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FAQs for Phage Display Peptide Libraries

GENERAL QUESTIONS

1. What is phage display?

Phage display describes an *in vitro* selection technique in which a peptide or protein is genetically fused to a coat protein of a bacteriophage, resulting in display of the fused protein on the exterior of the phage virion, while the DNA encoding the fusion resides within the virion. This physical linkage between the displayed protein and the DNA encoding it allows screening of vast numbers of variants of the protein, each linked to its corresponding DNA sequence, by a simple *in vitro* selection procedure called "biopanning." In its simplest form, biopanning is carried out by incubating the pool of phage-displayed variants with a target of interest that has been immobilized on a plate or bead, washing away unbound phage, and eluting specifically bound phage by disrupting the binding interactions between the phage and target. The eluted phage is then amplified *in vivo* and the process repeated, resulting in stepwise enrichment of the phage pool in favor of the tightest binding sequences. After 3 rounds of selection/amplification, individual clones are characterized by DNA sequencing.

2. What are the advantages of phage display screening over other methods of library screening?

The main advantage of phage display over other technologies is the ease with which one can screen large numbers of clones. Standard screening of cDNA libraries, such as those expressed in phage lambda, is limited by the number of plaques or colonies which can be screened by hybridization, typically on the order of 10^4 . Synthetic random peptide libraries are typically screened on grids of pins, where binding sequences are identified by position, or on beads in suspension, where bound sequences are identified by sequencing a tag affixed to the selected bead. These technologies also limit the maximum number of random peptides that can be screened to 10^3 - 10^4 different sequences. If synthetic peptides are screened in solution, libraries can contain as many as 10^{15} different sequences, but the requirement for sufficient material to sequence (~ 1 μ g peptide) requires such low stringency during binding that enrichments of only 100 to 1000-fold are possible, resulting in selection of an enormous pool of peptides with highly variable affinities. Using phage display, greater than 10^9 different displayed sequences can easily be screened, and since the selected phage pool can be amplified by propagation in *E. coli*, multiple rounds of selection can be carried out to iteratively select for the tightest binding sequences.

3. Can the Ph.D. libraries be used interchangeably with cDNA libraries?

The choice of library depends on whether your goal is to identify a sequence, natural or synthetic, which binds to your target tightly, or solely to identify the natural *in vivo* ligand for your target. Bear in mind that the Ph.D. kits are based on fully randomized peptide libraries, while cDNA expression libraries are limited to naturally occurring proteins. As a result, the Ph.D. libraries are most suitable for identifying novel ligands (e.g. receptor agonists) or mapping the interactions between two known proteins (e.g. antibody epitope mapping). Since the biopanning is carried out *in vitro*,

the selected sequence may bear little resemblance to any native ligands for your target. For routine identification of the native ligand for a protein, the yeast two-hybrid system, lambda gt11, or other cDNA libraries may be more appropriate.

4. Are phage display cDNA libraries available?

In general, M13 is not amenable to cDNA expression, due to the requirement for in-frame expression between the leader sequence (required for secretion) and the N-terminus of coat protein pIII or pVIII. The consequence of this requirement is that an insert must be in the correct reading frame at both ends ($p = 1/9$) and contain no in-frame stop codons ($p = [61/64]^{n/3}$, where n is the average insert length in base pairs) in order for the corresponding protein sequence to be properly fused to the coat protein. This results in a vanishingly small number of productive clones in M13 cDNA libraries. In contrast, expression of cDNA inserts as C-terminal coat protein fusions is possible in the T7Select phage display system available from Novagen. This system utilizes the lytic bacteriophage T7 instead of M13.

5. What are the advantages of M13 over other phage used for phage display applications?

M13 and the closely related filamentous bacteriophages fd and f1 are non-lytic, meaning that they do not lyse the host during phage production. This greatly simplifies the intermediate phage purification steps between rounds of panning, as a simple PEG precipitation step is sufficient to separate the phage from almost all contaminating cellular proteins. In contrast, other phage that have been used for phage display (T7, T4, lambda) are all lytic, necessitating additional time-consuming purification steps between rounds to avoid panning amplified phage in the presence of cellular proteins (including proteases which can degrade your target during panning).

6. What are the differences between pIII and pVIII display?

Filamentous phage display systems are generally based on N-terminal fusions to the coat proteins pIII or pVIII. pIII is present at 5 copies per virion, of which all 5 can be fused to short peptides without interfering with phage infectivity. The major coat protein pVIII is present at ~ 2700 copies per virion, of which $\sim 10\%$ can be reliably fused to peptides or proteins. As a result, peptides expressed as pIII fusions are present at low valency (1-5 copies per virion), while pVIII fusions are present at high valency (~ 200 copies per virion). The increased avidity effect of high valency pVIII display permits selection of very low affinity ligands, while low valency pIII display limits selection to higher affinity ligands. All of the Ph.D. libraries are pIII fusions (5 copies of the peptide per virion).

7. What is the size of pIII with/without leader?

The molecular weight of the unprocessed coat protein pIII (containing a leader sequence but no displayed peptide) is 44651 daltons. Without the leader sequence, the molecular weight of mature pIII is 42579. By SDS-PAGE, however, pIII usually runs with an apparent molecular weight of 60-65 kDa, possibly as a result of the unusual glycine-rich spacer regions between the domains of the protein [van Wezenbeek *et al.* (1980) *Gene* 11, 129-148]. The amino acid sequence of the leader peptide is MKKLLFAIPLVVPFYSHS (note that the initiator Met is encoded by a GTG codon).

8. Is the library cloning vector available?

The vector used for constructing all three Ph.D. libraries, M13KE, is available for sale. A derivative of M13mp19, M13KE has restriction sites engineered at the 5' end of gene III permitting construction of custom peptide libraries by insertion of a user-designed synthetic cassette. Because M13KE is a phage, rather than a phagemid vector, all 5 copies of pIII in the processed virions will carry the displayed peptide sequence. Since displayed peptides longer than 20-30 residues have a deleterious effect on phage infectivity, this vector is suitable only for display of short peptide libraries, rather than larger protein or cDNA libraries.

The vector is available, for research use only, as part of the Ph.D. Peptide Library Cloning System, (NEB #E8101). In addition to the vector, this product includes an extension primer for second strand synthesis of the randomized library insert and a detailed protocol for construction of random peptide libraries in M13KE.

9. Can the library be amplified for additional panning experiments?

We strongly recommend against amplification of the supplied libraries, as sequence biases *in vivo* will likely result in certain sequences being underrepresented in the resulting library, or absent altogether. Displayed peptides in the Ph.D. libraries are expressed as fusions to the coat protein pIII, which modulates infectivity by binding to the F-pilus of the recipient cell. As a result, there is a biological selection against certain displayed sequences during *in vivo* amplification, particularly sequences with multiple positive charges (which inhibit secretion) and unpaired cysteines. The supplied libraries have each been amplified only once following ligation, and all characterization (representative sequencing, panning etc.) carried out on this amplified selling stock. We cannot guarantee that the amino acid distribution data we report for each library as supplied will hold upon reamplification.

10. Are anti-M13 antibodies available?

We recommend the following anti-M13 antibodies, both available from GE Healthcare (800-526-3593). Both antibodies are polyclonal and recognize primarily pVIII.

Anti-M13 antibody #27-9420-01

Anti-M13 antibody, HRP conjugated #27-9421-01

APPLICATION

11. For what applications are these libraries ideally suited?

Over the last ten years, the Ph.D. libraries from New England Biolabs have become the dominant tools in this field, with hundreds of publications describing applications including epitope mapping/vaccine development (Youn, et al (2004) *FEMS Immunol. Med. Microbiol.* 41, 51-57; Eshaghi et al. (2006) *Mol. Immunol.* 43, 268-278), mapping protein-protein contacts (Carter et al. (2006) *J. Mol. Biol.* 357, 236-251) and identification of peptide mimics of non-peptide ligands (Hou and Gu (2003) *J. Immunol.* 170, 4373-9). Bioactive peptides, which can be used as cell-targeting or gene delivery agents, have been identified either by panning against purified receptors (De et al. (2006) *Biochem. Biophys. Res. Commun.* 342, 956-62) or against

intact cells or tissue samples, both *in vitro* and *in vivo* (Kragler (2000) *EMBO J.* 19, 2856-68). It is apparent that applications of the Ph.D. kits have been limited only by the imagination of the scientific community. Please see our applications page for additional references.

12. Can the Ph.D. libraries be used to find the natural ligands for a given protein?

Since the Ph.D. libraries consist of fully randomized peptides displayed on phage, a binding peptide identified in a particular panning experiment will not necessarily correspond to a "natural" ligand for the target. The biopanning procedure iteratively selects for those peptides which best bind the target under the panning conditions *in vitro*, without regard to the biological role of the target *in vivo*. For certain targets, such as antibodies with linear epitopes, the selected sequence will in all likelihood correspond to that region of your antigen recognized by the antibody. For targets which bind to large surfaces of a protein, or discontinuous regions of the primary sequence, the selected sequences are less likely to resemble the "natural" ligand. As a result, caution should be taken if you are planning on using the DNA corresponding to the selected sequences as probes when trying to clone any natural ligand proteins. In contrast, cDNA expression libraries are by nature limited to natural proteins, and as a result are much more likely to yield the native ligand for your protein. If your goal is to identify a sequence, natural or synthetic, which binds to your target tightly, then you should consider biopanning with the Ph.D. libraries. If you are only interested in identifying the natural ligand for your target, however, you should consider screening an appropriate cDNA library expressed in lambda gt11 or two-hybrid system.

13. Can the libraries be used to pan against intact cells?

Yes. There are numerous reports in the literature describing the use of libraries very similar to the Ph.D. libraries for identification of novel ligands for cell-surface receptors by panning against intact cells. Keep in mind, however, that a given cell type will have hundreds or thousands of different receptors, each capable in theory of pulling a ligand out of the library. As a result, simple panning against intact cells will likely yield a complex mixture of peptides with no clear consensus. To target the library to the receptor of interest, it is necessary either to elute bound phage with a known ligand for that particular receptor, or to carry out subtractive panning with cells that do not express the receptor. This is accomplished by having two cell lines, identical except for the presence or absence of the receptor of interest. The library is incubated with the cells without the receptor, and then the supernatant is added to cells expressing the receptor. Phage that bind to the second cell line are then amplified and taken on to the next round. Because of nonspecific binding of peptides to cell surfaces, however, we recommend carrying out the subtractive panning step beginning with the second round. For more details consult the following references:

Doorbar, J. and Winter, G. (1994). Isolation of a peptide antagonist to the thrombin receptor using phage display. *J. Mol. Biol.* 244, 361-369.

Goodson, R.J. *et al.* (1994). High-affinity urokinase receptor antagonists identified with bacteriophage peptide display. *Proc. Natl. Acad. Sci. USA* 91, 7129-7133.

Barry, M.A., Dower, W.J., and Johnston, S.A. (1996). Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting

phage libraries. *Nature Medicine* 2, 299-305.

Szardenings, M. *et al.* (1997) Phage display selection on whole cells yields a peptide specific for melanocortin receptor 1. *J. Biol. Chem.* 272, 27943-27948.

14. Can the Ph.D. libraries be used for *in vivo* screening?

An exciting development is the use of phage display to select organ-specific peptides *in vivo*. Peptides selected in this manner have been successfully used to specifically deliver drugs to tumor cells.

Pasqualini, R. and Ruoslahti, E. (1996). Organ targeting *in vivo* using phage display peptide libraries. *Nature* 380, 364-366.

Arap, W., Pasqualini, R. and Ruoslahti, E. (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* 279, 377-380.

Chen *et al.* (2006) Transdermal protein delivery by coadministered peptide identified via phage display. *Nat. Biotech.* 24, 455-460.

15. For epitope mapping, does the antibody need to be pure?

We recommend Protein A purification of antibodies for epitope mapping. However, the major component of crude serum or ascites fluid is serum albumin, which is used in the blocking step anyway. Following the direct coating method in the Manual, you can coat with serum or ascites fluid (diluted 1:10 in TBS) and omit the blocking step. If using the Protein A/Protein G bead capture protocol, use 1 μ L of serum or ascites fluid in place of the antibody in Step 5. In this case it is necessary to carry out the blocking step (Step 4) as described. However, given the variable level of antibody in serum or ascites, we cannot guarantee the crude antibody preps will work.

16. Can the antibody be polyclonal?

Maybe. A polyclonal antibody raised against a large antigen will likely contain numerous epitope specificities corresponding to different regions of the antigen and, if the antibody has not been affinity purified, other antigen specificities as well. When panning against such a heterogeneous population of antibodies, it is unlikely that a well-defined consensus epitope sequence will emerge. Rather, numerous sequences corresponding to the individual specificities will be selected, which will be difficult to discern against the background of non-binding sequences. For smaller antigens, the number of selected epitope sequences will likely be more manageable, particularly if the antibody is affinity purified. Experiments at New England Biolabs have demonstrated that polyclonal antibodies raised against peptide antigens yield clear consensus epitope sequences.

17. Can I pan against DNA to find sequence-specific DNA-binding peptides?

Probably not. In general, the sequence specificity of DNA binding proteins results from a series of precisely oriented hydrogen bonds between side chains of the protein and the nucleotide bases. This requires that the side chains responsible for sequence recognition be precisely fixed in position by the tertiary structure of the protein, which is not possible with short unstructured peptides. Additionally, the sequence specificity represents only a fraction of the overall binding energy, the bulk of which comes from nonspecific interactions with the phosphodiester backbone of the DNA. As a result, attempts to select for sequence-specific DNA-binding peptides by phage display have

generally resulted in peptides with multiple positively-charged residues that bind to the phosphodiester backbone of the DNA, but not sequence-specifically. Phage display has proven extremely successful, however, for selection of zinc finger domains with altered sequence specificity. Residues known to be important for sequence discrimination are randomized, and the resulting pool of specificity variants is biopanned against specific DNA sequences. See Choo and Klug (1995) *Curr. Opin. Biotech.* 6, 431-436.

CHOICE OF LIBRARY

18. Which of the three libraries should I buy?

The Ph.D.-7 library consists of randomized linear 7-mer peptides, fused to the coat protein pIII of M13 via a flexible linker, Gly-Gly-Gly-Ser. The first residue of the mature fusion protein is the first randomized position. The library contains 2.8×10^9 independent clones, sufficient to encode most if not all of the $20^7 = 1.28 \times 10^9$ possible 7-residue sequences. The Ph.D.-7 library is most useful for targets requiring binding elements concentrated in a short stretch of amino acids. It is the best characterized library we sell, and in the absence of other considerations we recommend trying this library first.

The Ph.D.-12 library consists of randomized linear 12-mer peptides, also fused to pIII via the flexible linker Gly-Gly-Gly-Ser. The library contains 1.9×10^9 independent clones, or only a small fraction (less than 1 millionth) of the $20^{12} = 4.1 \times 10^{15}$ possible 12-mer sequences. The Ph.D.-12 library can be thought of as having the equivalent diversity of a 7-mer library, but spread out over 12 residues. This is 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 ASDXXTXPY 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.

The Ph.D.-C7C library consists of randomized 7-mer peptides, each flanked by a pair of cysteine residues. In the absence of reducing agents, these cysteines spontaneously form a disulfide bond, resulting in each peptide in the library being constrained in a disulfide loop. The library contains 3.7×10^9 independent clones. Like the other libraries, the library is fused to pIII via the Gly-Gly-Gly-Ser spacer. The Ph.D.-C7C library is useful for targets whose native ligands are in the context of a surface loop, such as antibodies with structural epitopes. Additionally, imposing structural constraint on the unbound ligand results in a less unfavorable binding entropy, improving the overall free energy of binding compared to unstructured ligands. A major disadvantage of the Ph.D.-C7C library is that the disulfide constraint may "freeze out" a conformation required for target binding. The bottom line is that it is impossible to predict in advance which library is suitable for a given target. As a result, **we recommend that the Ph.D.-7 library be tried first, regardless of target.** Alternatively, all three libraries can be tried simultaneously, as it is very simple to carry out panning experiments in parallel using multiwell plates.

TROUBLESHOOTING

19. Can a different bacterial strain be used?

In theory other F⁺ strains containing the *supE* suppressor mutation (such as XL1-Blue and DH5aF') should work with our phage display system. However, we have not tested these strains with our libraries and do not know whether there will be any subtle effects on the expression or transport of certain peptides out of the cell. Since the Ph.D. libraries were made in ER2738 we know that all the peptides in the libraries can be successfully expressed in this strain. Therefore, we recommend this strain over any other one.

20. The supplied bacterial strain will not grow.

Glycerol stocks of ER2738 should be stored at -80°C and ideally, upon receipt of the kit, multiple fresh glycerol cultures should be made. These, never thawed, will last for many years in storage. Glycerol stocks have a shorter lifetime at -20°C. If colonies do not appear after streaking a plate, we recommend spreading 20-50 µL of ER2738 stock onto an LB/Tet plate. Alternatively, liquid culture can be inoculated and then a plate should be made by streaking turbid culture. If liquid cultures do not grow in LB/Tet, a fresh plate of ER2738 will usually solve the problem. Additional glycerol cultures of ER2738 (#E4104) can be ordered for the cost of shipping.

21. No plaques are visible when titering.

Unlike lambda, M13 is a non-lytic phage and does not produce clear plaques. M13 plaques are areas of diminished cell growth, not lysis, and consequently can be difficult to see. Try holding the plate up to a light. Also, since the vector used to prepare the library carries the *lacZa* gene, plaques will be blue, and easier to see, when using an a-complementing strain such as the supplied strain ER2738 and plating on Xgal/IPTG plates. Also, be sure the dilution range is appropriate for the phage you are titering. For amplified phage, plate 10 µL of 1:10⁹ - 1:10¹¹ dilutions; for unamplified panning eluates, try 1:10 - 1:10⁴ dilutions for early rounds, 1:10⁴ - 1:10⁷ for later rounds. If the phage is not sufficiently dilute, the plaques will be confluent on the plate and it will look like there are no plaques at all (or a bluish tinge when using Xgal plates). Occasionally after PEG precipitation, the phage will clump and not dilute properly. As a result, you might have a plate containing too many plaques merged together. Make sure to give the phage ample time to resuspend after precipitation (>1 hour) and vortex each dilution tube very well (~10 seconds).

22. All or most of the eluted phage plaques are white (colorless) on Xgal/IPTG plates.

If all of your plaques are white (colorless), it is possible that the Xgal/IPTG plates were incorrectly prepared. Test your plates by titering the naive unpanned library, using 10⁹ and 10¹⁰ dilutions. If both the unpanned library and your selected phage produce white plaques, then the plates are defective and should be carefully re-prepared. Also, the bacterial strain used for plating must be capable of a-complementation (*lacZDM15* or equivalent), such as the supplied strain ER2738, in order for blue/white screening to work. The most likely explanation for white plaques is that the pool of phage became contaminated with an environmental M13-like phage during panning and amplification. Display of foreign peptides as N-terminal fusions to

the infectivity protein pIII, as in the Ph.D. libraries, slightly attenuates infectivity of the library phage relative to wild-type M13. As a result, there is an *in vivo* selection for the contaminating phage during the amplification steps between rounds of panning. In the absence of a correspondingly strong *in vitro* binding selection during panning, even vanishingly small levels of contamination can result in a majority of the phage pool being wild-type phage after 3 (or especially 4) rounds of panning. The Ph.D.-C7C library is particularly susceptible to contamination, since phage infectivity is further attenuated by displayed cysteine-containing peptides. Contamination is an extremely common problem with any phage display system, but fortunately there are a few things you can do to minimize this problem:

- a. Use Xgal/IPTG plates for all titering steps, and if white plaques are evident, pick only blue plaques for sequencing.
- b. Use aerosol-resistant pipet tips and cotton-plugged pipets for all protocols described in the Manual.
- c. If contamination problems persist, all of the solutions used for panning should be autoclaved, with the exception of BSA-containing solutions which should be filter sterilized. Solutions used for phage display should not be used for anything else. Pipettors should be disassembled, the barrel autoclaved, and the internal plunger machinery soaked overnight in a detergent solution such as Count-Off™.
- d. Since wild-type phage are preferentially amplified during the amplification steps, pick plaques for sequencing directly after the 3rd round elution step. Do not amplify the 3rd round eluate and carry out a 4th round unless the third round sequences show no clear consensus.
- e. If all or most of the plaques are white (colorless) after 3 rounds of panning, it is possible that the library simply does not contain any clones that bind tightly to the target. The ideal ligand sequence may not be statistically represented in the library, or the target simply is not capable of binding to a short peptide sequence. In the case of the C7C library, where all the peptides are constrained in a disulfide loop, a ligand sequence where the imposed constraint allows a productive binding conformation will bind more tightly than the same linear sequence due to improved binding entropy. However, if the imposed constraint does not allow a productive binding conformation, than that sequence will likely not bind to the target at all. In this case either of our linear libraries may yield better results.

23. The amplified phage titer is low.

In order for M13 phage to be efficiently amplified, it is critical that cultures be well aerated, and that cultures be infected early in their growth phase. We recommend amplification in 20 mL cultures in 250 mL Erlenmeyer flasks, in a shaker set to 250 rpm. Amplification in smaller vessels, such as 50 mL conical tubes, will result in much lower yields of amplified phage. M13 phage should either be added to an early-log culture, $A_{600} < 0.01$, or to a 1:100 dilution of an overnight culture. Yield of amplified phage is maximal after 4.5-5 hours at 37°C; longer incubation may result in deletions and is not recommended. If carrying out nonspecific elution with pH 2.2 glycine buffer, the eluted phage must be neutralized as described in the Manual prior to amplification.

24. The phage DNA templates do not yield readable sequence.

The sequencing template purification protocol in the Manual should provide single-stranded template of sufficient purity for dideoxy sequencing with Sequenase™ (GE Healthcare), or automated cycle sequencing with dye-labeled terminators (ABI). The procedure should be followed exactly as described in the Manual: prolonged ethanol precipitation, precipitation at -20°C or centrifugation longer than 10 minutes will result in co-precipitation of salt and phage proteins, which will inhibit sequencing. Additionally, it is crucial that the phage pellet is thoroughly suspended in the iodide buffer prior to adding ethanol. If problems persist, or if another sequencing method is used, a phenol:chloroform extraction step can be added: Following suspension in Iodide Buffer, add 2 volumes of TE, extract once with phenol:chloroform (1:1) and once with chloroform, and ethanol precipitate. Another option is to isolate double-stranded template from the cell pellet by standard plasmid purification procedures. 5 µL of suspended template (approximately 0.5 µg) should be sufficient for sequencing; quantitation should be confirmed by agarose gel electrophoresis using 0.5 µg single stranded M13 DNA (NEB #N4040) as a standard.

25. The sequencing templates do not run where they should on a gel.

The sequencing templates prepared by the method in the Manual are single-stranded (approx. 7250 nucleotides), and as a result will not line up with double-stranded markers of the same length. The apparent size will vary depending on the applied voltage, ethidium and agarose concentration in the gel, and whether TBE or TAE is used as running buffer. We strongly recommend using single-stranded M13 DNA (e.g. single-stranded M13mp18, (NEB #N4040), as a marker.

26. The sequence does not have the cloning sites or insert as shown in Fig. 3.

If the sequence matches the sequence in Figure 3 from the primer back to the 3' G of the EagI site, but then deviates upstream from this position, you may have sequenced a wild-type M13 contaminant (see white plaques, above):

EagI

Fig. 3 sequence: ...GGTGGAGGTTTCGGCCGAAACTGTTGAA...

|||||

wt M13 sequence: ...TATTCTCACTCCGCTGAAACTGTTGAA...

Since the library phage are derived from the common cloning vector M13mp19, which carries the *lacZa* gene, phage plaques appear blue when plated on media containing Xgal and IPTG, providing an a-complementing strain such as ER2738 is used for plating. Environmental M13-like phage will typically yield white plaques when plated on the same media. These plaques are also slightly larger and "fuzzier" than the library phage plaques. We therefore recommend plating on LB/Xgal/IPTG plates for all titering steps and, if white plaques are evident, picking ONLY blue plaques for sequencing.

27. After 4 or more rounds of panning all clones are wild-type phage (white plaques).

In a typical round of biopanning, 2×10^{11} input phage are reacted with the target, and between 10^3 and 10^7 total phage are eluted off following washing. This corresponds to

an enrichment of 10^4 to 10^8 -fold per round. Since the library contains approx. 2×10^9 different clones, the eluted pool of phage should in theory be fully enriched in favor of binding sequences after only 2 or 3 rounds. Once this point is reached, further rounds of amplification and panning will result only in selection of phage that have a growth advantage over the library phage. For example, vanishingly small levels of contaminating environmental wild-type phage (less than one part per billion) will completely overtake the pool if too many rounds of amplification are carried out, regardless of the strength of the *in vitro* selection.

28. The sequence is fine back to the *KpnI* site, but then differs from Fig. 3 in the product manual, or the *KpnI* site is missing altogether.

If the sequence matches the sequence in Figure 3 starting from the primer back to the *KpnI* site, but then deviates upstream from the *KpnI* site (or the *KpnI* site is missing), the clone likely contains multiple inserts. All of the Ph.D. libraries were constructed by directional cloning of a synthetic randomized duplex into *KpnI* and *EagI* sites that had previously been engineered into the M13 genome. A small percentage (<1%) of clones in each library picked up more than one insert during ligation. Typically such clones contain 2-5 randomized inserts, with one or more inverted relative to the others. Preferential selection and amplification of these clones may occur when panning against targets which prefer longer ligands; consequently, selection of clones with multiple inserts is more likely to occur when using the Ph.D.-7 library. To properly characterize these clones, it is necessary to read the sequence back to the occurrence of a *KpnI* site preceded by the upstream vector sequence TTAGT, as shown in Figure 3. Starting from this *KpnI* site, the translated sequence Val-Pro-Phe-Tyr-Ser-His-Ser is the C-terminal end of the pIII leader sequence. Everything downstream from this sequence is displayed on the phage, and must be considered when identifying consensus binding elements. In experiments carried out at NEB, however, we have failed to identify meaningful consensus binding motifs from multiple insert clones, and typically ignore these clones when interpreting data. If all or most of your selected clones contain multiple inserts, we recommend repeating your panning with the Ph.D.-12 library.

29. The streptavidin control experiment did not yield the HPQ consensus sequence.

If you used low pH glycine rather than biotin to elute your phage, you will likely not get an HPQ consensus sequence. Due to the relatively low affinity of the peptide-streptavidin interaction, nonspecific elution is incapable of selectively enriching for HPQ-containing peptides. HPQ-containing peptides can be competitively eluted using the natural ligand biotin. If you used biotin to elute and still did not get a consensus sequence, the most likely explanation is that you did not carry out sufficiently rigorous washes. When you wash, pour the wash buffer in the plate from a bottle (don't gently pipet it in) and swirl it for about 10 seconds each time. The number of phage that you elute after the first round of biopanning should be in the range of 10^3 - 10^7 (closer to 10^3 for an ELISA well and closer to 10^7 for larger wells). If you are eluting more phage, you are not washing well enough and as a result, not getting sufficient enrichment. It also may help to add 0.1 $\mu\text{g/ml}$ streptavidin to the blocking buffer to complex any contaminating biotin in your BSA, which could otherwise complex the streptavidin on the plate during the blocking step.

30. The ELISA indicates that background binding to the plate is as high as binding to the target.

If panning against a polystyrene plate coated with the target (direct coating method), it is possible to inadvertently select peptides that specifically bind the polystyrene surface (see Adey *et al.* (1995) *Gene* 156, 27-31; Menendez. and Scott (2005) *Anal. Biochem.* 336, 145-157.). These peptides will yield identical ELISA signals in the presence and absence of target, since the ELISA plate is also made of polystyrene. Such "plastic binders" are typically rich in aromatic residues (Phe, Tyr, Trp, His), which often alternate (the sequence FHWTWYW is a plastic binder discovered and characterized at NEB). Selection of plastic binders often occurs in the absence of a strong target preference for peptide sequences present in the library: other libraries may yield the desired target-specific sequences. Selection of polystyrene-specific peptides can be avoided by using the bead capture protocol described in the Manual. The phage is reacted with the target in solution, and the phage-target complexes are then captured onto beads that specifically bind the target (protein A-agarose for antibody targets, glutathione-agarose for GST fusions, etc.). Unbound phage is removed by extensively washing the beads in a microfuge tube. Unlike polystyrene, neither the beads (typically crosslinked agarose) nor the microfuge tube (polypropylene) are likely to select specific peptide sequences from the library, although the species conjugated to the beads (protein A, glutathione, etc.) might. To avoid selection of bead-specific ligands, we suggest either alternating rounds between different beads specific for the target (e.g. protein A beads for rounds 1 and 3, protein G beads for round 2 for antibody targets), or adding a subtractive panning step, beginning with round 2, in which the phage pool is first reacted with the beads alone (no target), the beads discarded, and the supernatant from this step reacted with the target.

31. My selected sequences bind BSA, and the protocol does not select against BSA binders.

Most likely the "BSA-binding" sequences are actually binding to the polystyrene surface of the plate rather than the BSA (see previous answer). BSA is a soluble, monomeric globular protein without a defined ligand binding site. This means that the entire surface of the protein has evolved to specifically bind water (the definition of "soluble"), so it is unlikely that a peptide could bind specifically to the surface of the BSA in the presence of 55 M water during panning. Contrast this to the case where the target protein HAS a defined ligand binding site, such as an antibody. In this case, the surface of the protein has evolved to bind water, but the water in the ligand binding site is bound LESS tightly and can be displaced by the ligand. So when binding a phage library to an antibody, specific ligands in the library are able to displace the water in the ligand binding site of the antibody, but do not bind elsewhere on the surface of the antibody. This is why nonspecific elution (0.2 M glycine, pH 2.2) generally yields peptides that are specific for the ligand binding site of the antibody, even though the antibody has a vast surface area containing many more potential binding sites. The bottom line is that for small peptide ligands, there is generally not enough potential binding energy to displace water from the non-ligand-binding surface of proteins. The absence of a defined ligand binding site is precisely why BSA is generally used for blocking in phage display applications. In contrast, the

phenomenon of plastic binding peptides is well documented (see above).

32. Panning yielded a consensus sequence, but no ELISA signal.

When characterizing phage clones by the ELISA protocol in the Manual, it is difficult to add more than 10^{12} virions per 100 μ L well. This corresponds to a phage concentration of only 16 nM. At this concentration, an unambiguously positive ELISA signal can only be observed if the binding affinity is in the micromolar range or better. The iterative nature of phage selection permits identification of ligands with a broad range of affinities, from sub-nanomolar to 1 millimolar, so lower affinity ligands will not show a positive ELISA signal. In this case it is necessary to increase the concentration of the selected ligand, either by synthesizing a peptide corresponding to the selected sequence (be sure to include the spacer sequence GGGs at the C-terminus, and amidate the C-terminal carboxylate if possible), or by expressing the selected sequence as an N-terminal fusion to a smaller protein (e.g. an MBP fusion constructed with pMal-pIII, NEB #N8101). Alternatively, a sandwich ELISA can be carried out in which the selected phage is immobilized and an excess of target applied in the liquid phase. This procedure requires an antibody against the target protein, or some other means of detecting bound target protein. Coat the wells overnight with anti-M13 antibody (no HRP), wash, and add serial dilutions of each phage clone (one clone per row). After 1 hour, wash away unbound phage and add an excess of target protein (0.1 - 1 μ M) in TBST. Incubate 1-2 hours at RT^o, wash away unbound target, and detect bound target with an enzyme-linked antibody.

33. A synthetic peptide corresponding to an ELISA-positive sequence does not bind my target.

If a selected sequence binds the target in the context of intact phage, but not as a synthetic peptide, it is possible that the selected sequence requires additional elements from the adjacent spacer sequence for binding. Bear in mind that, while the N-terminus of the selected peptide sequence was free during panning, the C-terminus was fused to the phage. Furthermore, the C-terminal residue of the selected sequence did 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, which may completely abolish binding. When designing synthetic peptides corresponding to selected sequences, we recommend adding the spacer sequence Gly-Gly-Gly-Ser to the C-terminus, and if possible, amidating the C-terminal carboxylate to block the negative charge. For chemical conjugation of the peptide to a reporter enzyme, the C-terminal serine can be replaced with cysteine (if there are no other cysteines present in the sequence). The resulting peptide thiol can be easily coupled to maleimide-activated HRP or alkaline phosphatase (both available from Pierce).