<sup>R</sup>)/*fhuA2 glnV*  $\Delta$ (*lac-proAB*) *thi-1*  $\Delta$ (*hsdS-mcrB*)5. Supplied in 50% glycerol, tetracycline resistant. Store short term -20°C. Store long term (> 30 days) at -80°C, 0.2 ml

#### -96 gIII Sequencing Primer

5'-CCCTCATAGTTAGCGTAACG-3', 500 pmol, 10 pmol/μl

#### DYKDDDDK Mouse mAb

control panning experiment target, 15 μl

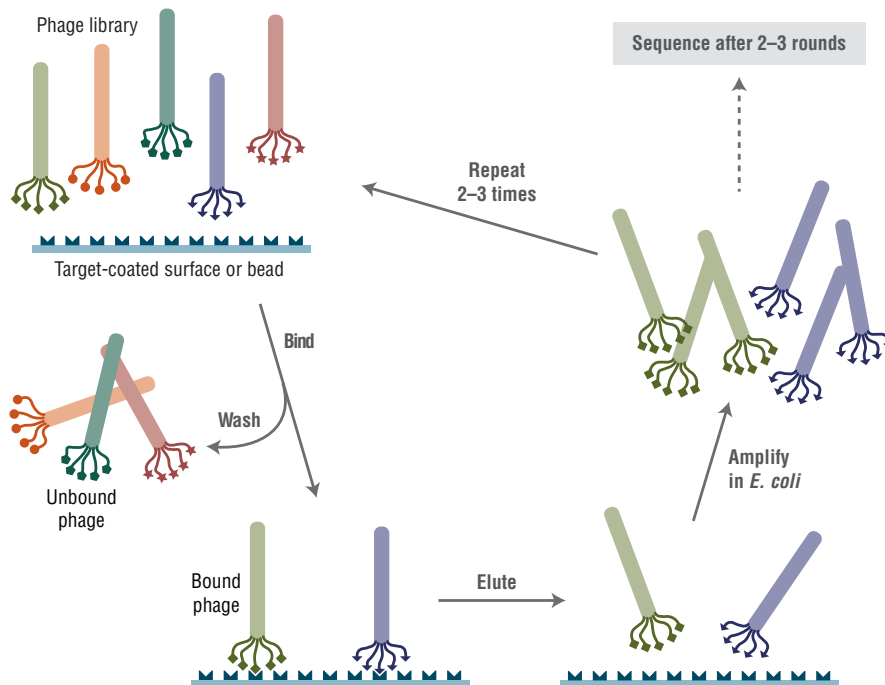
#### Protein G Magnetic Beads

0.15 ml

## Quick Start Guide – (see Figure 1)

1. Streak *E. coli* K12 ER2738 onto LB/Tet solid medium. Grow overnight at 37°C.
2. Select phage-target capture method: Prepare capture surface by coating with target and blocking, or select affinity (magnetic) beads method.
3. Incubate 10  $\mu$ l phage library (or  $10^{11}$  pfu) with target (on surface or free in solution, see Step 2), 10–60 min.
4. Wash unbound phage away with 10 x 1 ml washes of TBST.
5. Release bound phage with low pH buffer or with a known ligand to the target.
6. Amplify the selection's phage eluate in 20 ml *E. coli* culture for 4–5 hr.
7. Concentrate phage from culture supernatant by adding 1/5 volume of 20% PEG/2.5 M NaCl.
8. Titer a) unamplified eluate from Step 5, for future reference, and b) enriched phage pool from Step 7, to determine volume of input for round 2 selection.
9. Repeat steps 3–6, with  $10^{11}$  pfu input of enriched phage pool each time, increasing selection or wash stringency if desired.
10. After three rounds of selection, do not amplify elution. Instead, titer the third round elution to obtain individual plaques for phage sequencing reactions.
11. Post-panning analysis: Identify sequence motifs and/or carry out binding studies with selected clones.

**Figure 1. Panning with a pentavalent peptide library displayed on pIII.**



## Introduction

Phage display describes a selection technique in which a library of peptide or protein variants is expressed on the outside of a phage virion, while the genetic material encoding each variant resides on the inside (1–3). This creates a physical linkage between each variant protein sequence and the DNA encoding it, which allows rapid partitioning based on binding affinity to a given target molecule (antibodies, enzymes, cell-surface receptors, etc.) by an *in vitro* selection process called panning (4). In its simplest form, panning is carried out by incubating a library of phage-displayed peptides on a plate (or bead) coated with the target, washing away the unbound phage, and eluting the specifically bound phage (Figure 1). The eluted phage are then amplified and taken through additional binding/amplification cycles to enrich the pool in favor of binding sequences. After 3–4 rounds, individual clones are characterized by DNA sequencing and binding assays.

The Ph.D. (phage display) system is based on a simple M13 phage vector, modified for pentavalent display of peptides as N-terminal fusions to the minor coat protein pIII (5–8). This protein modulates phage infectivity by binding to the F-pilus of the recipient bacterial cell and is present in 5 copies clustered at one end of the mature M13 virion (2,9). If the displayed peptide is sufficiently short (< 50 residues), the infectivity function of pIII is not affected, and all 5 copies can carry displayed peptides without measurable attenuation of phage infectivity (6). As a result, the M13 genome contains only a single copy of gene III (gIII). This contrasts with phagemid systems, which provide both fused and unfused copies. The reduced valency of pIII libraries compared to pVIII libraries renders the Ph.D. system more suitable for the discovery of higher affinity ligands ( $K_d$  of 10  $\mu$ M or better). New England Biolabs offers 3 pre-made random peptide libraries, as well as the cloning vector M13KE (#E8101) for construction of custom libraries.

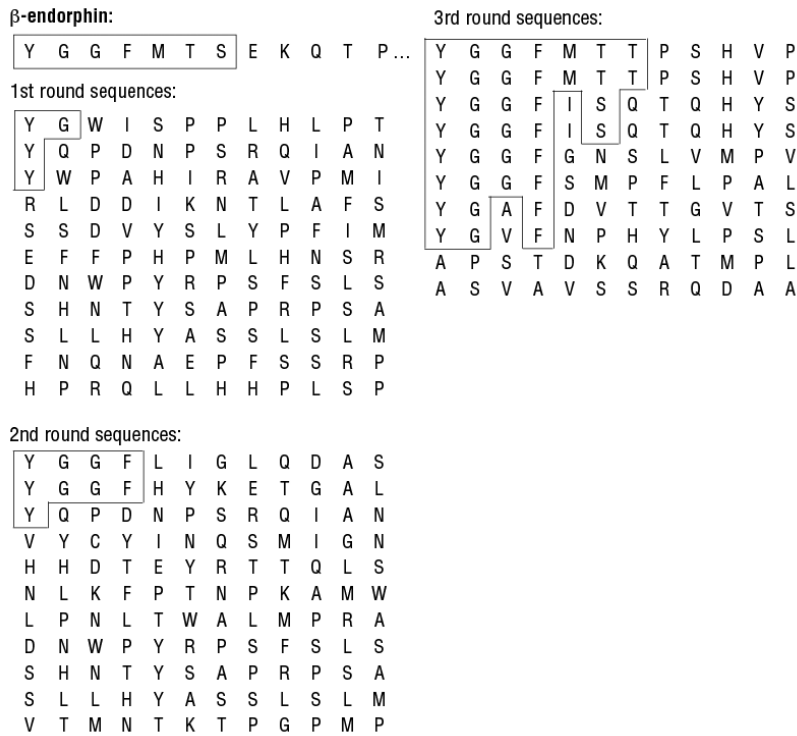
The premade libraries (Figure 2) consist of linear heptapeptide (Ph.D.-7) and dodecapeptide (Ph.D.-12) libraries, as well as a loop-constrained heptapeptide (Ph.D.-C7C) library. The randomized segment of the Ph.D.-C7C library is flanked by a pair of cysteine residues, which are oxidized during phage assembly to a disulfide linkage, resulting in the displayed peptides being presented to the target as loops. The libraries each have complexities on the order of  $10^9$  independent clones, which is sufficient to encode most if not all of the possible 7-mer ( $1.28 \times 10^9$ ) peptide sequences, but only a tiny fraction (less than 1 millionth) of the  $4.1 \times 10^{15}$  possible 12-mer sequences. The Ph.D. 12 library can thus be thought of as having the equivalent diversity of the Ph.D.-7 library but spread out over 12 residues. In both the Ph.D.-7 and the Ph.D.-12 libraries, the first residue at the N-terminus of the peptide-pIII fusion is the first randomized position, while the first randomized position in the Ph.D.-C7C library is preceded by Ala-Cys (Figure 2) at the N-terminus. All the libraries contain a short linker sequence (Gly-Gly-Gly-Ser) between the displayed peptide and pIII coat protein.

**Figure 2. NEB's Ph.D. Phage Display Peptide library forms. Fusions of randomized and linker sequences attached to pIII coat protein. Each phage particle has 5 copies of the pIII coat protein (not shown).**



Experiments at New England Biolabs have identified consensus peptide binding sequences against a variety of proteins including enzymes, cell-surface receptors, and monoclonal antibodies (Figure 3). In all cases the prepared libraries have been demonstrated to be of sufficient complexity to produce multiple DNA sequences encoding the same consensus peptide motifs. The system has been used elsewhere for myriad applications, including epitope mapping (12–16), anti-microbial/viral peptides (17–23), material-specific peptides (24–27), small molecule binders (10, 28–31) and novel enzyme substrates (32). The Ph.D. libraries have been used extensively for discovery of bioactive peptides through *in vitro* and *in vivo* panning approaches: peptide antagonists of VEGF-mediated angiogenesis (33), plasmodesmal trafficking peptides (34) and cell targeting peptides (35–40). In a particularly dramatic application, the Ph.D.-12 library was panned against Taxol, and the selected sequences were compared to a protein database to identify the natural target for the drug as Bcl-2 (10). This demonstrated that short peptides from an unstructured peptide library can mimic a three-dimensional ligand binding site, greatly increasing the potential utility of these libraries. Also along these lines, Ph.D. libraries have been used to map protein-protein interactions (41–47). It is apparent that applications of the Ph.D. kits have been limited only by the imagination of the scientific community. Phage display methods now and in the future will be revolutionized by Next Generation Sequencing (48–50).

**Figure 3. Epitope mapping of an anti- $\beta$ -endorphin monoclonal antibody (3E-7) with the Ph.D.-12 Phage Display Peptide Library.**



*The Ph.D.-12 Phage Display Peptide Library was panned in solution with affinity bead capture. A consensus sequence is enriched over three rounds of selection.*

## Panning Protocols

### Protocol 1. Solution-phase Panning with Affinity Bead Capture

This protocol also serves as the control panning experiment protocol (DYKDDDDK Mouse mAb target with Protein G Magnetic Beads). This protocol is a recommended starting point for any target that can be pulled out of solution with an affinity tag. A straightforward approach is recommended for initial experiments, but for complex or troublesome targets, the current literature for panning protocols in a particular application is invaluable. Refer to Appendix D for discussions on optimizing certain conditions for a given selection.

1. **Day culture.** Inoculate 10 ml of LB+Tet medium with *E. coli* K12 ER2738 for use in titrating (Step 10). Incubate culture at 37°C with vigorous shaking, ~250 RPM, for 4–8 hours. Culture should be turbid. Consider also starting a culture, if using Amplification method A (Step 7, part a) but only grow to OD<sub>600</sub> 0.01–0.05.
2. **Prepare beads.** Transfer 50 µl of a 50% aqueous suspension of affinity beads appropriate for capture of the target to a microfuge tube. Add 1 ml of TBS + 0.1% Tween (TBST). Suspend the beads by tapping the tube or GENTLY vortexing. Do not pipet up and down. Pellet the beads by magnetic capture if using magnetic beads or by centrifugation in a low-speed benchtop microcentrifuge for 30 seconds. Carefully pipette away and discard the supernatant, taking care not to disturb the bead pellet. Repeat bead wash two more times and set aside to use in Step 4. (Note: For some beads, a blocking step may be appropriate. Follow the manufacturer's instructions if blocking is recommended. NEB #S1430 Protein G Magnetic Beads are stored in BSA and do not need to be blocked.)
3. **Selection Step 1 (~2 pmol target + phage library).** Dilute 10 µl of the Ph.D. Phage Display Peptide Library (i.e., 10<sup>11</sup> pfu which is a 100-fold representation of a 10<sup>9</sup> complexity) and 3 µl DYKDDDDK Mouse mAb (NEB #E8004) (if using a different target, 2 pmol or 300–500 ng) to a final volume of 200 µl with TBST. The final target concentration is 10 nM. Incubate for 20 minutes at room temperature with intermittent mixing.
4. **Capture.** Transfer the phage–target mixture to the tube containing the washed beads from Step 2. Incubate for 15 minutes at room temperature, mixing occasionally.
5. **Wash.** Pellet the beads as in Step 2, discard the supernatant, and wash 10 times with 1 ml of TBST, pelleting the beads each time. Discard wash volume.
6. **Elution.** Elute the bound phage by suspending the beads in 1 ml of elution buffer. Intermittently mix for 10–60 min. For the control panning experiment, use Glycine Elution Buffer (see below).

#### Possible Elution Buffers:

- 0.2 M Glycine-HCl, pH 2.2, 1 mg/ml BSA; must be neutralized afterwards by adding 150 µl (15 µl, for microtiter wells) of sterile 1 M Tris-HCl, pH 9
  - A known ligand to the target in TBS (0.1–1 mM)
  - A free target in TBS (~100 µg/ml)
7. **Enrichment (amplification) of selected pool.** Retain a 5-10 µl volume of the eluate, immediately amplify the remaining eluate or store at 4°C for up to 2 weeks. Later rounds (with higher titers) may be stored at -20°C indefinitely with the addition of an equal volume of glycerol. Amplify the phage pool in a 250 ml Erlenmeyer flask (do not use a 50 ml conical tube) and culture by either method below:
    - a. **Amplification Method A:** Inoculate 20 ml *E. coli* K12 ER2738 LB culture with a small mass of cells or single colony. Shake flask at 250 RPM at 37°C for an hour or less to achieve OD<sub>600</sub> 0.01–0.05. The culture will not be turbid. Take care not to over grow. Add phage eluate to the culture within the recommended low OD<sub>600</sub> range, continue incubation with vigorous shaking for 4.5–5 hours.
    - b. **Amplification Method B:** Alternatively, amplify eluate the next day. Inoculate 10 ml of LB + Tet with *E. coli* K12 ER2738 and incubate overnight at 37°C with shaking. The next day, dilute the overnight culture 1:100 in 20 ml of LB and at the same time add the unamplified eluate. Incubate at 37°C with vigorous shaking for 4.5 hours. This approach does not call for monitoring OD<sub>600</sub>.
  8. **Remove cells.** Transfer the culture to a centrifuge tube and spin at 4°C for 10 minutes at 5,000 x g. Transfer the supernatant to a fresh tube and briefly re-spin.
  9. **Precipitate phage.** Pipette 16 ml of the culture supernatant to a fresh tube and add to it 4 ml (or 1/5 volume) of 20% PEG/2.5 M NaCl in a centrifuge tube. Mix gently and allow the phage to precipitate at 4°C for 2 hours or overnight. Spin the PEG precipitation at 12,000 x g for 15 minutes at 4°C (alternatively, 4,000 x g for 45 minutes). A fingerprint sized smear of phage pellet may be visible. Decant the supernatant. (If a phage pellet is not visible, save the supernatant at 4°C; the culture supernatant may be tittered later if troubleshooting of the amplification steps is necessary.) Add 1 ml of TBS to the phage pellet. Mix by gentle pipetting or vortex several times over 5 minutes. Transfer the suspension to a microcentrifuge tube and spin for 1 minute at maximum speed at 4°C to pellet residual cells. Transfer the supernatant to a fresh microcentrifuge tube and reprecipitate with 200 µl of 20% PEG/2.5 M NaCl. Incubate for 15–60 minutes on ice. Solution will rapidly appear cloudy. Microcentrifuge for 10 minutes at 20,000 x g at 4°C. Discard the supernatant, re-spin briefly, and remove residual supernatant with a micropipette. Suspend the pellet in 200 µl of TBS. Microcentrifuge at max speed (~20,000 x g) for 1 minute to pellet any remaining insoluble matter. Transfer the supernatant to a fresh tube. This is the amplified eluate.

10. **Determine pfu/ $\mu$ l of the amplified eluate by titration.** Titer the amplified eluate as described in General M13 Methods, Appendix C. Typically, the titer will be on the order of  $10^{10}$  pfu/ $\mu$ l (or  $10^{13}$  pfu/ml) for an amplified, PEG/NaCl concentrated stock.
11. **Selection round 2.** Using  $10^{11}$  pfu of the amplified eluate from the previous round, perform a second round of selection by repeating Steps 1–7, raising the Tween concentration in the binding and wash steps to 0.5% (v/v). If the amplified eluate titer is low, succeeding rounds of panning can be carried out with as little as  $10^9$  pfu of input phage, if need be.
12. **Additional selection steps.** Perform a third round of selection as above maintaining the Tween concentration at 0.5% (v/v) in the binding and wash steps. Store the eluate at 4°C.
13. Do not immediately amplify the third round eluate. Instead, obtain DNA sequences from 10–20 clones (see Rapid Purification of Sequencing Templates, page 7). Also, consider binding assays with synthetic peptides or whole phage ELISA (See Post Panning Protocols, page 8) to interrogate specificity for the target.

### Protocol 2. Surface-Phase Panning (Direct Target Coating)

The most straightforward method of affinity partitioning (panning) involves directly coating a plastic surface with the target of interest (by nonspecific hydrophobic and electrostatic interaction), washing away the excess, and passing the pool of phage over the target-coated surface (Figure 1). In this method, the target does not need an affinity tag. Depending on the available quantity of target molecule and the number of different targets being panned against simultaneously, panning can be carried out in individual sterile polystyrene petri dishes, 12- or 24-well plates, or 96-well microtiter plates. Coat a minimum of 1 plate (or individual well) per target. It is not productive to do a separate negative control panning experiment without target. Volumes given in the following procedure are for 60 x 15 mm petri dishes, with volumes for microtiter wells given in parentheses. For wells of intermediate size adjust volumes accordingly, but in all cases the number of input phage should remain the same ( $10^{11}$  pfu).

1. **Coating the surface.** Prepare a solution of 10–100  $\mu$ g/ml of the target in 0.1 M NaHCO<sub>3</sub>, pH 8.6. Alternative buffers (containing metal ions etc.) of similarly high ionic strength (e.g., TBS) can be used if necessary for stabilizing the target molecule. Add 1.5 ml of this solution (150  $\mu$ l if using microtiter wells) to each plate (or well) and swirl repeatedly until the surface is completely wet (this may take some effort as the solution may bead up). Incubate overnight at 4°C with gentle agitation in a humidified container (e.g., a sealable plastic box lined with damp paper towels). Store plates at 4°C in humidified container until needed. Plates coated with protein targets can usually be stored for several weeks (depending on target stability); discard if mold is evident on the paper towels.
2. **Day culture.** Inoculate 10 ml of LB + Tet medium with *E. coli* K12 ER2738 as described in Protocol 1 (Solution-phase Panning) Step 1, page 5. Also, consider starting a 20 ml culture for phage amplification (see Step 7 below and Protocol 1, Step 7 a/b).
3. **Block surface and wash.** Pour off the coating solution from each plate and firmly slap it face down onto a clean paper towel to remove residual solution. Fill each plate or well completely with Blocking Buffer. Incubate for at least 1 hour or overnight at 4°C. Then, discard the blocking solution. Wash each plate rapidly 6 times with TBST (TBS + 0.1% [v/v] Tween-20). Coat the bottom and sides of the plate or well by swirling, pour off the solution, and slap the plate face down on a section of dry paper towel each time. (If using a 96-well microtiter plate, an automatic plate washer may be used.) Work quickly to avoid drying out the plate.
4. **Selection Round 1.** Dilute 10  $\mu$ l of the Ph.D. Phage Display Peptide Library (i.e.,  $10^{11}$  pfu, a 100-fold representation of a  $10^9$  complexity) with 1 ml of TBST (100  $\mu$ l, if using microtiter wells). Pipette diluted phage onto coated plate and rock gently for 10–60 minutes at room temperature.
5. **Wash.** Discard nonbinding phage by pouring off liquid and slapping plate face-down onto a clean paper towel. Wash plate with 10 x 1 ml (100  $\mu$ l) TBST.
6. **Elution.** Elute the bound phage by applying 1 ml (100  $\mu$ l, if using microtiter wells) elution buffer to the well or plate. Rock the elution mixture gently for 10–60 minutes at room temperature. Pipette eluate into a microcentrifuge tube.

#### Possible Elution Buffers:

- 0.2 M Glycine-HCl, pH 2.2, 1 mg/ml BSA (must be neutralized afterwards, add 150  $\mu$ l (15  $\mu$ l, for microtiter wells) of sterile 1 M Tris-HCl, pH 9)
  - A known ligand to the target in TBS (0.1–1 mM)
  - A solution of the free target (~100  $\mu$ g/ml in TBS)
7. **Enrichment of selected pool.** Go to Protocol 1: Solution Phase Panning Step 7. First choose either Amplification Method A or B. Proceed with amplification and subsequent steps above, 1–6, using the target treated surface.

## Post-Panning Protocols

### Rapid Purification of Single-Stranded DNA Templates for Sequencing Reactions

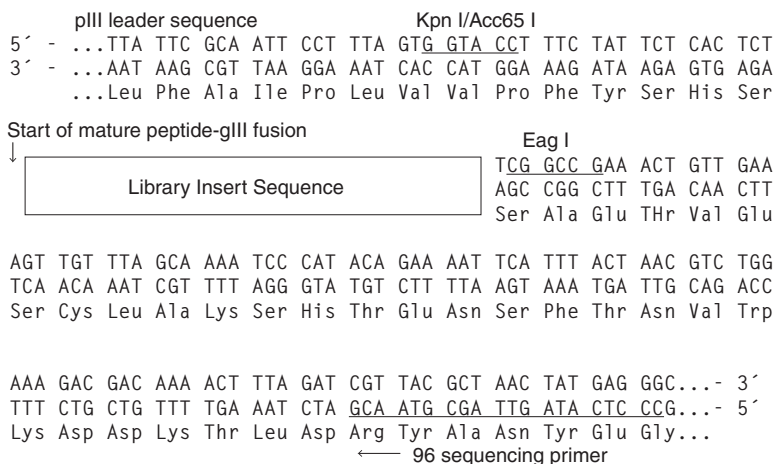
Our preferred, extremely rapid procedure (51) produces template of sufficient purity for manual or automated dideoxy sequencing, without the use of phenol or chromatography. Alternatives would be to purify double-stranded DNA from infected cell pellets by standard mini prep or use PCR to make DNA sequencing templates.

1. **Amplify phage from individual plaques.** Titer phage stock of interest to obtain plaques (Appendix C, General M13 Methods-titering). Use plates that are not older than 3 days, have less than 100 plaques and have been stored at 4°C, if not fresh. The next day, dilute an overnight culture of *E. coli* K12 ER2738 1:100 in LB. Dispense 1 ml of diluted culture into culture tubes, one for each clone to be sequenced. Use a sterile wooden stick or pipette tip to stab, i.e., pickup, a blue plaque and transfer phage particles to media in culture tube. Pick well-separated plaques. This will ensure that each plaque contains a single DNA sequence. Shake cultures at 37°C at 250 rpm for 4.5–5 hours (no longer).
2. **Precipitate phage.** Transfer cultures to microcentrifuge tubes, and microfuge at max speed (~20,000 x g) for 30 seconds. Transfer 500 µl of the phage-containing supernatant to a fresh microfuge tube. Add 200 µl of 20% PEG/2.5 M NaCl. Invert several times to mix and let stand for 10–20 minutes at room temperature. *Note: Supernatant from phage infected cultures may be stored at 4°C for several weeks or, with the addition of an equal volume of glycerol, at -20°C for several years. Phage with added PEG/NaCl may be stored for 1 week at 4°C.*
3. **Precipitate nucleic acids.** Microfuge at max speed (~20,000 x g) for 10 minutes and discard the supernatant. The phage pellet may not be visible. Suspend the pellet thoroughly in 100 µl of Iodide Buffer by vigorously tapping the tube. Add 250 µl of ethanol and incubate 10–20 minutes at room temperature. This short incubation at room temperature will preferentially precipitate single-stranded phage DNA, leaving most phage protein in solution. Spin in a microfuge at max speed for 10 minutes at 4°C and discard the supernatant. Wash the pellet with 0.5 ml of 70% ethanol (stored at -20°C), re-spin, discard the supernatant, and briefly dry the pellet under vacuum or for several hours on the bench top. Suspend the pellet in 30 µl of TE buffer; store at 4°C or -20°C.
4. Quantify product by 1% TBE agarose gel electrophoresis. 5 µl of resuspended template should give a band of comparable intensity to 0.5 µg purified single-stranded M13mp18 DNA (NEB #N4040). Follow the commercial sequencing facility's instructions to submit reactions for sequencing; typically 0.5 µg DNA template and 1 pmol of primer are required.

### Processing Sequencing Data and Notes

The raw sequence data generated with the -96gIII sequencing primer corresponds to the anticodon strand of the gIII region of the template. Obtain the reverse complement of the data and compare the result against the top strand of the insert sequence shown in Figure 4. Due to the design of the Ph.D. Phage Display Peptide Library oligonucleotides the third nucleotide of each codon in the randomized region should be either G or T. *Note: TAG stop codons are suppressed by glutamine in ER2738 (glnV). If the library was amplified in this strain or any glnV (also known as supE) strain, TAG should be considered a glutamine codon when translating.* Keep in mind that point mutations in a clone's genome may present translations that are not exactly like the expected formats. Insertless and multiple insert clones may be observed. Sequences with stop codons in the middle are mis-translated or not accurately sequenced.

**Figure 4.**



*The hybridization position of the -96 sequencing primer is indicated. To process sequencing data, take the reverse complement of the raw data and the search for GGTACC and CGGCCG sites to locate the library insert.*



## Use of Synthetic Peptides in Binding Studies

For more detailed binding or inhibition studies, it may be necessary to synthesize selected sequences as free, soluble peptides. This allows precise control of peptide concentration, without the avidity artifacts associated with pentavalent display on phage. Additionally, without the phage attached, the peptide can be used at much higher concentrations and can be used *in vivo*. When designing a synthetic peptide corresponding to a selected binding sequence, it is important to realize that, while the N-terminus of the displayed sequence on phage is free and fully protonated in the solvent, the C-terminus is embedded in the phage particle. Hence, the C-terminal residue of the selected sequence does NOT have a free negatively charged carboxylate during panning, so a simple synthetic peptide with a free carboxy terminus will introduce a negatively charged group at a position occupied by a neutral peptide bond during panning. Depending on the nature of the target-ligand interaction, this negative charge can completely abolish binding. It is, therefore, recommended that the C-terminal carboxylate of the synthetic peptide be amidated to block the negative charge. Additionally, if the library insert was designed to include a peptide spacer between the random sequence and pIII (as the Ph.D. libraries are designed with Gly-Gly-Gly-Ser spacer), this spacer sequence should be added to the C-terminus of the synthetic peptide. For chemical conjugation of the peptide to a reporter enzyme or a solid support, the peptide can be designed with a C-terminal cysteine (if there are no other cysteines present in the sequence). The resulting peptide thiol can be easily coupled to maleimide-activated HRP, alkaline phosphatase or agarose beads.

## Plaque Amplification for ELISA Samples

Selected phage clones can be identified by DNA sequencing, and target specificity can be confirmed by phage ELISA. The number of phage particles in an ELISA should be maximized. First, 20 ml phage amplifications are carried out to obtain at least  $10^{12}$  pfu for each clone or pool to be used in ELISA.

1. **Phage Amplifications.** Dilute an overnight culture of *E. coli* K12 ER2738 1:100 in LB to a total volume of 20 ml in a 250 ml Erlenmeyer flask, one for each clone to be tested. A clone selected at random from a titer plate of Ph.D. Phage Display Peptide Library itself can be a negative control. Alternatively, instead of analyzing individual clones, a characterization of an entire pool of selected phage can be examined for binding activity. To detect signal, it is generally necessary to amplify the third round of eluted phage, as unamplified titers are typically no more than  $10^7$  pfu/ml. To amplify a pool of phage, add 10  $\mu$ l of the unamplified eluate to 20 ml of diluted overnight culture.
2. **Amplification of Individual Clones: pick plaques.** Use a sterile wooden stick or pipette tip to stab a blue plaque from a titering plate (note: plates should be no more than 3 days old, stored at 4°C and have < 100 plaques) and transfer to a flask containing the diluted *E. coli* K12 ER2738 culture. Pick well-separated plaques. (Note: instead of single plaques, add 5–10  $\mu$ l of a concentrated phage to obtain more of that stock.) Follow with phage amplification cell harvest and PEG/NaCl precipitation (Go to Protocol 1: Solution Phase Panning Step 7).
3. **Resuspension.** Resuspend final stocks in 50  $\mu$ l instead of 200  $\mu$ l. Concentrated phage stocks may be stored at 4°C for several weeks with little loss of titer. For long-term storage (up to several years), dilute 1:1 with sterile glycerol and store at -20°C. Titer the stocks for ELISA (Appendix C, General M13 Methods). The titer should be approximately  $10^{14}$  pfu/ml.

## Phage ELISA Binding Assay with Direct Target Coating

It is useful to include a phage ELISA in any panning experiment since artifacts of the panning process cannot always be anticipated or prevented. The following ELISA protocol is sufficient for rapidly determining whether a selected phage clone binds the target, without the need for an antibody specific for the target. In this procedure a microtiter plate is coated with the target at high density, and each purified phage clone is applied to the plate at various dilutions. Bound phage is then detected with an anti-M13 antibody (Thermo Fisher Scientific, PA1-26758 or MA1-34468). The amount of target coated on the plate is not quantifiable but is present at sufficiently high density to allow multivalent binding to the phage. This method will not determine whether the selected phage binds with high or low affinity. The method is useful for qualitative determination of relative binding affinities for a number of selected clones in parallel and will distinguish true target binding from binding to the plastic support. **Prepare stocks of phage to assay as described in the section above. You will have 50  $\mu$ l of  $\sim 10^{13-14}$  pfu/ml stocks for each.**

1. Coat one row of ELISA plate wells for each clone or pool to be characterized with 100–200  $\mu$ l of 100  $\mu$ g/ml of target in 0.1 M NaHCO<sub>3</sub>, pH 8.6. Incubate overnight at 4°C in an air-tight humidified box (e.g., a sealable plastic box lined with damp paper towels).
2. Shake out excess target solution and slap plate face-down onto a paper towel. Fill each well completely with blocking buffer. Additionally, one row of uncoated wells per clone to be characterized should also be blocked to test for binding of each selected sequence to BSA-coated plastic (*this test for background signal is extremely important, especially if panning was carried out on a polystyrene surface*). A second, fully uncoated microtiter plate should be blocked for use in serial dilutions of phage (Step 5) before addition to the target-coated plate. Dilutions are done in a separate blocked plate to ensure that phage are not absorbed onto the target while performing dilutions, which would result in a sudden “falling-off” of signal as the phage is diluted. Incubate the plates filled with blocking buffer for 1–2 hours at 4°C.



3. Shake out the blocking buffer and wash each plate 6 times with TBST, slapping the plate face-down onto a clean section of paper towel each time. The percentage of Tween should be the same as the concentration used in the panning wash steps.
4. In the separate blocked plate, carry out four-fold serial dilutions of the phage in 200  $\mu$ l of TBST per well, starting with  $10^{12}$  virions in the first well of a row and ending with  $2 \times 10^5$  virions in the 12<sup>th</sup> well.
5. Using a multichannel pipettor, transfer 100  $\mu$ l from each row of diluted phage to a row of target-coated wells, and transfer 100  $\mu$ l to a row without target. Incubate at room temperature for 1–2 hours with agitation.
6. Wash plate 6 times with TBST as in Step 4.
7. Follow manufacturer instructions for antibody application and development.

## Appendix A

### Media and Solutions

#### LB Medium

Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl. Autoclave, store at room temperature.

#### IPTG/Xgal Stock:

Mix 1.25 g IPTG (isopropyl-b-D-thiogalactoside) and 1 g Xgal (5-Bromo-4-chloro-3-indolyl-b-D-galactoside) in 25 ml DMF (dimethyl formamide). Store at -20°C.

#### LB/IPTG/Xgal Plates:

1 liter LB medium + 15 g/l agar. Autoclave, cool to < 70°C, add 1 ml IPTG/Xgal Stock per liter and pour. Store plates at 4°C in the dark.

#### Top Agar:

Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 7 g Bacto-Agar (or electrophoresis grade agarose). Autoclave, dispense into 50 ml aliquots. Store solid at room temperature, melt in microwave as needed.

#### Tetracycline Stock (suspension):

20 mg/ml in 1:1 Ethanol:Water. Store at -20°C. Vortex before using.

#### LB+Tet Plates:

LB medium + 15 g/l Agar. Autoclave, cool to < 70°C, add 1 ml Tetracycline Stock and pour. Store plates at 4°C in the dark. Do not use plates if brown or black.

#### Blocking Buffer:

0.1 M NaHCO<sub>3</sub> (pH 8.6), 5 mg/ml BSA, 0.02% NaN<sub>3</sub> (optional). Filter sterilize, store at 4°C.

#### TBS(T)

50 mM Tris-HCl (pH 7.5), 150 mM NaCl. Autoclave, store at room temperature. TBST, 0.1% or 0.5 % Tween-20 as noted in protocol.

#### PEG/NaCl:

20% (w/v) polyethylene glycol-8000, 2.5 M NaCl. Autoclave, mix well to combine separated layers while still warm. Store at room temperature.

#### Iodide Buffer:

10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M sodium iodide (NaI). Store at room temperature in the dark. Discard if color is evident.

## Appendix B

### Ph.D. Phage Display Peptide Library Complexity and Choosing a Library for an Application

The Ph.D. Peptide Display Cloning System (NEB #E8101) manual describes the construction methods of NEB's Ph.D. libraries and may be ordered to build custom libraries. Library **complexity** refers to the number of unique clones in the library. Generally, the more complex or diverse the library the more powerful a tool it is. The complexity of Ph.D. Phage Display Peptide Libraries is estimated by titration of representative post-electroporation outgrowths. For example, if there are 160 pfu on the plate made with 10  $\mu$ l of a  $10^3$  dilution of the outgrowth and this represents 1 electroporation out of 100 total, then the library complexity is  $1.76 \times 10^9$  pfu:

$$(160 \text{ pfu}/10 \mu\text{l}) \times (10^3 \text{ dilution}) \times (1100 \mu\text{l outgrowth}) \times (100 \text{ electroporations}) = 1.76 \times 10^9 \text{ pfu}$$

After the outgrowths period, the library is amplified in *E. coli* culture for five hours to enrich the library with many copies of each clone. Historically, a 100-fold representation of a peptide library (or approximately 100 copies of each unique clone) is the recommended input for a selection. The recommended initial input for library selection using a Ph.D. Phage Display Library is  $10^{11}$  pfu.

Extensive sequencing of naïve libraries prepared in this vector system have revealed little sequence bias apart from selection against unpaired cysteine residues (unpublished observations) and the expected reduced levels of arginine (but not lysine) residues. The reduced arginine levels are likely caused by the secY-dependent secretion of pIII (11).

**The choice of library to be used in a particular experiment is dependent upon many factors and is not typically application dependent.** Unfortunately, in the absence of detailed structural information about the target-ligand interaction, it is impossible to predict in advance which type of library will yield the most productive ligands. The Ph.D. 7 library consists of randomized linear 7-mer peptides and may be most useful for targets requiring binding elements concentrated in a short stretch of amino acids. The Ph.D.-12 library consists of randomized linear 12-mer peptides, but with a diversity equivalent to the Ph.D.-7 library. It may be useful for targets requiring 7 or fewer defined residues for binding, but which cannot be contained within the 7-residue “window” of the Ph.D.-7 library. For example, the motif ASDXXXTXPY has only six defined positions, but cannot be present in the Ph.D.-7 library. Additionally, 12-mers are long enough to fold into short structural elements, which may be useful when panning against targets that require structured ligands. A caveat is that the increased length of the randomized segment may allow your target to select sequences with multiple weak binding contacts, instead of a few strong contacts. Note, that lengthier random peptides will not necessarily reveal lengthier binding sequences since only 3–5 residues are involved in most peptide binding interactions.

We recommend using one of the linear libraries first. If a looped structure is a desired characteristic for a downstream step, the structurally constrained Ph.D.-C7C library may be used. Such libraries are especially useful for targets whose native ligands are in the context of a surface loop, such as antibodies with structural epitopes. Additionally, imposing structural constraints on the unbound ligand may result in a less unfavorable binding entropy, improving the overall free energy of binding compared to unconstrained ligands (53). A major disadvantage of structurally constrained libraries is that the constraint may “freeze out” a conformation required for target binding, preventing binding outright rather than improving affinity (54).

## Appendix C

### General M13 Methods and Notes

It is important to note that unlike phage lambda, M13 is not a lytic phage. Plaques are caused by diminished cell growth rather than cell lysis and are turbid rather than clear. Plating on Xgal/IPTG media is strongly recommended to facilitate visualization of plaques.

### Strain Maintenance

1. The recommended host strain *E. coli* K12 ER2738 (*F'* *proA*<sup>+</sup>*B*<sup>+</sup> *lacI*<sup>q</sup>  $\Delta$ (*lacZ*) *M15* *zzf::Tn10(Tet<sup>R</sup>)/fhuA2* *glnV*  $\Delta$ (*lac-proAB*) *thi-1*  $\Delta$ (*hds-mcrB*)5 is particularly well-suited for M13 propagation. *E. coli* K12 ER2738 is a *recA*<sup>+</sup> strain, but we have never observed spontaneous *in vivo* recombination events with M13 or phagemid vectors. Commercially available *F*<sup>+</sup> strains such as DH5 $\alpha$ *F'* and XL1-Blue can probably be substituted for *E. coli* K12 ER2738 but have not been tested with our vector system. Any strain used should be *supE* (*GlnV*) to suppress amber (UAG) stop codons within the library with glutamine.
2. Because M13 is a male-specific coliphage, it is recommended that all cultures for M13 propagation be inoculated from colonies grown on media selective for presence of the F-factor, rather than directly from the glycerol culture. The F-factor of ER2738 contains a mini-transposon which confers tetracycline resistance, so cells harboring the F-factor can be selected by plating and propagating in tetracycline-containing medium. Tetracycline does not need to be added to media during phage amplification but should be used on all solid media growth and overnight liquid cultures. Streak out *E. coli* K12 ER2738 from the supplied glycerol culture onto an LB + Tet plate. Invert and incubate at 37°C overnight and store wrapped with parafilm at 4°C in the dark for a maximum of 1 month. We recommend that fresh glycerol stocks of *E. coli* K12 ER2738 be prepared from liquid cultures and archived at -80 C for extended Ph.D. Library projects.
3. Serial dilution of *E. coli* K12 ER2738 cultures for infection will give erratic results. If titer or amplification results are inconsistent, first streak a fresh *E. coli* K12 ER2738 /LB + Tet plate.

### Avoiding Phage Contamination

The library cloning vector M13KE differs from wild-type filamentous phage vector in that the *lacZ* $\alpha$ -peptide cloning sequence (which permits blue/white screening) has been inserted in the vicinity of the (+) strand origin of replication, resulting in a longer replication cycle. In addition, display of foreign peptides as N-terminal fusions to pIII (which mediates infectivity by binding to the F-pilus of the recipient bacterium) may attenuate infectivity of the library phage relative to wild-type M13. As a result, there is the possibility of *in vivo* selection for any contaminating wild-type phage during the amplification steps between rounds of panning. In the absence of a correspondingly strong *in vitro* binding selection, even vanishingly small levels of contamination can result in a majority of the phage pool being wild-type phage after three rounds of panning.

1. The potential for contamination with environmental bacteriophage can be minimized by always using aerosol-resistant pipette tips and wearing gloves for all protocols.

2. Because the library cloning vector M13KE is derived from the common cloning vector M13mp19, which carries the lacZ $\alpha$  gene phage plaques appear blue when plated on media containing Xgal and IPTG. Environmental filamentous phage will typically yield colorless plaques when plated on the same media. These plaques are also larger and “fuzzier” than the library phage plaques. We strongly recommend plating on LB/IPTG/Xgal plates for all titering steps and, if white plaques are evident, pick ONLY blue plaques for sequencing. Note, many times the presence of some white or clear plaques is unavoidable.
3. Severe contamination (white plaques present in large numbers) can lead to contamination of subsequent panning experiments. To prevent this, all solutions should be re-autoclaved if possible; any solutions containing heat-labile components should be remade. The work area including incubators should be wiped down with ethanol. Pipettors should be disassembled and the parts soaked in detergent, rinsed carefully with sterile water, and reassembled.

### Phage Titering

The number of plaques will increase linearly with added phage only when the multiplicity of infection (MOI) is much less than 1 (i.e., *E. coli* cells are in considerable excess). For this reason, it is recommended that phage stocks be titered by diluting prior to infection, rather than by diluting cells infected at a high MOI. Plating at low MOI will also ensure that each plaque contains only one DNA sequence.

1. **Day culture.** Inoculate 5–10 ml of LB with *E. coli* K12 ER2738 from a plate and incubate with shaking 4–8 hrs until mid-log phase, OD<sub>600</sub> ~ 0.5.
2. **Prepare top agar and LB/IPTG/Xgal plates.** While cells are growing, melt Top Agar in microwave and dispense 3 ml into sterile culture tubes, one per expected phage dilution. Maintain tubes at 45–50°C. Pre-warm, for at least 30 minutes, one LB/IPTG/Xgal plate per expected dilution at 37°C until ready for use.
3. **Phage Dilutions.** Prepare 10 to 10<sup>3</sup>-fold serial dilutions of phage in LB; 1 ml final volumes are convenient. Suggested dilution ranges: for amplified-PEG/NaCl concentrated phage culture supernatants, 10<sup>8</sup>–10<sup>11</sup>; for unamplified panning eluates, 10<sup>1</sup>–10<sup>4</sup>. Use aerosol-resistant pipette tips to prevent cross-contamination and use a fresh pipette tip for each dilution.
4. **Pre-plating Infections.** When the culture in Step 1 is turbid, dispense 200  $\mu$ l into microfuge tubes, one for each phage dilution. To carry out infection, add 10  $\mu$ l of each phage dilution to each tube of cells, vortex quickly, and incubate at room temperature for 1–5 minutes.
5. **Plating.** Transfer the infected cells one infection at a time to culture tubes containing warm Top Agar. Vortex briefly and IMMEDIATELY pour culture onto a pre-warmed LB/IPTG/Xgal plate while maintaining sterile conditions. Gently tilt and rotate plate to spread top agar evenly. Allow the plates to cool for 5 minutes, invert, and incubate overnight at 37°C.
6. **Count plaques the next day.** Count blue plaques on plates. Multiply each number by the dilution factor for that plate to get phage titer in plaque forming units (pfu) per 10  $\mu$ l. For example, if there are 91 pfu on a 10<sup>9</sup> dilution plate, the titer result would be 9.1 x 10<sup>9</sup> pfu/ $\mu$ l for the stock.

### Storing of M13 Phage Solutions

Panning experiments may be interrupted at several points in the protocol. Phage in suspension with NaCl/PEG may be stored for several weeks at 4°C. Eluted phage in neutralized buffer may be stored at 4°C for up to 1 week. Amplified phage may be stored in neutral buffer for up to 3 weeks with, either the addition of 0.02% NaN<sub>3</sub> or incubation at 65°C for 15 min to kill residual *E. coli*. Amplified phage may be stored long term, 5 years or more, by adding an equal volume of sterile glycerol, vortexing and placing at -20°C. It is not necessary to store phage at temperatures below -20°C, however, if required, single use aliquots of phage stocks may be flash frozen and thawed once without significant loss of titer. This follows for either glycerol-free -20°C storage or -70/-80°C storage with or without glycerol.

## Appendix D

### Optimizing Peptide Binding Interactions/Panning Interactions

There are several variables affecting the stringency of selection during panning. Depending on the interaction being studied, adjustment of the stringency of selection or elution may be necessary to obtain a consensus binding sequence.

1. **A negative selection step can be added if there are concerns about non-specific binding to a resin, cell surface or cell lysate.** Do not carry out a negative selection prior to the first round of selection. To remove phage that non-specifically bind to a resin: Prepare an additional 50  $\mu$ l of washed, blocked resin as in Step 2 of Protocol 1, page 5. Pellet the resin and wash 4 times with TBST. Dilute the phage input for round 2 (or later) in 200  $\mu$ l of TBST. Add the diluted phage to the washed, blocked resin and incubate for 15 minutes at room temperature with occasional mixing. Spin out the resin (or capture magnetically) and transfer the phage-containing supernatant to a fresh microfuge tube and proceed with the selection
2. **Detergent:** The presence of detergent (typically Tween 20) in the binding and wash buffers reduces non-specific hydrophobic interactions between the phage and the target and/or blocking agent (BSA), which lead to higher levels of background binding. Lower Tween concentrations in early rounds will result in higher eluate titers, and the stringency can be gradually increased with

each round by raising the Tween concentration stepwise to a maximum of 0.5%. In side-by-side experiments; however, we have obtained identical consensus sequences when Tween concentrations were held constant (0.5%) or increased stepwise (0.1, 0.3, 0.5%) in 3 rounds of panning. The use of lower Tween concentrations in earlier rounds is recommended when the interaction under study is so specific that the eluate titer (i.e., the number of bound sequences) in early rounds is expected to be very low.

3. **Salt:** Hydrophobic interactions are favored at high ionic strength, while ionic interactions are favored at low ionic strength. Nonspecific ionic interactions between surface charged groups on the target and phage, which would lead to high background binding, are thus avoided by the use of a high salt buffer such as TBS in the binding steps. Since it is impossible to predict in advance, whether the peptide-target interaction will be largely ionic or hydrophobic in nature, it may be necessary to test for the optimum salt concentration of the binding buffer.
4. **Target Concentration:** If panning against the target in solution, the stringency can be increased by lowering the concentration of target (54). An initial target concentration of 10 nM is recommended; this can be lowered to 1 nM in later rounds for selection of ligands with high binding affinity. If no consensus sequence is evident, it is possible that the best sequences in the library bind with very low affinity. Even with the avidity effects associated with multivalent display, target concentrations in the nanomolar range may simply be too low to see any binding. In this case it may be necessary to raise the target concentration to 1–2  $\mu\text{M}$  in all rounds of panning (32,55,56). Also, low affinity binders present in low numbers in the initial unpanned library might be lost in the first round of solution-phase panning, regardless of target concentration. In this case it may be helpful to carry out the first round by panning against target which has been immobilized by direct coating (Surface Panning, page 6), and then carrying out subsequent rounds in the solution phase.
5. **Number of Rounds:** With each round of panning and amplification, the pool of phage becomes enriched in favor of sequences that bind to the target. Maintaining a constant input phage concentration in each round results in a stepwise increase in the number of particles displaying a given sequence until a point is reached where most or all the eluted particles display a consensus binding sequence. Depending on the interaction being studied and the applied stringency, this usually happens after 2 or 3 rounds. If no clear consensus sequence emerges after 3 rounds, the 3rd round eluate can be amplified and a fourth round of panning carried out.
6. **Solution phase panning capture method.** As a general alternative to panning against a target that has been immobilized on a surface, the library can be reacted with the target in solution, followed by affinity capture of the target–phage complexes onto an affinity matrix (bead) specific for the target protein. For example, if the target protein has a GST, MBP or polyhistidine affinity tag, the target-phage complexes can be captured on glutathione, amylose or chelated nickel beads, respectively. If the target is an antibody, Protein A and/or Protein G beads can be used for capture. In addition to requiring substantially less target per experiment than surface panning, solution panning can result in improved accessibility of the putative ligand binding site to phage-displayed peptides, as well as avoiding partial denaturation of the target on a plastic surface. Fortuitous selection of peptide sequences that specifically bind the bead can be avoided by employing a **negative selection** beginning with Round 2, in which the amplified phage is pre-incubated with the bead in the absence of target. The supernatant from this step is then reacted with the target in a positive selection.

## Appendix E

### Phage Display Troubleshooting Guide

PROBLEM AREA	PROBLEM	CAUSE	SOLUTION
<b>Titering M13</b>	No plaques	F' of <i>E. coli</i> K12 ER2738 lost	<ul style="list-style-type: none"> <li>Re-streak <i>E. coli</i> K12 ER2738 LB/Tet plate</li> <li>Check Tet stock</li> </ul>
		Poor bacterial lawn growth	<ul style="list-style-type: none"> <li>Plate incubator must be 37°C</li> <li>Agarose top must be ≤ 54°C</li> </ul>
	Inconsistent titer results and/or blue tinged (confluent) plates	Phage solutions not dilute enough	<ul style="list-style-type: none"> <li>Use 10<sup>9</sup>–10<sup>11</sup> dilutions for amplified stocks</li> <li>Elution titers will vary widely</li> </ul>
		Cross-contamination during dilution steps or plating	<ul style="list-style-type: none"> <li>Use aerosol barrier pipet tips, change at each step</li> </ul>
	<i>E. coli</i> K12 ER2738 liquid culture or plate contaminated with phage	<ul style="list-style-type: none"> <li>Re-streak <i>E. coli</i> K12 ER2738 on LB/Tet plate</li> <li>Include bacteria alone negative control-plate for titer</li> </ul>	
Smear blue coloring on plates	Excess moisture in agarose top or IPTG/Xgal plates	<ul style="list-style-type: none"> <li>Allow plates to sit at RT or 37°C for 4–8 hrs to dry</li> <li>Difficult to eliminate totally</li> </ul>	
<b>Amplification</b>	No phage pellet (failed or low yield amplification)	<i>E. coli</i> have lost F'	<ul style="list-style-type: none"> <li>Re-streak <i>E. coli</i> K12 ER2738 LB/Tet plate</li> <li>Check Tet stock</li> </ul>
		Cells overgrown prior to addition of phage	<ul style="list-style-type: none"> <li>Use 1:100 dilution of overnight culture or add phage at 0.01–0.05 OD<sub>600</sub> of same day culture</li> </ul>
		Failed PEG/NaCl precipitation	<ul style="list-style-type: none"> <li>Titer supernatant to check</li> <li>Use PEG-8000, PEG/NaCl stock should be homogeneous</li> </ul>
		Poor culture conditions	<ul style="list-style-type: none"> <li>Use ≤ 5 g NaCl per L LB media</li> <li>Aeration must be high (e.g., 250 ml flask for 20 ml culture)</li> </ul>
	Clear plaques predominate after amplifying elution pool	Environmental contamination from contaminated media, buffers, target or work area	<ul style="list-style-type: none"> <li>Re-make solutions and autoclave</li> <li>Wipe down work area with dilute ethanol or bleach</li> <li>Check <i>E. coli</i> K12 ER2738 plate for contamination</li> </ul>
		Too many rounds of selection (> 4–5)	<ul style="list-style-type: none"> <li>Modify panning protocol to make binding conditions more stringent</li> <li>Refine elution method</li> </ul>
<b>AFTER SELECTIONS</b>			
<b>Sequencing phage clones</b>	High A <sub>280</sub> /A <sub>260</sub> for purified ssDNA	Protein contamination, A <sub>280</sub> /A <sub>260</sub> of 3–8 is typical from NaI ssDNA protocol	<ul style="list-style-type: none"> <li>Sequencing reactions should still work</li> <li>For cleaner templates include PCI extractions or use miniprep kit for ss or dsDNA</li> </ul>
	Poor or no sequencing data	Residual NaI interfering with reactions	<ul style="list-style-type: none"> <li>Try diluting templates 3-fold</li> </ul>
		If only in random region it is a mixture of templates	<ul style="list-style-type: none"> <li>Pick only well-spaced plaques from plates 1–2 days old maximum</li> </ul>
	Multiple bands on agarose gel, unexpected sizes	Single- and double- stranded DNA mixture, ssDNA will not migrate with dsDNA of same size	<ul style="list-style-type: none"> <li>Multiple bands is typical -use M13mp18 (NEB #N4040) as control</li> <li>It is difficult to separate completely ss from ds phage DNA; and not necessary for this application</li> </ul>
	My sequencing facility requires more template or primer than in NEB's protocol		<ul style="list-style-type: none"> <li>We recommend following instructions from the facility. However, we typically use 1 pmol primer and 0.5–1 µg ss template per reaction.</li> </ul>

AFTER SELECTIONS (continued)			
<b>Sequencing phage clones (continued)</b>	I cannot find random region (displayed peptide codons) in my data	Did not look at reverse compliment of raw data from -96 gIII Sequencing Primer	<ul style="list-style-type: none"> <li>Take reverse compliment, search for KpnI and EagI sites. Compare what is in between these with Fig 4 in manual</li> </ul>
		Clone has no insert or multiple inserts	<ul style="list-style-type: none"> <li>Usually these are nonspecific binders; phage ELISA will confirm</li> </ul>
	I am out of sequencing primer solution that came in my kit	Kits provide enough primer to sequence ~100 clones	<ul style="list-style-type: none"> <li>NEB does not sell primers separately</li> <li>Order oligo from your preferred supplier (-96 gIII Sequencing Primer: 5'-CCCTCATAGTTAGCGTAACG)</li> </ul>
<b>Post-panning analysis</b>	No consensus sequence after 3 rounds	Conformational motif, single clones with specificity	<ul style="list-style-type: none"> <li>Carryout phage ELISA or other binding assay</li> <li>Carryout 4<sup>th</sup> round of selection</li> </ul>
	No ELISA signal despite consensus sequence	Selected clones are weak binders	<ul style="list-style-type: none"> <li>Phage ELISA binding is not sensitive enough to detect weaker (mM) interactions</li> </ul>
	No consensus and no hits from phage ELISA	Selected phage are weak binders, target unrelated selections or no phage were bound	<ul style="list-style-type: none"> <li>Consider modifying panning strategy to eliminate target unrelated binding activity</li> </ul>
	Synthetic peptides do not bind despite phage particle binding	Phage display 5-copies of each peptide	<ul style="list-style-type: none"> <li>Due to avidity affects, multimers may be required for affinity to the target</li> </ul>
		Synthetic peptide design is dissimilar to phage	<ul style="list-style-type: none"> <li>C-terminus should not be charged; consider amidation</li> <li>Consider using GGGG linker</li> </ul>

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## Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/22

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