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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.

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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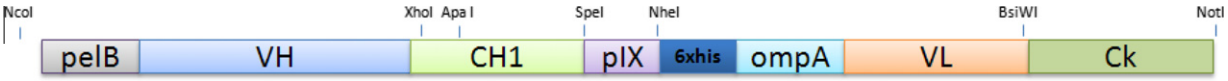

Fig. 1. Diagram of Fab-pIX display vector coding region. A lac promoter (not pictured) drives expression of a pelB-Fab heavy chain fused to pIX at the C-terminus followed by an out-of-frame 6×-his tag. Following the ompA leader peptide is the light chain. Digestion with SpeI and NheI removes pIX from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

then 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. Expression plates were washed four times with TBST and 50 μL of anti-kappa:HRP (1:5000) was added per well for 1 h. Antigen was serially diluted and these various concentrations of the biotinylated antigen was added to the antigen binding plate(s) for 1 h. Antigen binding plates were washed four times with TBST and 50 μL high-sensitivity Streptavidin-HRP (Pierce) (1:5000) was added per well for 1 h. All plates were washed four times with TBST and signal was generated with 50 μL PoD per well and detected with a luminescence detection plate reader (EnVision, Perkin Elmer). A binding curve for antigen binding was derived from the level of signal seen with the antigen dilution series. From this curve an EC50, antigen concentration with half maximal binding signal, was calculated.

2.3. Epitope binning using Octet

2.3.1. Micropurification of Fabs

Fabs need to be partially purified for analysis on Octet. Yield of purified Fab is greatest when isolating protein from cell pellets as opposed to the small amount that is secreted into the media supernatant. We utilized a 6x histidine-tag at the C-terminus of the heavy chain to facilitate purification of Fab fragments (Fig. 1). Expression of Fabs was done as described above and cell pellets from the induced cultures were lysed with addition of 100 μL of Bugbuster (Novagen) lysis buffer containing benzonase (1 U/mL) and incubation at RT for 20 min. Four hundred microlitre of 10 mM Imidizole in 1×PBS was added per sample and mixed. Cell debris was then centrifuged at 3500 rpm for 10 min. Two millilitre of MagnaHIS beads (Promega) were added to 18 mL of 1×PBS, and 200 μL of a 1:10 dilution of MagnaHIS beads were added to each sample. Beads were washed with 700 μL of 10 mM imidizole in 1×PBS, then Fab protein was eluted with 100 μL of 150 mM imidizole in 1×PBS.

2.3.2. Epitope binning on Octet

The micro-purified Fab samples are examined in a sandwich formatted assay (Fig. 2) on the Octet (ForteBio). Amine-reactive sensor tips were placed in a 96-well polycarbonate plate containing 200 μL MES pH 4.0 buffer per well for 10 min. A sample plate was arrayed as follows: baseline, activation (1:50 of EDC/NHS to MES pH 4.0), loading (Fab 1 in MES pH 4.0), quenching (100 mM Ethanolamine), baseline-2 (1×PBS), loading antigen in 1×PBS, baseline-3 (1×PBS), and loading-3 (Fab 2 in 1×PBS). All required buffers were included in the Amine reaction kit (Fortebio). The tip sensor plate/tray and the sample plate were placed into the appropriate positions in an Octet machine. In the data acquisition software under kinetics assay, the corresponding positions are selected for the tip sensors and for the sample plate and the reagents are defined. The length of time for each step is shown in Fig. 1b. The assay is run with tip vibration at 1000 rpm as recommended by ForteBio and at 30 °C.

2.4. In-line maturation of Fabs

2.4.1. Light chain stock library construction

We utilized four human germline gene scaffolds, A27, B3, L6 and 012, for light chain libraries in our primary library design, with a theoretical diversity of 10^7 potential clones. Diversification of the light chains was achieved by PCR with degenerate oligonucleotides as described previously [10]. Diversified fragments of the light chain variable region were combined in an overlap-extension PCR using flanking primers. The 3′ primer was biotinylated to allow for strand separation of the megaprimer.

The full-length light chain library fragment is gel isolated (Qiagen). This fragment was then phosphorylated with T4 polynucleotide kinase (NEB). The single-stranded megaprimer was isolated by strand separation. DNA is incubated with Dynal M280 streptavidin beads. Beads were washed twice with 2x B&W (10 mM Tris (7.5), 1 mM EDTA, 2 M NaCl, 0.1% Tween). The unbiotinylated strand is isolated by denaturation with 0.15 M NaOH. This megaprimer was then precipitated with LiCl (8 M) and Ethanol and re-suspended in dH_2O. A palindromic sequence containing two XbaI restriction sites, TAACCGGGTCTAGACCCCCTGAAAACAGGGGGTCTAGACCCGGTTA, was inserted into the "Framework 3" region of the V_L for 012, A27, L6 and B3 in the pIX phagemid, pCNTO. A 3:1M ratio of V_L megaprimer:pCNTO:V_L-loop ssDNA was mixed and annealed (95 °C for 5 min, cooling by 0.2 °C/s to 55 °C, incubating at 55 °C for 2 min followed by rapid cooling to 4 °C). After annealing, reaction buffer was added, along with T7 DNA polymerase (NEB) and T4 DNA ligase (Invitrogen). The reaction was incubated 2 h at 20 °C, 2 h at 15 °C, followed by heat inactivation at 65 °C for 20 min and storage at 4 °C. The dsDNA product was electroporated into *E. coli* MC1061F' cells, then allowed to recover for 30 min in 16 mL of SOC media and tittered for transformation efficiency. One twenty millilitre of 2×YT with Carbenecillin (100 μg/ml) and Glucose (0.1%) was added to the cells. The cells were grown for 3–4 h to O.D = 0.6. One third of the culture was spun down and re-suspended in 2×YT (Carbenecillin – 100 μg/mL) with 20% glycerol and stored at −80 °C as a glycerol stock. The remaining 40 mL was grown overnight at 37 °C. Plasmid DNA was isolated by maxiprep (Qiagen) for use as a template for heavy chain cloning.

2.4.2. In-line maturation library construction

Light chain libraries were digested with NcoI and ApaI. The cut vector band, 4.5 kb, was gel isolated (Qiagen) for heavy chain cloning. Heavy chain variable regions were PCR amplified from each primary Fab as described [10]. These fragments were gel isolated (Qiagen) and digested with NcoI and ApaI, followed by column purification (Qiagen). The heavy chain variable region fragments were then ligated into a matching light chain library at a vector:insert molar ratio of 1:3, with 500 ng of vector DNA. Ligated DNA was phenol:cholorform extracted and precipitated with 1/10 volumes LiCl (8 M) plus 2 volumes Ethanol and re-suspended in 20 μL dH_2O. MC1061F' cells were electroporated with the ligated DNA and then allowed to recover for one hour in 10 ml of SOC media at 37 °C. Bacteria were serially diluted and plated onto LB agar plates supplemented with Carbenecillin (100 μg/mL) and glucose (1%) to determine transformation efficiency. One twenty millilitre of 2×YT with Carbenecilin (100 μg/ml) and Glucose (0.1%) were added to the remaining cells, which were then grown for 3–4 h until OD_{600nm} = 0.6. Cells were infected with 10 mL VCSM13 helper phage (10^{10} pfu/mL) for 30 min at 37 °C. One twenty millilitre of 2×YT/Carbenecillin (100 μg/ml)/Kanamycin (15 μg/ml)/IPTG (1 mM) was added and grown overnight at 30 °C with shaking. The following morning, phage was precipitated with 20% Polyethylene Glycol (PEG)/0.25 M Sodium Chloride (NaCl) solution and re-suspended in 3 mL PBS and stored in 3, 1 mL aliquots at −80 °C. Phage was then used for affinity maturation selections.

2.4.3. Affinity maturation phage selections

Selections were done as described for the naive libraries except that the stringency was increased by lowering antigen concentration (10 nM for the first round, followed by 1 and 0.1 nM for subsequent rounds) and increasing washes to 10 times in PBS-T with an overnight wash in the third round.

2.4.4. Screening for affinity-matured clones

A binding EC50 was determined for primary hits by ranking ELISA (see above). Screening of maturation library output was then done at the average EC50 antigen concentration. Parental Fabs are included in each corresponding library Fab expression plate to serve as a reference for affinity improvement. Methods for ELISA are the same as

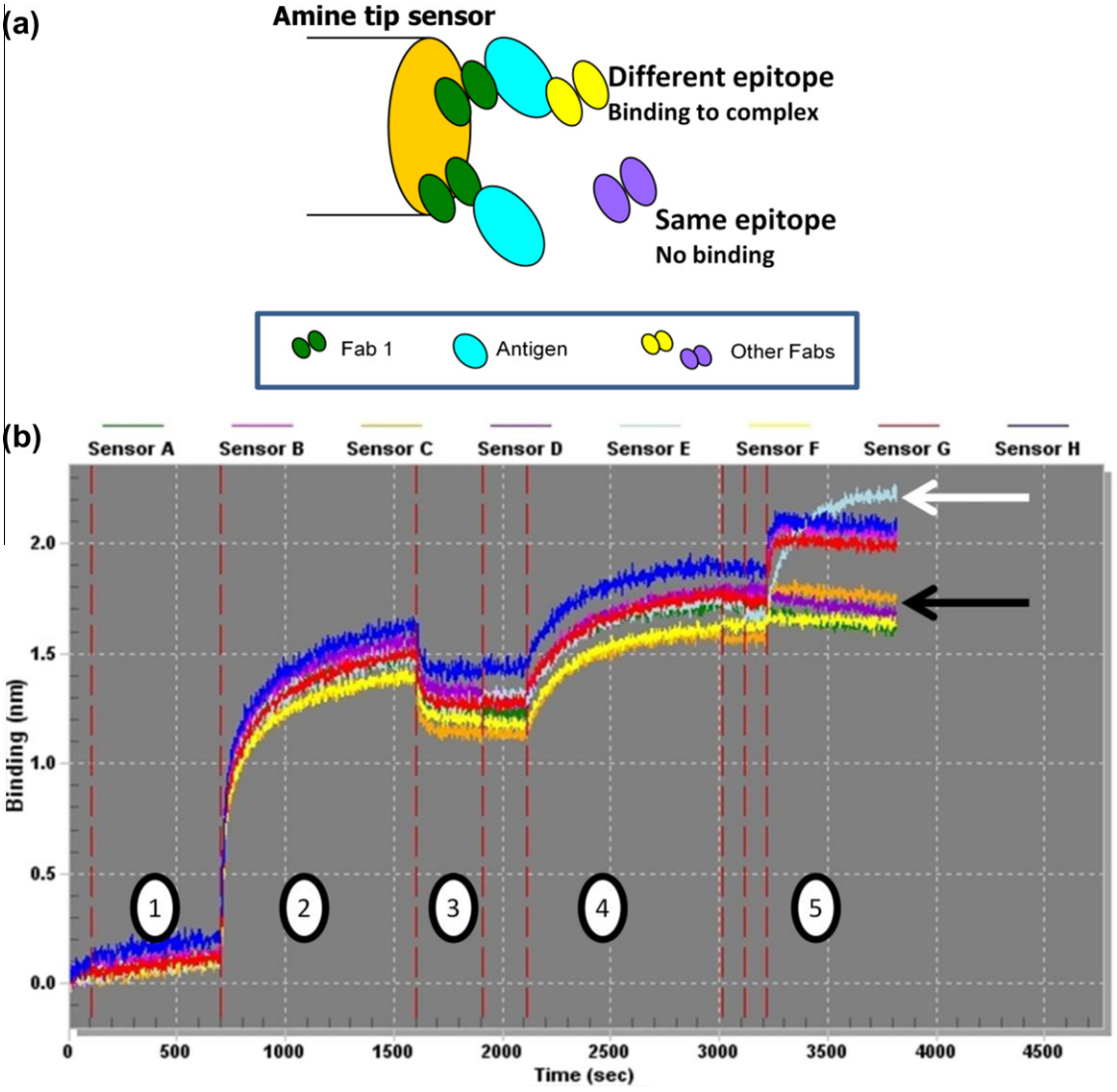

Fig. 2. (a) Schematic of the epitope grouping assay. Fab 1 is captured on the amine-reactive Octet sensor tip. Antigen is bound to the captured Fab 1. Fabs of interest are then added to each tip and assessed for binding. If binding is seen, noted by increase in mass on the tip, then the Fab recognizes a distinct epitope relative to Fab 1. (b) A sensorgram showing activation (1), loading of Fab1 onto the amine reactive sensor tip surface (2), quenching (3), loading or binding antigen target (4), and loading or binding other Fabs (5). Spaces in between steps 1–5 are baselines that contain the buffer similar to the next step in this experiment. The white arrow is pointing to a Fab (light blue) having a different epitope to that of Fab1. The black arrow is pointing to a Fab (orange) having the same epitope or overlapping epitope to that of Fab1.

for primary screening with the following differences. Two Maxisorp plates were coated with anti-human Fd as described. Fabs were captured on two anti-human Fd coated plates. Biotinylated antigen was applied to one plate at the pre-determined EC50 concentration (Section 2.2.2), followed by detection with Streptavidin-HRP. Fab expression was detected on the other plate with anti-kappa-HRP. The ratio of antigen-binding to expression signal was determined for each plate and clones were ranked relative the parental clone(s) from each library. The clones showing improved binding signal relative to parent(s) from each library were sequenced and further ranked using the same Fab-capture ELISA format with a titration of biotinylated antigen. Top affinity clones are then converted to full IgG for analysis in functional assays.

3. Results and discussion

3.1. Selection of antigen-binding Fabs

The first step in the process of identifying Fabs from the naive pIX-displayed Fab library is phage panning. Antigen presentation for the library is a critical aspect for isolation of antibodies to functional epitopes. As adsorption of protein to charged polystyrene can distort primary structure [15,17], it is preferred to display protein through use of a tag or label or to expose the library to antigen in solution and pull-down the Fab-phage/antigen complex with a solid support. Display of Fc-fusion proteins via an anti-Fc antibody is one method of displaying native antigen. More commonly, biotinylation of antigen and capture with streptavidin-coated beads is utilized, primarily due to the broad applicability and high affinity of such an interaction. Antigen is generally chemically biotinylated through primary amines reaction with *N*-Hydroxysulfosuccinimide (Sulfo-NHS) esters on biotin (Pierce). Antigen biotinlylation is directed towards a goal of one biotin per molecule. We have used both antigen display on streptavidin-coated magnetic beads and solution capture of biotinylated antigen followed by pull-down with streptavidin-coated magnetic beads. For *de novo* discovery, we find that display of antigen on beads produces a more diverse panel of binders possibly due to high local concentration of antigen and lack of competition from un-biotinylated antigen. We pooled our synthetic naïve Fab libraries by light chains, keeping each

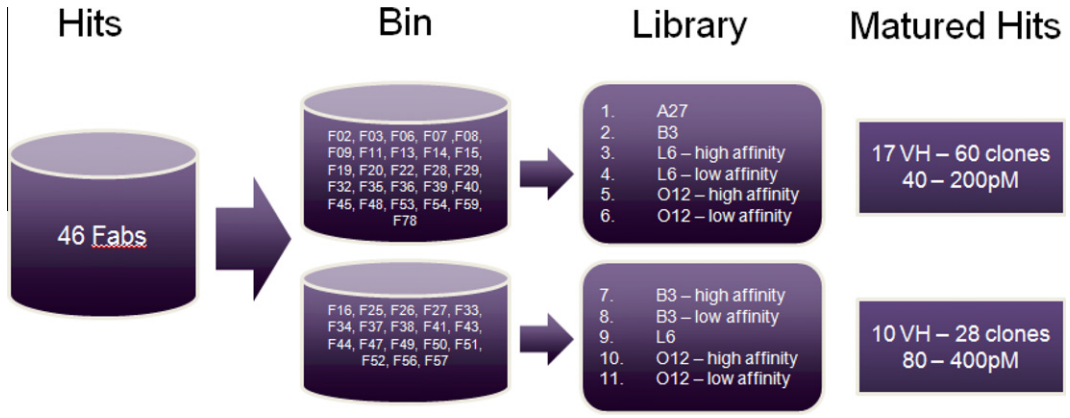

Fig. 3. In-line maturation. Fabs were separated into epitope bins. Fabs were further separated by light chain gene and relative affinity as determined by a ranking ELISA. (Libraries without low/high affinity designations consisted of either single Fabs or clones with similar relative affinities). Libraries were then constructed in eleven pools and panned separately. Fabs were screened from each library output for improved affinity. 88 clones were isolated representing both epitope bins and 27 of the primary V_H clones that were cloned into the maturation libraries.

heavy chain separate. Panning was done as described [10] against a soluble receptor ECD fused to human Fc, with soluble human Fc present as a blocking agent at 10-fold higher concentration than the receptor-Fc fusion.

3.2. Screening for antigen-binding Fabs

Screening soluble Fab for binding is the most reliable method to assess binding of phage selected antibodies, as Fab-phage screening can have a high background and/or high rate of false positives. To produce soluble Fabs from our libraries, we removed the pIX coding sequence by restriction digestion and re-ligation of the compatible overhangs. Fab is then expressed and secreted into the *E. coli* media supernatant for direct screening in ELISAs. We isolated 67 unique Fabs to a receptor ECD-Fc fusion protein, representing all three heavy chain scaffolds from our libraries. We further ranked these Fabs for relative affinity to the receptor ECD using a capture ELISA with a range of antigen concentrations. This ranking method allowed us to separate clones into high and low relative affinity groups. "High" affinity clones would exhibit a *Kd* of 0.5–10 nM while the "low" affinity clones had a *Kd* of 20–100 nM. Some clones exhibited minimal binding in this format and were not further characterized, reducing the number of clones to 46.

3.3. Epitope binning of Fabs

The Octet instrument is well suited for analysis of competitive binding of different proteins [11]. We developed a "sandwich" format for examining such binding using small-scale preparations of Fabs, allowing for high-throughput analysis of library selection output. Micro-purification of Fab protein was performed from 1 mL cultures, yielding between 5–50 μg of 85% pure protein. These purified candidates were then suitable for both kinetic assays, such as off-rate ranking, and epitope grouping.

The micro-purified Fab samples are examined in a sandwich formatted assay (Fig. 1a), where the Fab showing the best binding activity in the ranking ELISA (Fab1) is placed on the tip sensor. Next, the antigen is added and monitored for binding. Fab 2 is added in the final step. If Fab 2 shows binding, then it has a different epitope than Fab 1 and if Fab 2 does not bind then it has the same epitope or an overlapping one to that of Fab 1. We analyzed the 46 Fabs for binding in this format and were able to identify two epitope groups (Fig. 1b).

3.4. In-line maturation of primary Fabs

Primary hits from naive antibody libraries generally require affinity optimization for thorough assessment in functional assays. While state-of-the-art libraries are capable of generating some clones in the sub-nanomolar affinity range, they may not be to the proper epitope for functional activity, or may have liabilities for expression and solubility. Therefore it is critical to have a broad panel of diverse sequences and epitopes from which to identify clones for further development. Using sequence data along with epitope grouping, we devised a scheme to promote affinity maturation of clones in a manageable high-throughput manner, while preserving diversity. We have termed this process "in-line" maturation as it is a seamless process from primary panning and screening to maturation library construction and selection, requiring no clone-specific library design. As clones from primary screening were binned to different epitopes, we also separate clones based on V_L germline gene and relative affinity. The 26 heavy chains from the two epitope binds were isolated and cloned into V_L libraries, yielding 11 libraries, each containing approximately 10^8 clones (Fig. 3).

3.5. Screening maturation library panning

We performed a stringent maturation panning with these libraries and screened for improved Fabs. We were able to isolate 88 improved clones, 60 clones were from epitope bin-1 and 28 clones were from epitope bin-2. Twenty seven of the original 46 heavy chain sequences were isolated during maturation. Clones were converted to IgG and assessed in functional assays and in SPR for affinity. Parental clones demonstrated affinities from 1–100 nM to the target and matured clones demonstrated affinities from 40–400 pM to the target.

4. Conclusion

We show that *de novo* Fab libraries displayed on pIX are an efficient approach to discover and optimize human antibodies. Use of an in-line maturation process, where the V_H scaffold from selected clones was paired with the fully diversified library of its corresponding V_L was very effective at isolating clones in the 10–100 pM range. To apply this to a large panel of clones and maintain diversity in sequence and epitope, we developed an epitope binning method for Fabs on Octet and applied this information to mat-

uration library construction. Through this effort we are able to isolate matured Fabs with diverse sequences and epitopes, thus improving chances of isolating candidates that can be developed as therapeutics.

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