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Generating conformation-specific synthetic antibodies to trap proteins in selected functional states

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Abstract

A set of phage display sorting strategies and validation methodologies are presented that are capable of producing high performance synthetic antibodies (sABs) with customized properties. Exquisite control of antigen and conditions during the phage display selection process can yield sABs that: 1) recognize conformational states, 2) target specific regions of the surface of a protein, 3) induce conformational changes, and 4) capture and stabilize multiprotein complexes. These unique capabilities open myriad opportunities to study complex macromolecular processes inaccessible to traditional affinity reagent technology. We present detailed protocols for *de novo* isolation of binders, as well as examples of downstream biophysical characterization. The methods described are generalizable and can be adapted to other *in vitro* direct evolution approaches based on yeast or mRNA display.

Keywords

synthetic antibody; phage display; protein-protein interactions; conformation specific

1. Introduction

Over the past decade, the accumulation of detailed knowledge of antibody structure and function has enabled phage-displayed antibody technology to emerge as a powerful *in vitro* alternative to traditional hybridoma methods [1–4]. In this context, antibody fragments are expressed and displayed on the phage surface as fusions to a coat protein. Phage pools containing billions of unique antibodies (~1010) are used in affinity selections (Fig. 1A) to isolate antibody variants against the antigens of interest, and the sequence of each antibody can be decoded by sequencing of the viral DNA of the isolated phages (Fig. 1B). Thus, the process directly links the genotype with its phenotype since the selection process not only yields functional antibodies, but also DNA sequences from which they are produced in bacteria. Combining rational *in silico* library design, modern protein engineering tools and sophisticated downstream characterization methods has resulted in generating customized antibodies that recognize conformations or discrete states of target molecules [5–7].

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Below is discussed how combining *in silico* designed libraries with exquisitely controlled selection conditions can generate a class of high performance affinity reagents that can be exploited in myriad of biological and biophysical applications.

1.1. Synthetic antibody (sAB) phage display

The antigen recognition domains of antibodies contain six loops that constitute the complementary determining regions (CDRs). The CDRs are the source of the sequence diversity that is central to their antigen binding properties; three of these loops are contained in the light chain (L1, L3, L3) and three in the heavy chain (H1, H2, H3). Although there are a number of antibody-based phage-display libraries available, a particularly powerful set of synthetic antibody (sAB) libraries is based on a novel "reduced genetic code" concept. Such libraries provide a way for a large number of positions in the CDR loops to be diversified compared to traditional phage-display libraries with no loss in function [8–11]. Another distinction is that antibodies from natural sources contain a considerable level of sequence diversity in their framework, making them each a unique scaffold [12,13], so it is difficult to predict how well they will express or what their stability will be.

Synthetic antibody libraries have been constructed using a scaffold from a humanized Fab 4D5 fragment engineered for high stability and good phage display (Fig. 1B) [8,14]. This allows modular design of sAB libraries where the scaffold can be enhanced for stability, expression or even efficient crystal lattice formation. Further, the antigen-binding interface can be maximized for binding potency step by step, similarly to "synthetic" approaches exploited by chemists. Such systematic and modular optimization is much more difficult with monoclonal antibodies and antibody phage-display libraries derived from natural immune repertoires. Natural repertoires are limited in generation of antibodies against self-antigens, but synthetic antibodies are constructed entirely *in vitro* and thus, are not biased against natural proteins, regardless of source or sequence. For therapeutic applications, optimized human frameworks can be used to minimize immunogenicity, thus obviating the need for humanization. Furthermore, design features can be incorporated to allow facile affinity maturation and adaptation to a high throughput format. For comprehensive review of synthetic antibodies please see Miersch S. *et al. [15]*.

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1.2. Advantages of synthetic antibodies

Economical production and permanent storage of DNA clones are some of the advantages of the sAB approach. Monoclonal antibodies can be reproduced, but the maintenance and large-scale culture of hybridoma cells are cumbersome and expensive. Properties of a monoclonal antibody from the same hybridoma line can gradually change due to the clonal drift [16]. The requirement of sacrificing animals in large animal facility is controversial and in many cases wasteful. In contrast, sABs can be readily produced in *E. coli* and they can be stored economically in the form of an expression vector. Because the amino acid sequences of all sABs can be easily determined by DNA sequencing, any sAB can be reproduced even in the event that the expression clones are lost. Furthermore, the use of a single stable antibody fragment makes it straightforward to reformat a sAB into a full length IgG construct or a single chain Fv.

An important attribute of the sAB phage display approach is the ability to design selection strategies to generate antibodies with customized functions [6,7], which can be classified based on activity or mode of binding. For instance, it is possible to generate sABs that: 1) preferentially recognize a specific conformational state and thus, have the potential to induce a specified conformational change [7]; 2) target specific regions of the surface of the target protein ("regio-specific") [17]; 3) specifically recognize multi-protein complexes

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(unpublished results), and 4) capture and stabilize weak protein-protein interactions (unpublished results) (Fig. 2).

1.3. Applications

sABs can be used in essentially any application where monoclonal antibodies are commonly used: western blots, ELISA and related plate based immunoassays, immunostaining, immunoprecipitation and chromatin immunoprecipitation (ChIP). However, a higher level of power is in their ability to perform more sophisticated functions that utilize the custom designed properties to probe both the static and dynamic features of a protein system [18,19]. Importantly, selection procedures can be designed to generate multiple unique sABs that target a single protein or its ligand bound state, a multi-protein complex, a nucleic acidprotein complex or folded RNAs [6,7,20]. Further, sAB scaffolds with a known structure can be used as effective crystallization chaperones of RNAs and proteins inherently recalcitrant to generate stable crystal lattices [17,20,21]. Moreover, it has been shown that once diffraction quality crystals are produced, the sAB can be used as a molecular replacement model to obtain the initial phasing information speeding up the process of structure solution[17].

2. Experiment design

sABs with desired properties can be obtained from two different types of selection: i) negative or ii) positive. The choice of type of selection is mainly dictated by the properties of the system under study and the desired outcome, since subtraction and competition schemes lead to different outputs.

The basis for subtractive selection is presented in Fig. 3A where negative and positive selections are applied in a sequential manner. First the library is “pre-cleared” to remove unwanted binders by incubating the phage library with the unwanted protein component immobilized on solid support (bead or plate). Most phage binders to the unwanted component are captured and effectively eliminated from the library. This pre-cleared library then is used for positive selection with the primary target without any further modification of the protocol.

In the competitive selection strategy, negative and positive selections are performed in a single step (Fig. 3B). The competitor (unwanted component) is introduced in a soluble form in large excess to the mixture of an affinity-tagged target antigen and the phage-sAB library. This effectively “sponges up” all binders to the competitor that might cross-react with the desired target antigen. The affinity-tagged target is then pulled down using magnetic beads coated with a capture agent, bringing along only target-specific sAB-phages. Because the competitor is not tagged, it and any of the sABs bound to it are removed in the wash steps. The competition selection scheme usually produces higher numbers of clones due to smaller number of steps required to complete the experiment and it is generally better suited to systems with clear separation of conformations or where high affinity components are used.

2.1. Target design for competitive selections

For competitive selections to work, it is essential that the “competitor” closely mimic the property that is being selected against. For discriminating the apo- form from the ligand-bound form of a protein, the competitor is the ligand-bound form of the protein added in solution with the apo- form immobilized on the solid surface, as described above. The strategy is reversed to generate sABs to the ligand-bound form (Fig. 3). The competition selection strategy can be picked only if dissociation of ligand is negligible on the time scale of the experiment.

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The strategy to generate sABs that bind exclusively to a molecular complex comprising of targets A and B is very similar. Target A is immobilized on a magnetic bead and then a saturating concentration of target B is added. This ensures the formation of the A-B complex, even if the affinity is low, while the sABs binding to uncomplexed target B are not captured.

For generating regio-specific sABs several strategies can be used. If the structure is known, strategic mutations or truncations can be made and used as competitors to direct binding to a specific region on the protein surface. If the protein target has a known binding partner, there are two possible outcomes. When the partner is added in excess at the end of the selection round, it will compete off sABs that share its epitope, while leaving the targetbound sABs that bind to other epitopes. Thus, the sABs can be categorized as either sharing or having an independent binding epitope.

2.2. Library design

For phage display selections, we use synthetic Fab fragment libraries built on a single antibody fragment framework and standard phage display generation methods [8,22,23]. In these libraries, limited amino acid diversity is introduced into the 3 heavy chain CDRs: H1, H2, and H3 as well as the third CDR of the light chain: L3 (Fig. 1B). The combined naïve library contains >1010 unique clones. Alternative scaffolds and libraries with similar designs can also be used in the approach described here. Nevertheless quality of the library will strongly influence selection process and is essential to success of antibody generation.

3. sAB selection and screening

This section describes the concepts and detailed protocols for isolation of synthetic antibodies from combinatorial phage-display libraries. Protocols for phage library preparation and subsequent affinity maturation have been previously described [8,22,24]. Below we focus on experimental design, phage display selection schemes, methodology for sAB expression, characterization and troubleshooting.

Fig. 4 illustrates the workflow involved in the sAB generation and validation pipeline. Prior to the phage display selection process (Section 6.2), the target has to meet a set of quality control (QC) criteria; in particular, homogeneity, stability and efficient bead capture. Once the QC is passed, the targets are ready to enter the phage-display sorting pipeline (Section 6.3) following the scheme set out in Section 2. If the selection process is deemed successful, based on increased round to round enrichment of specifically binding sABphages, primary validation can commence (Section 3.2). The goal of primary validation is to provide a rapid high-throughput test to distinguish good clones and triage poor ones. At this point, additional characterization can be performed to rank the relative performance of individual clones in designated high-end applications. The most promising clones are carried on to secondary validation in which their quality and versatility are tested to a much more stringent level (secondary screening - Section 3.3).

3.1. Target protein preparation

Effective selection relies on a specific capture and release process utilizing an affinity tag to maximize solution capture of phage displaying the desired sABs. It is crucial that the tag binds to the capture beads at an extremely high affinity so as to withstand the rigorous washing steps that are essential for removing nonspecific binders present in the library. One of the most generic affinity tags relies on chemical modification of amines or exposed Cys residues with biotin-derivatized reagents with linkers that can be cleaved by reducing agents (Section 6.1) [8]. Random surface biotinylation allows accessing a higher number of epitopes by not restricting the orientation of molecule during the selection and screening

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process. Chemical biotinylation is the method of choice for proteins that are obtained from natural sources due to lack of recombinant protein or the presence of post translational modifications (PTM). Thus, if the protein sample is stable and readily available this method should be used as the first approach. Biotinylated protein samples have to be rigorously tested for the level of biotinylation in pull-down assay described in Section 6.2 (example on Fig. 5A).

A downside to this approach is that many proteins, eukaryotic ones in particular, are not stable enough to withstand chemical biotinylation or their solubility can be compromised due to introduction of hydrophobic biotin moiety. In select cases biotinylation can impair protein function inadvertently affecting its conformation. An alternative approach relies on *in vivo* or *in vitro* biotinylation by the biotin ligase BirA of an AVI-tag fused to the protein of interest [25]. This method is recommended when working in a high throughput environment and has some advantages over chemical modifications. In either case, it is critically important to determine the state of the target protein before library sorting. Denatured and/or aggregated protein samples are the principal problem for why sAB generation fails. Thus, after biotinylation it is important to test for physical state and function. For soluble proteins, one-dimensional ^{1}H NMR spectra can be useful to establish the overall quality of the sample. For enzymes, target validation should include enzymatic activity assays. Determining the oligomeric state by dynamic light scattering in combination with size exclusion chromatography is also an important analytical metric.

3.2. Library sorting and primary screening

Phage library sorting (Section 6.3) is conducted based on the selection strategy chosen as outlined in Section 2. The process combines both automated and manual steps and usually takes several days depending on the number of sorting rounds required. The steps can be readily adapted to high-throughput formats that can greatly expand the scope of the selections. Not only can many more targets be included simultaneously, but variants of the targets and conditions can be screened side by side allowing for direct comparisons of subtle, but important differences that can lead to dramatic changes in the outcomes of the selections.

3.2.1 Phage display selections—The process utilizes a KingFisher magnetic bead handler and involves the following steps: 1) capturing sAB-displaying phages that bind to the target which is immobilized on magnetic beads, 2) vigorous washing to eliminate non-binding phages, and 3) release of the captured phages by cleaving the disulfide linkage within the biotinylation reagent [8] (when a non-cleavable biotinylation reagent is used, phages are released with an acidic solution). The progress of selection can be monitored by phage titering to track the enrichment of targetbinding phages round to round relative to background (Fig. 5B). Phage titering provides simplest diagnostic readout where count of phage particles expressed in colony forming units or plaque forming units can be assessed and compared to the relevant controls (Section 4). Recovered phages are amplified in E. coli and the sorting cycle is repeated. The selective pressure that favors the tightest binders is produced by the successive reduction of the concentration of the target protein usually starting with 100 nM in the first round down to 10 nM in the final round. However, for difficult targets, 10 nM may be too stringent, requiring readjusting the initial and final target concentrations.

3.2.2 Primary screening—Typically, three rounds of library sorting are sufficient to generate a good repertoire of sABs. In cases where few binding clones are isolated in round 3, additional rounds of library sorting can be performed. The initial step to identify binders with desired properties usually employs standard competitive phage ELISA (Section 6.4)

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modified to include compounds or partner molecules present in the selection process. 96 individual ("monoclonal") phages are tested. In some cases fewer clones can be picked, but with a concomitant loss in quality of the final pool. Usually single point competitive ELISA is performed with 20 nM competitor. This allows selection of clones with K_D values of 20 nM or lower. In addition, the long ELISA incubations and multiple wash steps favor clones with the low k_{off} values. Clones for secondary validation are chosen based on several different parameters derived from ELISA data: i) overall strength of ELISA signal (indicating affinity and display level), ii) ability to compete with WT protein (competition ratio - fraction of the signal retained upon protein coating the ELISA plate competing for sAB-phage binding sites with WT non-biotinylated protein added in solution; indicating affinity), and iii) combination of both these values measured for each competitor used in selection (Fig. 5C).

Clones that pass an established threshold are then analyzed by DNA sequencing to determine their uniqueness (Section 6.5). Determining their CDR H3 sequences is usually sufficient for this purpose because this loop is most diverse and should contain unique sequence signatures. If the project involves analyzing data from many targets, for instance in high throughput environments, then dedicated software linked to the database can be used, especially if closely related targets are screened and duplicate sequences need to be pruned across the data sets.

3.3. Secondary screening

Phage displayed sABs obtained in primary screening need to be converted to the format that will allow efficient expression of protein in soluble form. This step is essential to ensure that properties of sABs observed in primary screening also translate to similar properties in protein format later used in all downstream applications. This task can be achieved either by introduction of a stop codon between sAB and phage protein P3 by the means of mutagenesis (Section 6.5) or cloning of the sAB fragment into the bacterial expression vector. To establish the performance of sAB clones several methods provide simple, but robust evaluation of the level of performance as customized affinity reagents. In most of the cases, sABs can be validated with methods similar to those routinely used for monoclonal antibodies [26].

3.3.1. sAB expression—Most of the initial validation steps can be performed using sABs expressed at small scale (Section 6.6) utilizing the phoA promoter system also used in phage display (Fig. 1B). However, full secondary validation usually requires large scale production of sABs (Section 6.7). Both small and large scale methods follow similar steps employing Protein A affinity purification yielding sABs of a least 95 % purity. Yields vary typically from 5 to 20 mg of pure sAB from 1 L of media. Large scale purification can be readily scaled up to 12 L for applications requiring a large amount of antibody. sABs can also be subcloned into other dedicated vectors to introduce additional tags aiding the validation process or even to convert them into a full IgG format.

3.3.2. Biophysical characterization—The principal set of techniques relies on various biophysical methods, some of which are described below.

i. Surface Plasmon Resonance (SPR): This technique is used to determine binding kinetics and affinity [27,28], as well as to map the epitopes with which the sAB interacts with antigen. SPR measurements can be performed by utilizing the same compounds, protein mutants or fragments that were used in phage selections and can provide more quantitative results compared to primary validation by ELISA (Fig. 5D).

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ii. Differential Scanning Fluorimetry (DSF): Useful information can be provided by a fluorescence-based thermal shift assay using DSF [29]. This is an established method for the identification of inhibitors of target proteins from small molecule libraries [30] and can be adapted to screening of sAB-mediated stabilization effects. The assays require a small amount of sample and can be performed in a microplate format using a typical RT-PCR instrument [29,31]. Since majority of proteins unfold at much lower temperatures than sABs (Tm ~80 °C) changes in their stability can be easily detected and are proportional to sAB affinity and concentration (Fig. 5E) [32].

iii. Size exclusion chromatography and light scattering-based approaches: Size exclusion chromatography can provide information about sample homogeneity and size distribution of molecules. This method is particularly useful to monitor sAB complex formation prior to crystallization or to analyze large multi-protein complexes. This technique is useful because sABs typically induce distinct changes in elution volumes upon binding due to their large molecular weight (~50 kDa) (Fig. 5F). Two other analytical high-throughput light scattering-based methods that can be used for sAB validation are differential static light scattering (DSLS) or size exclusion chromatography in conjunction with multi-angle light scattering (SEC-MALS) [33].

3.3.3. Epitope binning—There are many applications where it is advantageous to have a collection of sABs that bind to non-redundant epitopes. Most of the methods in use look to establish whether two sABs can bind simultaneously to the antigen. sABs that recognize non-overlapping epitopes are linked in pairs. These pairs can subsequently be used to group sABs displaying similar binding profiles into bins. The sAB pipeline utilizes single point competition ELISA screening, as described above. Each individual phage-sAB is screened against every other sAB that is used as a competitor following the standard competitive ELISA protocol. An alternative approach is epitope binning using SPR. This experiment provides much higher fidelity of detection although it is more time consuming. For the example and description of the method see [34].

4. Anticipated results

The performance of phage display selection is usually characterized by an enrichment parameter defined as the ratio of the phage count recovered from the target sorting compared to the count of background binding phages in the negative control. Significant enrichment after round 3 and 4 of the library sorting step combined with the results of primary screening can generally gauge the performance of the sAB generation process. As a general rule, the expected enrichment after round 3 should fall within 2 – 100 fold and 10 – 1000 after round 4 (Fig. 5B). Lower or no enrichment, especially in selections where a competitor was not used, indicate poor selection performance which in turn drastically affects success rates of obtaining sABs for the desired conformation, complex or region of the molecule.

The follow up primary screening is performed using a single point competitive ELISA and involves analysis of 96 clones. A successful experiment typically yields 25 – 80 % positive clones in this assay. Because the goal is to generate sABs that perform quite sophisticated functions, there are usually fewer clones that meet the stringent criteria compared to standard phage display selections that capture all binders. Depending on the set thresholds, a successful secondary assay usually yields 10 – 50 % of clones with the desired properties (Fig. 5C).

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4.1. Troubleshooting

Antigen quality is a principal source of problems in selection. Prior knowledge of the biochemical properties of the target is necessary, especially if the selection is performed on an unstable protein or a protein that requires cofactors to maintain its activity. Cysteine-rich proteins can be problematic, as well as proteins with metal binding sites, especially if metals undergo electrochemical reactions with paramagnetic particles such as those used during the selection. It is absolutely essential that the selection should include a positive control using a "standard" target that has known characteristics and selection output. Problems in the selection can be detected earlier in the process if enrichment levels can be compared with the control. If several of the targets show substantially lower levels of enrichment than the control, it can be a signal that those targets are problematic and their preparation or handling has to be re-evaluated; however, there were instances where we were able to obtain good sABs where the enrichment was low.

For problematic targets, there is often a trade-off between affinity and specificity. For instance, a sAB may have good selectivity to a particular conformation, but the affinity may be too low to be useful. As a general rule, it is advised to perform another selection with optimized stringency to obtain binders with the desired characteristic directly from the affinity selection; however, low affinity clones can be "rescued" by performing an affinity maturation step. Since the overall structure and sequence of each isolated sAB is known, this process is straightforward. A library can be designed to introduce a higher level of diversity and several additional cycles of sorting can be run.

Besides affinity issues, there are other possible sources of low recovery of usable clones. A potential troublesome area is the efficiency of the pull-down steps (Fig. 5A) resulting from a non-optimal level of biotinylation. Both too little and too much biotinylation have undesirable consequences. This is where *in vitro* or *in vivo* biotinylation in bacteria during expression can provide an advantage. *In vivo* biotinylation provides high throughput means for biotinylation of proteins utilizing BirA enzyme coexpressed in the same cells [35]. There are also cases where enrichment is high, but the sequence diversity of captured clones is low and dominated by one or a few clones that most likely have expression advantage over the rest of the clones in the pool. This can happen for difficult targets where the pool of "winners" is inherently small and more rounds (>3) were required to pull them out. Ideally clones should be characterized on earliest round possible to not compromise diversity of the pool.

Protein mutants or compounds that stabilize a particular conformational state might unintentionally interfere with the selection process. In such cases, further investigation is required to find a better mutant or compound. One might consider switching to a subtractive selection scheme to achieve a better separation of states. We have successfully used different compounds during screening, mainly small molecule drugs. Usually compounds that are stable and soluble in aqueous solutions do not pose significant problems in phage selections. Low affinity compounds should be avoided and possibly replaced with higher affinity variants if available. The use of organic solvents like DMSO at concentrations higher than 5 % is not recommended, especially if the impact on the target protein cannot be tested. Also, the use of compounds for the phage elution (Section 6.3) should be treated with caution, since bacteriostatic activity might affect phage propagation in the bacterial host. Additionally low affinity compounds might be ineffective in eluting sABs with sub nM affinity. In the case of membrane proteins many commonly used detergents can be used above their CMC without significant loss of infectivity of phage.

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4.2. Limitations of the system

This recombinant system has multiple advantages over the traditional monoclonal based approaches. Nevertheless there are several types of protein systems that inherently produce fewer high affinity sAB clones.

i. Selections on multi-protein systems or proteins with substrates or DNA bound are possible; nevertheless they pose several technical difficulties: namely low affinity or non-specific binding based on electrostatics. Well characterized binary interactions with affinity as low as μM can be stabilized utilizing sAB energy of binding by driving the equilibrium towards complex formation. The feasibility of this is directly linked to the stability of the interactions. Selection schemes targeting higher multimers involving transient interactions become complex and have a negative impact on the selection efficiency lowering the number of unique clones recovered.

ii. Charge: Proteins with high isoelectric points (pI > 10) are problematic because the M13 phage coat is acidic and binds nonspecifically to the target. There are several solutions to this problem, but none is fail-safe [36].

iii. Hot-spots: Not surprisingly, we have found that many proteins have antigenic interaction hot-spots that generate a highly disproportional number of sABs against a single epitope. It is also common that such sites are natural binding sites and sABs targeting them can be very useful in other biological studies. To overcome this obstacle, competitive selections that use variants of the protein with engineered mutations at the hot-spot, or masking the epitope with a characterized sAB, have generally been successful in producing new sABs to other regions of the target's surface. However, sABs that target the overlapping epitope based on the binning experiment described above (Section 3.3) should not be readily discarded since they might still have desired properties.

iv. Conformational flexibility: While all proteins display dynamic behavior, some systems that are extremely flexible only produce low affinity sABs because of the entropic cost they pay to bind the target. To increase a chance of generating sABs against these targets, high energy conformations of the target protein must be "stabilized" during selection by either optimizing the chemical milieu (pH, ions) or addition of ligands or other protein components. In many cases one should aim for much higher recovery of binders rather than affinity geared selections. In such cases it is appropriate to expand the range to include sAB clones that bind at the 40 – 60 nM range, that can be further improved through an affinity maturation step. However, selecting antibodies with poor affinity (in μM range) should be generally avoided. Such sABs are either not specific or target high energy state, where a large fraction of the binding energy is used towards the shift in equilibrium between the conformational states. It has to be noted that peptides are not good targets for antibody generation using either recombinant or traditional hybridoma approaches. Peptides are highly flexible and provide relatively few possible sites of interaction that can be exploited by multiple CDR loops. This is not to say that sABs (or monoclonal antibodies) cannot be found to bind to peptides, however much more clones need to be screened.

v. Post translational modifications (PTMs): Competitive selection approaches are potentially very powerful to differentiate between proteins with and without a particular PTM, especially if it is induces some type of local conformational change. However, if there are multiple PTMs, the probability of deciphering the specificity of the recovered sABs becomes virtually unachievable. The issue of making glycosylation specific sABs, even at a single site, is also problematic

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because of the heterogeneity of the sugar groups. However, it is possible in some cases to make sABs to highly glycosylated proteins by targeting epitopes on the protein surface that are devoid of glycosylation sites. Alternatively, protein can be treated with PNGase to remove all glycans prior to the selection. However, the sABs have to be validated on the untreated sample to ensure they still bind to the native glycosylated protein. The same logic applies to phosphorylation of Ser, Thr and Tyr residues, where phospomimics can be used followed by validation by ELISA using the WT protein.

5. Materials

- EZ-Link NHS-SS-PEG_4-Biotin: NHS ester of biotin with an ethyl-1,3-dithioproprionate and tetraethyleneglycol spacer (Thermo Scientific, 21442)
- EZ-Link Biotin-HPDP: Pyridyldithiol-activated biotin with a hexylenediamine spacer (Thermo Scientific, 21341)
- Streptavidin MagneSphere Paramagnetic Particles (Promega, Z5482)
- dithiothreitol (Sigma, BP172)
- tertracycyline (USB, 22105 100 GM)
- kanamycin (USB, 17924 25 GM)
- ampicillin (USB, 11259 100 GM)
- D-biotin (Sigma-Aldrich, B4639-5G)
- 2xYT media (Fisher, BP2466-10)
- TB media 1 L: - 24 g Yeast extract, 12 g Tryptone, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, (Fisher, BP2468-1), 5 g glycerol (Fisher BP229-4)
- 55244 *E. coli* expression cells: *tonA ptr3 deltaphoA deltaE15 delta(argF-lac)169 degP41 deltaompT KanR* (ATCC, 55244)
- XL1-Blue *E. coli* selection and screening cells: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacIqZΔM15 Tn10 (TetR)*] (Stratagene, 200249)
- M13 KO7 helper phage (NEB, N0315S)
- HRP/anti-M13 antibody conjugate (GE Healthcare, 27-9421-01)
- NeutrAvidin (Thermo Scientific, 31050)
- TMB substrate (Thermo Scientific, 34021)
- Omega E-Z 96 Fastfilter Plasmid Kit (Omega Bio-Tek D1097-01)
- ELISA High-Binding 96-Well Flat Bottom MICROLON 600 Plate (Greiner, 655081)
- 96-Well Deep Well Plates, Round Wells (Axygen, P-DW-20-C-S)
- Costar Polypropylene Plates V-bottom (Corning, 3357)
- 96 well 1.3 mL filter plates NUNC (Thermo Scientific, 278011)
- Axygen 24 Square Deep Well Plate 10mL (Axygen, P-DW-10ML-24-C-S)
- B-PER (Thermo Scientific, 78248)

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- CRAP-Pi sAB expression media 1 L: 53.6 g Yeast Extract (Fisher, BP1422), 53.6 g Hy-Case SF Casein (Sigma, C9386), 35.7 g $(NH_4)_2SO_4$ (Fisher, BP212-212), 7.1 g sodium citrate x2 H_2O (Sigma, C3434), 10.7 g KCl (Sigma, P9541), fully dissolved and treated with 100 mM NH_4OH (aq.) (Fisher, A669-212) and 100 mM $MgCl_2$, (Fisher, M36-212) filtered and supplemented with 110 mM MOPS (VWR,BDH4174-5kg), 7 mM $MgSO_4$,(Fisher, M65-500), 0.55 % Glucose (Sigma, G8270) adjusted to pH 7.4
- Hi-Trap rProtein A FF 5ml (GE Healthcare, 17-5080-01)
- Resource S 1ml (GE Healthcare, 17-1178-01)
- rProtein A Sepharose Fast Flow (GE Healthcare, 17-1279-01)
- KingFisher Flex (deep well head) (Thermo Scientific, 5400630)

6. Methods

6.1. Chemical biotinylation

1. Dialyze or desalt the protein of interest to the buffer of choice. A non-amine buffer of pH 7 – 8 should be used for biotinylation reactions using NHS ester; salt, detergents or stabilizing cofactors or compounds can be added, do not add reducing agents. The starting protein concentration should be preferably in 1–100 μM range. Total amount of protein used in phage display and primary testing can be as little as 5 nmols.

   Nevertheless, losses can occur due to precipitation during labeling and extra protein might be necessary for troubleshooting.

2. Dissolve biotinylation reagent reagent EZ-Link NHS-SS-PEG_4-Biotin for amine labeling or EZ-Link Biotin-HPDP for Cys labeling in dry DMSO or buffer at 10 mg/mL. Avoid exposure of reagent powder to humidity, and prevent condensation of water on the reagent bottle, you can safely store the reagent in DMSO for about 2 weeks without noticeable drop in activity, or conversely reagent dissolved in water has to be used immediately.
3. Mix the EZ-Link biotinylation reagent solution and the protein to obtain 2:1 – 20:1 molar ratio. Adjust the ratio based on molecular weight (Lys content) and efficiency – ideally no more that 3 biotins on average per 20 kDa molecule should be attached. For biotin-HPDP Cys labeling lower ratios can be used and protein should be in fully reduced state and freshly desalted from excess reducing agent immediately before the reaction. Keep the reaction at room temperature and occasionally mix it by gently flipping the tube upside down. Do not place on ice since biotinylation reagent is insoluble in low temperatures.
4. After 60 minutes of incubation, quench the reaction by adding TRIS buffer (pH 7.5 – 8) (10 mM final) (this step is not required for EZ-Link biotin-HPDP reagent). Transfer the reaction to the dialysis tubing. Incubation time longer than 60 min or excessive vortexing is not recommended. Dialyze the reaction mixture against a buffer of choice overnight, preferably for 48 h. Alternatively ion exchange or affinity chromatography can be used to completely remove excess reagent and speed up the process. Gel filtration chromatography can be also used for desalting nevertheless in select cases it might not allow for efficient removal of excess biotinylation reagent.
5. Determine the protein concentration by the method of your choice and proceed to pull-down test using streptavidin-coated beads. Efficiency of biotinylation can be

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also tested by mass spectrometry, HPLC chromatography or HABA test (Pierce) according to the manufacturer recommended procedures.

6.2. Pull-down test to determine the extent of biotinylation and efficiency of antigen capture

1. Wash 50 μL of Streptavidin MagneSphere particles slurry with 3 volumes of binding buffer (buffer of choice used for biotinylation reaction).
2. For a protein of up to ~150 kDa mix up to 5 μg of biotinylated protein with 50 μl of streptavidin magnetic beads suspension washed with binding buffer. Note: more protein can be loaded for smaller proteins.
3. Incubate for 15 min and collect the supernatant (unbound fraction). Keep the total volume of reaction at 10 μL so the whole sample can be loaded on the gel for testing. For a large membrane proteins use up to 200 μL of beads (washed and resuspended in binding buffer containing detergent).
4. Incubate for 15 min and recover supernatant, use magnet to collect the beads at the side of the tube.
5. Wash the beads once with 50 μL of binding buffer.
6. Add 10 μL 100 mM fresh DTT, mix gently with a pipette tip and allow to incubate for 15 min in room temperature. Collect eluate in separate tube.
7. Wash the beads once with 50 μL of binding buffer and keep the beads.
8. Run SDS-PAGE gel with all collected fractions and determine percentage of protein biotinylated and captured on the beads (load unbound fraction, washes, elution and beads after elution). Usually ~80 % pull-down efficiency and elution from magnetic beads should be obtained. Lower efficiencies make the solution capture during library sorting troublesome and should be avoided. Negative results are usually indication of incomplete desalting of reaction mixture from free biotinylation reagent or the reagent is inactive due to hydrolysis of NHS ester.
9. Biotinylated protein can be aliquoted in tubes and frozen at −80 °C or stored at 4°C.

6.3. Competitive and subtractive phage display selection

A. Round 1 selection

   1. In 250 mL shaker flask inoculate 10 mL of 2xYT media supplemented with tetracycline 20 μg/mL with 200 μL of overnight starting culture of XL1-Blue cells (starting culture should be prepared the night before the experiment from single bacterial colony inoculated into 2 mL of 2xYT media supplemented with tetracycline 20 μg/mL) and grow at 37 °C shaking intensely for 3 h or until OD_{600} reaches 0.5. Culture should be grown in parallel to the steps below so the cells can be used for phage infection immediately after the sorting step is complete.
   2. In a 50 mL conical tube combine 16 mL PBS, 3.5 mL PEG / NaCl (20 % PEG 8000, 2.5 M NaCl filter sterilized), 1 mL of 2×10^{12} cfu/mL sAB phage library stock in glycerol and place on ice for 30 min. Spin down at 8000 rpm for 20 min, discard supernatant immediately, flash spin the tube and aspirate remaining supernatant. Resuspend the pellet in 1 mL of PBST-BSA (PBS, 0.05 % Tween, 0.2 % BSA).

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a. For competitive selection: add competitor preferably at 1 μM concentration and incubate for 15 min before adding to the "Protein binding plate".

b. For subtractive selection: add 100 μl of 1 μM biotinylated competitor to 100 μL of washed Streptavidin MagneSphere paramagnetic particles, wash once with one volume of PBST-BSA, place on magnet for 1 min, remove the supernatant and add resuspended phage library. Incubate for 15 min on the rotator. After the incubation is complete magnet down the beads and carefully collect the phage library and add it to the "Protein binding plate".

Note: The amount of sAB library specified is sufficient for sorting of 8 targets, scale up the phage precipitation if more library is required. PBST-BSA should be supplemented with compounds that are going to be used in screening or replaced with selection buffer of choice containing BSA and detergent. In competition experiment soluble competitor molecules interact with phages bearing sABs selective for the competitor before they are transferred to the well in "Protein binding plate" containing target of interest. The incubation time should be sufficient to capture both low and high affinity binders and effectively make them unavailable for binding to the selection target. Also the competitor concentration should be high enough to favour formation of the complex between competitor and sAB-phage in the presence of the target. In subtractive selection sAB-phages specific for the competitor are separated from the library in separate incubation step. Phage pre-clearing as outlined on Fig. 4A should be performed immediately before adding the phage to the "Protein binding plate". Care needs to be taken to not carry over any beads from the pre-clearing step. Usually competitive selection allows for finer control of the conditions. If the aim of the experiment is also maximum binder recovery, competition should be performed starting from round 2.

3. Prepare KingFisher selection plates. Note: Up to 48 targets can be selected at the same time, dispense solutions to the number of wells required.

   a. Protein binding plate: 100 μL of 1 μM biotinylated target protein, and 100 μL of washed Streptavidin MagneSphere paramagnetic particles.

   b. Biotin block plate: 950 μL of PBST-BSA and 50 μL of 100 μM biotin.

   c. Beads wash plate: 1000 μL of PBST-BSA.

   d. Library plate: 1000 μL of resuspended phage to one of eight wells.

   e. Wash 1 plate: 1000 μL of PBST-BSA.

   f. Wash 2 plate: 1000 μL of PBST-BSA.

   g. Bead release plate: 500 μL of PBST-BSA.

   h. Tip load plate: place KingFisher Flex comb in empty plate.

Note: Compounds or partner proteins with low affinity should be included in all wash wells. Buffer composition should be adjusted to match the

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selection objectives. Preferably all competitors should be added in concentration 100 times higher than target molecule.

4. Run KingFisher protocol:

   a. Load tip comb from 'Tip load plate'.

   b. Mix the beads in 'Protein binding plate' at slow speed for 15 min. Collect beads for 30 s 5 times.

   c. Release the beads into 'Biotin block plate' and mix at medium speed for 1 min. Collect beads for 30 s 5 times.

   d. Release the beads into 'Beads wash plate' and mix at medium speed for 1 min. Pause and combine contents of 8 wells into single well of 96 well plate. Collect beads for 30 s 5 times.

   e. Release the beads into 'Library plate' and mix at medium speed for 1 h in room temperature. Collect beads 30 s 5 times.

   f. Release the beads into 'Wash 1 plate' and mix at fast speed for 1 min. Collect beads 30 s 5 times.

   g. Release the beads into 'Wash 2 plate' and mix at fast speed for 1 min. Collect beads 30s 5 times.

   h. Release the beads into 'Bead release plate' and mix at fast speed for 1 min do not collect the beads.

   i. Drop the comb back into 'Tip load plate'.

5. Transfer 500 μL of beads from 'Bead release plate' to 5 mL log phase XL1-Blue cells (freshly grown in step 1) in 250 mL baffled flask. Agitate slowly in room temperature for 20 min. Add 30 mL of prewarmed to 37 °C 2xYT media supplemented with ampicillin 100 μg/mL and 10^{10}/mL M13 KO7 helper phage. Shake at 300 rpm in 37 °C for 20 h. Note: In described method phage is propagated in single flask for 8 individual targets, therefore same phage sample will be used for individual target on round 2.

6. Transfer the phage cultures to conical tubes and spin down the cells at 8000 rpm, 4 °C for 10 min. Collect the supernatant to a fresh tube with 6 mL of chilled PEG / NaCl solution. Gently mix and incubate on ice for 30 min. Spin down at 8000 rpm, 4 °C for 10 min and discard supernatant immediately. Flash spin to remove remaining supernatant. Resuspend the phage in 0.5 mL of buffer (PBST-BSA or buffer of choice supplemented with compounds or competitors). Spin again at 12000 rpm, 4 °C for 10 min and transfer the supernatant to the new tube. Prepped phage will be used as an input for round 2.

   a. For competitive selection: add competitor preferably at 1 μM concentration and incubate for 15 min before adding to the "Protein binding plate".

   b. For subtractive selection: add 100 μl of 1 μM biotinylated competitor to 100 μL of washed Streptavidin MagneSphere paramagnetic particles, wash once with one volume of PBST-BSA, place on magnet for 1 min, remove the

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supernatant and add resuspended phage library. Incubate for 15 min on the rotator. After the incubation is complete separate the beads with a magnet and carefully collect the phage library to be added to the “Protein binding plate”. Note: Phage clearing steps can be also programmed on the KingFisher magnetic bead hadler.

B. Round 2 to Round 4 selection. Note: All remaining rounds follow the same protocol, only amount of protein and phage is modified to control the stringency of selection. Note: When Avi-tagged constructs are used it might be necessary to pre-clear the library using biotin blocked Streptavidin MagneSphere paramagnetic particles to lower the non-specific background in selection.

1. Prepare the log phase cells as described in Section 6.3 A) step 1.

2. Prepare KingFisher selection plates. Note: Each target requires 2 wells on all selection plates listed below: one for target selection “+” and the second one for negative control where no target protein is added “–”. Place the wells “+” and “–” next to each other in plates to speed up the phage pipetting and titering. Compounds should be added to “Protein binding plate” at concentration at least 10 times higher than expected K_D to obtain saturation of binding. Low affinity compounds should be also added in all wash plates. Adding protein competitor in all wells is advised.

   a. Protein binding plate: 5 μL of 1 μM biotinylated target for round 2 and 1 μL for rounds 3 and 4, add 20 μL of phage solution for round 2 (prepared in Section 6.3 A step 6) and 10 μL for round 3 and 4 (prepared in Section 6.3 B step 8). Bring the total volume of the well to 100 μL with PBST-BSA.

   b. Bead plate: 20 μL of washed magnetic beads for round 2 and 10 μL for round 3 and 4, add PBST-BSA to the final volume of 100 μL.

   c. Biotin block plate: 95 μL of PBST-BSA and 5 μL of 100 μM biotin.

   d. Wash 1 plate: 100 μL of PBST-BSA.

   e. Wash 2 plate: 100 μL of PBST-BSA.

   f. Wash 3 plate: 100 μL of PBST-BSA.

   g. Elution plate: 100 μL of PBST-BSA containing 100 mM DTT. Note: Always use freshly prepared DTT solution. Alternatively if AVI-tag construct is used add 100 mM Glycine-HCl pH 2.1. (Solution needs to be neutralized after sorting is complete with 1M HEPES pH 7.5 before phage infection step).

   h. Tip load plate: place KingFisher Flex comb in empty plate.

3. Run KingFisher protocol:

   a. Load tip comb from ‘Tip load plate’.

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   - **b.** Mix the beads in 'Protein binding plate' at medium speed for 15 min.
   - **c.** Premix beads at fast speed in 'Bead plate', collect for 10 s 3 times and release to 'Protein binding plate', mix for 15 min at fast speed and collect for 10 s 3 times.
   - **d.** Release the beads into 'Beads plate', mix beads at fast speed for 30 s and collect beads for 10 s 3 times.
   - **e.** Release the beads into 'Biotin block plate', mix at fast speed for 30 s and collect beads for 10 s 3 times.
   - **f.** Release the beads into 'Wash 1 plate', mix at fast speed for 30 s and collect beads for 10 s 3 times.
   - **g.** Release the beads into 'Wash 2 plate', mix at fast speed for 30 s and collect beads for 10 s 3 times.
   - **h.** Release the beads into 'Wash 3 plate', mix at fast speed for 30 s and collect beads for 10 s 3 times.
   - **i.** Release the beads into 'Elution plate', mix at fast speed for 15 min and collect beads for 10 s 3 times.
   - **j.** Release the beads into 'Biotin block plate'.
   - **k.** Drop the comb back into 'Tip load plate'.

7. Infect bacteria and make serial dilutions to determine the enrichment ratios.

   - **a.** Dispense 100 μL of log phase XL1-Blue cells (freshly grown in step 1) into rows A and E of 96 well plate, fill the remaining wells with 90 μL of 2xYT media. Serial dilutions will be prepared in top and bottom half of the plate so 12 targets can be titered on single 96 well plate.
   - **b.** Transfer 50 μL (round 2) or 10 μL (rounds 3 and 4) of eluted phage from 'Elution plate' to rows A and E maintaining original order of "+" and "–" wells from the 'Protein binding plate'. Agitate slowly in room temperature for 20 min and make 3 ten-fold serial dilutions each time transferring 10 μL of material and mixing well. Note: Change tips between the serial dilution steps to prevent nonspecific carryover of cells on the outside of the tip.
   - **c.** Plate 10 μL of each of the serial dilutions for wells "+" and wells "–" in sections of the single ampicillin agar plate, do not discard serial dilution plate.
   - **d.** Transfer contents of the wells "+" to the individual wells of 24 well deep well block filled with 3 mL 2xYT media supplemented with ampicillin 100 μg/mL and 10^{10}/mL M13 KO7 helper phage. Seal the plate with breathable film and shake at 300 rpm in 37 °C for 20 h.

8. Spin down the cells in 24 deep well blocks at 4000 × g, 4 °C for 20 min. Transfer the supernatants to the fresh plate filled with 600 μL of

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chilled PEG / NaCl solution. Gently mix and incubate on ice for 30 min. Spin down at 4000 × g, 4 °C for 1 h and very gently discard the supernatant. Flash spin the plates and aspirate remaining supernatant, avoid aspirating the pellet. Resuspend the phage in 100 μL of buffer (PBST-BSA or buffer of choice supplemented with compounds or competitors) and transfer to the regular 96 well plate. Spin again at 4000 × g, 4 °C for 20 min and transfer the supernatant to the new plate.

  a. For competitive selection: add competitor at 1 μM concentration and incubate for 15 min before transferring to the "Protein binding plate".

  b. For subtractive selection: add 100 μl of 1 μM biotinylated competitor to 100 μL of washed Streptavidin MagneSphere paramagnetic particles, wash once with one volume of PBST-BSA, place on magnet for 1 min, remove the supernatant and add resuspended phage library. Incubate for 15 min on the rotator. After the incubation is complete separate the beads with a magnet and carefully collect the phage library to be added to the "Protein binding plate". Note: Phage clearing steps can be also programmed on the KingFisher magnetic bead hadler.

9. Examine the agar plates with phage titers after overnight growth. In case of lack of enrichment perform another round of sorting (go to Section 6.3 B step 1). If a significant enrichment ratio is observed (more than 20) plate the remaining volume of the wells from serial dilution plate and incubate at 37 °C until colonies reach ~0.5 mm in size. Proceed to clone picking (Section 6.4. step 1) and ELISA testing.

6.4. Competitive phage ELISA

1. Pick 96 single colonies into 96 deep well block filled with 400 μL 2xYT supplemented with ampicillin 100 μg/mL and 10^{10}/mL M13 KO7 helper phage. Seal the plate with breathable film. Grow for 20 h, shaking fast at 300 rpm in 37 °C.

2. Coat 2 ELISA plates with 50 μL 2 μg/mL NeutrAvidin dissolved in PBS buffer. Incubate for 1 h in 37 °C or 16 h at 4 °C.

3. Discard coating solution and add 150 μL of blocking buffer (BSA 0.5 % in PBS) and incubate for 1h in 37 °C or 16 h 4 °C.

4. Spin down the phage growth plate form step 1 and transfer supernatant to a fresh plate.

5. Prepare two phage dilution plates as follows:

  a. Dilution A: Add 7 μL of phage supernatant to 63 μL of PBST-BSA (PBS, 0.05 % Tween, 0.2 % BSA).

  b. Dilution B: Add 7 μL of phage supernatant to 63 μL of PBST-BSA with a 20 nM soluble competitor (non biotinylated target protein) and incubate for 15 min. Note: Competitor concentration can be adjusted from 10 to 100 nM; in case of AVI-tagged proteins add 1 μM biotin to block residual binding sites on the plate; phage can be more diluted, up to 50 fold - the goal is to obtain a moderate signal from dilution A.

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6. Wash blocked ELISA plates (prepared in step 3) 3 times with PBST (PBS, 0.05 % Tween) on ELx405 plate washer and add 50 μL of 20 nM biotinylated target protein.
7. Incubate for 15 min and wash 3 times with PBST.
8. Apply diluted phage transferring 50 μL from dilution A and B plates (step 5) to the coated ELISA plates.
9. Shake for 15 min at room temperature and wash 3 times.
10. Add 50 μL of 5000x diluted HRP/anti-M13 antibody and incubate for 30 min with shaking.
11. Wash 3 times and add 50 μL of TMB substrate.
12. Incubate from 5 to 15 min with shaking and stop the reaction with 50 μL of 1M H_3PO_4.
13. Read the absorbance at 450 nm in a plate reader.
14. Plot signal of dilution A as function of the ratio of dilution B to dilution A.
15. For further analysis pick the clones with the highest signal from dilution A and lowest ratio of dilution B to A signals.

6.5. Clone sequencing and sequence analysis

1. Prepare log phase XL1-Blue cells as described in Section 6.3. A) step 1.
2. Dispense 10 μL of log phase XL1-Blue cells into each of the wells of 96 well deep well block.
3. Cherry pick primary assay positive sAB phage clones from the cultures used for the ELISA experiment (Section 6.4 step 4) by transferring 1 μL of supernatant to the new plate with XL1-Blue cells.
4. Add 900 μL of TB media to the plate and shake overnight at 37 °C, 250 rpm.
5. Isolate plasmid DNA using Omega E-Z 96 Fastfilter Plasmid Kit according to the manufacturer's protocol. Elute the dsDNA with 100 μL of sterile water adjusted to pH 8.5.
6. Sequence both HC and LC of sAB.
7. Analyze sequencing data using multiple alignment tool such as MAFFT [37]. Compare identity of the randomized CDR loops and check entire sAB scaffold for mutations.
8. After isolating clones with unique CDR loop sequences perform site-directed mutagenesis using QuikChange method to convert sAB from phage display format to protein expression format by introduction of a stop codon in the linker connecting heavy chain to phage P3 protein. This step can be also performed using Kunkel mutagenesis [38].

6.6. Small scale sAB purification

1. Transform 10 μL of chemocompetent 55244 cells with 1 μL of sAB plasmid DNA in PCR plate. For protocols describing the preparation and transformation of chemically competent cells, refer to Maniatis et al. [39]. Do not plate the transformants.

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2. Transfer the cells to the 24 well deep well block and bring up the volume to 5 mL with 2xYT supplemented with 100 μg/mL ampicillin. One 96 well of transformants has to be split into 4 24 deep well blocks for growth.

3. Grow 8 h at 30 °C shaking at 200 rpm, pellet the cells at 3000 × g for 20 min, decant media and resuspend the cell pellets in 5 mL of CRAP-Pi supplemented with 100 μg/mL ampicillin.

4. Grow overnight at 30 °C shaking at 200 rpm.

5. Pellet the cells at 3000 × g for 20 min and discard the media.

6. Add 200 μL of lysis buffer containing 90 % B-PER, 0.5 M NaCl, 0.2 mg/mL lysozyme, 0.2 mg/mL DNaseI and shake vigorously to resuspend at room temperature for 30 min and transfer to the 96 well plate.

7. Pellet insoluble debris by spinning at 3000 × g for 1h at 4 °C and carefully harvest clarified cell lysate for affinity purification.

8. Dispense 60 μL of rProtein A Sepharose Fast Flow resin (50 % slurry) into each of the wells of the NUNC filter plate.

9. Equilibrate resin with 1 mL of PBS and apply whole cell lysate to resin and let it drip through, avoid using vacuum to speed up the process to prevent foaming.

10. Wash with 0.5 mL of PBS once and twice with 0.25 mL of PBS buffer.

11. Place the block on vacuum manifold and vacuum for 1 min at 70 mbar to remove excess of buffer, alternatively spin over the waste plate at 600 × g, 2 min.

12. Elute with 100 μL of 0.2 M acetic acid to the collection microplate by passing it twice through the beads allowing for about 15 min of total contact time. You can spin down the filter plate to collect the dead volume of the resin and frit.

13. Neutralize the elute with 100 μL of 1 M HEPES pH 7.4 and store at 4 °C.

14. Regenerate binding plate by wash with 500 μL of 1 M acetic acid and 500 μL H_2O. Equilibrate the resin with 500 μL of PBS. For storage and future use dry the bottom of plate by pressing on a clean paper towel, tape bottom of the plate, apply 100 μL of PBS to all wells, and seal the plate. Store at 4 °C.

15. Proceed with protein quantification using Bradford assay in microplate format according to the manufacturer protocol.

6.7. Large scale sAB expression and purification

1. Transform 10 μL of 55244 chemocompetent cells with sAB plasmid DNA and directly inoculate starter culture of 50 mL 2xYT supplemented with ampicillin 100 μg/mL and kanamycin 10 μg/mL at 37 °C, 250 rpm for 16 h. Note: always start from fresh transformation; transform late during the day so the culture will not overgrow.

2. Use 100 % of the overnight culture to inoculate 1 L 2xYT supplemented with ampicillin 100 μg/mL. Grow for 8 h in a baffled flask at 37 °C, 250 rpm.

3. Pellet the cells 5000 × g, 10 min and resuspend in main culture media. Use 1 L CRAPPi media supplemented with ampicillin 100 μg/mL in 2.8 L non-baffled Fernbach flask (36 % v/v).

4. Grow culture for 16 h at 30 °C shaking at 200 rpm.

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5. Pellet the cells 5000 × g, 10 min, 4 °C and discard the supernatant. Do not freeze the pellet.
6. Resuspend pellets in lysis buffer containing 50 mM TRIS, 500 mM NaCl, 0.5 % Triton X-100, DNaseI 0.01 mg/mL, 0.5 mM $MgCl_2$ pH 8.0. Use 5 mL of lysis buffer per 1 g wet cell pellet.
7. Lyse the cells using the high pressure homogenizer (eg. Emulsiflex C3). Pass lysate through microfluidizer twice to ensure complete lysis.
8. Heat the whole cell lysate to 65 °C in water bath for 30 min and immediately chill on ice for 5 min.
9. Clear the lysate by spinning it down at 20000 × g, 1 h, 4 °C. Collect the supernatant and filter through 0.22 μM filter before loading it onto the purification column.
10. Regenerate Hi-Trap rProtein A FF 5 ml with 3 column volumes of 1 M acetic acid. Note: For best results it is advised to perform all liquid chromatography steps on FPLC systems eg. AKTA Purifier 10.
11. Equilibrate column with 10 volumes of running buffer containing 50 mM TRIS, 500 mM NaCl pH 8.0.
12. Load supernatant onto column at ~10 mL/min using peristaltic pump and immediately wash with 10 column volumes of running buffer 50 mM TRIS, 500 mM NaCl pH 8.0 or until absorbance 280 nm reaches baseline.
13. Elute protein with 0.1 M acetic acid at 5 mL/min collecting 2 mL fractions. Note: The eluted solution can be directly loaded onto ion exchange column (step 16) without neutralization or desalting step. The sAB is typically more than 95 % pure following Protein A purification, and for most applications, it is sufficient. If further purification is required proceed to step 15.
14. Regenerate Hi-Trap rProtein A FF 5 mL column with 5 column volumes of 1M acetic acid and equilibrate with 5 column volumes of running buffer for short term storage.
15. Recharge the Resource S 1 mL according to the manufacturer protocol and equilibrate with with ion exchange running buffer A containing 50 mM sodium acetate pH 5.0 at 5 mL/min. Note: It is necessary to use high performance column for best separation results.
16. Load fractions containing sAB from step 13 onto column at 2 mL/min.
17. Wash column with 5 column volumes of buffer A at 5 mL/min or until baseline is reached.
18. Elute sAB with a linear gradient 0 – 50 % of buffer B containing 50 mM sodium acetate, 2 M NaCl pH 5.0 over 45 mL at 2 mL/min collecting 1 mL fractions.
19. Recharge the column with 100 % buffer B and equilibrate with water for short term storage. Note: To extend life of the column proceed with column regeneration according to the manufacturer protocol.
20. Run SDS-PAGE on fractions of interest, pool and dialyze fractions containing pure sAB to the buffer of choice.

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7. Conclusions

We have devised strategies and generated extensive proof of concept data demonstrating the capabilities of a high throughput phage-display pipeline to produce multipurpose synthetic antibodies to challenging protein systems. The pipeline includes all steps from target preparation, to phage display selections and several levels of validation. The pipeline processes are organized to allow most steps to be automated and run in a high throughput mode. The duty cycle of all operations takes about two weeks, start to finish and can be multiplexed to potentially accommodate over one hundred targets at a time. However, the protocols are equally applicable to running in a mode where researchers have only one, or a few targets to screen.

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Abbreviations

sAB	synthetic antibody
CDR	complementarity determining region
ELISA	enzyme linked immunosorbent assay
PTM	post translational modification
WT	wild-type
CMC	critical micelle concentration
ChIP	chromatin immunoprecipitation

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Highlights

- We present a streamlined method of generating synthetic antibodies by phage display
- *In vitro* selections allow for unique control over the antibody selection process
- Synthetic antibodies can be made that specifically target functional states of molecules
- Trapping of discrete protein conformations is possible

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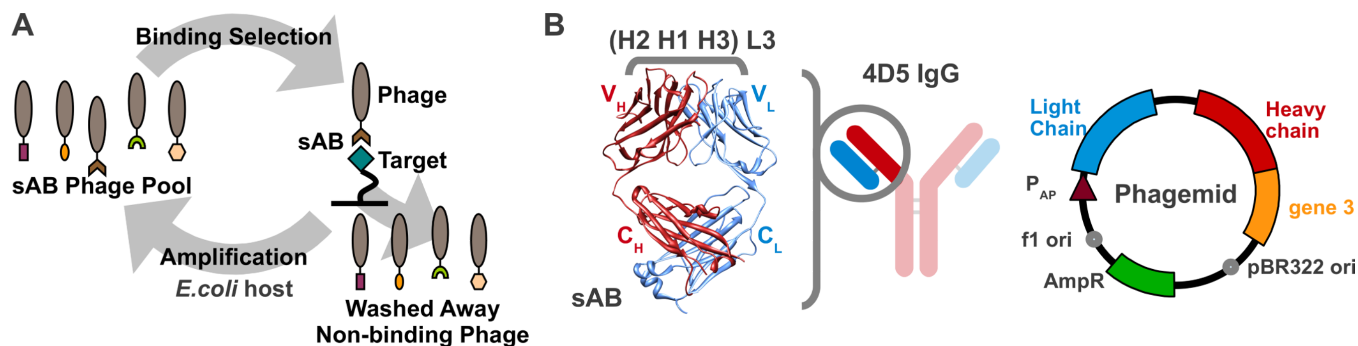

Fig. 1. sAB phage display

A) sAB binders undergo standard phage affinity selection and amplification in host E. coli cells. **B)** sAB - optimized Fab fragment of anti-Her2 mAB scaffold where CDR loops L3, H1, H2, H3 are randomized, and the entire antibody fragment is encoded on the phagemid. The sAB is fused to the p3 coat protein, allowing for display on the surface of the phage.

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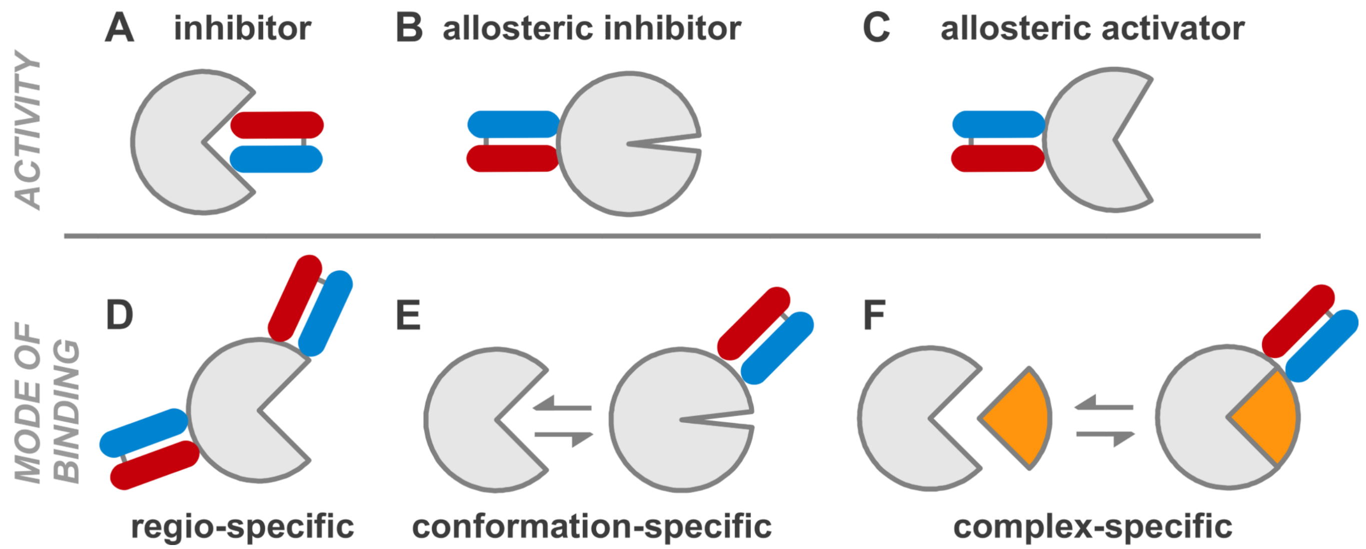

Fig. 2. sAB binding characteristics

Phage display selections can be tuned to generate sABs with multiple activities **A), B), C)** based on either targeting a particular surface epitope **D)**, conformational state **E)** or stabilizing a transient complex **F)**.

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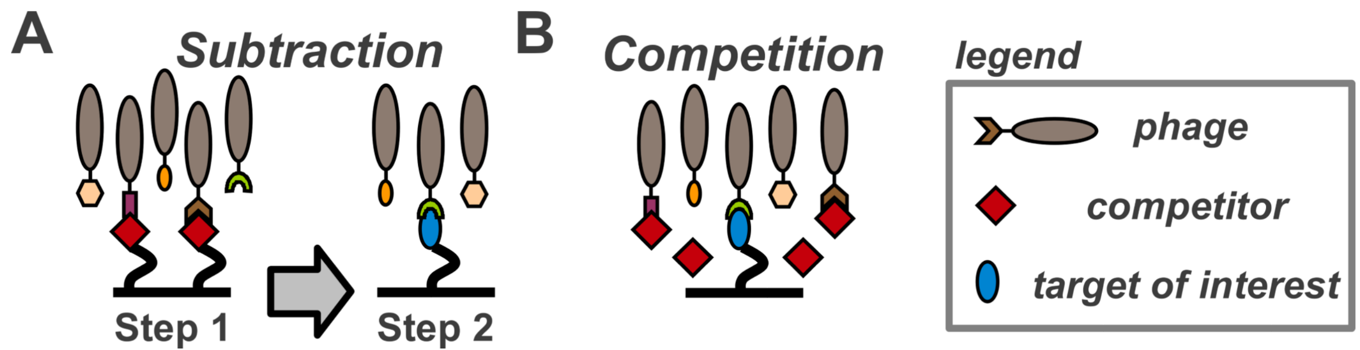

Fig. 3. sAB selection strategies

A) Subtractive selection - competitor (red diamond) in step 1 (negative selection) is added to the beads, library is pre-cleared and added in step 2 (positive selection) to the new beads containing target of interest (blue circle), **B)** competition selection - competitor is added in excess in solution along with captured biotinylated target.

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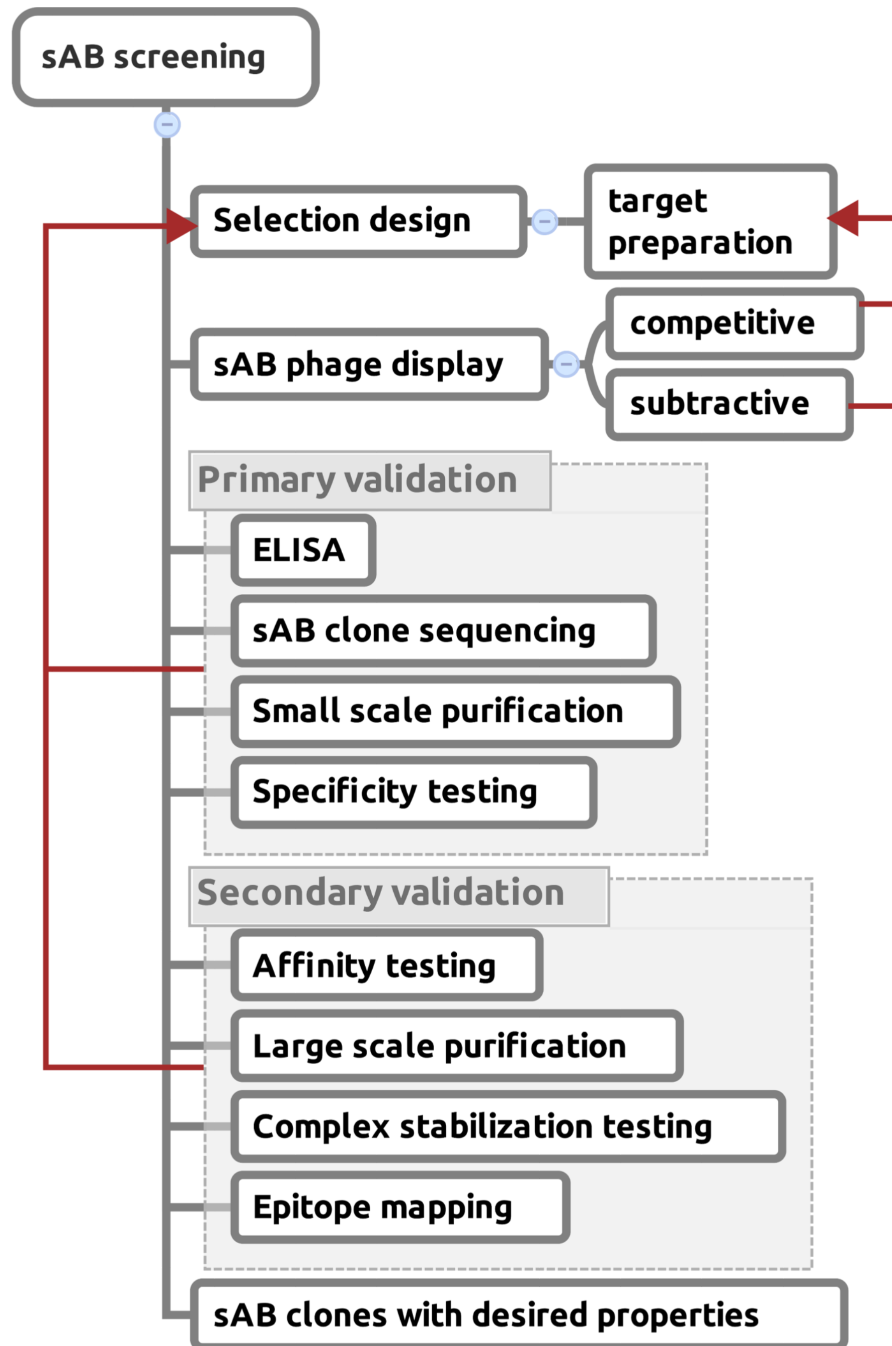

Fig. 4. Process work flow chart

sAB screening starts with selection design (Section 2) which is followed by phage display selection (Section 3). Information acquired during selections can be used to further enhance experiment design and introduce necessary modifications (for troubleshooting see Section 4). Primary and secondary screening proceeds immediately after (Sections 3.2 and 3.3).

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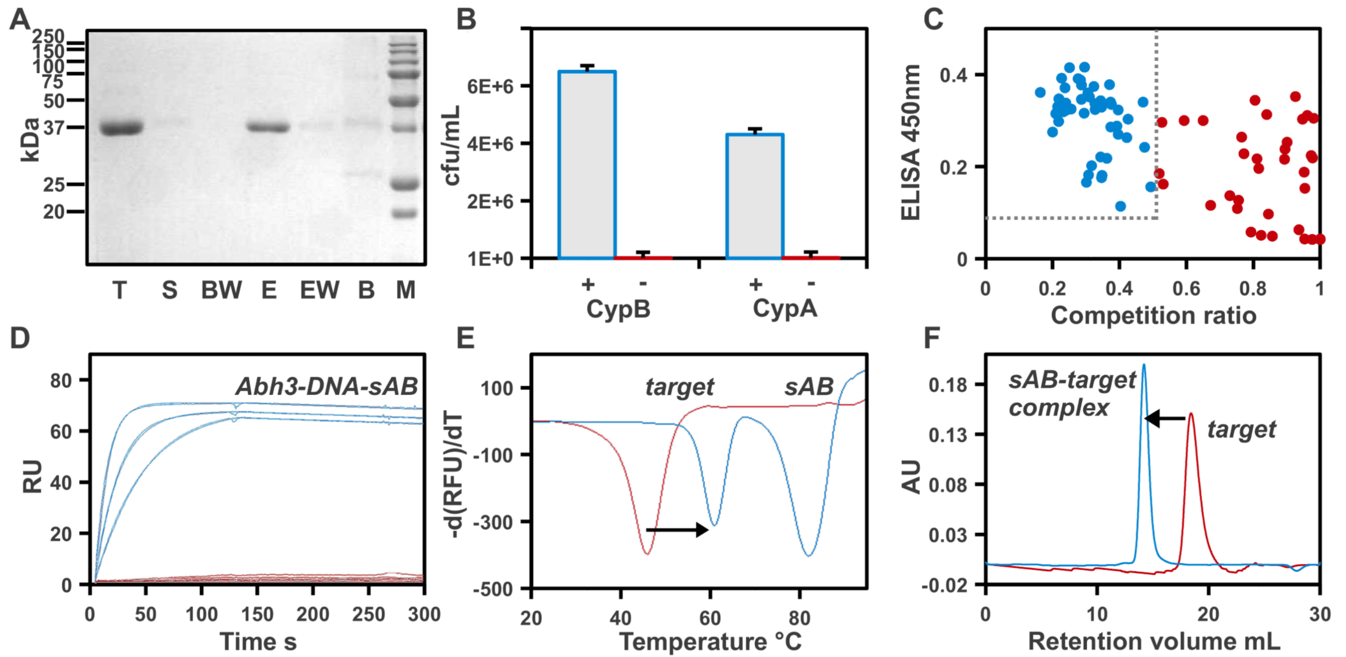

Fig. 5. Examples of primary and secondary validation

A) Pull-down assay; SDS-PAGE gel: T -total biotinylated protein before loading on the beads, S - supernatant (fraction of the protein not captured by streptavidin), BW - bead wash, E - DTT elution, EW - wash after elution, B - beads, M - protein marker. Almost 90 % of the protein is captured and eluted in pull-down. **B)** phage selection enrichment check; bars marked with "+" represent count of the phages eluted in selection experiment"" phages obtained from negative control where protein of interest was not added. **C)** Single point competition ELISA - scatter plot showing ELISA signals of target binding (x-axis) and competition with soluble protein (y-axis) - clones located in top left corner of the plot (high ELISA 450 nm signal and low competition ratio; positive clone threshold - dashed line in gray) are picked for secondary screening (marked in blue). **D)** SPR - affinity measurement; plot represents SPR trace of sAB injection (3 analyte concentrations) over 4 channels on a chip - binding is detected only when target protein (Abh3) is crosslinked to its ligand (DNA) - immobilized on channel 2 (blue lines), sABs do not bind to the reference channel or ligand alone and only residual binding (small signal change) is observed for protein without the ligand (red lines). **E)** DSF - thermal shift assay: red line - target, blue line - target-sAB complex melt curves, increased stability of target protein is observed in the presence of the excess of sAB indicated by thermal shift (black arrow, 15 °C shift). **F)** size exclusion chromatography - complex formation is observed by large volumetric shift (black arrow) of target-sAB (blue line) complex on elution profile.