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Review Article

Selection and maturation of antibodies by phage display through fusion to pIX

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ABSTRACT

Antibody discovery and optimization by M13 phage display have evolved significantly over the past twenty years. Multiple methods of antibody display and selection have been developed – direct display on pIII or indirect display through a Cysteine disulfide linkage or a coiled-coil adapter protein. Here we describe display of Fab libraries on the smaller pIX protein at the opposite end of the virion and its application to discovery of novel antibodies from naive libraries. Antibody selection based on pIX-mediated display produces results comparable to other in vitro methods and uses an efficient direct infection of antigen-bound phages, eliminating any chemical dissociation step(s). Additionally, some evidence suggests that pIX-mediated display can be more efficient than pIII-mediated display in affinity selections.

Functional assessment of phage-derived antibodies can be hindered by insufficient affinities or lack of epitopic diversity. Here we describe an approach to managing primary hits from our Fab phage libraries into epitope bins and subsequent high-throughput maturation of clones to isolate epitope- and sequence-diverse panels of high affinity binders. Use of the Octet biosensor was done to examine Fab binding in a facile label-free method and determine epitope competition groups. A receptor extracellular domain and chemokine were subjected to this method of binning and affinity maturation. Parental clones demonstrated improvement in affinity from 1–100 nM to 10–500 pM.

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1. Introduction

Display of antibody fragments on pIII of M13 bacteriophage has evolved to include Cys-display through a disulfide linkage to pIII [1] and adapter display through a coiled-coil heterodimer [2]. The pIII protein is responsible for phage infection of *Escherichia coli* [3] and thus proteins fused to pIII can interfere with phage infection. Additionally, display of antibody fragments can also be achieved by fusion to the smaller pIX protein at the opposite end of the virion [3,4]. Because it is not involved in the infection process, display on pIX allows for efficient recovery of phage by direct incubation with *E. coli* cells. Additionally, some evidence suggests that affinity selections are more efficient with antibodies displayed on pIX ([5] and personal observations). Yeast and mammalian cell surface display have also been utilized for antibody discovery and optimization [6,7]. These technologies are at a disadvantage for *de novo* discovery due to low transformation efficiency of those cell types. Antibody library designs range from natural source, immunized or non-immune, to semi-synthetic and completely synthetic designs. All approaches share the need for highly efficient mutagenesis and cellular transformation. For synthetic approaches, degenerate oligonucleotides [8], trinucleotide mixtures [1] and solid-phase ligation-based strategies [9] are used to introduce defined panels of amino acids at particular positions within the antibody CDRs. As diversity at multiple positions within the antibody variable region multiply, theoretical diversity increases exponentially, oftentimes beyond what can be captured by molecular methods. Restriction cloning, while inefficient, has been used with brute force to achieve libraries of 10^9–10^{10} members [1,9]. Additionally, Kunkel's mutagenesis [10] has been used to achieve higher transformation efficiencies [11]. We generated a synthetically diversified human antibody library on a panel of germline gene scaffolds, $3V_H$ genes $\times$ $4V_L$-kappa genes by a modified Kunkel's mutagenesis [12] for display on pIX (Fig. 1).

Phage panning is typically done by either direct immobilization of antigen to a polystyrene surface, indirect display through biotin–streptavidin or antibody–antigen complexes, or through solution-capture and "pull-down". Antigen display and solution-capture are preferred methods as alterations to antigen conformation are minimized relative to direct adsorption to polystyrene. Screening for antigen binding is commonly done by ELISA. Use of the Octet (ForteBio) allows for facile epitope binning and off-rate ranking [13]. This information can assist the functional characterization of clones along with affinity maturation. The strategy employed for affinity maturation of antibodies identified from naive libraries is dependent upon library design. Individual CDRs are often randomized in functional lead clones. Light chain shuffling is another

Abbreviations: VH, heavy chain variable region; VL, light chain variable region; RT, room temperature.

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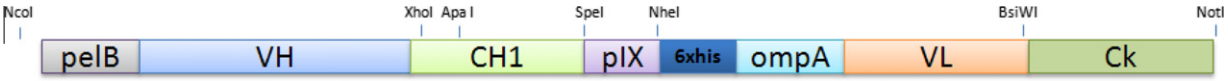

Fig. 1. Diagram of Fab-pIX display vector coding region. A lac promoter (not pictured) drives expression of a pelB-Fab heavy chain fused to pIX at the C-terminus followed by an out-of-frame 6×-his tag. Following the ompA leader peptide is the light chain. Digestion with SpeI and NheI removes pIX from the Fab sequence and re-ligation results in addition of the his-tag to the C-terminus of the heavy chain constant region.

method for maturation [14,15]. Our primary libraries were constructed separately to allow for parallel panning and subsequent maturation of pools of clones. We developed an "in-line" maturation process where selected heavy chain sequences are combined with a library of the corresponding light chain, conceptually similar to prior reports for chain shuffling [14,15].

Diversifying pools of clones and performing high stringency selection can result in a loss of primary hit sequences and, thus, a loss in epitopic diversity [16]. To mitigate this, we developed a facile, label-free epitope binning step for Fabs using the Octet (ForteBio) apparatus. We propose that separately applied maturation libraries to clones in different epitope bins will help maintain epitopic diversity through high stringency selections.

2. Materials and methods

2.1. Selection of antigen-binding Fabs

A human receptor ECD (extracellular domain)-Fc fusion was expressed and purified from HEK293 cells. The protein was biotinylated using an EZ-link NHS Chromogenic-Biotin (Pierce) to approximately one biotin per molecule. Phage panning was done as follows: MC1061F' [11] cells were grown in 25 mL 2×YT (Tetracycline – 15 μg/mL) at 37 °C until log phase (OD_{600nm} = 0.6). Two hundred microlitre of each library phage stock was added to 700 μL PBST-M (D-PBS supplemented with 0.05% Tween-20 and 3% non-fat dehydrated skim milk) plus human Fc (10 μg/mL) and incubated on rotator for one hour at room temperature (RT). Streptavidin-coated Dynabeads® M280 (Invitrogen) were washed two times with PBST (D-PBS supplemented with 0.05% Tween-20) and incubated in PBST-M for 30 min. Biotinylated antigen was added to the blocked beads to a final concentration of 100 nM and incubated on a rotator for one hour at RT. Antigen-bound beads were washed one time with PBST, then blocked in 100 μL PBST-M per library for 30 min at RT. Blocked antigen-coated beads were then added to blocked phage and incubated on a rotator for 1 h. Beads were then washed five times with PBST followed by one wash with PBS. Phage were eluted by adding 400 μL of MC1061F' cells (OD_{600nm} = 0.6) to the beads during a 30 min incubation at 37 °C. The bead mixture was then placed on a magnet and the bacterial suspension was withdrawn for amplification and phage output tittering. Phage-infected cells were serially diluted and each dilution was spotted onto LB agar plates supplemented with 50 μg/mL carbenicillin + 20% glucose (Teknova). The remaining bacteria from each library/Ag combination was grown in 10 mL 2×YT (Carbenecillin – 100 μg/ml) at 37 °C. When OD_{600nm} = 0.6, the bacteria were infected with 1 mL VCSM13 (Stratagene) helper phage at concentration of 10^{10} pfu/mL – 30 min at 37 °C. Phage was then amplified by growth in 10 mL total volume 2×YT (Carbenecillin – 100 μg/ml/Kanamycin – 15 μg/ml/IPTG – 1 mM) overnight shaking at 30 °C. Phage supernatant was isolated by centrifugation of *E. coli* cells at 9000 rpm for 10 min. Two hundred microlitre of phage in the media supernatant is used for the subsequent round of panning. Three additional rounds of panning were performed, maintaining an antigen concentration of 100 nM. For the final round of panning the phage infected *E. coli* were plated across 3 LB-agar (Carbenecillin/Glucose) 150 mm plates and incubated at 37 °C overnight.

2.2. Screening Fabs for binding

2.2.1. Primary screening

To express soluble Fabs, we removed the pIX-encoding sequences from our vector, leaving an in-frame his-tag at the C-terminus of the heavy chain (Fig. 1). Cultures were scraped from the LB-agar (Carbenecillin/Glucose) plates from the final round of panning into 2 mL 2×YT (Carbenicillin/20%glycerol) broth per plate. Fifty microlitre of the harvested bacteria were subjected to miniprep of phagemid DNA (Qiagen). The remaining culture was frozen and stored at −80 °C. The miniprep DNA is digested with NheI and SpeI and the vector band of approximately 5 kb was isolated on an agarose gel (0.8% agarose in TBE, BioRad) and extracted using a gel extraction kit (Qiagen). Two hundred nanogram of the extracted DNA was then ligated with T4 DNA ligase, purified (Qiagen PCR clean-up) and transformed into MC1061F' cells.

Fabs were then expressed and assessed for binding by ELISA. 96-well deep-well plates, filled with 500 μL 2×YT (Carbenecillin – 100 μg/mL), were inoculated with individual bacterial colonies until cells reached log phase. Fifty microlitre of bacteria from each well was then replicated into a second 96-well plate filled with 450 μL 2×YT (Carb). The primary plate was then induced to express Fab by addition of IPTG to a concentration of 1 mM. The induction plate was grown overnight at 30 °C and the replica plate is grown overnight at 37 °C. Maxisorp ELISA plates (Nunc) are coated with sheep anti-human Fd (The Binding Site) at 1 μg/mL or streptavidin (Promega) at 2 μg/mL and stored overnight at 4 °C. The anti-Fd coated plate is used to assess Fab expression for each clone screened and the streptavidin-coated plate is used to determine antigen binding of each clone. On the next day, glycerol was added to the replica plates to a final concentration of 20%/well and are stored at −80 °C. The induction plates were centrifuged at 3500 rpm for 10 min to pellet the bacteria. Maxisorp (Nunc) ELISA plates coated with streptavidin are washed once with TBST (Tris-buffered saline supplemented with 0.05% Tween-20). Fifty microlitre of biotinylated antigen (1 μg/mL) was added to each well of the plate and incubated at RT for 1 h. ELISA plates coated with streptavidin plus antigen and anti-human Fd were washed once with TBST. Wells were blocked with 200 μL PBST-M at RT for 1 h. Plates were washed once with TBST and 50 μL media supernatant per well was added to each plate and incubated for 1 h at RT. Plates were washed four times with TBST, then HRP-conjugated Goat anti-human kappa (Southern Biotech) was diluted 1:5000 in PBST and added for 1 h. Plates are washed four times with TBST and signal is generated with 50 μL chemiluminescence substrate PoD (Roche) per well and detected with a luminescence detection plate reader (EnVision, Perkin Elmer). This method of detection provides a broad dynamic range with a 10- to 1000-fold signal-to-noise ratio. Fabs demonstrating positive expression and binding signals are then cherry-picked and sequenced. Unique clones are consolidated and re-examined for binding to antigen without binding to streptavidin or biotin.

2.2.2. Fab ranking ELISA

Two Maxisorp ELISA plates (Nunc) were coated with anti-human Fd (1 μg/mL) and stored overnight at 4 °C. One plate was used to examine expression of each Fab clone and the other was used to detect Fab binding to antigen. Wells were washed one time with TBST

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