EZnet™ Phage Display cDNA Library

Construction Kit (PDL-5001)
Screening Kit (PDL-3001)

Instruction Manual

version 3.2
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Introduction

Maxim’s EZnet™ Phage Display Libraries, Library Screening, and Detection/Expression kits provide a convenient and sensitive method to isolate rare and low-copy-number cDNA. Our system maximizes the benefits of in vivo and in vitro technologies to ensure you success in locating the cDNA transcripts of your interest. We offer a complete, high-titer phage display library which is ready to be screened with our Library Screening kit or you may choose to create your own library. The protein-ligand complex can be detected by screening the library with MBI’s Screening kit. Screening of phage display libraries provides a means to locate the cDNA which encodes the protein. EZnet™ cDNA Libraries are constructed in pHage3.2 vector. The cDNA/gene III fusion products are displayed on the filamentous phage particle tip, which makes interaction with an immobilized bait ligand easy. Several rounds of biopanning enrich for the interacting clones and then ELISA can be used to identify individual positive clones and confirm positive interactions.

EZnet™ Phage Display cDNA Libraries are ready-to-screen by panning. With their high complexity (1010 pfu/ml), EZnet Libraries enhance the possibility of finding cDNAs for rare or low-copy-number transcripts. You can conveniently screen up to 10⁸-10¹⁰ phage clones in one panning experiment. Premade EZnet Libraries are made from poly A+RNA isolated from a wide selection of human tissues and cell lines.

EZnet™ Phage Display Libraries Screening Kit includes all the necessary materials you need for performing the library panning and ELISA procedures. We also include control reagents so you can familiarize yourself with the screening procedure before you try it with the library and your bait. The control system includes microtiter wells precoated with a control bait protein (the bcl-2 fusion proteins), a positive control phage clone known to interact with the control bait, and a negative control (noninteracting) phage clone. The EZnet™ Library Screening Kit and protocol have been optimized for use with EZnet™ Libraries.

EZnet™ Phage Display Expression Kit provides the HB2151 nonsuppressor E. coli host strain for in vivo expression of Myc-tagged proteins without the pIII (gene III coat protein) fusion moiety. This kit also provides the materials you need for detecting Myc-tagged proteins, including a titered anti-Myc monoclonal antibody (mAb), and myc-tag based protein purification system. This kit has been optimized for use with EZnet Phage Display Libraries.

Panning

Panning is used to enrich libraries for specific clones by exposing pooled phage clones to a bait-coated well followed by a series of washings to remove unbound phage particles (Kay et al, 1996). All phages that express a protein that can interact with the bait will adhere to the well. The TG1 host cells are then applied to the well so they can become infected by the bound particles. Since the presence of ampicillin favors plasmid amplification, recombinant phagemids are then amplified by growing transformants on an ampicillin-containing medium. To maximize enrichment M13 phage particles are superinfected with helper phage and phagemid DNA. The entire panning process can be executed in one well of a 96-well microtiter plate and can be completed in only one day.
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Positive clones and interactions can be confirmed by screening individual clones by ELISA; using the same bait ligand as in the panning. Detection of bound phae is achieved by applying an antibody against the pVIII protein. Additionally, positive protein-ligand interactions can be further verified by using an anti-myc monoclonal antibodies in ELISA and molecular weight of fusion proteins can be determined by Western Blot. Then, myc-tagged proteins can be over-expressed from the original vector and purified with an anti-myc mAB affinity column. It is also possible to perform additional studies on the purified Myc-tagged proteins.

Vectors:

Maxim has designed a multifaceted, M13-derived phagemid vector: pHage 3.2. Vector carries the pUC replication origin and the ampicillan resistance gene which permits growth and selection. They also contain an amber stop codon between the myc-tag sequence and the pIII gene which allows for heproduction of geneIII fusion proteins. The gene III leader sequence is strategically fused to the N-terminus so that the gene III fusion proteins can be secreted into the periplasmic space (Kang et al., 1991).

Helper phage:

The helper phage M13K07 conveniently infects TG1 cells, that have been transformed with phage2.1 or pHage 3.2, at room temperature or 37. The helper phage express wild-type gene III proteins and secrete them into the periplasmic space where they are packaged into phage particles with pIII fusion proteins. Transcription is under the control of the lac promoter which keeps fusion gene transcription levels low in the absence of IPTG. It is important to maintain low transcription levels so that only one or two copies of the pIII fusion proteins are displayed on each phage particle surface. The presence of multiple copies of wild-type pIII coat proteins ensures efficient packaging and infectivity. Each vector contains an M13 replication origin so in the presence of M13K07 helper phage the phagemid is replicated as a normal M13 phage.

After a positive clone has been identified and isolated, the foreign protein can be directly overexpressed from pHage3.2 without the pIII fusion moiety in the nonsuppressing E.coli host strain HB2151. High-level expression is inducible with IPTG. No subcloning is necessary, and no proteolysis is required to separate the cloned protein form pIII. In addition, because the foreign protein is still fused to the gene III leader sequence, the expressed protein is secreted into the periplasm and, in some cases, can be purified directly from the culture medium without lysing the cells.

Low-valency, gene III fusion system:

pIII is a minor coat protein that is usually located in groups of five at one end of the filamentous rod. pIII is an integral component in the adsorption of the virus particle to the bacterial pilus (Sambrook et al, 1989). Since only a few copies of the hybrid pIII molecules are produced by each phage particle, large fusion moieties can be put onto pIII without causing steric interference with protein-ligand interactions.
Introduction

The low valency of pIII hybrids tends to select for high-affinity interactions (e.g., Kd’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 (see Section VIII, Troubleshooting Guide). Another possible limitation of this system is the full-length cDNA 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.

Applications:

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 cell’s 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 geneIII 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 use this powerful technology to identify sequences that are expressed in nature and presumably encode proteins with specific biological functions. For example, an allergenic protein form Aspergillus fumigatus was identified (and its cDNA cloned) by panning an A. fumigatus cDNA phage display library against immobilized serum immunoglobulin E from sensitized individuals (Cramer 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; Mc Cafferty 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 1010 independent clones in small aliquots (Marks et al., 1992; Bass et al., 1990; Jacobsson & Fryberg, 1995; Gram et al., 1993; McCafferty et al., 1991; Cramer et al., 1994; Cramer & Suter, 1993). Phage display and yeast two-hybrid library screening are complementary approaches for identifying novel proteins that interact with known bait. The EZnet Phage Display Library Screening Kit and Libraries are the system of choice if your application involves:
Introduction

- √ looking for protein-protein interactions that normally occur on the cell surface or in the cytoplasm;
- √ 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 post-translational modifications unique to eukaryotes, or that are particularly weak or transient, you should use a MATCHMAKER Two-Hybrid System (Allen et al., 1995). Phage display library screening requires a small amount of bait material (preferably purified), which is not required for a two-hybrid library screening. However, phage display library screening requires much less bait than plaque screening on filters using a conventional immunoscreening procedure.
## Product Information

EZnet™ Library Screening Kit (Cat. No.: PDL-3001)

### Components

The materials provided are sufficient for probing 88 bait wells and 8 total control wells in 96-well microtiter plates.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control phage clone (BAD)</td>
<td>Interacts with the bait protein bcl-2 (10⁹ cfu/ml in PBS/20% glycerol)</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Negative control phage clone</td>
<td>Does not interact with bait (10¹⁰ cfu/ml in PBS/20% glycerol)</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>M13K07 helper phage</td>
<td>10¹⁰ pfu/ml in PBS/20% glycerol</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Strips coated with bcl-2 positive control protein</td>
<td>Interacts with provided positive phage</td>
<td>1 strip</td>
</tr>
<tr>
<td>Anti-pVIII polyclonal antibody</td>
<td>Raised in goats (50 μg/ml)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>HRP-conjugated antibody</td>
<td>Rabbit anti-goat; 50X</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>HRP substrate A</td>
<td>Includes H₂O₂</td>
<td>6 ml</td>
</tr>
<tr>
<td>HRP substrateB</td>
<td>Solution B; includes 3,3',5,5'-tetramethyl-benzidine [TMB]</td>
<td>6 ml</td>
</tr>
</tbody>
</table>
EZnet™ Library Construction Kit (Cat. No.: PDL-5001)

Components:

Materials necessary are provided to construct phage-display library.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHage 3.2 Vector</td>
<td>Phage-display Vector (0.1 ug/ul)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TG1</td>
<td>E. coli host cells in Stab agar</td>
<td>1 vial</td>
</tr>
<tr>
<td>HB2151</td>
<td>E. coli host cells in Stab agar</td>
<td>1 vial</td>
</tr>
<tr>
<td>M13K07 helper phage</td>
<td>$1 \times 10^{10}$ pfu/ml in PBS/20% glycerol</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Manual</td>
<td></td>
<td>one copy</td>
</tr>
</tbody>
</table>

Kit Storage:

Short-term storage: Less than 3 months. Store components at 4°C.
Long-term storage: Greater than 3 months. Store host cells at 4°C. Store all other components at -70°C.

Recommendations:

**Host strain:** TG1 can also be used to titer the library and the helper phage. Upon arrival, prepare a TG1 stock culture in 20% glycerol and store at -70°C. Also, maintain a working stock plate on an M9/+ Thi minimal medium to keep selection on the F’ episome. The TG1 genotype: supEthi-1((lac-proAB) hsd(5[F’ traD36+ proAB+ lacIq lacZ(M15] (Sambrook et al., 1989).

**Host strain:** HB2151

Prepare a stock culture in 20% glycerol for long-term storage and maintain a working stock plate on an M9/+ Thi minimal medium.
The HB2151 genotype: nalr thi-1 ara ( (lac-proAB [F’ proAB+ laciq lacZ(M15]

**Helper Phage:** M13K07. Prepare a fresh stock of helper phage before procedure.
EZnet Phage Display Libraries

Components:

Materials are sufficient for up to 20 primary library pannings using 20 bait-coated wells in a 96-well microtiter plate (or an equivalent surface area).

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage library</td>
<td>Dissolved in PBS/20% glycerol</td>
<td>0.2ml</td>
</tr>
<tr>
<td>TG1</td>
<td>E. coli host cells; lyophilized from 1 ml of saturated culture</td>
<td>1 vial</td>
</tr>
<tr>
<td>M13K07 helper phage</td>
<td>1x10^10 pfu/ml in PBS/20% glycerol</td>
<td>0.5ml</td>
</tr>
<tr>
<td>PEG solution</td>
<td></td>
<td>1 vial</td>
</tr>
</tbody>
</table>

Library Storage:

Short-term storage: Less than 3 months. Store components at 4°C.

Long-term storage: Greater than 3 months. Divide the library into working aliquots (~20µl each) and store at -70°C. Store all other components at -20°C. Avoid freeze/thaw cycles.

Recommendations:

Titer: The titer is the number of colony-forming units (cfu) per ml of culture. Retiter the library upon arrival to verify the titer since the titer value stated on the label is the titer at the time of construction. Titers are usually stable at -70°C for at least one year, but may drop slightly when stored at 4°C. If the library titer is at least 10^8 pfu/ml, it is representative. However, if it drops below 10^8 cfu/ml, repeat the titer. If the titer is still low, please contact our Technical Support Department immediately.

Host strain: TG1 can also be used to titer the library and the helper phage. Upon arrival, prepare a TG1 stock culture in 20% glycerol and store at -70°C. Also, maintain a working stock plate on an M9/Thi minimal medium to keep selection on the F’ episome. The TG1 genotype: supEthi-1((lac-proAB) hsd(5[F’ traD36+ proAB+ lacIq lacZ/M15]) (Sambrook et al., 1989).

Helper Phage: M13K07. Prepare a fresh stock of helper phage before each panning procedure.
**Library Information**

**Library Construction:**

cDNA is synthesized using random primers and a modified Gubler & Hoffman procedure (1983). The cDNA is size-fractionated by gel electrophoresis; the 0.3-3.0 kb fraction is extracted. The cDNA is blunt-ended and ligated into the EcoRV site of pHage3.2. Recombinant phagemids are packaged into infectious M13 phage particles upon superinfection with helper phage M13K07. The phage is precipitated from supernatants and resuspended in PBS/20%/glycerol at a high titer (>10^{10} cfu/ml).

The primary library (see Figure 2) is amplified by growth of the E. coli culture in liquid medium for 3 hr before it is frozen or superinfected with M13K07 helper phage. The superinfection and packaging in M13 also amplify the library. No further amplification of the library is performed or recommended.

**Library Quality Control:**

**Independent clones:**

The number of independent clones is equal to the number of recombinant ampicillan resistant colonies that were produced when the vector was transformed into TG1.

**Library titer:**

Library titer (generally >10^{10} cfu/ml) is determined after the phage particles are precipitated with PEG and resuspended in PBS/20% glycerol. Although the library is provided in the form of M13 phage particles, the titer is given in units of cfu/ml (rather than pfu/ml) because pHage3.2 phagemids are not packaged and do not form plaques in the absence of helper phage.

**Insert:**

The average insert size and range are estimated by running the cDNA on a gel prior to cloning and comparing the profile to MW size markers. The insert size of 15 randomly selected clones is determined by PCR using primers designed for this vector.

Note that even though the libraries are constructed using size-selected cDNA, some of the inserts may be <300 bp.
Library Construction

Note: The methods for phage-display library construction in this manual is started from ds DNA.

**DNA Fragmentation:**

1. ds cDNA is synthesized using random primers and a modified Gubler & Hoffman procedure (1983). Fragmentate cDNA or genomic DNA by sonication at 4°C using a microprobe. The optimal conditions for sonication should be worked out in each laboratory to obtain suitable size of DNA. The blunt-ends cutter enzymes can be used for DNA fragmentation. The sticky-ends cutter enzymes may also be used for DNA fragmentation if proper strategy can be figured out here.

2. Isolate the fragment from 500bp to 3000kb by gel electrophoresis or sizing column.

3. Polish the blunt-end of DNA fragments using Klenow of DNA Pol. I. Set up reaction as follows:
   - cDNA fragments x ul
   - 2 mM dNTPs 1 ul
   - 10X Klenow Buffers 2.5 ul
   - Klenow 2-5 u
   - H2O add to final 25 ul
   - Incubate for 30 min. at 37°C.

4. Extract above DNAs twice with phenol, pheno/chloroform, chloroform.

5. Precipitate DNAs with ethanol. It may be necessary to use glycogen as a carrier to precipitate DNA. Don't use tRNA as a carrier because tRNA may be ligated into the vector.

   - Add 1/10 volume of 3M sodium acetate (pH5.5) and 2 volumes of cold ethanol
   - Mix well and store 30 min. at -20°C.
   - Centrifuge for 30 min. at 12,000 x g.
   - Discard supernatant.
   - Rinse the DNA pellet with cold 70% ethanol, and centrifuge for 10 min. at 12,000 x g.
   - Dry DNA pellet and resuspend in a suitable volume of H2O.
Library Construction

(continue)

Ligate DNA Fragments into Vector:

(1) Digeste pHage 3.2 vector with EcoRV to generate blunt-ended vector. Or proper sticky-end enzymes if applicable.

(2) Dephosphorylate linear vector with alkaline phosphatase under recommended condition.

(3) Extract the digested vector DNAs twice with phenol, pheno/chloroform, chloroform.

(4) Incubate for 30 min. at 37°C.

(5) Extract above DNAs twice with phenol, pheno/chloroform, chloroform.

(6) Precipitate DNAs with ethanol. Add 1/10 volume of 3M sodium acetate (pH5.5) and 2 volumes of cold ethanol Mix well and store 30 min. at -20°C. Centrifuge for 30 min. at 12,000 x g. Discard supernatant. Rinse the DNA pellet with cold 70% ethanol, and centrifuge for 10 min. at 12,000 x g. Dry DNA pellet and resuspend in a suitable volume of H2O.

(7) Ligate DNA fragments into the vector at ratio 2:1. (We recommend to use high concentration of T4 ligase from Bio-lab)

(8) Transform the ligation reaction into TG1 cells by electroporation.

Transformation (electroporation)

(1) Thaw competent TG1 cells on ice or use freshly made competent cells.

(2) Add 1 ul DNA vector (salt-free) in 50 ul of cells and transfer to a pre-chilled 0.2 cm cuvette. Place on ice for 1 min.

(3) Program electroporator according to Manual. Dry outside of the curette with Kimwipes. Place it in the electroporator chamber. Pulse once.

(4) Immediately add 0.95 ml 2x YT medium containing 2% glucose to the curette and cover to resuspend the cells. Transfer the transformed cells to a 10 ml disposable culture tube.

(5) Grow the cells for 1 hour at 37°C with shaking at 225 rpm.
Library Construction

(continue)

5) Grow the cells for 1 hour at 37°C with shaking at 225 rpm.

6) Take 10 ul for determination of independent clones.

7) Grow the rest of cells for 6 hours in 2x YT containing 2% glucose & 100 ug/ml ampicillin. This is primary library. It may be stored at 4°C for a few days.

8) Take 0.5 ml library and add glycerol to final concentration 20%. Stored at -70°C until use.

Rescue of Recombinant Phage-display cDNA Library

1) Add 9.4 ml 2x YT containing 2% glucose & 100 ug/ml ampicillin and 100 ul 10^{10} pfu M13KO7 to the rest 0.5 ml library culture above (or take library stock from -70°C) and continue to grow for 1-2h.

2) Centrifuge the culture at 2,000 x g for 10 min. Discard the supernatant.

3) Resuspend the cell pellet in 10 ml of 2x YT containing 100 ug/ml ampicillin & 100 ug/ml kanamycin.

4) Incubate at 37°C for 12-20 h (overnight) with shaking at 250-300 rpm.

5) Centrifuge at 10,000 x g for 20 minutes. Take the supernatant and recentrifuge. Note: The supernatant contains the recombinant phage.

6) Transfer the supernatant to a sterile tube and store at 4°C or proceed panning. The typical phage yield is 10^{10} to 10^{11} ampicillin-transducing units per ml. For long-term storage, the phage-display library should be stored in 20% glycerol at -70°C.

Determination of Independent Transformation Clones

The number of independent clones is the number of recombinant ampicillin-resistant colonies when the vector transformed into TG1 cells. An average of 10^6 independent clones are usually desired.

Calculation:
# of independent clones = # of colonies x dilution factor x 10 x 10
I. Plating, Storing and Culturing

A. Reconstitution
Reconstitute the lyophilized culture of E. coli provided with your EZnet™ Kit or Library.
To reconstitute lyophilized cultures:
1. Add 1 ml of 2X YT
2. Incubate on shaker (250rpm) overnight at 37°C.
3. Use this culture to prepare a glycerol stock culture for freezing and a primary streak plate.

B. Glycerol Stock Culture
To prepare a glycerol stock culture, remove 0.75 ml of the overnight-reconstituted culture from Step I above to a fresh, pre-labeled tube. Add glycerol to a final concentration of 20% and store at -70°C.

C. Primary streak plate
Using a sterile loop, streak out the overnight culture from Step I above on a 2X YT agar plate for single colonies. Incubate at 37°C overnight.
Wrap the primary streak plate in Parafilm and store it at 4°C. Prepare a fresh primary plate from the frozen glycerol stock at 3-month intervals (or sooner if contamination is evident).
To recover the frozen cells, streak a small portion (~5ul) of the frozen glycerol stock onto a 2X YT plate. Incubate at 37°C overnight and proceed as in Step III above.

D. Working Stock Plate
To prepare a working stock plate, pick a single, isolated colony from the primary streak plate and streak it on an M9/+ Thi plate. Incubate at 37°C overnight. Wrap the plate in Parafilm and store at 4°C for up to 2 weeks. This plate will be your source of fresh colonies for inoculating liquid cultures and for preparing your next fresh working stock plate.
Be sure to prepare a fresh working stock plate each week 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.

E. Log-phase liquid culture
Choose an isolated colony from the working stock plate and use it to inoculate 5 ml of 2X YT (or LB) broth in a 50-ml test tube and incubate at 37°C with shaking at 250 rpm until the OD600 reaches 0.4-0.6 which is mid-log phase (approx. 6-8hr). After 4 hours of incubation, check the OD every half hour.
Note: Stationary-phase bacterial cultures tend to lose F’ episomes (Sambrook et al., 1989). Therefore, culture should be chilled to 4°C and placed in storage at 4°C before it reaches saturation.
Immediately chill the culture on ice and then store it at 4°C for up to one week. This chilled, log-phase culture may be used for phage titering and plating.

Additional Information:
The genes coding for enzymes involved in proline biosynthesis have been deleted from the chromosomes of TG1 and B2251. Thus, only those bacteria carrying the F’ plasmid [F’ traD36 proAB+ lacIq lacZ(M15)] or [F’ proAB+ lacIq lacZ(M15)] will form colonies on medium lacking proline.
Bacteria grown on minimal medium plates will not survive long at 4°C. Also, bacteria grow much slower on minimal plates.
Titering

II. Titering: Host cells

Required Reagents & Materials

- Prechilled, log-phase TG1 liquid culture
- Sterile, 13 X 100-nm test tubes
- 2X YT liquid medium (Appendix A)
- Sterile, glass spreading rod or bent Pasteur pipette
- Four 2X YT/amp agar plates (100 nm plates)

Preparation:

1. Always prewarm agar plates to 37°C; 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 37°C incubator to warm just prior to use. (Freshly prepared plate at room temperature will be warmed to 37°C in 10-15 min; plates that have been stored at 4°C will require about 1 hr to warm. Do not overdry the plates.

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

If you have obtained a premade EZnet Phage Display Library, aliquot and store it at -70°C. The titer of your library must be greater than 10^8 cfu/ml to obtain optimal results in a phage display library panning.

3. Prepare the following dilutions of the phage display library in 2X YT medium. You will need 100 µl of a 10^-6, a 10^-7, a 10^-8, and a 10^-9 sample.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µl undiluted library + 198 µl</td>
<td>10^2</td>
</tr>
<tr>
<td>20 µl 10^-2 dilution + 180 µl</td>
<td>10^3</td>
</tr>
<tr>
<td>20 µl 10^-3 dilution + 180 µl</td>
<td>10^4</td>
</tr>
<tr>
<td>20 µl 10^-4 dilution + 180 µl</td>
<td>10^5</td>
</tr>
<tr>
<td>20 µl 10^-5 dilution + 180 µl</td>
<td>10^6</td>
</tr>
<tr>
<td>20 µl 10^-6 dilution + 180 µl</td>
<td>10^7</td>
</tr>
<tr>
<td>20 µl 10^-7 dilution + 180 µl</td>
<td>10^8</td>
</tr>
<tr>
<td>20 µl 10^-8 dilution + 180 µl</td>
<td>10^9</td>
</tr>
</tbody>
</table>
Panning & Screening Preparation

4. Transfer 100\(\mu\)l of the \(10^{-6}\), \(10^{-7}\), \(10^{-8}\), and \(10^{-9}\) dilutions of the phage library to fresh, prelabeled tubes.

5. Add 100 \(\mu\)l of prechilled log-phase TG1 cells to each tube from Step 2 above. Mix gently and incubate at room temperature for 5 min to allow infection; M13 phage adsorbs rapidly to TG1 cells.

6. 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.

7. Incubate plates up-side-down at 37\(\degree\)C overnight.

8. Count the number of the colonies on the plates that have between 30 and 300 colonies.

9. Worksheet

**WORKSHEET 1**

Plating the infected cells on ampicillin-containing medium ensures that only cells harboring a pHage3.2 phagemid 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 = the titer (pfu/ml) of the library.

Calculate the titer (cfu/ml) of the infected cells and hence of the phage display cDNA library:

\[
\text{cfu/ml} = \frac{\text{(# colonies on the plate)} \times \text{dilution factor}}{0.5 \times 0.1 \text{ ml}}
\]

\[
\text{_________ cfu/ml} = \frac{_____ \text{ colonies} \times 10^{-}}{0.5 \times 0.1 \text{ ml}}
\]
Titering

II. Titering: M13K07 Helper Phage Stock

**Required Reagents & Materials**

- Prechilled, log-phase TG1 cells in 2X YT (Section III.A.6)
- Melted 0.7% top agarose (in 2X YT) equilibrated to 47°C in a water bath or heating block; allow 3 ml per dilution to be plated. When plating bacteria/phage mixtures using melted top agar, the melted top agar should be at 45-47°C; higher temperatures will kill the bacteria.
- Sterile, 13 X 100-mm test tubes
- Three 2X YT agar plates (100-mm plates)

**Preparation:**

1. The helper phage titer sometimes drops during prolonged storage. Therefore, titer the helper phage M13K07 stock provided before you use it to prepare the fresh helper phage stock.

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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2μl undiluted library + 198μl</td>
<td>10²</td>
</tr>
<tr>
<td>20μl 10⁻² dilution + 180μl</td>
<td>10³</td>
</tr>
<tr>
<td>20μl 10⁻³ dilution + 180μl</td>
<td>10⁴</td>
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<tr>
<td>20μl 10⁻⁴ dilution + 180μl</td>
<td>10⁵</td>
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<td>20μl 10⁻⁵ dilution + 180μl</td>
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<td>20μl 10⁻⁶ dilution + 180μl</td>
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<td>20μl 10⁻⁷ dilution + 180μl</td>
<td>10⁸</td>
</tr>
<tr>
<td>20μl 10⁻⁸ dilution + 180μl</td>
<td>10⁹</td>
</tr>
</tbody>
</table>

3. Transfer 100μl of the 10⁻⁷, 10⁻⁸, and 10⁻⁹ dilutions of the helper phage to sterile, 13 X 100-mm tubes.

4. Add 100μl 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).
II. Titering: M13K07 Helper Phage (Cont.)

5. To each tube of infected TG1, add 3 ml of melted top agarose (47°C), mix gently, and immediately pour on a 2X YT plate. Gently rock the plate to evenly distribute the melted top agarose.

6. Allow the top agarose to harden at room temperature for 5 min. Then, invert the plates and incubate them at 37°C for 8-10 hrs or overnight.

7. Count the plaques to determine titer (pfu/ml)
   **Note:** Because M13K07 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.

   \[
Pfu/ml = \frac{(# \text{ plaques on the plate}) \times \text{dilution factor}}{0.5 \times 0.1 \text{ ml}}
   \]

8. Use Parafilm to seal one of the plates having distinct plaques and store the plate at 4°C. This is your working stock plate for helper phage preparation.
**Helper Phage Stock Preparation**

### Required Reagents & Materials

- Fresh working stock plate of M13K07
- Prechilled, log-phase TG1 cells
- 2X YT/kan medium

### Preparation:

1. You will need 3 ml of fresh helper phage stock (10⁹ pfu/ml) to carry out a complete phage display library screening experiment.
2. Use a sterile, yellow plastic pipette tip to pick up the agar plug containing a single, isolated plaque of helper phage M13K07 from the working stock plate. Transfer the plaque to 3 ml of 2X YT/kan medium in a sterile 17 X 100-mm tube.

   **Fresh plaques that are less than 1 month old will give best results.**

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

4. Incubate at 37°C for 12-16 hr (overnight) with shaking at 250 rpm.

5. Transfer the cells to a sterile centrifuge tube. Centrifuge at 12,000 x g for 2 min at 4°C.

6. Transfer the supernatant to a fresh, labeled tube and store at 4°C for up to 1 year.

7. Measure the titer of the M13K07 stock by plaque formation on a TG1 lawn.

   **NOTE:** The titer of particles containing single-stranded plasmid DNA is normally >5 x 10¹⁰ pfu per ml of bacterial culture. During propagation of M13K07, there is selection for bacteriophage genomes that have lost the p15A origin and the Tn 903 transposon. Therefore do not pass the bacteriophage serially. Use stocks of M13K07 derived directly from a single plaque for superinfection.
Bait Coating

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 and 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. Alternatively, oligonucleotides may be synthesized with a primary amine at one end, and then covalently attached to the surface of a specially prepared plate precoated with a layer of reactive N-oxysuccinimide esters (e.g., Costar’s DNA-BindTM), or maleic anhydride (e.g., Pierce’s Reacti-BindTM).

Whole cells in suspension or cell membranes may be used as a bait source for panning in solution; do not attempt to attach these baits to a panning surface. Monolayer cell cultures may be used as an immobilized bait.

Required Reagents & Materials

- Bait (protein) solution, diluted in PBS
- Flat-bottom, 96-well polystyrene microtiter plate or multiwell strips to coat with the bait for panning
- Wash buffer: PBS containing 0.05% Tween-20
- Blocking buffer: Wash buffer containing 1% BSA

Note: Multiwell plates and strips are available from several commercial suppliers. We recommend the high-capacity LabSystems ELISA plates (Cat.# 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).
Panning & Screening Preparation

Procedure:
1. If the bait protein is more concentrated than 10 µg/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°C overnight (16hr).
   Note: Use a 4°C 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 overnight (16hr).
   Note: Use a 4°C 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°C for up to 1 month.
   Note: Most peptides and some bait proteins are not stable enough to store and should be used immediately.
Please review the following guidelines before starting this procedure.

Guidelines:

1. For best results, repeat the panning procedure at least three times on each library aliquot. ELISA screening is generally not recommended before the third round of panning because the percentage of positives in the enriched population will still be too low (<1%) in most cases.

2. For each subsequent panning, use a fresh bait-coated well; a well may not be reused since the phage coats remain attached to the bait in this procedure. Note: “spent” library aliquots may be saved after panning for use in future studies. However, due to possible loss of clones that cross-react with different baits, it is not recommended to reuse the spent library to pan against different bait.

3. In this protocol, the bound phage is gently and efficiently rescued by simply allowing them to infect TG1 host cells. However, if you prefer, the bound phage can also be eluted using buffers with decreasing pH, as described in Wrington et al. (1996). Be aware that extremes of pH (pH2) can adversely effect phage viability.

4. After each panning cycle, you must amplify and superinfect a portion of the infected, enriched TG1 cells if you plan to perform a subsequent panning.

5. After the third panning, you have the option of screening individual clones for positives by ELISA using the Anti-pVIII polyclonal antibody provided in the EZnet Library Screening Kit. Alternatively, you may wish to directly repeat the amplification/panning/plating process for one or two more cycles before you perform the ELISA screening. The extra cycles will require 2-3 more days, but may save time overall if it turns out that additional panning cycles are required to identify positives.

6. If >30% of the clones are positive after three rounds of panning, many of them may be duplicated of the same insert; bias may be introduced into the population due to differences in infectivity, relative growth rates, or both. In this case, you may find a wider variety of clones after fewer rounds of enrichment. For this reason, it is prudent to store the enriched library at -20°C after each panning. If necessary, you can use them as a source of individual clones for ELISA screening.

Controls:

1. After each panning, plate out a diluted sample of the enriched TG1 library for single colonies on appropriate selection medium and count the resulting colonies. This will indicate whether you have successfully recovered phagemid DNA in the host cells. In addition, the number of phage trapped on the plate and recovered, assuming one phage will infect one host cell.
Controls (cont):

[Recommended] Titer the enriched phage library. Although you need not wait for this information before proceeding to the next panning cycle, it will be necessary if you wish to calculate the percent of phage trapped in the panning.

[Optional] To estimate the percent of enriched library clones that have been selected because of nonspecific binding to the surface, pan a 10 µl aliquot of the enriched library against a well precoated with BSA (bovine serum albumin; Sigma, Fraction V).

### Required Reagents & Materials

- EZnet Phage Display Library
- The following materials are included in the EZnet Library Screening kit and with EZnet Phage Display libraries:
  - M13K07 helper phage
  - TG1 cells (prechilled, log phage cells)
  - Microtiter plate wells precoated with the desired bait material
  - PBS (Appendix)
  - Wash buffer: PBS containing 0.05% Tween-20
  - Blocking buffer: wash buffer containing 1% BSA.
  - 2X YT/amp/kan broth (Appendix)
  - 2X YT/glucose broth (Appendix)
  - Ampicillin stock solution (50 mg/ml)
  - Sterile 20% PEG 8000 solution
- For estimating recovery of trapped phage: 2X YT broth and 2X YT/amp/glucose/MgCl2 agar plates
- For titering the purified, enriched phage library: 2X YT/amp plates or long-term storage of enriched TG1 library and enriched purified phage library: Sterile glycerol solution (at least 50% v/v)
Panning:

1. Add library aliquot to the bait-coated well.

2. **First panning cycle**: use an aliquot of the original library. Usually, panning $10^9$ library clones against one coated well (1 cm²) is sufficient to identify a cDNA for a rare or moderately abundant transcript. For example, for an EZnet Phage Display 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.

   **Subsequent rounds of panning**: use up to 50 µl of the purified, enriched phage library from the previous panning.

3. Incubate the library on the coated surface at 37°C for 1hr. Alternatively, for less stable proteins, incubate at room temperature or 4°C for up to 4 hr.

4. 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) for higher-stringency binding conditions.

Rescuing Trapped Phage:

1. Add 0.1 ml of prechilled, log-phase TG1 cells to the panning well.

2. Incubate at 37°C for 30 min. Immediately place the plate on ice or at 4°C.

   **Note**: Longer incubation at 37°C 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. Dilute a small aliquot of the enriched TG1 library and plate it out to determine the titer and estimate recovery of trapped phage. **(Recommended)**

4. Transfer the enriched TG1 library to fresh microcentrifuge tubes. Store at 4°C for up to 3 days, or add sterile glycerol to a final concentration of 15%, and store at -70°C for up to 1 yr.

   **Helpful Tip**: If you plan to repeat the panning within days, keep 50 µl of the enriched TG1 library at 4°C and store the rest at -70°C.
If you just finished your 1st or 2nd round of panning, repeat the panning process. If you just finished your 3rd round of panning, proceed to the ELISA screening.

To estimate recovery of trapped phage:

1. Prepare $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilutions of the enriched TG1 library from Step E.4 above in 2X YT medium.

2. Plate 100 µl of each cell dilution onto a 2X YT/amp/glucose/MgCl$_2$ plate. Invert the plates and incubate at 37°C overnight.

3. Count colonies, calculate the total number of cells in the enriched TG1 library, and estimate percent of phage recovered in panning.

$$\text{cfu/ml} = (#\text{colonies on plate}) \times \text{dilution factor}$$

$$\text{Ml}$$

Total # cfu = cfu/ml x total vol. of enriched TG1 library

Percent of phage recovered = \frac{\text{total # cfu}}{\# \text{cfu}^* \text{ applied to panning surface}}$

*Note that the library titer is expressed in cfu/ml (see Section III.B).

Expected recovery rates

Because the TG1 cells have time, at most, to undergo one generation before they are placed at 4°C (Step E.2 above), the total number of cells in the enriched TG1 library is a rough estimate of the number of phage rescued.
Panning, Rescue & Superinfection

After only one or two panning rounds, the percentage of phage trapped is expected to be very low ((1%) for most rare-to-moderately abundant transcripts. The percent recovery is an indication of the stringency of selection. Thus, if your recovery is >1%, the panning conditions may not be stringent enough for your particular bait (see Troubleshooting Guide, Section VIII).

Repeat Panning:

Transfer 50 μl of the enriched TG1 library to a sterile, 17 X 100-mm tube. Store the remainder at -70°C.

1. Add 1 ml of 2X YT/glucose to the 50 μl aliquot of enriched TG1 library.

2. Incubate at 37°C for 1 hr with shaking at 250 rpm.

3. Add ampicillin to a final concentration of 100 μg/ml (e.g., 2 μl of a 50 mg/ml amp stock). Immediately add 5 x 10⁹ pfu of M13K07 (~0.1 ml of fresh phage stock) to the cell culture (superinfection step).
   Note: The multiplicity of infection (MOI) should be about 20:1 (phage:cells).

4. Incubate at 37°C for 1 hr with shaking at 250 rpm.

5. Transfer culture to a centrifuge tube. Centrifuge at 2,000 x g for 10 min at 4°C or room temperature. Remove and discard the supernatant.
   Note: Be sure to remove all the supernatant. Traces of glucose will repress the expression and hence, display, of fusion proteins.

6. Resuspend the cell pellet in 10 ml of 2X YT/amp/kan.

7. Incubate at 37°C overnight with shaking at 250 rpm.

8. Centrifuge culture at 10,000 x g for 20 min at 4°C. Transfer the supernatant (which contains the enriched phage library) to a fresh tube.

9. Recentrifuge the supernatant at 10,000 x g for 10 min to further clarify it. Transfer the supernatant to a sterile polypropylene tube: this is the enriched library in a crude phage preparation.
   Note: If necessary, the enriched crude phage library may be stored at 4°C for up to 1 month. However, this is not recommended because of potential reduction of titer.
10. Purify the enriched phage library as follows:

Note: Because the suppression of the amber stop codon is only about 80% efficient in TG1 (Sambrook et al., 1989), some cDNA-coding proteins with only signal sequences will be expressed and secreted to the medium. If not removed from the phage preparation, these soluble proteins will compete for binding sites in the bait-coated wells and interfere with the next panning.

a. Add 10 ml of blocking solution to 10 ml of the enriched (crude) phage library. (Use an equal volume of blocking solution and phage library.)

b. Incubate at 4°C for 10 min.

c. Add 4 ml of 20% sterile PEG solution. Incubate at 4°C for 30-60 min to precipitate the phage.

d. Centrifuge at 10,000 x g for 20 min at 4°C. Discard the supernatant and resuspend the pellet in 1 ml of PBS.

e. Transfer the resuspended pellet to a sterile microcentrifuge tube and centrifuge at 14,000 rpm (top speed) for 10 min at 4°C. This time, save the supernatant; this is the enriched (purified) phage library.

11. Titer the enriched phage library on 2X YT/amp plates (Section III.B). Titering will provide important information, as described in Section IV. B.

12. The enriched (purified) phage library may be stored at 4°C for up to 1 month. If you plan to keep the enriched (purified) phage library for >1 month, add sterile glycerol to a final concentration of 20%, and store it at -70°C for up to 1 yr.

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

If you have performed only one or two rounds of panning, then return to the panning procedure.
# Required Reagents & Materials

- M13K07 helper phage
- Control protein and phage clones
- Anti-pVIII polyclonal antibody (affinity purified)
- HRP-conjugated secondary antibody
- HRP substrates A & B
- Enriched phage display library in TG1
- ELISA plates precoated with the desired bait
- Notes: If you have purchased the EZnetExpression and Detection Kit, use the 96-well microtiter plates provided.
- 2X YT/amp/glucose/MgCl2 agar plates (Appendix )
- 2X YT/amp/glucose broth (Appendix )
- 2X YT/amp/kan broth (Appendix )
- Ample supply of 1.5-ml microcentrifuge tubes or deep-well microtiter array tubes and holder
- Sterile glycerol solution (at least 50%)
- Sterile 1X PBS (Appendix A)
- Wash buffer: PBS containing 0.05% Tween-20
- Blocking buffer: Wash buffer containing 1% BSA.
- Sterile 20% PEG 8000 solution

**Optional:** for quantitation of results 1 N H₂SO₄ and an ELISA plate reader, such as the ThermoMax Plate Reader from Molecular Devices.
Preparation of Candidate Phage Clones for ELISA:

Dilute an aliquot of the enriched TG1 library after the third panning and plate out 100 µl of each dilution on 2X YT/amp/glucose/MgCl₂ for single colonies. Incubate at 37°C overnight.

1. Add 400 µl of 2X YT/amp/glucose medium to prelabeled 1.5-ml microcentrifuge tubes or separate tubes of a 96-deep-well microtiter array. Label these tubes “PC1” + a number to designate the individual clone.

2. Using sterile pipette tips, pick 44-88 well-isolated, single colonies from the plates above and use each to separately inoculate the medium in the small tubes.

3. Incubate tubes at 37°C overnight shaking at 250 rpm. These are the master stocks of the individual phagemid clones in TG1; we refer to these culture as “PC1”

4. Transfer 50 µl of each overnight PC1 culture to a fresh tube prelabeled “PC2” + the number designating the individual clone. To the remaining 350 (l of PC1 culture, add sterile glycerol (final concentration 15%) and store at -70°C. Save all PC1 cultures until you have obtained the ELISA results.

5. Add 400 µl of 2X YT/amp/glucose medium containing 5 x 10⁸ pfu of M13K07 (~10 µl of fresh phage stock to each tube labeled PC2.

6. Incubate tubes at 37°C for 1-2 hr with shaking at 200-250 rpm.

7. Centrifuge samples at room temperature:

8. Centrifuge 1.5-ml microcentrifuge tubes at 14,000 rpm for 5 min.

9. For a 96-deep-well microtiter array, centrifuge at 2,000 x g for 10 min using a centrifuge adapted for multi-well plates.

10. Discard the supernatants. Resuspend each cell pellet in 400 µl of 2X YT/amp/kan. Note: Be sure to remove all the supernatant. Traces of glucose will repress the expression and, hence, display of fusion proteins.

11. Incubate the wells or tubes at 37°C overnight with shaking at 250 rpm. These are the crude supernatants of the superinfected individual phagemid clones; we refer to these cultures as “PC2”.
12. Purify phage clones
13. Centrifuge PC2 cultures as in Step 8 above.
14. Transfer 300 µl of each supernatant (which contains the phage) to fresh tubes prelabeled “PC3” + the number designating the individual clone.
15. Add 300 µl of blocking buffer.
16. Incubate at 4°C for 10 min
17. Add 120 µl of sterile 20 PEG solution to each tube.
18. Incubate at 4°C for 30 min.
19. Centrifuge at 10,000 x g for 20 min at 4°C.
20. 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°C for 1 month. For storage > month, add glycerol to 20% final concentration and place at -70°C.

Screening:

1. Apply 50 µl of each PC3 phage prep to a well precoated with your bait protein. We strongly recommend to apply 20-50 µl of each positive control phage clone to a well in a positive trip precoated with control bait protein (provided in kit) at same time.

2. Incubate at room temperature for 2 hr. Note: Use a 4°C 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 polyclonal antibody to 2 µg/ml in blocking buffer. Add 100 µl of diluted anti-pVIII antibody to each well of the plate. Note: The anti-pVIII antibody can be used at a higher concentration (up to 10 µg/ml) if necessary, to boost a weak signal.
ELISA

Screening (cont):

5. Incubate the plate at room temperature 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 secondary antibody 1:50 in blocking solution
   Note: Do not use sodium azide in the blocking solution; it inhibits HRP activity.

8. Add 100 µl of the diluted HRP conjugate to each well.

9. Incubate the plate at room temperature for 1 hr. 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 Solution B. Add
    100 µl of the substrate solution into each well.

11. Incubate the plate at room temperature until a suitable blue color has developed (i.e., when
    the color intensity in the positive control wells appears to be approx. 2X background. For
    the positive control clone provided in the kit, color development should take 30 min. For
    library clones, color development can take from 30 min. to 12 hr, depending on the relative
    abundance of the transcript and on the affinity of the displayed protein for the bait. At this
    point, take a photograph if desired and make a note of the color intensity in the positive wells
    relative to the positive controls. Proceed to Step 12 if you wish to obtain quantitative results;
    otherwise, proceed to Step 13.

12. Stop the color reaction in all the wells at the same time by adding 10 µl of 1 N H2SO4 to
    each well. (Blue wells will turn yellow.) Read the absorbance at 405 or 410 nm (preferably)
    on a plate-reader.

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.
Troubleshooting

No Positive Library Clones Obtained After Panning & Screening

If not positive clones are obtained in the ELISA screening of the enriched library, it could be due to problems with the panning procedure, the ELISA, or both. Problems with the ELISA are generally easier to pinpoint, especially if you are using the control materials provided with the EZnet Phage Display Library Screening Kit. This is contains control protein and positive and negative control phage clones to help you ascertain if the ELISA screening is working properly. In addition, the anti-pVIII antibody produced with the EZnet Library Screening Kit has been affinity purified and pretested for performance in the ELISA assays.

Elisa Tips

If you have not done so already, be sure to perform ELISA assays on at least 88 clones. Remember, the more clones you screen, the greater your chances of finding displayed proteins encoded by rare transcripts.

Try using a higher concentration of the pVIII polyclonal antibody and/or purified phage clone in the ELISA assays. The purified anit-pVIII antibody provided with the EZnet Library Screening Kit may be used within a range of 1-10 µg/ml; the higher end of the range may be more suitable for displayed proteins that have a relatively weak affinity for the bait.

The wells may not be optimally coated with the bait protein. Check the concentration of the protein solution used to coat the wells; it should be approximately 10mg/ml. If available, use an antibody specific for the bait to confirm that a sample well is evenly coated with bait.

Panning Tips

If you have not already done so, be sure to check the titer of the library used in the panning. If the titer is lower than expected, you may be starting the panning experiment with a suboptimal number of library clones. Use a larger volume of library if necessary to provide at least $10^9$ clones.

If you have not done so already, perform at least three rounds of panning before you prepare individual clones for ELISA screening. If you have performed three rounds, perform one or two more rounds and rescreen individual clones.

The wells may not be optimally coated with bait protein.

Check the viability of the TG1 culture used in the phage rescue. Check for the presence of the F' episome by plating on M/9+Thi minimal medium. Remember that the host cells must contain an F' episome to be efficiently infected by M13, and they can lose the episome if they are grown to saturation under nonselective conditions.
Troubleshooting

If you have not done so already, check the titer of the M13K07 helper phage; if the titer is $< 10^9$ pfu/ml, prepare a fresh stock of helper phage.

If you have no don so already, check the percent phage recovery. Checking the percent page recovery will also tell you the titer of the enriched library.

If phage recovery was poor (i.e., $< 0.001\%$), you may not have used the optimal number of phage (i.e., $\sim 10^9$ particles) in the subsequent panning. Use more of the enriched library when you repeat the panning. Alternatively, decrease the stringency of the washing conditions. For example, if you used more than five washes, reduce the number to five. (However, do not use fewer than five washes, or else you may have problems with nonspecific binding.) You may want to try changing the formulation of the washing buffer to decrease its stringency; please refer to a laboratory manual on immunoassays for suggestions.

If phage recovery was very high (i.e., $> 1\%$), but the number of positive clones still very low, increase the number of washes (up to a maximum of 20). You may want to try changing the formulation of the washing buffer to increase its stringency. However, be aware that most measures that will increase stringency (such as using a higher detergent concentration or more extreme pH) will have a deleterious effect on phage viability. Therefore, if you change the formulation of the washing buffer, be sure to test the viability of the phage in the new buffer before you use it in a panning experiment.

If phage recovery was $>0.001\%$, but $< 1\%$, use more panning rounds (at least five total).

For rare transcripts, you can use more library clones and a correspondingly larger panning surface.

Many of the Positive Clones Do Not Bind Bait Specifically

Upon further analysis, if clones do not bind specifically to the bait, it could be due to nonspecific interactions with the wells, or the specific interactions with other components in the bait preparation.

To reduce nonspecific binding to wells, check the percent phage recovery. If recovery is $>1\%$, increase the stringency of the wash conditions. If recovery is $<1\%$, use more panning rounds (at least five total). Check your blocking agent, such as 1% nonfat dry milk instead of BSA, or try adding both BSA and nonfat dry milk to the buffer. (Note that nonfat dry milk contains biotin, which will interfere with biotin-based detection systems.)

It may be possible to prevent phage particles from binding to non-bait components in the panning process. However, use of proper controls in the initial analysis of candidate positives will eliminate the non-bait binding clones from further analysis.
Troubleshooting

Background too High in the ELISA Assay

Try a different blocking buffer

Use a higher dilution (i.e., less concentrated) solution of the secondary antibody conjugate.

Do not let the HRP color reaction go longer than 20 min; in most cases, 10 min should be sufficient.

Cannot Find Overexpressed Protein

If you have not done so already, look for the overexpressed protein in the medium, the periplasmic extract, and a whole-cell extract.

If you have not done so already, optimize the induction conditions by taking several time points over a period of at least 20 hr. If the overexpressed protein is particularly unstable or insoluble, it may help to lower the incubation temperature. Temperature as low as 4°C may be used, but you may need a correspondingly longer induction period (at least 20 hr).


References


References


**pHage 3.2** (a phagemid vector derived from M13) is designed for construction of phage-display libraries. Transcription is under control of the lac promoter. The secretion of target-gene III fusion protein is directed by the gene III signal sequence. The phage coat protein geneIII helps to display targets on phage tips. An amber stop codon in frame with and immediately following Myc-tag permits the expression of the foreign protein as a fusion with gene III in an amber suppressor host (TG1) or as a fusion with only the Myc-tag in a non-suppressor host strain HB2151.

### Gene III Leader

<table>
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<th>GTG</th>
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**Sal I**

**EcoRV**

**Bgl II**

**Apa I**

### Myc tag

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### Gene III

| GAG | CTC | GCT | GAA | ACT | GTT | GAA | AGT | TGT | TTA | GCA | AAA | CCC | CAT | ACA |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
Appendix II

Molecular Cloning Manual by Sambrook et al. contains useful information on how to grow TG1 cells and prepare competent cells properly. Following is a procedure we recommend:

Preparation of Competent Cells

1. Grow E.coli host TG1 cells in LB Broth medium (500ml-1litre) with single colony from a minimal plate. Incubate at 37°C overnight with shaking at 250 rpm.
2. Dilute the overnight culture 1:50 in LB-Broth medium. Incubate at 37°C with shaking at 250 rpm until a A600 of 0.4-0.6 is reached (approximately 2-2.5 hours).
3. Chill the cells on ice for 10 minutes. Spin at 4,000 x g for 15 minutes at 4°C. Discard the supernatant and resuspend the cell pellet in 1 litre of ice-cold sterile 1mM Hepes (pH 7.0). (Wash Step 1)
4. Spin as described above, Discard the supernatant and resuspend the cell pellet in 500 ml of ice-cold sterile 1mM Hepes (pH 7.0). (Wash Step 2)
5. Spin as described above, Discard the supernatant and resuspend the cell pellet in 500 ml of ice-cold sterile 1mM Hepes (pH 7.0). (Wash Step 3)
6. Spin as described above, Discard the supernatant and resuspend the cell pellet in 20 ml of sterile 10% glycerol in water. (Wash Step 4)
7. Spin as described above, Discard the supernatant and resuspend the cell pellet in a total volume of 2-3 ml 10% glycerol in water. Dispense in 50-100 ul aliquots, freeze on dry ice and store at -70°C until use.