Ready-To-Use Phage Display Library

Manual

Ready-To-Use Phage Display cDNA Library

Phage Display Library Screening Kit

FOR REASEARCH USE ONLY

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I. Introduction

Display cDNA on the surface of M13 phage

Phage Display Libraries and Library Screening Kit give you the tools you need to search cDNA libraries for displayed proteins that interact with an immobilized bait ligand. Phage display screening provides immediate access to the cDNA encoding the interacting protein. The Phage Display System is based on proven technology that combines the benefits of *in vivo* expression in *E. coli,* M1 3 phage displayed proteins, and *in vitro* (solid-support) "biopanning" (Kay *et al.,* 1996; Dunn, 1998; O'Neil & Hoess, 1995; Smith & Scott, 1993; Parmley & Smith, 1988; Smith, 1985). cDNA libraries are constructed in a state-of-the-art phagemid vector (pHD9). cDNA/gene III fusion products are displayed on the lip on the filamentous phage particle, making them available for interaction with an immobilized bait ligand. Several rounds of panning (or biopanning) enrich for the interacting clone(s); subsequent ELISAs identify individual positive clones and confirm positive interactions. Phage Display cDNA Libraries and Library Screening Kit help you achieve success with different phases of the phage display screening procedure. Our high-quality, premade Phage Display cDNA Libraries are made to screen by panning. With their high complexity (>=10^8 independent clones) and high titer (>10^{10} pfu/ml), the libraries optimize your chances of finding cDNAs for rare or low-copy-number transcripts. You can easily screen 10^9 phage clones in one panning experiment with a minimal amount of preparation and hands-on time. Premade libraries are made from poly A^+RNA isolated from a wide selection of human tissues and cell lines.

Phage Display Library Screening Kit provides all the biological materials you need for performing ELISA screening phases of the procedure; anti-pVIII *(*gene VIII coat protein) antibody for binding (and detecting) phage particles, an HRP-conjugated secondary antibody, and a colorimetric HRP substrate. Library Screening Kit and protocol have been optimized for use with phage display libraries.

Simple panning procedure-obtain results in one week

Panning is the process whereby the library is enriched for clones of interest by applying an aliquot of the pooled phage clones to a bait-coated surface and subsequently washing off unbound phage particles (Kay et al., 1996; Smith & Scott, 1993). If the phage clone expresses a protein that can interact with the immobilized bait, it will form a stable complex and be retained. The TG1 *E. coli* host is then added to the well to permit infection by the bound phage particles. The efficiency of recovery is very high with our protocol because the bound phages are not eluted under the harsh conditions used in other phage display systems. The recombinant phagemids are amplified by growing the transformants on ampicillin-containing medium, which favors plasmid amplification, not phage production. To further enrich for positive clones, phagemid DNA is packaged in M13 phage particles by superinfection with helper phage (Sambrook *et al*., 1989) and the panning procedure is repeated with the same or

increasing stringency. The panning step can be performed in one well of a 96-well microtiter plate and takes only one day.

To identify positive clones and to confirm a positive interaction individual clones from the enriched library are screened by ELISA using the same immobilized bait that was used for the library panning. Bound phages are detected using an antibody against the pVIII phage coat protein.

PHD9 is a versatile, bifunctional phagemid vector

Vector pHD9 carries the pUC replication origin and ampicillin resistance (Amp^r) gene for growth and selection in transformed E coli. The gene III coding regions in pHD9 permits expression of the foreign proteins as N-terminal pIII (gene VIII coat protein) fusions in host strain TGI. Because of the gene III leader sequence fused to the N-terminus, gene III fusion proteins are secreted to the periplasmic space (Kang et al., 1991).

Helper phage M13K07 infects host cells (e.g., TG1 transformed with pHD9 library plasmids) easily and rapidly at room temperature or 37^0C. Superinfected host cells are not lysed. Wild-type gene III proteins are expressed from the helper phage and are secreted to the periplasmic space, where they are assembled into phage particles along with the pIII fusion proteins. Fusion gene transcription levels from the *lac* promoter are kept relatively low (in the absence of IPTG) when the goal is to display only one or two copies of the pIII fusion displayed protein on the surface of each phage particle. The very low average number of pIII fusions displayed per phage particle makes this a "low valency" system (valency is discussed further below). The simultaneous presence of three or four copies of the wild-type pIII coat protein (encoded by the helper phage) ensures that the phage will be efficiently assembled and will be infective. PHD9 contains an M13 replication origin so, in the presence of helper phage, phagemid DNA is replicated in the normal M13 fashion. However, M13KO7 carries a gene II mutation that renders it 50-fold less efficient than the recombinant phagemid vector at producing progeny (+) strands for packaging (Sambrook *et al.,* 1989). Thus, the vast majority of phage particles contain the phagemid vector, not the M13KO7 genome.

Low-valency, gene *III* fusion system

pIII is a minor coat protein; typically, five copies of pIII are located at one end of the filamentous rod, with the N-terminus of the proteins extended beyond the phage surface. Upon infection, pIII is involved in the adsorption of the virus particle to the end of the bacterial pilus (Sambrook *et al., 1989).* Gene III fusion vectors like pHD9 generate pIII molecules with a fusion moiety at the N-terminus. Because only one or two copies of the hybrid pIII molecules are normally produced per phage particle, relatively large fusion moieties can be added onto pIII without resulting in steric interference with protein-ligand interactions. For example, proteins as large as ~60-kDa have been identified in a cDNA phage display library. Furthermore, infective M13

phage displaying biologically active enzymes have been reported (for example, see Corey *et al.,* 1993).

The low valency of pIII hybrids tends to select for high-affinity interactions (e.g., K_d's in the micromolar range; Kay *et al.,* 1996; Cesarini, 1992). Thus, low-affinity interactions may be missed in this system unless the binding and/or washing conditions are modified to decrease the stringency. Another possible limitation of this system is that full-length cDNAs containing in-frame stop codons TAA and/or TGA will not be expressed as pIII fusions and thus will not be displayed or found in the screening. However, if the only stop codon is an amber stop (i.e., TAG), then the entire cDNA may be expressed as a pIII fusion in the amber suppressor host strain TG1.

Broad range of proven applications for phage display technology

An important application of phage display cloning technology is in receptor/ligand research (Wrighton *et al*., 1996). In fact, the display of proteins on the surface of a phage is one of the most effective methods for identifying cell-surface proteins that recognize specific baits. In addition, because it is not necessary for the interacting proteins to be localized to the cells' nucleus, phage display technology is suitable for identifying interacting cytoplasmic proteins that may be overlooked in a two-hybrid library screening. Signal transduction research, new drug development and screening, and antibody engineering are areas of proven application of phage display technology.

Phage display peptide libraries made with synthetic oligonucleotides have been used successfully to identify peptides that interact with many different kinds of bait ligands, including proteins, peptides, RNAs, and oligonucleotides (reviewed in Kay *et al.*, 1996; Dunn, 1996; Smith & Scott, 1993; Cesarini, 1992). For example, phage display peptide libraries using *gene III* as the fusion moiety have been used to screen the epitopes of antibodies (Devlin *et al.,* 1990; Cwirla *et al*., 1990; Scott & Smith, 1990; Oldenburg *et al.,* 1992; Scott *et al.,* 1992).

The recent development of phage display cDNA libraries enables researchers to this powerful technology to identify sequences that are expressed in nature presumably encode proteins with specific biological functions. For example, an allergenic protein from *Aspergillus fumigatus* was identified (and its cDNA cloned) panning an *A. fumigatus* cDNA phage display library against immobilized serum immunoglobulin E from sensitized individuals (Crameri *et al.,* 1996). Other examples are the specialized libraries constructed using cDNAs for single-chain antibodies (scFv) and Fab antibody fragments (Griffiths *et al.,* 1993; Nissim *et al.,* 1994; McCafferty *et al.,* 1990; Clackson *et al.,* 1991; Winter *et al.,* 1994). Phage display technology offers an advantage for identifying rare cDNAs because it allows screening of up to 1 x 10^{10} independent clones in small aliquots (Marks e*t al.,* 1992; Bass *et al.,* 1990; Jacobsson & Fryberg, 1995; Gram *et al.,* 1993; McCafferty *et al.,* 1991; Crameri *et al.,* 1994; Crameri & Suter, 1993).

Phage display library screening is for identifying novel proteins that interact with a bait. The Phage Display Library Screening Kit and Libraries are the system of choice if your application involves:

- looking for protein-protein interactions;
- using a nonproteinaceous bait or very small amounts of bait material;
- screening a very large library quickly.

However, if you are looking for protein-protein interactions that depend on posttranslational modifications unique to eukaryotes, or that are particularly weak or transient, you should use a Two-Hybrid System (Allen *et al.,* 1995). Phage display library screening requires a small amount of bait material. However, phage display library screening requires much less bait than does plaque screening on filters using a conventional immunoscreening procedure.

II. Product Information

A. Phage Display Library Screening Kit List of Components

The materials provided are sufficient for probing 4x 96 bait wells (384 total).
Store all components at 4^0C.

- 0.5 ml — Anti-pVlll monoclonal antibody (The antibody was purified by affinity chromatography and tested for use in phage display library screening)
- 0.5 ml — HRP-conjugated secondary antibody
- 14 ml/each — HRP substrates

B. Premade Phage Display Libraries

1. List of Components

Package size is sufficient for up to 10 primary library pannings using bait-coated wells in a 96-well micro-titer plate (or an equivalent surface area).

For short-term storage (<2 months), store all components at 4^0C.
For long-term storage (>12 months), divide the library into 10 working aliquots (-20 µl each) and store it at -70^0C before placing. Avoid multiple freeze/thaw cycles. Store TG1 and M13KO7 at -70^0C. Store sequencing primers at -20^0C.

- 0.2 ml — Phage library resuspended in PBS & 20% glycerol
- 1 vial — TG1 *(E. coli* host cells; 1ml of saturated culture/20%glycerol)
- 0.5 ml — MI 3KO7 helper phage (>I 0^{10} pfu/ml in PBS/ 20% glycerol)
- 50 µl/each — Sequencing primers

2. Important Notes

Upon receipt, retiter Phage Display Library. Titers are usually stable at -70^{0}C for at least one year. If the library titer is at least ten-fold higher than the number of independent clones stated, the library is representative, and you should proceed with screening. If the titer is <10^8 cfu/ml, repeat the titer. If the titer is still low, please contact our Technical Support Department or your local distributor.

- TG1 is an amber suppressor *E. coli* host strain used for expressing foreign proteins as fusions with the pIII coat protein and thus is used in the panning process. TGI can also be used to titer the library and the helper phage. Prepare a TG1 stock culture in 20% glycerol after receipt and maintain a working stock plate of TG1 on M9/+Thi minimal medium to keep selection on the F' episome.
- M13KO7 is used to help package recombinant phagemid DNA into infective M13 phage (Sambrook *et al,* 1989). It can also be used to produce single-stranded DNA from phagemid-transformed cells for sequencing. M13KO7 carries the gene for kanamycin resistance. Pre-pare a fresh stock of helper phage before you begin the panning experiment.

3. Library Construction Information

cDNA is synthesized using random primers and a modified Gubler & Hoffman procedure (1983). The cDNA is size-fractionated by gel electrophoresis; the 0.2->=2.0kb fraction is extracted. The cDNA is normalized to remove high abundant cDNA (high expressed genes). Then the cDNA is blunt-ended and ligated into the *EcoR* V site of pHD9 vector. Recombinant phagemids are packaged into infectious M13 phage particles upon superinfection with helper phage M13KO7. The phage is precipitated from supernatants and resuspended in PBS/20% glycerol at a high titer (>l 0^{10} cfu/ml).

III. Preparation for Library Panning and Screening

A. Bacterial Plating, Storing, and Culturing

See Appendix A for media recipes.

1. Culture of *E. coli* provided with the Library. Incubate in 2X YT at 37^{0}C overnight with shaking at 250 rpm. Use this culture to prepare a glycerol stock culture for freezing and a primary streak plate.
2. To prepare a glycerol stock culture, remove 0.75 ml of the over night culture from Step A. 1 above to a fresh, labeled tube. Add glycerol to a final concentration of 20% and store at -70^{0}C.
3. Prepare a primary streak plate:

a. Using a sterile loop, streak out the overnight culture from A.1 above on a 2X YT agar plate for single colonies. Incubate at 37^{0}C overnight.

b. Wrap the primary streak plate in Parafilm and store it at 4^0C. Prepare a fresh primary plate from the frozen glycerol stock at 3-month intervals (or sooner if contamination is evident).

c. To recover the frozen cells, streak a small portion (-5 µl) of the frozen glycerol stock onto a 2XYT plate. Incubate at 37^0C overnight and proceed as in Step A.3.a above.

4. To prepare a working stock plate, pick a single, isolated colony from the primary streak plate and streak it onto an M9/+Thi plate (Appendix A). Incubate at 37^0C overnight. Wrap the plate in Parafilm and store at 4^0C for up to 2 weeks. This plate is used as a source of fresh colonies for inoculating liquid cultures and for preparing the next fresh working stock plate.

Notes:

• Bacteria grow much more slowly on minimal media than on rich media and do not survive prolonged periods of storage on minimal medium (even at 4^0C).

5. At 1-week intervals, prepare a fresh working stock plate from the previous working stock plate, so you will always have a source of fresh colonies. If you suspect contamination on your current working stock plate, prepare a new primary streak plate from the frozen culture.

6. To prepare a log-phase liquid culture:

a. Pick a single isolated colony from the working stock plate (Step A.4 above) and use it to inoculate 5 ml of 2X YT (or LB) broth in a 50-ml test tube.

b. Incubate at 37^0C with shaking at 250 rpm until the OD_{600} of the culture reaches 0.4-0.6 (i.e., mid-log phase). Check the OD at 0.5-hr intervals, starting after 4 hr of incubation. It typically takes 6-8 hr for the culture to reach the desired OD.

Note: Stationary-phase bacterial cultures tend to lose F' episomes (Sambrook at al, 1989). The liquid culture should therefore be chilled to 4^0C and placed in storage at 4^0C before it reaches saturation.

c. Immediately chill the culture on ice and then store it at 4^0C for up to one week. This chilled, log-phase culture may be used for phage titering and plating.

B. Titering the Phage Display Library

Reagents and Materials Required:

- Prechilled, log-phase TGI liquid culture
- Sterile, 13 x 100-mm test tubes
- 2X YT liquid medium (Appendix A)
- Sterile, glass spreading rod or bent Pasteur pipette
- 2X YT/amp agar plates (at least 4 100-mm plates; Appendix A)

Note. Pre-warm the agar plates to 37^0C before use; make sure the agar surface is free of excess moisture droplets. To dry the plates, remove the lids and shake off excess droplets from the inside of the lids. Then place the agar plates-up-side-down and partially uncovered--in a 37^0C incubator to warm just prior to use. (Freshly prepared plates at room temperature will be warmed to 37^0C in 10-15 min; plates that have been stored at 4^0C will require about 1 hr to warm. Do not over dry the plates.)

Use the following protocol to titer the phage display library before you use it-even if you have obtained a premade (and pretitered) library.

Notes:

- If you have obtained a premade Phage Display Library, aliquot and store it at -70^{0}C before use it.
- Sometimes library titer can drop during prolonged storage. The titer of your library should be $\geq 10^8$cfu/ml to obtain optimal results in a phage display library panning.

1. Prepare ten-fold serial dilutions of the phage display library in 2X YT medium. You will need 100 µl of a 10^{-6}, 10^{-7}, a 10^{-8}, and a 10^{-9} dilution.

	Medium	Dilution factor
2 µl undiluted library stock	+ 198 µl	10^2
10 µl 10^{-2} dilution	+ 90 µl	10^3
10 µl 10^{-3} dilution	+ 90 µl	10^4
10 µl 10^{-4} dilution	+ 90 µl	10^5
10 µl 10^{-5} dilution	+ 90 µl	10^6
20 µl 10^{-6} dilution	+ 180 µl	10^7
20 µl 10^{-7} dilution	+ 180 µl	10^8
20 µl 10^{-8} dilution	+ 180 µl	10^9

2. Transfer 100 µl of the 10^{-6}, 10^{-7}, a 10^{-8}, and a 10^{-9} dilutions of the phage library to fresh, prelabeled tubes.
3. Add 100 µl of prechilled log-phase TG1 cells to each tube. Mix gently and incubate at room temperature for 5 min to allow infection; M13 phage adsorbs rapidly to TG1 cells.
4. Using a sterile glass spreading rod or bent Pasteur pipette, spread each phage/cell mixture on a 2X YT/amp plate. Set plates at room temperature for 5 min to allow the inoculum to be absorbed by the agar.
5. Incubate plates upside-down at 37^{0}C overnight.
6. Count the number of the colonies on the plate having between 30 and 300 colonies.

Note: Plating the infected cells on ampicillin-containing medium ensures that only cells harboring a pHD9 phagamid will grow. The low multiplicity of infection ensures that a host cell will be infected by only one phage. Thus, the titer (cfu/ml) of infected cells correlates to the titer (pfu/ml) of the library.

7. Calculate the titer (cfu/ml) of the infected cells, and hence of the phage

cfu/ml (may = pfu/ml) = [(# colonies on the plate)x dilution factor]/(0.5x 0.1ml)

C. Titering the M13KO7 Helper Phage Stock

Reagents and Materials Required:

- Prechilled, log-phase TGI cells in 2X YT

- Melted 0.7% top agarose (in 2X YT) equilibrated to 47^0C in a water bath or heating block; allow 3 ml per dilution to be plated (Recipe in Appendix A)

Note. When plating bacteria/phage mixtures using malted top agar. The malted top agar should be at 45-47^0C; higher temperatures will kill the bacteria.

- Sterile, 3 x 100-mm test tubes
- 2X YT agar plates (at least three 100-mm plates; Appendix A)

The helper phage titer sometimes drops during prolonged storage. Therefore, titer the helper phage M13KO7 stock provided before you use it to prepare the fresh helper phage stock.

1. Prepare ten-fold serial dilutions of the helper phage stock provided in 2X YT medium. You will need 100 µl of a 10^{-7}, a 10^{-8}, and a 10^{-9} dilution.

	Medium	Dilution factor
2 µl undiluted phage stock	+ 198 µl	10^2
10 µl 10^{-2} dilution	+ 90 µl	10^3
10 µl 10^{-3} dilution	+ 90 µl	10^4
10 µl 10^{-4} dilution	+ 90 µl	10^5
10 µl 10^{-5} dilution	+ 90 µl	10^6
20 µl 10^{-6} dilution	+ 180 µl	10^7
20 µl 10^{-7} dilution	+ 180 µl	10^8
20 µl 10^{-8} dilution	+ 180 µl	10^9

2. Transfer 100 µl of the 10^{-7}, 10^{-8}, and 10^{-9} dilutions of the helper phage to sterile, 13 x 100-mm tubes.
3. Add 100 µl of prechilled, log-phase TG1 culture to each tube from Step 2 above. Mix gently and incubate at room temperature for 5 min (infection; M13 phage adsorbs rapidly to TG1 cells).
4. To each tube of infected TG1, add 3 ml of melted top agarose (47^0C), mix gently, and immediately pour on a 2X YT plate. Gently rock the plate to evenly distribute the melted top agarose.
5. Allow the top agarose to harden at room temperature for 5 min. Then, invert the plates and incubate them at 37^0C for 8-10 hr or overnight.
6. Count the plaques to determine titer (pfu/ml)

Note: Because M13KO7 does not lyse the host cells, the plaques are cloudy rather than clear. They are small (~1-mm) circular areas of less dense bacterial growth, as seen against a bacterial lawn.

pfu/ml = [(# plaques on the plate) x dilution factor]/ (0.5 x 0.1 ml)

7. Use Parafilm to seal one of the plates having distinct plaques and store the plate at 4^0C. This is your working stock plate for helper phage preparation.

D. Preparation of M13KO7 Helper Phage Stock

Reagents and Materials Required:

• Fresh (<I -month-old) working stock plate of M13KO7

• Prechilled, log-phase TG1 cells

• 2X YT/kan medium (Appendix A)

You will need 3 ml of fresh helper phage stock (>I 0^9 pfu/ml) to carry out a complete phage display library screening experiment.

1. Use a sterile, yellow plastic pipette tip (bigger end) to pick up the agar plug containing a single, isolated plaque of helper phage M13KO7 from the working stock plate. Transfer the plaque to 3 ml of 2X YT/kan medium in a sterile 17 x I 00-mm tube.

Notes:

• Fresh plaques (<1 month old) will give best results.

• To ensure that you will obtain a successful phage culture, start two or three such cultures from isolated plaques.

2. Incubate at 37^0C for 12-16 hr (overnight) with shaking at 250-300 rpm.

3. Transfer the cells to a sterile centrifuge tube. Centrifuge at 12,000 x g for 2 min at 4^0C. Transfer the supernatant to a fresh, labeled tube and store at 4^0C for up to 1 year.

4. Measure the titer of the M13KO7 stock by plaque formation on a TGI lawn described as before.

E. Coating Support Surface with Bait Protein

Many different types of substances may be attached to a solid surface for use as bait ligands in a phage display experiment (see Section I for examples and references). The method used to attach the ligand to the surface depends on the nature of the ligand. We provide a typical protocol for coating polystyrene microwells with a bait protein. However, the procedure may need to be modified for certain types of bait proteins or for nonproteinaceous baits. For information about coating surfaces with bait ligands, refer to a source book on ELISA methods (e.g., Harlow & Lane, 1988; Kerr & Thorpe, 1994).

Proteins and peptides are most commonly used as bait material. Most peptides are inherently less stable than most proteins and must be treated with care to avoid degradation during the coating and panning procedures. DNA, RNA, and oligonucleotide bait ligands are typically conjugated to a protein carrier (such as BSA), and the protein carrier is then attached to the panning surface according to the guidelines for unconjugated protein.

Whole cells in suspension or cell membranes may be used as a bait source for panning in solution.

Reagents and Materials Required:

- Bait (protein) solution, diluted in PBS (PBS recipe in Appendix A)
- Flat-bottom, 96-well polystyrene microliter plate or multiwell strips to coat with the bait for panning

Note: Multiwell plates and strips are available from several commercial suppliers. We recommend the high-capacity LabSystems ELISA plates (Cal.# 950-2920-00p), which are pretreated for high-capacity protein binding. Note that plates from other manufacturers may behave differently (e.g., with respect to the adsorption of the bait substance and nonspecific binding).

• Wash buffer: PBS containing 0.05% Tween-20
• Blocking buffer: Wash buffer containing 1% BSA

1. If the bait protein is more concentrated than 10 mg/ml, dilute it to this concentration in 1X PBS.
2. Coat each well with 50-200 µl of solution (equivalent to 0.1-1 µg of protein).
3. Incubate at room temperature for 2 hr, or at 4^0C overnight (16 hr).

Note: Use 4^0C incubation if the bait protein is unstable at room temperature.

4. Discard or save the coating solution and wash the wells three times with wash buffer.
5. Add 200 µl of blocking buffer to each well.
6. Incubate at room temperature for 2 hr, or at 4^0C overnight (16 hr).

Note. Use 4^0C incubation if the bait protein is unstable at room temperature.

7. Discard blocking buffer and wash the wells three times with wash buffer.
8. Dry the plate and wrap it in plastic wrap. Store at 4^0C for up to 1 month.

Note: Most peptides and some bait proteins are not stable enough to store and should be used immediately.

IV. Panning, Phage Rescue, and Superinfection

A. Reagents and Materials Required

- Phage display library
- M13KO7 helper phage
- TG1 (log phase cells)
- Microtiter plate wells precoated with the desired bait material
- PBS (Appendix A)
- Wash buffer: PBS containing 0.05%Tween-20
- Blocking buffer: washing buffer containing 1%BSA
- 2x YT/amp/kan broth (Appendix A)
- 2x YT/glucose broth (Appendix A)
- Ampicillin stock solution (50 mg/ml)
- Sterile 20% PEG 8000 solution (Appendix A)
- For tittering the purified, enriched phage library: 2x YT/amp plates (Appendix A)
- For long-term storage of enriched TG1 library and enriched purified phage library: Sterile glycerol solution (at least 50% v/v)

B. Panning Protocol

1. Add library aliquot to the bait-coated well

- For the first panning cycle, use an aliquot of the original library. Panning 10^9 library clones against one coated well (1 cm^2) is usually sufficient to identify a cDNA for a rare or moderately abundant transcript. For example, for a phage display cDNA library with a titer of 10^{10} cfu/ml, dilute 10 µl of the library in 90 µl of blocking buffer and apply this to the well. More phage display cDNA library can be used for panning.
- For subsequent rounds of panning, use 50 µl of purified, enriched phage library from previous panning.

2. Incubate the library on the coated surface at 37^0C for 1 hr. Alternatively, for less stable proteins, incubate at room temperature (25^0C) or 4^0C for up to 4 hr.
3. Remove the spent library and save it if you wish. Wash the coated surface at least 5 times with wash buffer (~200 µl each wash).

Note: use more washes (up to 20 times) for higher stringency binding conditions.

C. Rescuing Trapped Phage

1. Add 0.1 ml of prechilled, log-phase TG1 cells to the panning well.
2. Incubate at 37^0 C for 30 min. Immediately plate on ice or 4^0C.

Note: Longer incubation at 37^0C will allow the infected TG1 cells to duplicate and will make it more difficult to use cell number to estimate percent of phage rescued.

If you have just completed the first round of panning, this is the **1st round enriched TG1 library**. If you have just completed the second round of panning, this is the **2nd round enriched TG1 library**, etc.

3. (recommended) Dilute a small aliquot of the enriched TG1 library and plate it out to determine the titer and estimate recovery of trapped phage.
4. Transfer the enriched TG1 library to fresh microcentrifuge tubes. Store at 4^0C for up to 3 days, or add sterile glycerol to a final concentration of 15%, and store at –70^0C for up to 1 yr. If you plan to repeat the panning within 3 days, keep 50 µl of the enriched TG1 library at 4^0C and store the rest at –70^0C.
5. If this was your first or second round of panning, repeat the panning process (Section IV.B). If this was your third or fourth round of panning, proceed to Section V (ELISA Screening of Enriched Library).

D. Preparation of Enriched Library for Repeat Panning

1. If you have not done so, transfer 50 µl of the enriched TG1 library to a sterile, 17x 100-mm tube. Store the rest at –70^0C.
2. Add 1 ml of 2x YT/glucose to the 50-µl aliquot of enriched TG1 library.
3. Incubate at 37^0C for 1 hr with shaking at 250 rpm.

4. Add ampicillin to a final concentration of 100 µg/ml. Immediately add 5x 10^9 pfu of M13KO7 (~0.1 ml of fresh phage stock) to the cell culture (superinfection step).
5. Incubate at 37^0C for 1 hr with shaking at 250 rpm.
6. Transfer culture to a centrifuge tube. Centrifuge at 2,000 x g for 10 min at 4^0C or room temperature. Remove and discard the supernatant.

Note: Be sure to remove all the supernatant. Trances of glucose will repress the expression and hence, display, of fusion proteins.

7. Resuspend the cell pellet in 10 ml of 2x YT/amp/kan.
8. Incubate at 37^0C overnight with shaking at 250 rpm.
9. Centrifuge culture at 10,000 x g for 20 min at 4^0C. Transfer the supernatant (which contains the enriched phage library) to a fresh tube.
10. Recentrifuge the supernatant at 10,000 x g for 10 min at 4^0C to further clarify it. Transfer the supernatant to a sterile polypropylene tube: this is the enriched library in a crude phage preparation.
11. Purify the enriched phage library

a. Add 10 ml blocking solution to 10 ml of the enriched phage library (Use an equal volume).
b. Incubate at 4^0C for 10 min.
c. Add 4 ml of 20%PEG solution. Incubate at 4^0C for 30 min to precipitate the phage.
d. Centrifuge at 10,000 x g for 20 min at 4^0C. Discard the supernatant and resuspend the pellet in 1 ml of PBS.
e. Transfer the resuspended pellet to a sterile microfuge tube and centrifuge at 14,000 rpm (top speed) for 10 min at 4^0C. This time, save the supernatant: this is the **enriched (purified) phage library**.
f. (recommended) Titer the enriched phage library.

12. The enriched phage library may be stored at 4^0C for up to 1 month. For long-term storage (1 year), add sterile glycerol to a final concentration of 20%, and store at -70^0C.

Note: You will need 50 µl of the enriched phage library for the next round of panning.

13. If you have performed only one or two rounds of panning, then return to repeat the panning cycle. We suggest to perform fourth round of panning.

V. ELISA Screening the Enriched Phage Library

A. Reagents and Materials Required

- M13KO7 helper phage
- Anti-pVIII antibody
- HRP-conjugated secondary antibody
- HRP substrates
- Enriched phage library
- ELISA plate wells precoated with desired bait
- 2x YT/amp/glucose/MgCl2 agar plates

- 2x YT/amp/glucose agar plates
- 2x YT/amp/kan agar plates
- Glycerol solution
- PBS
- Wash buffer
- Blocking buffer
- 20% PEG solution
- 1.5-ml microcentrifuge tubes
- (Optional) 1 N H2SO4 and an ELISA plate reader, such as the ThermoMax Plate Reader from Molecular Devices.

B. Preparation of Candidate Phage Clones for ELISA

1. If you have not done so already, dilute an aliquot of the enriched TG1 library after the third or fourth panning and plate out each dilution on 2x YT/amp/glucose/MgCl2 for single colonies. Incubate at 37^0C overnight.
2. Add 400 µl of 2x YT/amp/glucose medium to prelabeled 1.5-ml microcentrifuge tubes. Label these tubes as "**PC1**" +a number designate the individual clone.
3. Using sterile tip, pick 44 – 88 well-isolated, single colonies from the plate above and use each to separately inoculate the medium in the small tubes.
4. Incubate tubes at 37^0C overnight with shaking at 250 rpm. These are the master stocks of individual phagemid clones in TG1; we refer to these cultures as "**PC1**".
5. Transfer 50 µl of each overnight PC1 culture to a fresh tube prelabeled "PC2" + a number designate the individual clone. To the remaining 350 µl of PC1 culture, add sterile glycerol (final concentration 15%) and store at $–70^0$C. Save all PC1 culture until you have obtained the ELISA results.
6. Add 400 µl of 2x YT/amp/glucose medium containing 5x 10^8 pfu of M13KO7 helper phage (~ 10 µl of fresh phage stock) to each labeled PC2.
7. Incubate tubes at 37^0C for 1-2 hr with shaking at 200 – 250 rpm.
8. Centrifuge samples at 14,000 rpm for 5 min at 25^0C.
9. Discard the supernatant. Resuspend each cell pellet in 400 µl of 2x YT/amp/kan.

Note: Be sure to remove all the supernatant. Trances of glucose will repress the expression and hence, display of fusion proteins.

10. Incubate the tubes at 37^0C overnight with shaking at 250 rpm. These are the crude supernatant of the superinfected individual phagemid clones; we refer these as PC2.
11. Purify phage clones

a. Centrifuge PC2 tubes as Step 8 above.

b. Transfer 300 µl of each supernatant (which contains the phage) to fresh tubes prelabeled "PC3" + a number designate the individual clone.

c. Add 300 µl of blocking buffer.

d. Incubate at 4^0C for 10 min.

e. Add 120 µl of sterile 20% PEG solution to each tube.

f. Incubate at 4^0C for 30 min.

g. Centrifuge at 10,000 xg for 20 min at 4^0C.

h. Discard the supernatant and resuspend the phage pellet in 100 µl of PBS. These are the individual purified phage clones; we refer to these preparations as "**PC3**". PC3 phage preps may be stored at 4^0C for 1 month. For longer storage (up to 1 year), add glycerol to 20% final concentration and place at -70^0C.

C. ELISA Screening

1. Apply 50 µl of each PC3 phage prep to a well precoated with your bait protein.
2. Incubate at 25^0C for 2 hr. Use a 4^0C incubation overnight if bait is unstable.
3. Remove the phage solution and wash the wells three times with wash buffer (~200 µl each wash).
4. Dilute the anti-pVIII antibody 1:50 of in blocking buffer. Add 50 µl of diluted anti-pVIII antibody to each well of the plate.
5. Incubate the plate at 25^0C for 1 hr. Remove the primary antibody and discard it.
6. Wash the plate three times with wash buffer (~200 µl each wash).
7. Dilute the HRP-conjugated second antibody 1:50 in blocking buffer. Do not use sodium azide in the blocking solution; it will inhibit HRP activity.
8. Add 50 µl of the diluted HRP conjugate to each well.
9. Incubate the plate at 25^0C for 1 hr. Remove the second antibody and discard it. Wash the plate three times with wash buffer (~200 µl each wash).
10. Prepare the HRP substrate solution: mix 1 part of solution A and 1 part of solution B. Add 50 µl of the substrate solution into each well.
11. Incubate the plate at 25^0C until a suitable blue color has developed. For phage library clones, the color development can take up to 1 hr, depending on the affinity of the displayed protein for the bait.
12. Stop the color reaction in all the wells at the same time by adding 10 µl of 1 N H2SO4 to each well. The blue wells will turn to yellow. Read the absorbance at 405 or 410 nm (preferably) on a plate-reader or spectrophotometer.
13. On the basis of the ELISA results, decide which of the PC1 and PC3 clones you wish to keep and discard the rest. Keep all of the positive clones and a few of the negative clones for later use as controls.

VI. Analysis of Positive Clones

A. Eliminate clones that do not specifically interact with the bait

Some of the clones identified as positives in the ELISA may bind to non-bait components in the bait preparation. Measure to eliminate these clones depend on the nature of the bait used. For

example, if your bait protein was generated as GST or MBP fusion, test your positive clones for binding to wells coated with GST or MBP moiety only. To reduce these clones, the phage library should incubate with GST or MBP moiety only before panning.

B. Isolation of Plasmid from Positive Clones

Recombinant phagemid (plasmid) vector can be isolated from the positive PC1 clones. Grow the positive clones in 2x YT/amp medium. Isolate plasmid using a standard mini-prep method or commercial available kit.

C. Sequencing

The isolate plasmid can be sequenced with two provided primers.

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Appendix A   Media and Solution Formulations

Basic Medium

- **LB broth**

  10 g Bacto-tryptone

  5 g Bacto-yeast extract

  10 g NaCl

  Deionized, distilled H2O to a total volume of 1 liter.

  Adjust pH to 7.0 with 5N NaOH. Autoclave. Allow medium to cool to ~55^{0}C before adding ampicillin, kanamycin, IPTG, or MgCl2.

- **LB agar plate**

  Prepare LB broth as above, but add Bacto-agar (18g/L) before autoclaving. Cool to 50^{0}C. Pour plate using 25-30 ml per 100-mm plate. Store sleeved at 4^{0}C.

- **2x YT broth**

  17 g Bacto-tryptone

  10 g Bacto-yeast extract

  5 g NaCl

  Deionized, distilled H2O to a total volume of 1 liter.

  If necessary, adjust pH to 7.0 with 5N NaOH. Autoclave. Allow medium to cool to ~55^{0}C before adding ampicillin, kanamycin, IPTG, or MgCl2.

- **2x YT agar plate**

  Prepare 2x YT broth as above, but add Bacto-agar (18g/L) before autoclaving. Cool to 50^{0}C. Pour plate using 25-30 ml per 100-mm plate. Store sleeved at 4^{0}C.

- **2x YT Top Agarose**

  Prepare 2x YT broth as above, but add agarose (7 g/L) before autoclaving. Cool to 47^{0}C before using to plate cell/phage mixtures.

- **M9/+Thi minimal medium**

  For nutrition selection of E.coli strains (such as TG1) carrying F' episomes with the proAB genetic marker.

  Prepare 1 liter of M9 medium as directed in Sambrook et al. (1989). To prepare agar plate, add agar (20 g/L) prior to autoclaving. After autoclaving, allow medium to cool to 55^{0}C. Then add 1 ml of 1 M thiamine-HCl stock.

Medium With Additives

Prepare medium according to one of the basic recipes above. After autoclaving medium, cool to 55^{0}C and add one or more of following components to the indicated final concentration:

- **Ampicillin (amp; 50 µg/ml)**

  To select for E.coli host cells transformed (or transduced) with pHD9 derived phagemides.

- **Glucose (dextrose; 2%)**

To suppress fusion protein expression from Plac promoter of pHD9; used to reduce possible toxicity effects of fusson protein on host cells.

- **MgCl2 (10 mM)**

  To increase efficiency of phage adsorption to host cells (infection).

- **Kanamycin (kan; 50 µg/ml)**

  To select for host cells tranduced with M13KO7 helper phage.

Stock Solutions

- **Ampicillin (50 mg/ml in distilled water)**

  Sterilize by filtration and store at -20^{0}C.

- **Glucose (40%)**

  Filter sterilized

- **Kanamycin (25 mg/ml in distilled water)**

  Sterilize by filtration and store at -20^{0}C.

- **MgCl2 (1 M)**

  Filter sterilized

- **Thiamine-HCl (1 M)**

  Filter sterilized

Other Solutions

- **Wash Buffer**

  PBS (Sambrook et al.,1989) contains 0.05% Tween-20.

- **Blocking Buffer**

  Wash buffer contains 1% BSA (w/v).

- **20% PEG (polyethylene glycol; avg. mol. Wt. = 8000)**

  Dissolve 100 g of PEG and 73 g of NaCl in 500 ml of distilled water. Heat up to 50^{0}C to dissolve and autoclave.

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