Antibody Design LABORATORIES

MIM™ Phage Display Libraries

INSTRUCTION MANUAL

MIM™ Phage Display Libraries

Version: A1.5 – February 2023

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Description

Introduction

Antibody Design Labs' premade MIM™ phage display peptide libraries are based on the fADL™-2blue phage vector. The fADL™-2blue vector is a type 3 phage display vector (Smith 1997) derived from the phage vector fd-tet (Zacher1980). fADL-2blue is well tolerated by the host and gives large colonies and small plaques, simplifying the screening of libraries and the handling of single clones. The peptides are fused to the N-terminal end of the pIII gene, resulting in a pentavalent display on the phage head. The multivalent display creates a situation of high avidity that enables detection of peptide binding even in the low micromolar range. Biopanning is carried out as described in the original George Smith's publication (Scott 1990). In brief, the library is incubated with a target immobilized on a plate or on beads, the unbound phages are washed away, and the bound phages are eluted. The eluted virions are further amplified to create a new library. Iteration of the process leads to the progressive enrichment of peptide binders. After a few rounds, usually 3 or 4, binding is confirmed by ELISA on single clones, the peptides are analyzed by DNA sequencing, and binding motifs are identified by homology comparison.

Content, Shipping & Storage

Kit Component

COMPONENT	DESCRIPTION	AMOUNT
MIM LIBRARY	100 µl phage, ~ 2 x 10^{12} cfu/ml. Supplied in TBS with 10% glycerol Enough for 10 screening campaigns	100 µl
PHI8S3 PRIMER	5'-CAAGCTGTTTAAGAAATTCACCTCG, 1 nmol, 10 µM, 10 pmol/µl	100 µl
PSIR2 PRIMER	5'-CGTTAGTAAATGAATTTTCTGTATGAGG, 1 nmol, 10 µM, 10 pmol/µl	100 µl
TG1	Phage-Competent™ TG1 cells, 20 OD_{600}/ml, >2 x 10^{10}cfu/ml	0.5 ml
SS320	Phage-Competent™ SS320 cells, 20 OD_{600}/ml, >2 x 10^{10}cfu/ml	0.5 ml

Associated Products

PRODUCT	COMPOSITION	AMOUNT
AL101	MIM™-C10 Phage Display Peptide Library	1 kit
AL103	MIM™-12 Phage Display Peptide Library	1 kit
PD021	fADL™-2blue Phage Vector. 20 µl at 0.5 µg/µl of DNA vector in DNA Conservation Buffer (Tris-HCL 5 mM, EDTA 0.1 mM, pH 8.5)	10 µg
PC001	Phage-Competent™ TG1 cells, 20 OD_{600}/ml, >2 x 10^{10}cfu/ml	10 x 0.5 ml
PC002	Phage-Competent™ SS320 cells, 20 OD_{600}/ml, >2 x 10^{10}cfu/ml	10 x 0.5 ml

Shipping & Storage

The MIM™ libraries are shipped on dry ice. Upon receipt, store immediately at -80°C.

For research use only; not intended for any animal or human therapeutic or diagnostic use.

Libraries & Vector Maps

Peptide Phage Display Library

Summary

LIBRARY	CATALOG #	DESCRIPTION	SEQUENCE
MIM™-C10	AL101	Cyclic 10-mer, trimer codon-based random library	AEGCX_{10}CGGGSGPGGLRGGS-pIII
MIM™-12	AL103	Linear 12-mer NNK-based random library	X_{12}GGGSGPGGLRGGS-pIII

MIM™-12 Peptide Phage Display Library

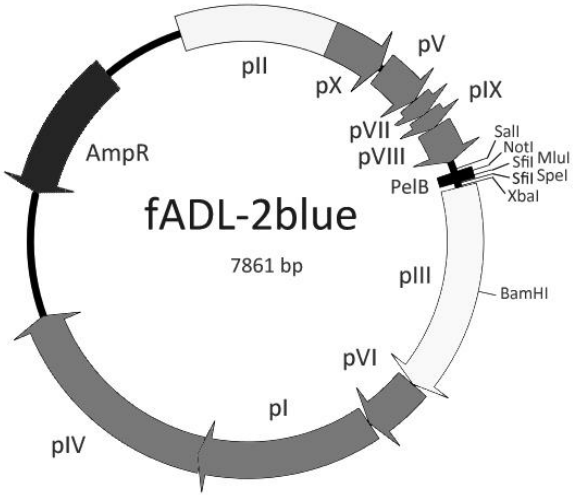
MIM-12 is a 12-mer N-terminal random peptide library. The sequence of the library is X_{12}, where X indicates amino acids encoded by the degenerate NNK codons. The 12-mer random peptide X_{12} starts immediately after the pelB leader peptide. Therefore, the first N-terminal amino acid of the peptide is random. The library is fused to pIII through the linker GGGSGPGGLRGGS. The library was created using the bacterial strains MC1061 and SS320, which are both non-amber suppressive. The library size is ~1 x 10^9.

MIM™-C10 Peptide Phage Display Library

MIM-C10 is a 10-mer cyclic peptide library. The sequence of the library is AEGCX_{10}C, where X indicates amino acids encoded by a mix of the 19 trimer codons, excluding the cysteine codon. The library is fused to pIII through the linker GGGSGPGGLRGGS. The library was created using the bacterial strains MC1061 and SS320. The library size is ~1 x 10^9.

fADL™-2blue Phage Vector

The figure below illustrates the main features of fADL™-2blue vector (cat# PD021). A detailed manual as well as the vector sequences are available on the **Antibody Design Labs** website at **http://www.abdesignlabs.com**under product number PD021.



Experimental Procedures

Working with Phage

General Molecular Biology Techniques

Molecular cloning and phage display should be conducted under the supervision of a qualified instructor trained in standard safety practice in a molecular biology laboratory environment. Standard molecular biology procedures are described in detail in general molecular biology handbooks such as Sambrook *et al*, 1989.

Working with Filamentous Phage & Avoiding Phage Contamination

Keep the bench clean. Regularly wipe the bench surface with 2% bleach to limit phage cross-contamination. Only use filtered micropipette tips to prevent aerosol contaminations. Phages are known to survive standard autoclaving conditions and are not removed by 0.22 µm filtration. Phages are killed either by heat-treating dry, autoclaved materials in an oven for 4 hours at 105°C (Barbas III 2001) or by incubation in 2% bleach for at least 1 hour. Extensively wash all glassware and plasticware with hot water, then submerge (tubes) or incubate (flasks) the items in2% bleach for at least one hour. Heat-resistant glassware can then be sterilized in an autoclave that is never used for biological waste, while sensitive plasticware can be used directly or heat-treated as described above.

Bacterial Strains

Bacterial Strains

We recommend using the *E. coli* strains SS320 with the fADL-2blue phage vector. *E. coli* strain TG1 can be used as well. Both *E. coli* strains SS320 and TG1 have been widely used for phage display and are well documented in the literature. Both strains can be made highly competent for transformation by electroporation. The phenotypes are highlighted below. SS320 derives from MC1061 by addition of a tetracycline-resistant episome. TG1 can suppress the amber codon, but not MC1061 or SS320.

SS320 *hsdR2 mcrA0 araD139 Δ(araA-leu)*7697 Δ*lac*X74 *galK16 galE15(GalS) λe14⁻ rpsL150(Str^R) spoT1 thi F'[proAB+lacIqlacZΔM15 Tn10 (tet^r)]*

MC1061 *F⁻hsdR2 mcrA0 araD139 Δ(araA-leu)*7697 Δ*lac*X74 *galK16 galE15(GalS) λe14⁻ rpsL150(Str^R) spoT1 thi*

TG1 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$) F' [traD36 proAB⁺lacI^q lacZΔM15]*

Bacterial Stocks & F-factor Maintenance

We recommend a round of selection for the F-factor every time single colonies are isolated from bacterial stocks or when a bacterial culture is started for subsequent transduction.

TG1

The TG1 episome complements the proline pathway, allowing for selection and maintenance of the F-factor in the bacterium's *proAB*$^-$ background. Streak out TG1 on an M9 minimal agar plate supplemented with thiamine (1 mg/ml) and incubate at 37°C overnight until colonies are visible. Inoculate 2xYT medium with a single colony and grow to late log phase. Add sterile glycerol to 10%-20% v/v final concentration, aliquot, and freeze at -80°C.

SS320

Streak out SS320 on an agar plate supplemented with tetracycline (20 µg/ml) and incubate at 37°C overnight until colonies are visible. Inoculate 2xYT medium with a single colony and grow to late log phase. Add sterile glycerol to 10%-20% v/v final concentration, aliquot, and freeze at -80°C.

MC1061

Streak out MC1061 on an agar plate supplemented with streptomycin (25 µg/ml) and incubate at 37°C overnight until colonies are visible. Inoculate 2xYT medium with a single colony and grow to late log phase. Add sterile glycerol to 10%-20% v/v final concentration, aliquot, and freeze at -80°C.

Phage Purification

The components and recipes of all reagents and solutions employed in the following procedures are listed under Material, Reagents & Recipes.

Procedure 1. Small-scale Phage Preparation by Polyethylene Glycol (PEG) Precipitation

1. Transfer 1500 µl of the bacterial culture containing the phage particles to a microfuge tube.
2. Spin down bacteria by microcentrifugation for 2 min at 13,000 x g.
3. Transfer 1200 µl of the supernatant to a clean microfuge tube. Do not disturb the bacterial pellet with the pipet tip.
4. Add 300 µl of **PEG/NaCl 5x** and mix thoroughly by inversion. Do not vortex.
5. Incubate the tube on ice for5 min. Subsequently, wipe the tube with a clean tissue and expose the supernatant to indirect light. Gently rock the tube back and forth and check for PEG-precipitated virions. If a precipitate is visible, proceed to step 6, otherwise continue the incubation on ice for up to 60 min. Note that long incubations may promote proteolysis, especially when epitope tags are present, and it is recommended to shorten the precipitation as much as possible.
6. Pellet the virions by microcentrifugation for 3 min at 13,000 x g.
7. Cautiously remove the bulk of the supernatant with a large pipet tip and carefully discard the liquid in a designated container. Spin again for 1 min at 13,000 x g, remove all residual supernatant with a 100-µl pipet tip and discard the tip. The second centrifugation is essential to collect all phage particles at the bottom of the tube.
8. Add 120 µl of **Tris-buffered saline (TBS) (1x)** (1/10th of the initial culture volume) and resuspend the pellet by vortexing. If resuspension remains incomplete, let the mixture stand at room temperature for a few minutes, and vortex again. Incubate the resuspended virions on ice for 30 – 60 min. This incubation is essential to prevent a loss of particles in step 9.
9. Vortex vigorously again and clear the phage solution by microcentrifugation for 1 min at 13,000 x g. Transfer the supernatant (containing the phage) to a clean microtube and proceed to virion quantification.

Procedure 2. Large-scale Phage Preparation by PEG Precipitation

The protocol outlined above can be adapted to larger scale preparations by adjusting the centrifugation time while keeping the relative centrifugal force to 13,000 x g, as indicated in the following table.

PROCEDURE STEP	MINI (1-2 ML)	MIDI (20-50 ML)	MAXI (100-400 ML)
BACTERIAL CLEARANCE (STEP 2)	2 min	5 min	10 min
CENTRIFUGATION (STEP 6)	3 min	10 min	20 min
PHAGE CLEARING (STEP 9)	1 min	5 min	10 min

Phage Storage

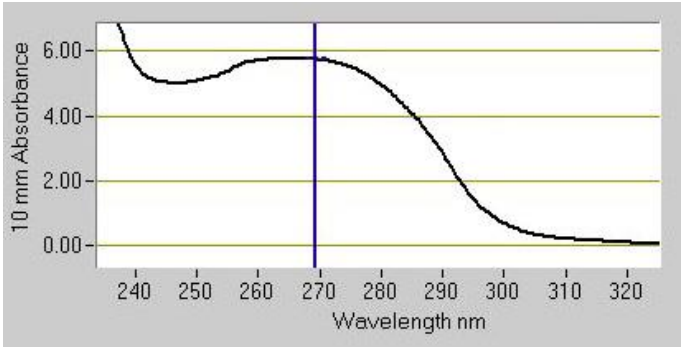
Filamentous virions are extremely resistant and can survive exposure to prolonged heat without losing infectivity. A heat exposure at 65°C for 15 min may therefore be used to kill residual bacterial host cells in the phage preparation. However, displayed polypeptides are sensitive to proteolysis, and contamination by proteases must therefore be avoided. Culture supernatants containing virions should be tested rapidly in the first few days following harvest.

Eluted phages in neutralized buffer and purified virions in TBS may be stored at 4°C for a few days to a week without loss of infectivity. For prolonged storage of phage preparations, freezing is recommended. Purified virions in TBS can be frozen directly without apparent loss of infectivity. However, if multiple freezing/thawing cycles are likely to occur, addition of a cryoprotectant such as glycerol (to 10% v/v final concentration) is recommended. For very long-term preservation of libraries and single virions, a storage temperature of -80°C is recommended.

Phage Quantification by Spectrophotometry

Introduction

Measuring phage particles represents an essential step in phage display. Absorption spectrophotometry offers a rapid and simple way to measure the concentration of virion preparations. The technique is based on the constant relationship between the length of viral DNA and the amount of the major coat protein VIII, which, together, are the major contributors of the phage absorption spectrum in the UV range. A typical UV absorption spectrum of filamentous phage purified by PEG precipitation and dissolved in TBS is depicted below. The spectrum exhibits a broad maximum at around 269 nm.



The relationship between virion number and absorption is given by the following formula:

$$\text{virions/ml} = \frac{(A_{269} - A_{320}) \cdot 6 \times 10^{16}}{\text{number of bases/virion}}$$

This formula was established by George Smith, and the calculation is based on the measurements of Day and Wiseman (Day & Wiseman, 1978). At 320 nm, phage chromophores have very little absorption.This value is therefore used to distinguish between light scattering from phage and absorption from non-phage particulate contaminants.

Note the diminished absorption at around 245 nm.When contaminants are present in the preparation, this decrease in absorption at 245 nm is absent from the spectrum. This is more likely to occur when the preparation contains fewer virions and indicates that a second PEG precipitation may be required to increase virion yield and to decrease the level of contaminants.

Method

1. Blank the spectrophotometer between 240 nm and 320 nm with clean TBS.
2. Replace the TBS with an appropriate dilution of the phage preparation in TBS. If the phage was 10 times concentrated after the PEG precipitation, dilute the preparation 10-fold.
3. Measure the absorption at 269 nm versus 320 nm. You can estimate the apparent virion concentration using the formula given above. Sum the length of the phagemid vector and the length of the insert to determine the number of bases per virion.
4. Measure the absorption spectrum of the phage preparation between 240 nm and 320 nm to verify purity.

Notes

- If the optical density at 269 nm(A_{269nm}) is ≥ 2, the preparation is too concentrated. Use a higher dilution factor.
- Undiluted preparations can be measured directly on microvolume spectrophotometers.
- Virion preparations with absorption measurements of $A_{269nm} – A_{320nm} ≥ 10$ should be diluted for safe long-term storage.
- Always verify the quality of the spectrum. If the decrease in absorption at around 245 nm is not present or an increase in absorption at 320 nm is observed, the phage preparation is likely of low quality, rendering the quantification inaccurate.

Phage Titration

This procedure measures the phage concentration as a phage titer (number of transducing units [TU] or colony forming units [cfu] per ml). The method is primarily suitable for preparations of very low virion concentrations, such as those typically obtained during biopanning, or when the preparation's infectivity (number of TUs per virion, or number of successful infections per virion) needs to be determined. Precise titrations require that the multiplicity of infection (MOI, or number of virions per bacterial host cell) is < 1.

1. Serially dilute virion preparations in 2xYT medium, to obtain the required low MOI. Serial 10-fold dilutions are recommended. Make dilutions in duplicate.

2. Mix an aliquot of each phage dilution with an aliquot of fresh TG1or SS320 bacteria grown in 2xYT at 37°C to mid to late log phase (A_{600nm} between 0.4 and 0.5). Incubate for 45 min at 37°C and 250 rpm. Alternatively, instead of growing your own cells, you can use ready-to-use Phage-Competent TG1 or SS320 (cat# PC001 and PC002).
3. Spread 100 µl of each suspension on a pre-warmed 2xYT agar plate supplemented with ampicillin 100 µg/ml.
4. Incubate overnight at 37°C.
5. Count bacterial colonies and correct for the dilution factors to determine the phage titer (cfu/ml).

Detecting Contaminations Using Blue/White Selection

The fADL-2Blue vector carries a copy of the lacZ α gene inducible by IPTG. Colonies grown on 2xYT media supplemented with X-gal, IPTG and ampicillin appear light blue. Working X-gal concentrations are typically 20 µg/ml. Doubling this concentration to 40 µg/ml will produce colonies with a darker blue color. IPTG between 200 µM and 1 mM is required for the blue color to appear.

Sequencing of Peptide Inserts

Forward or Sense Primers

phi8S3 5'-CAAGCTGTTTAAGAAATTCACCTCG

phiS2 5'-ATGAAATACCTATTGCCTACGG

Backward, Antisense, or Reverse Primers

psiR2 5'-CGTTAGTAAATGAATTTTCTGTATGAGG

psiR3 5'-GCGTAACGATCTAAAGTTTTGTCG

Nested Sequencing

Often it is easier to sequence an insert by PCR on the bacterial culture supernatant or directly from a colony rather than on tediously isolated plasmids. Use the outward primers phi8S3 and psiR3 together with a DNA polymerase not inhibited by bacterial cultures such as TAQ polymerase for the PCR and sequence the insert with the nested reverse primer psiR2. Use less than 1 µl of bacterial culture supernatant (or the touch of a toothpick on a colony) as DNA template per 50 µl of PCR reaction.

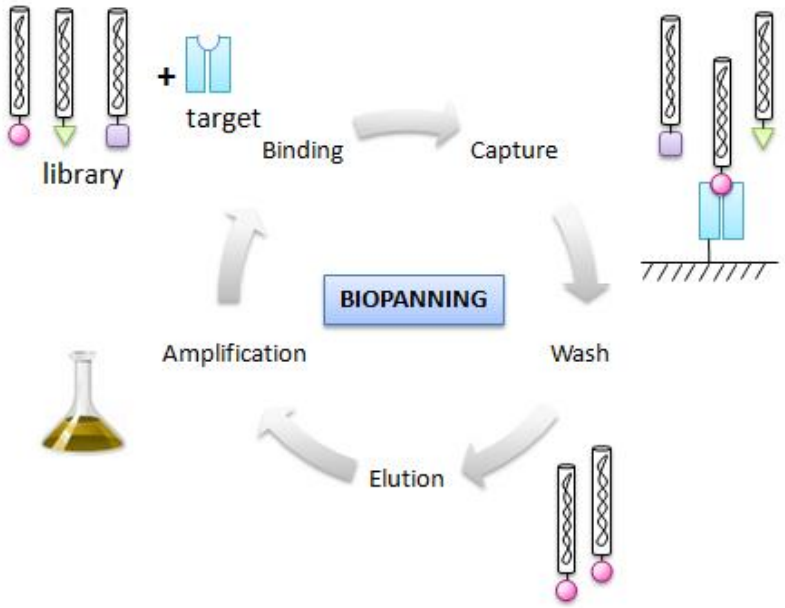
Direct Sequencing from Phagemids

Because TG1, SS320, and MC1061 contain DNA-specific endonuclease I (genotype *endA*$^+$), DNA hydrolysis must be carefully avoided during plasmid isolation. Most DNA isolation kits provide an additional washing buffer to remove this enzyme during plasmid preparation. Alternatively, virions from culture supernatants may be used to directly transduce *endA*$^-$, F$^+$*E coli* strains lacking the endonuclease.

Library Biopanning

Screening Workflow

Biopanning was a term created by George Smith (Parmley 1988) to designate a selection procedure where “binders” are progressively enriched through iterative rounds of selection. In this process, binder/target complexes are captured on a solid surface and selectively eluted after a thorough wash to eliminate non-specific clones; elution and amplification of the bound phage allows further rounds of selection and enrichment.



It is useful to distinguish various phage populations to adjust screening conditions; for example, one often considers non-binding phages, non-specific binding phages, low-affinity binding phages, and high affinity binding phages (Menendez 2001). Because biopanning is a dynamic and competitive process, the preferential enrichment of one population may inhibit competitively the growth of the others. During a biopanning experiment, the primary goal will be to control non-specific phages in order to identify the first positive, specific clones; if the stringency is too high from the initial round of panning, binders may get lost.

In “solid-phase capture”, the target is attached to the solid phase; binding to the target and capture on the solid phase occur at the same time in a process intrinsically multivalent. This method of screening is particularly well-adapted for the first round of selection to enrich even the lowest affinity binders. fADL-2blue is a multivalent type-3 phage vector mainly used for the isolation of low-to-moderate affinity binders typically found in peptide libraries. The use of a similar multivalent phage vector has also been reported to improve the screening of antibody libraries (O’Connell 2002).

In “solution-phase capture”, the target is incubated with the target in solution followed by the capture of the phage/target complexes on a solid surface. Binding in solution can be helpful to prevent selection of non-specific binders. Varying the concentration of the target during binding is a way to control selection stringency. The biotin/streptavidin system is often used for capture. This opens many possibilities to control selection; for example, adding an excess of non-biotinylated

target can be used during affinity maturation to prevent re-binding and operate a K_{Off} selection, or adding an excess of competitors where binding is not desired can be helpful for negative selection.

Typical screening of a MIM™ library starts with high-avidity solid-phase capture rounds to ensure that all binders present in the primary library at very low concentrations are enriched during the first rounds and the lowest affinity clones are further selected during the subsequent rounds. Enrichment of a population of selective binders is often accompanied by an increase in the number of eluted phage (selection yield = total eluted phage / total input phage). If enrichment is seen, a mock round without target made in parallel can confirm that the increase in output phage is target-specific. The ratio of phage outputs for positive selection versus negative selection is a good indicator on how strong the ongoing selection is. Even if no enrichment is seen, one should nevertheless test a few clones after 3 – 4 rounds for binding. To guide the selection toward the highest affinity binders, the first round can be followed by multiple rounds of solution-phase captures at varied target concentrations, e.g., between 100 nM and 100 pM.

Panning Procedures

The following guidelines are general recommendations and not a substitute for the multiple books, chapters, reviews, and publications on the matter.

Solid-Capture Surfaces

Small Petri dishes have been used with success for the first round in the early hours of phage display. Immunotubes (Nunc™ Maxisorp 444202 ImmunoTubes) are extremely popular for solid phase-capture assays, although of limited binding capacity. We prefer high-binding capacity surfaces found on some ELISA/RIA microplates; multiple wells can be combined if a large surface is desired.

Solution-Capture

The use of the interaction between biotin and streptavidin is the preferred option for solution capture. Streptavidin can be adsorbed on a plastic surface but pre-coated magnetic beads offer elegant alternatives. A classical solution to the problem of non-specific binders is to alternate capture surfaces between rounds to prevent their enrichment.

Blocking and Washing Buffers

Most protocols recommend phosphate-buffered saline (PBS) or TBS for blocking and during binding, with either bovine serum albumin (BSA) (1%– 3%) or non-fat dry milk (3% – 5%) with Tween 20 (0.5‰ – 1.5‰). Tween 20 is usually incompatible with screening on cell surface. Washing is usually extensive (5 -10 times) using the same buffer base without blocking agent. We recommend washing 5 times with a buffer containing Tween 20 followed by 5 times without Tween 20 before elution. Many non-specific plastic binders exhibit a Tween-dependent binding and the use of different washing conditions helps eliminate them.

Thawing the Library

The library kit contains 100 µl of virion preparation or the equivalent of 10 library inputs of 10 µl each. The first time the library is thawed, it is suggested to make aliquots of 10 or 20 µl and store them immediately at -80ºC. Storing the library in liquid state at -20ºC is possible over very short periods of time.

Library Input

There are 3 ways to count virions: virion particles, TUs, and phage optical density (OD_{269}). Virion particles are usually seen by electron microscopy. TUs count successful transduction events during infection, either as plaques (pfu) or colonies (cfu) like with fADL-2blue. If we know the infectivity (I) of a phage, we can deduce the exact number of virions: v = TU/I.

Unfortunately, infectivity may vary with display, especially on pIII; on the other hand, getting a precise knowledge of infectivity during screening is time consuming and not very informative. It is more convenient to assume infectivity, e.g. 5% for fADL-2blue, to convert TUs into virions and *vice versa* on a routine basis. Since virions are made of DNA and protein in a ratio that mostly depends on the length of the virions and therefore on the size of the phage genome, it is possible to estimate phage concentration by spectrophotometry (phage OD) (see above). Practically, we recommend around 0.1 – 0.2 phage OD or 7.5 x 10^{11} – 1.5 x 10^{12} virions/ml or 4 x 10^{10} – 8 x 10^{10} cfu/ml for input; these numbers are concentrations and the absolute number of virions in your biopanning experiments will vary with the volume of incubation.

The MIM libraries are provided at a concentration of ~2 x 10^{12}cfu/ml. This corresponds to ~5.5 phage OD with an infectivity of 5%. We recommend using 10 µl of the library in a screening volume of 500 µl or 0.11 phage OD. The number of cfu will be ~2 x 10^{10} cfu or a theoretical coverage of 20 times of the MIM libraries, which have a size of 1 x 10^{9}.

Elution

Multiple methods have been reported for elution.

- Direct infection. Phages are not eluted and transduction is done directly at the capture surface. Multivalent interactions may interfere with infection and this method is not recommended with fADL-2blue.
- Trypsin elution. Trypsin elution is the recommended method for elution. fADL-2blue has a Leucine-Arginine sequence in the linker between the fusion polypeptide and p3 that is trypsin-sensitive. Trypsin elution is specific since phages not interacting through the fusion protein will not be released; also, binders not eluted at low pH will be now recovered. Noticeably, removal of the display will eliminate bias during infection created by the interference of fusion polypeptides with the transduction process. Elution conditions must be carefully established with positive controls. We recommend using commercial preparations such as Trypsin-EDTA (0.25%) that is also used in cell culture for an incubation period of 10 to 20 min at 37°C. The wash prior to trypsinization is done in PBS. There is no need to neutralize trypsin after elution. The eluate can be kept at 4°C for 24 h without loss of infectivity.
- Acidic elution. This is the most frequently used technique, classically by incubation for 10 min with shaking in the presence 0.1 M glycine/HCl buffer, pH 2.5 with BSA 1 mg/ml. The eluate is neutralized with Tris buffer 1 M, pH 8.0 – 8.5. Use pH paper and different volumes of Tris buffer to determine the best conditions for neutralization.
- Basic elution. Usually done with triethylamine (TEA); note that exposure to basic conditions tends to denature phage rapidly.

Sub-Library Amplification

The eluate is stable in liquid state a few days at 4°C or frozen several weeks at -20°C. Typically the entire eluate is used for re-amplification after the first round and only part of it during the next rounds. Analysis of single clones is preferably done on colonies prepared directly from the eluate rather than on colonies obtained after re-amplification of the library. For amplification, mix part of the eluate or all of it with a volume equal or superior of F^+ bacterial culture in mid to late log phase (OD_{600} between 0.5 and 1.0); incubate with shaking at 37°C for 45 min to 1 h; mix with a volume of 2xYT at least 20 times larger, supplement with ampicillin 100 µg/ml, and incubate overnight at 37°C. Prepare virions the morning after.

To count virions in the eluate, proceed to appropriate dilutions in 2xYT after the 45 min initial incubation, plate on 2xYT/agar plates supplemented with ampicillin 100 µg/ml, and incubate overnight at 37°C.

Pre-made, stabilized, Phage Competent™TG1 and SS320 bacteria are available from **Antibody Design Labs** under the catalog numbers PC001 & PC002. Phage Competent bacteria are ready for transduction and can be thawed and frozen multiple times without loss of infectivity. Their use brings reliability, eliminates contaminations, and shortens experimental time drastically.

Procedure 1. Amplifying Sub-libraries Using Phage-Competent™ Cells

1. Thaw a tube (0.5 ml) of Phage-Competent™TG1or SS320 cells at 37°C for 5 min and place it immediately on ice. Dilute the cells with 2xYT medium. One 0.5 ml tube of cells will yield 20 ml bacterial suspension at A_{600nm} 0.5. If larger volumes of cells are needed, dilute the Phage-Competent™ TG1 cells in the required volume of 2xYT medium and place them in a 37°C incubator at 250 rpm. The doubling of TG1 cell density is 33 min and may vary with laboratory conditions.
2. Mix the bacterial cells and the phage eluate. The cell density should be betweenA_{600nm}0.5and 1 OD_{600}. Incubate for 45 min at 37°C and 250 rpm. The volume of the bacterial cells should always be equal or larger than the volume of the phage eluate.
3. Prepare a 100-fold dilution of the incubated bacteria/phage mixture (10 µl bacteria/phage mix + 990 µl 2xYT). Serially dilute this suspension 10-fold in 2xYT and spread 100 µl of each dilution onto a 2xYT agar plate supplemented with 100 µg/ml ampicillin. Incubate overnight at 37°C in a dry incubator.
4. Expand the cells to the desired volume and incubate overnight.
5. On the second day, prepare frozen stocks from cells pelleted at low speed (~3,000 – 4,000g on a benchtop centrifuge).
6. In parallel, count the colonies on the dilution plates; correct for dilution and initial volume of the eluate and estimate the total number of eluted cfu. Store the dilution plates with well-individualized colonies at 4°C for up to 2 days before starting a new bacterial culture. Colonies on plates that have been stored hermetically wrapped at 4°C can still be used for colony PCR after several weeks of storage.
7. Finally, isolate the virions by PEG precipitation and resuspend in a volume of TBS between 1/10 and 1/50 the initial culture volume.

Procedure 2. Amplifying Phagemid Sub-Libraries Using Home-made Cells

Use bacterial cells freshly grown to mid to late log phase (optical density of A_{600nm}=0.4 – 0.5) - do not use an overnight bacterial culture for transduction. Freshly grown cells may be kept on ice before transduction. Mix virions and cells and incubate immediately for 45 min at 37°C.Proceed to step 4 of the procedure above to prepare the library.

Biopanning Protocols

Protocol 1 – Surface Panning Procedure

This is the most straightforward method to screen the MIM libraries. The target is passively adsorbed on a plastic surface. After incubation in the presence of the library, the unbound phages are washed away and the bound phage are eluted by trypsin. Binders are progressively enriched after each cycle. In the following protocol, we are using high binding capacity Corning® 96-well EIA/RIA plates, cat# 3369, but other brands can be preferred.

DAY 0 – ROUND I

1. Coat 10 wells with 50 µl of the target at 10 µg/ml in PBS. Cover the plate and incubate o/n at 4˚C.

DAY 1

1. The morning after, wash the plate two times with PBST and block the wells with 200 µl of Blocking Buffer (PBST with non-fat milk 5% w/v) at 37°C for 1 h.
2. Thaw the phage library and dilute 10 µl of phage in 500 µl of Blocking Buffer. Wash the plate two times with PBST, add 50 µl of the phage solution to each well and incubate for 2 h at 37°C.

- ❖ A clearance step to eliminate non-specific binders prior to the selection can be done at this stage. Block three times 10 wells on a different plate with Blocking Buffer for 1 h. After removing the Blocking Buffer in the first series of 10 wells, add the diluted phage to the wells and incubate at 37°C for 30 min (Clearance 1). Transfer the phage to the next row of wells and proceed to Clearance 2 and Clearance 3, respectively. Finally, transfer the cleared phage to the positive selection plate as in step 2.

3. Wash the plate five times with PBST and five time with PBS.
4. Distribute 50 µl of freshly defrosted Trypsin-EDTA (0.25%) from Gibco™ to each well. Incubate for 15 min at 37°C and 250 rpm and collect all the phage eluates into a single tube.
    - ❖ It is best to aliquot and freeze Trypsin-EDTA in advance and use a fresh 1 ml aliquot each time.
5. Mix 500 µl of eluate and 100 µl of Phage-Competent SS320 cells with 1400 µL of 2xYT (2 ml total volume, OD_{600} 1.0) and incubate for 45 min at 37˚C and 250 rpm.
6. Prepare four 10-fold dilutions to calculate the number of eluted cfu and spread 100 µl of each onto pre-warmed ampicillin-supplemented agar plates.
7. Transfer the remaining of the culture to 25 ml 2xYT in a 125-ml flask, add ampicillin to a final concentration of 100 µg/ml and incubate o/n at 37˚C and 250 rpm.
    - ❖ Spread another agar plate with 100 µl from the 25 ml, undiluted culture.

DAY 2

1. Count the colonies and calculate the number of eluted cfu.
    - ❖ Retrospectively calculate the MOI using the estimate number of virions (not cfu) and assuming ~1 x 10^9 cells for TG1 and half less for SS320 per one OD_{600} x ml. If the MOI is above 0.1, there were likely more virions than bacteria and the counts are not accurate. The issue usually occurs during subsequent rounds. Proceed to the transduction again using a lower amount of eluate (or more bacteria).
2. Prepare the phage by PEG-precipitation and resuspend in 1 ml of TBS.
3. Measure OD_{269} and store the phage at -80°C.

DAY 3 AND AFTER - SUBSEQUENT ROUNDS

For round 2 and after, follow the selection procedure listed above with the following modifications:

- ❖ Coat two extra wells for negative control selection in parallel, e.g. coat with 50 µl streptavidin or Bovine Serum Albumin (BSA) at 10 µg/ml in PBS.
- ❖ Use an input phage of 0.2 OD_{269}.
- ❖ Use a fraction of the eluate (e.g. 50% on Round 2, 10% on Round 3) to re-amplify the library; carefully determine the MOI during re-amplification.
- ❖ Amplify 50 µl of the negative elution in 0.5 mL culture. Plate no dilution and 1:10, 1:100, 1:1000 dilution.

After Round 3 or when an enrichment becomes visible, proceed with a 96-well clone screening and phage ELISA.

Protocol 2 - 96-Well Plate Screening & Phage ELISA

DAY 1

1. Add 600 µl 2xYT medium supplemented with ampicillin 100 µg/ml to each well of a 2-ml 96-deep-well plate.
    - ❖ Note: Use only deep-well plates with square wells.
2. Seed the plate with 96 individual colonies from the counting plates at the end of the screening or from fresh colonies generated from leftover eluates.
3. Incubate the plate o/n at 37˚C and 250 rpm.
4. Coat two 96-well high-binding capacity ELISA plates with 50 µl of the target or a negative control in each well at 2 µg/ml in PBS. Cover the plate and incubate o/n at 4˚C.

DAY 2

1. Wash the ELISA plates two times with PBST. Block each well with 100 µl Blocking Buffer (PBST with non-fat milk 5% w/v) at 37°C for 1 h.
2. In parallel, centrifuge the deep-well plate with the overnight culture at 4000 rpm for 15 min.
3. In a separate 96-well storage plate, transfer 30 µl of the culture supernatant in each well and combine with 90 µl of Blocking Buffer to make a 1 to 4 dilution.
4. Wash the ELISA plates two times with PBST. Transfer 50 µl of the diluted supernatant to each well and incubate at 37°C for 2 h.
5. In parallel, transfer 100 µl of supernatant to one or more storage plates and freeze at -80°C for future assays. Resuspend the cell pellet in the leftover supernatant and transfer to a storage plate containing glycerol to a final concentration of 10% and freeze at -80ºC for long term storage. Finally, seed a pre-warmed agar plate with the 96 colonies in preparation for sequencing. Incubate at 37˚C until the colonies are visible.
6. Prepare the secondary anti-M13 g8p antibody HRP conjugate (cat# AS003-100 or AS003-500). Dilute to 1:4000 in Blocking Buffer, wash the ELISA plates two times with PBST, add 50 µl to each well, and incubate at 37˚C for 1 h.
7. Wash the ELISA plates five times with PBST. Add 50 µl of Tetramethylbenzidine (TMB) substrate to each well and develop the signal till satisfactory.
8. Read OD at 600 nm. If the signal is weak, add 25 µl of HCl 2 M to each well and read at 450 nm.

DAY 3

1. Sequence the positive clones with the primer psiR2 by rolling circle amplification (RCA).

Biopanning Examples

Biopanning of the MIM-12 Library with the FLAG® Tag Antibody M2

The M2 antibody recognizes the FLAG® tag (DYKDDDDK sequence). The panning was done on plastic surface using high binding capacity Corning® 96-well EIA/RIA plates, cat# 3369. A total of 10 wells was used on round 1 and less for the subsequent rounds. Coating was done in PBS with M2 10 µg/ml. A starting phage OD of 0.2 was used during the screening. PBST with 5% milk was used as blocking buffer. Elution was done with Trypsin-EDTA (0.25%) from Gibco™. After 3 rounds, the enrichment ratio was ~10,000 in the presence of M2. Sequencing of 16 randomly picked clones shows a clear motif that matches perfectly the FLAG sequence on the N-terminal side where M2 is known to bind and a high diversity:

```
      DYKDDDDK
  DIVYDYKDVDSV
  HTIRDYKDFDKC
FHWKFDDYKDGD
   LQMDYKVDDLWI
   LISDYKHEDGFF
FKCVTEDYKSED
    CDDYKHEDHLVA
    SADYKCNDLPYN
  CLMYDYKCMDAS
    HCDYKTMDVLSI
   TLDDYKVFDSVL
   SYFDYKFGDNSD
FVDTCTDYKCAD
   NQCNYKCGDPNH
       YKNLDALSTITM
       YKQFDPVIHITPGGGS
```

Biopanning of the MIM-C10 Library with the FLAG® Tag Antibody M2

Panning of the MIM-C10 library was conducted in a similar way as for the MIM-12 library. The enrichment was modest in comparison, but this did not come as a surprise since the FLAG tag is a linear motif. Thirty clones were sequenced. The FLAG motif is seen in most of them, but the cystine loop clearly imposes constraint on the position of the matching amino acids. The diversity is lower and redundancy is seen in multiple clones. A glutamate immediately after the last cysteine, resulting for faulty nucleotide synthesis, is often selected. Also, short motifs not seen in the FLAG sequence were selected.

```
            DYKDDDDK
AEGCIGPSWESWKNCGGGS          7
AEGCIGTPVNDYKLCDGGS          1
AEGCERKPFVDYKDCDGGS          4
AEGCAISSHNDYKLCDGGS          3
AEGCERTGVDDYKQCDGGS          1
AEGCQHEAWPGYKNCGGGS          1
AEGCKFSMWGDHKDCDGGS          1
AEGCYAEEWPQYKGCGGGS          3

      DYKDDDDK
AEGCFIYKDEVISDCGGGS          2
AEGCWIYKDEVISDCGGGS          2
AEGCWIYKDEFISDCGGGS          1
AEGCNSLEQVTYANCGGGS          4
```

Material, Reagents & Recipes

Buffers

Tris-Buffered Saline (TBS) 10x

TBS 10X, of commercial origin. Use 1x after dilution in pure water.

Phosphate-Buffered Saline (PBS) 10x

PBS 10X, of commercial origin. Use 1x after dilution in pure water.

Reagents

PEG/NaCl 5xStock Solution

PEG-8000 20%w/v, NaCl 2.5 M. Dissolve 100 g PEG-8000 and 75 g NaCl in 400 ml double-distilled water (ddH_2O). Add ddH_2O to a final volume of 500 ml, while stirring at room temperature. The solution can be autoclaved (optional), but mixing during the cooling period is required to prevent a phase separation. Store at room temperature.

Media

2xYT Medium

Use a commercial preparation. Dilute 31 g/l of dH_2O and autoclave.

M9 Minimal Agar Plates

Use 2x commercial preparation, usually already supplemented with thiamine. Warm to 37°C and mix with an equal volume of 2x melted agar (32 g/l in water).

2xYT-Amp Agar Plates

Autoclave agar (16 g/l) in 2xYT medium. Mix well after removal from the autoclave. Add ampicillin 100 µg/ml when temperature is at 50°C (at this temperature, bare hands can painlessly touch and remain on the vessel's surface). Store at 4°C in the dark.

Supplements

Ampicillin 100 mg/ml Stock Solution 1000x

Dissolve ampicillin sodium salt to 100 mg/ml in pure water and filter-sterilize through a 0.22 µM filter. Store at -20°C.

Streptomycin 50 mg/ml Stock Solution 1000x

Dissolve streptomycin sulfate salt to 50 mg/ml in pure water and filter-sterilize through a 0.22 µM filter. Store at -20°C.

Tetracycline 20 mg/ml Stock Solution 1000x

Dissolve tetracycline 20 mg/ml in 70% ethanol and store at -20°C.

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Appendix

MSDS Information

MSDSs (Material Safety Data Sheets) are available on the **Antibody Design Labs** website at the corresponding product page.

Quality Control

Specifications and quality control are detailed on the online product page. **Antibody Design Labs** certifies that the product will perform according to these specifications.

Technical Support

Visit **Antibody Design Labs'** website at **www.abdesignlabs.com** for technical resources, including manuals, vector maps and sequences, application notes, FAQs, etc.

For more information or technical assistance, call, write, fax, or email us at:

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