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Creating Highly Specific Chemically Induced Protein Dimerization Systems by Phage Selection of a Combinatorial Single-Domain Antibody Library

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Phillip Steindel, Ph.D.
Review Editor
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Dear Dr. Steindel:

Enclosed please find our revised manuscript entitled "*Creating Highly Specific Chemically Induced Protein Dimerization Systems by a Stepwise Phage Selection of a Combinatorial Single-Domain Antibody Library*" by Gomez, *et al.* for publication in JoVE. We also provided point-by-point responses to the issues raised by each reviewer and the editor.

We thank reviewers for the helpful comments and hope that you will find this manuscript suitable for publication in JoVE.

Sincerely,

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TITLE:

Creating Highly Specific Chemically Induced Protein Dimerization Systems by Stepwise Phage Selection of a Combinatorial Single-Domain Antibody Library

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KEYWORDS:

chemically induced dimerization, combinatorial antibody library, nanobody, cannabidiol, phage display, enzyme-linked immunosorbent assay

SUMMARY:

Creating chemically induced protein dimerization systems with desired affinity and specificity for any given small molecule ligand would have many biological sensing and actuation applications. Here, we describe an efficient, generalizable method for de novo engineering of chemically induced dimerization systems via the stepwise selection of a phage-displayed combinatorial single-domain antibody library.

ABSTRACT:

Protein dimerization events that occur only in the presence of a small-molecule ligand enable the development of small-molecule biosensors for the dissection and manipulation of biological pathways. Currently, only a limited number of chemically induced dimerization (CID) systems exist and engineering new ones with desired sensitivity and selectivity for specific small-molecule ligands remains a challenge in the field of protein engineering. We here describe a high throughput screening method, combinatorial binders-enabled selection of CID (COMBINES-CID), for the de novo engineering of CID systems applicable to a large variety of ligands. This method uses the two-step selection of a phage-displayed combinatorial nanobody library to obtain 1) “anchor binders” that first bind to a ligand of interest and then 2) “dimerization binders” that only bind to anchor binder-ligand complexes. To select anchor binders, a combinatorial library of

over 10^9 complementarity-determining region (CDR)-randomized nanobodies is screened with a biotinylated ligand and hits are validated with the unlabeled ligand by bio-layer interferometry (BLI). To obtain dimerization binders, the nanobody library is screened with anchor binder-ligand complexes as targets for positive screening and the unbound anchor binders for negative screening. COMBINES-CID is broadly applicable to select CID binders with other immunoglobulin, non-immunoglobulin, or computationally designed scaffolds to create biosensors for in vitro and in vivo detection of drugs, metabolites, signaling molecules, etc.

INTRODUCTION:

CID systems, in which two proteins dimerize only in the presence of a small-molecule ligand (**Figure 1**), offer versatile tools for dissecting and manipulating metabolic, signaling, and other biological pathways[1]. They have demonstrated the potential in biological actuation, such as drug-controlled T cell activation[2] and apoptosis[3,4], for improving the safety and efficacy of adoptive T cell therapy. Additionally, they provide a new methodology for in vivo or in vitro detection of small-molecule targets. For example, CID proteins can be genetically fused with fluorescence reporter systems (e.g., fluorescence resonance energy transfer (FRET)[5] and circularly permuted fluorescent proteins)[6] for real-time in vivo measurements, or serve as affinity reagents for sandwich enzyme-linked immunosorbent assay (ELISA)-like assays.

Despite their wide applications, creating new CID systems that can be controlled by a given small-molecule ligand has major challenges. Established protein binder engineering methods including animal immunization[7], in vitro selection[8,9], and computational protein design[10] can generate ligand binding proteins that function via binary protein-ligand interactions. However, these methods have difficulties creating a ligand-induced ternary CID complex. Some methods create CID by chemically linking two ligands that independently bind to the same or different proteins[11-16] or rely on selecting binder proteins such as antibodies targeting preexisting small molecule-protein complexes[17,18], and thus have a limited choice of ligands.

We recently developed a combinatorial binders-enabled selection of CID (COMBINES-CID) method for de novo engineering of CID systems[19]. This method can obtain the high specificity of ligand-induced dimerization (e.g., an anchor-dimerization binder dissociation constant, K_D (without ligand)/K_D (with ligand) > 1,000). The dimerization specificity is achieved using anchor binders with flexible binding sites that can introduce conformational changes upon ligand binding, providing a basis for the selection of conformationally selective binders only recognizing ligand-bound anchor binders. We demonstrated a proof-of-principle by creating cannabidiol (CBD)-induced heterodimers of nanobodies, a 12–15 kDa functional antibody fragment from camelid comprising a universal scaffold and three flexible CDR loops (**Figure 2**)[20], which can form a binding pocket with adaptable sizes for small-molecule epitopes[21,22]. Notably, the in vitro selection of a combinatorial protein library should be cost-effective and generalizable for CID engineering because the same high-quality library can be applied to different ligands.

In this protocol and video, we focus on describing the two-step in vitro selection and validation of anchor (**Figure 3A**) and dimerization binders (**Figure 3B**) by screening the combinatorial nanobody library with a diversity higher than 10^9 using CBD as a target, but the protocol should

be applicable to other protein libraries or small-molecule targets. The screening of CID binders usually takes 6–10 weeks (**Figure 4**).

PROTOCOL:

1. Library construction

1.1. Use a synthetic combinatorial single-domain antibody library with a diversity of ~1.23–7.14 x 10^9, as previously described[19]. While this protocol does not include library construction, it can be applied to other combinatorial binder libraries.

2. Biotinylation of ligand target or ligand

2.1. Biotinylate the selected ligand, for example, CBD and tetrahydrocannabinol (THC)[19], via various chemical synthesis strategies, depending on the suitable biotinylation sites of a target.

3. Anchor binder screening

3.1. Beginning of selection

3.1.1. Begin every round of selection by inoculating a single TG1-cell colony, freshly grown in 6 mL of 2YT at 37 ˚C and 250 revolutions per minute (rpm) to a 600 nm (OD_{600}) absorbance of ~0.5. Incubate the cells on ice for the use in step 3.5.1.

3.2. Negative selection with biotin-bound streptavidin beads

3.2.1. Prepare the "negative selection beads" by washing 300 µL of streptavidin-coated magnetic beads using a magnetic separation rack, 3x with 0.05% phosphate-buffered saline with Tween buffer (PBST, 1 x PBS with 0.05% vol/vol Tween 20%) and 2x with 1 x PBS.

3.2.2. Resuspend the beads with 1 mL of 1% casein in 1 x PBS (pH = 7.4), and saturate the beads by adding 5x the reported binding capacity using **biotin**. Incubate at room temperature (RT) on a rotator for 1 h.

3.2.3. Wash the beads 5x using 0.05% PBST and 3x using 1 x PBS, for a total of eight washes.

3.2.4. Add ~10^{13} phage particles in 1% casein/1% BSA in 1 x PBS (pH = 7.4) and incubate at RT on a rotator for 1 h.

3.2.5. After incubation, collect the supernatant to be used in step 3.3.6.

3.3. Positive selection with biotinylated ligand-bound streptavidin beads

3.3.1. Prepare the “positive selection beads” using 1/2 the volume of the beads used for the “negative selection beads” following steps 3.2.1 to 3.2.3.

3.3.2. Saturate the beads by adding 5x the full binding capacity calculated based on the manual using the **biotinylated ligand** of choice. Incubate at RT on a rotator for 1 h.

3.3.3. Wash the beads 5x using 0.05% PBST and 3x using 1 x PBS, for a total of eight washes.

3.3.4. Block the beads with 1 mL of 1% casein/1% BSA in 1 x PBS (pH = 7.4) and incubate at RT on a rotator for 1 h to prevent nonspecific binding between the phages and the streptavidin-coated magnetic beads.

3.3.5. Wash the streptavidin-coated magnetic beads 3x using 0.05% PBST and one time using 1 x PBS, for a total of four washes.

3.3.6. Resuspend the streptavidin-coated magnetic beads using the unbound phages taken from step 3.2.5 and incubate at RT on a rotator for 1 h.

3.3.7. Extract the supernatant without disturbing the magnetic beads. Save the unbound phages as input, to be used in step 3.5.1.

3.3.8. Wash the beads 10x using 0.05% PBST and 5x using 1 x PBS. In between every three washes transfer them to a new tube to avoid phages nonspecifically bound to the tube walls.

3.4. Elution of phage-displayed nanobodies

3.4.1. Competitively elute bound phages by adding 450 µL of the **non-biotinylated ligand,** using a concentration in the micromolar range (e.g., 10–50 µM) and incubating at RT on a rotator for 30 min. The selected ligand concentration for the competitive elution of bound phages is dependent on desired K_D of the “anchor binder”. Ligand concentrations can be relatively high in initial selection rounds and then decreased in later rounds.

3.4.2. Collect supernatant and save the eluted phages as output, to be used in step 3.5.2.

3.5. Input/output titrations and infection

3.5.1. For input titration, prepare 10x serial dilutions in 1 x PBS up to 10^9-fold with the input phage from step 3.3.7. Use the 10^7–10^9 serial dilutions to do infections by transferring 10 µL input phage from each dilution to 70 µL TG1 cells (OD_{600} of ~0.5). Incubate at 37 ˚C for 45 min, plate the infected TG1 cells on three 90 mm 2YT-agar dishes containing 100 µg/mL ampicillin and 2% (wt/vol) glucose, and incubate overnight at 37 ˚C. From the overnight plates, phage input can be calculated as follows:

$$\frac{(\text{colony count}) \times (\text{dilution factor}) \times (\text{total volume of phage used in biopanning, in } \mu\text{L})}{\text{volume used to infect 70 } \mu\text{L of TG1 cells}}$$

3.5.2. For output infection and titration, transfer the eluted phages from step 3.4.2 to 3 mL of TG1 cells (OD_{600} of ~0.5). Incubate in a water bath at 37 ˚C for 45 min. Then prepare 10x serial dilutions in 2YT up to 10^3-fold, plate each dilution on 90 mm 2YT-agar dishes, and incubate overnight at 37 ˚C. From the overnight plates, phage output can be calculated as follows:

$$\frac{(\text{colony count}) \times (\text{dilution factor}) \times (\text{total volume of phage used in biopanning, in } \mu\text{L})}{\text{volume used to infect 70 } \mu\text{L of TG1 cells}}$$

3.5.3. Divide the remaining infected TG1 cells on three 150 mm 2YT-agar plates containing 100 µg/mL ampicillin and 2% (wt/vol) glucose. Incubate plates overnight at 37 ˚C.

3.6. Library amplification and recovery for further rounds of selection

3.6.1. Add 3 mL of 2YT per plate, scrape with a sterile cell scraper and collect all cells in a 50 mL conical tube. Mix the collected cells with sterile glycerol (20% wt/vol final concentration). Measure the OD_{600} of the mixture and make 3–5 stock aliquots. Store at -80 ˚C for long-term storage.

3.6.2. For phage rescue, dilute the phagemid-containing TG1 bacterial mixture using 25 mL of 2YT media supplemented with 2% glucose and 100 µg/mL ampicillin to an OD_{600} of ~0.1. Culture cells at 37 ˚C and 250 rpm to an OD_{600} of ~0.5.

3.6.3. Superinfect the cells by adding CM13 helper phage at 5 x 10^9 pfu/mL and incubate at 37 ˚C and 250 rpm for 45 min. The CM13 helper phage provides required phage coat proteins for the assembly of complete phage particles.

3.6.4. Centrifuge the culture at 8,000 x *g* for 10 min to remove the glucose. Resuspend the cells using 50 mL of 2YT media supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin and incubate at 25 ˚C and 250 rpm overnight.

3.6.5. Centrifuge the cells from the overnight culture at 9,000 x *g*, 4 ˚C for 30 min. Transfer supernatant to a new tube and precipitate phages in the supernatant using 1/5 volume PEG/NaCl solution (20% wt/vol polyethylene glycol-6,000 and 2.5 M NaCl). Mix gently and place on ice for 1 h.

3.6.6. Collect phage particles by centrifugation using 12,000 x *g* at 4 ˚C for 30 min. Resuspend the pellets using 1 mL of 1 x PBS, and transfer the suspension to a microcentrifuge tube. Centrifuge the tube at 20,000 x *g* and 4 ˚C for 10 min to remove residual bacteria.

3.6.7. Transfer the supernatant to a new microcentrifuge tube without disturbing the bacterial pellet. Use a 1:100 dilution to measure the absorption at 269 nm and 320 nm. The total number of phages can be calculated using the following formula[23]:

$$\frac{phages}{ml} = \frac{(A_{269} - A_{320}) \cdot 6 \times 10^{16}}{vector\ size}$$

3.6.8. Store phage library at 4 ˚C for short-term use or with 25% glycerol at -80 ˚C for long-term storage.

3.6.9. Repeat rounds of selection (steps 3.1–3.6) for 3–6 rounds or until desired enrichment is observed (refer to Results section). Plate and pick single clones (section 4) in order to characterize their affinity and specificity to the ligand (sections 5–7).

4. Single clone isolation

4.1. To isolate individual clones from an enriched sublibrary, prepare 10x serial dilutions of the phage-infected TG1 cells (step 3.5.2). Plate serial dilutions on 90 mm 2YT-agar dishes containing 100 µg/mL ampicillin and 2% (wt/vol) glucose and incubate at 37 ˚C overnight.

4.2. From the overnight plates, pick single colonies into 250 µL of 2YT media supplemented with 100 µg/mL ampicillin per well in sterile deep-well plates and grow at 37 ˚C overnight.

4.3. From the overnight cultures, inoculate 10 µL into 500 µL of fresh 2YT media supplemented with 100 µg/mL ampicillin.

4.4. Grow cells to an OD_{600} of ~0.5, add CM13 helper phage at 5 x 10^9 pfu/mL and incubate at 37 ˚C and 250 rpm for 45 min.

4.5. Add 500 µL of 2YT media supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin. Incubate at 25 ˚C and 250 rpm overnight.

4.6. Centrifuge the deep-well plates from the overnight cultures at 3,000 x *g* for 10 min. Collect the supernatant containing the phage particles without disturbing the cell pellet.

4.7. Phage particles can be used for ELISA to determine the specificity of the selected clones to the ligand. Biotin or a structural homolog of the target can be used as a negative control.

5. Anchor binder validation by ELISA

5.1. Coat 96 well ELISA plates using 100 µL of 5 µg/mL streptavidin in coating buffer (100 mM carbonate buffer, pH = 8.6) at 4 ˚C overnight.

5.2. Wash the ELISA plates 3x using 0.05% PBST and add 100 µL of 1 µM biotinylated target to the target wells. Add 100 µL of 1 µM biotin or target homolog to the control wells. Incubate at RT for 1 h.

5.3. Wash plates 5x using 0.05% PBST and block nonspecific binding by adding 300 µL of 1% casein in 1 x PBS. Incubate at RT for 1 h.

5.4. Wash the ELISA plates 3x using 0.05%-PBST and add the purified phage supernatant. Incubate for 1 h at RT.

5.5. Wash the ELISA plates 10x using 0.05% PBST and add 100 µL horseradish peroxidase (HRP)-M13 major coat protein antibody (1:10,000 dilution with 1 x PBS with 1% casein). Incubate at RT for 1 h.

5.6. Wash the ELISA plates 3x using 0.05% PBST and add 100 µL tetramethylbenzidine (TMB) substrate. Incubate for 10 min or until a visible color change is observed. Stop the reaction by adding 100 µL of 1 M HCl. Read the plate at 450 nm on a spectrophotometer.

5.7. For protein expression and purification, choose the clones showing high affinity and specificity for the target (see Discussion).

6. Protein expression, purification, and biotinylation

6.1. As previously reported[19], subclone selected clones from section 5 and express as C-terminal Avi-tagged and His-tagged nanobodies.

6.2. Express selected nanobodies in the periplasm of E. coli WK6 cells (typically in 1 L culture), release by osmotic shock, and purify using a nickel-NTA column (see **Table of Materials**).

6.3. Exchange buffer with a desalting column (1 x PBS with 5% glycerol; see **Table of Materials**).

6.4. Biotinylate nanobodies using a commercial kit (see **Table of Materials**) for further use.

7. Anchor binder characterization by BLI

7.1. Analyze the binding affinity and kinetics of selected anchor binders by immobilizing 200 nM biotinylated anchor binders on streptavidin biosensors (see **Table of Materials**) with binding assay buffer (1 x PBS (pH = 7.4), 0.05% Tween 20, 0.2% BSA, 3% methanol).

7.2. Calculate dissociation constants (K_D) of anchor binder-ligand interactions by steady-state analysis using data analysis software (see **Table of Materials**). Obtained K_D values typically range from single- to double-digit micromolar.

8. Dimerization binder screening

NOTE: The biopanning screening of "dimerization binders" is similar to that of anchor binders, except for two critical steps: 1) Dimerization binders are selected using a selected biotinylated anchor binder and the anchor binder-ligand complex for the negative and positive selections, respectively. 2) During the elution step, 100 mM triethylamine is used to elute positively selected phages that were only bound to the anchor binder--ligand target complex. The 100 mM trimethylamine solution (pH = 11.5) is used to elute positive clones by disrupting the protein interactions.

8.1. Beginning of selection

8.1.1. Begin every round of selection by inoculating a single TG1 cell colony, freshly grown on a minimal media, in 6 mL 2YT at 37 ˚C and 250 rpm to an OD_{600} of ~0.5. Incubate cells on ice.

8.2. Removal of negatively selected nanobodies

8.2.1. Prepare the "subtraction tube" by using 400 µL of streptavidin-coated magnetic beads and follow step 3.2. However, instead of saturating with biotin, add 5x the calculated full binding capacity using the selected **biotinylated anchor binder** and save the unbound phages to be used in step 8.3.3.

8.3. Selection of positively selected nanobodies

8.3.1. Prepare the "capturing tube" by using 1⁄2 the volume of streptavidin-coated magnetic beads used for the "subtraction tube" and following steps 3.3.2 to 3.3.3. However, instead of saturating with the biotinylated ligand, add five times the calculated full binding capacity using the selected **biotinylated anchor binder**.

8.3.2. To form the anchor binder-ligand complex for the positive dimerization binder selection, add a high enough concentration of **non-biotinylated ligand.** This will allow most streptavidin-bound anchor binder to form the ligand-bound complex.

8.3.3. Follow steps 3.3.3 to 3.3.8, using the unbound phages taken from the "subtraction tube".

8.4. Elution of positively selected nanobodies

8.4.1. Elute the phages bound to the anchor binder-ligand complex by adding 450 µL of 100 mM triethylamine, and incubating at RT on a rotator for 10 min.

8.4.2. Collect the competitively eluted phages and follow steps 3.4.1 to 3.4.2.

8.5. Further rounds of dimerization binder selection

8.5.1. Follow steps 3.5 and 3.6 to amplify and recover the library in order to perform further

rounds of selection. Repeat rounds of selection for 3–6 rounds or until desired enrichment is observed. Plate and pick single clones (refer to section 4) in order to characterize their affinity and specificity to the target.

9. Dimerization binder characterization by ELISA

9.1. Follow the steps in section 4 to isolate individual clones for characterization via ELISA.

9.2. To test the affinity of dimerization binder candidates to the anchor binder-ligand complex, coat the ELISA target plate using 100 µL of 100 nM biotinylated anchor binder. After incubation for 1 h, add 1 µM of the ligand target to form the anchor binder-ligand complex.

9.3. The control plate should be coated using the biotinylated anchor binder alone to screen out clones that can also bind to the free anchor binder. Add 100 µL of 100 nM biotinylated anchor binder and incubate at RT for 1 h.

9.4. Follow sections 5.3–5.7.

10. Dimerization binder characterization by BLI

10.1. The binding affinity and kinetics of dimerization binders for the anchor binder--ligand complex can be analyzed by immobilizing biotinylated dimerization binders on streptavidin (SA) biosensors with the binding assay buffer and then assayed with 1 µM anchor binder pre-equilibrated with serial dilutions of the ligand. The K_D, k_{on}, and k_{off} of the interactions can be calculated using our reported method[19].

REPRESENTATIVE RESULTS:

We describe the two-step in vitro selection and validation of anchor and dimerization binders by screening the combinatorial nanobody library with a diversity higher than 10^9 using CBD as a target. Assessing the enrichment of the phage biopanning during the successive rounds of selection for both anchor and dimerization binders is important. Typical enrichment results after 4–6 rounds of selection as shown in **Figure 5** are a good indication that there is a high ratio of potential hits in the sublibraries, so further rounds of selection might not be necessary.

Single-clone ELISA is suitable for analyzing the relative binding affinity and selectivity of anchor and dimerization binders. **Figure 6A** is a representative anchor binder selection result after six rounds of biopanning. Clones showing high (e.g., #87) or low (e.g., #27) ligand selectivity can be compared. High selectivity clones should be chosen as anchor binder candidates. Likewise, **Figure 6B** shows the dimerization binder selection results after four rounds of biopanning. We typically observed clones that formed a heterodimer with the immobilized anchor binder only with the ligand (e.g., #49) or without (e.g., #80). The former, showing dimerization specificity, should be selected for further validation.

Anchor binder ELISA relies on the use of the biotinylated target. Thus, we need to use BLI to further confirm the binding to the non-labelled target. BLI also allows the characterization of binding kinetics. Representative BLI results of anchor and dimerization binders are shown in **Figure 7A** and **7B**, respectively. The left panels show the ligand concentration-dependent binding, suggesting that they are suitable for constructing a CID system. The right panels show the negative controls. The calculated K_D of anchor and dimerization binder interactions in the presence of the ligand typically ranged from double-digit nanomolar to double-digit micromolar. They might vary depending on the ligand and the combinatorial library of choice.

Analytical size-exclusion chromatography (SEC) was performed to confirm the heterodimer formation between anchor and dimerization binders. A dimerization peak was observed when the anchor and dimerization binders and CBD were mixed (**Figure 8A**, red line). In contrast, no dimerization peak was detected in the absence of CBD (**Figure 8A**, blue line) or when each binder was loaded to the column alone (**Figure 8B**). The chemical crosslinking was used to stabilize CID complexes, and crosslinked nanobodies have slightly increased sizes corresponding to earlier eluted peaks.

FIGURE AND TABLE LEGENDS:

Figure 1. Mechanism of chemically induced protein dimerization.

Figure 2. Schematic of the generation of a synthetic nanobody combinatorial library. The library is constructed by using a universal nanobody scaffold and incorporating designed distributions of amino acids to each randomization position in three complementarity-determining regions (CDRs) by a Trinucleotide Mutagenesis (TRIM) technology [24].

Figure 3. Flowchart of (A) anchor and (B) dimerization binder screening.

Figure 4. Timeline of COMBINES-CID.

Figure 5. Enrichment of phage titers following each round of biopanning for the anchor binder selection.

Figure 6. Representative ELISA results showing positive (+) and negative (*) clones. (**A**) Anchor binder ELISA results from 96 randomly picked clones after six rounds of selection. (**B**) Dimerization binder ELISA results of 96 randomly picked clones after four rounds of selection.

Figure 7. Anchor and dimerization binder kinetic analysis by BLI. (**A**) Analysis of the anchor binder with unlabeled CBD (left) and THC (right). Biotinylated anchor binder was immobilized on Super Streptavidin (SSA) biosensors titrated with different concentrations of CBD. Measured data for CBD binding (red curves) were globally fitted (grey lines). (**B**) Left, BLI analysis of an SA biosensor-immobilized dimerization binder binding to the anchor binder preequilibrated with different concentrations of CBD. Right, the anchor binder concentration was titrated and bound to the immobilized dimerization binder in the absence of CBD.

Figure 8. SEC analysis of the heterodimerization between the anchor and dimerization binders. (**A**) The dimerization and anchor binders (5 μM each) in the presence or absence of CBD were crosslinked by 100 μM bis-*N*-succinimidyl-(pentaethylene glycol) ester for 30 min at RT before the analysis. Elution volumes of protein standards are marked by triangles. (**B**) Non-crosslinked anchor and dimerization binders (30 μM each) were injected separately. Chromatograms in A and B are shown in different Y scales.

DISCUSSION:

It is critical to choose the correct concentrations of input phage libraries for different rounds of biopanning. We typically started from an input library of ~10^{12}–10^{13} phage particles with a diversity >10^9, allowing ~100–1,000 copies of each phage clone to be presented in the pull-down assay. If the phage concentration in a binding assay is too high or low, the likelihood of nonspecific binding or loss of positive clones will increase. The anchor or dimerization binder selection normally consists of three to six rounds of biopanning, and the output phage counts usually start from ~10^4 and increases to ~10^8–10^9. It is suitable to pick single clones for ELISA validation after observing such enrichment. Additional rounds of biopanning might decrease the chances of identifying suitable low-abundance positive clones.

It is important to set up suitable negative controls and selections to enhance the success of the selections. For example, the use of structural analogs of ligand targets will facilitate the selection of ligand specificity. In our work, a highly similar analog, THC, was used as a control for CBD in the ELISA and BLI validation of anchor and dimerization binders[19]. In the dimerization binder selection, if anchor binders have relatively low ligand binding affinity, both free and ligand-bound anchor binders can be presented as targets in the positive selection. Thus, it is important to thoroughly remove binders that bind to free anchor binders during the negative selection. This can be achieved by performing multiple rounds of the subtraction with free anchor binders.

A limitation of our protocol is that target molecules need to be biotinylated for the anchor binder selection and only one or a few anchor binders can be used for the dimerization selection. The use of biotinylated targets can enrich binders that partly bind to the linker between biotin and targets. Thus, it is important to validate hits using unlabeled targets by BLI or other techniques. Choosing a single or a few anchor binders for dimerization binder selection can decrease the chance of identifying CID systems with suitable sensitivity and specificity. Thus, the multiplexing capability of the selection awaits further improvement by coupling to other techniques—for example, single-molecular-interaction sequencing (SMI-Seq) which enables a “library-by-library” protein-protein interaction screening[25].

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DISCLOSURES:

A provisional patent related to this work has been filed by the University of Washington.

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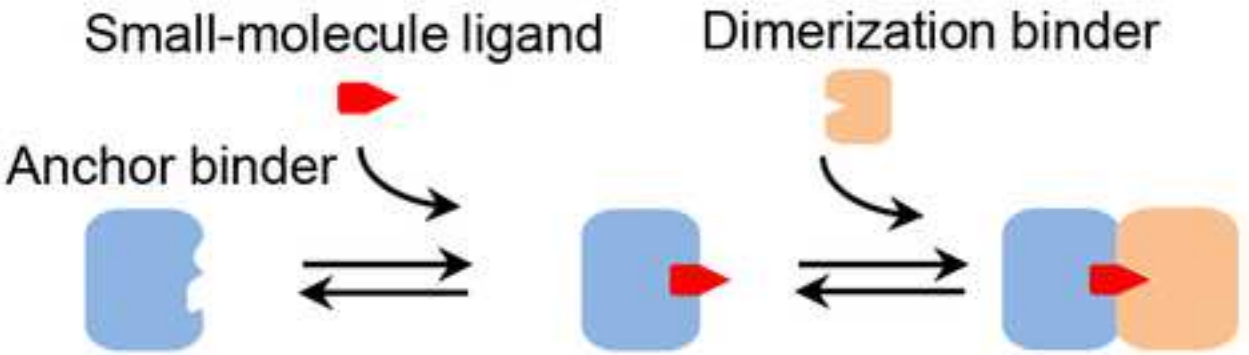
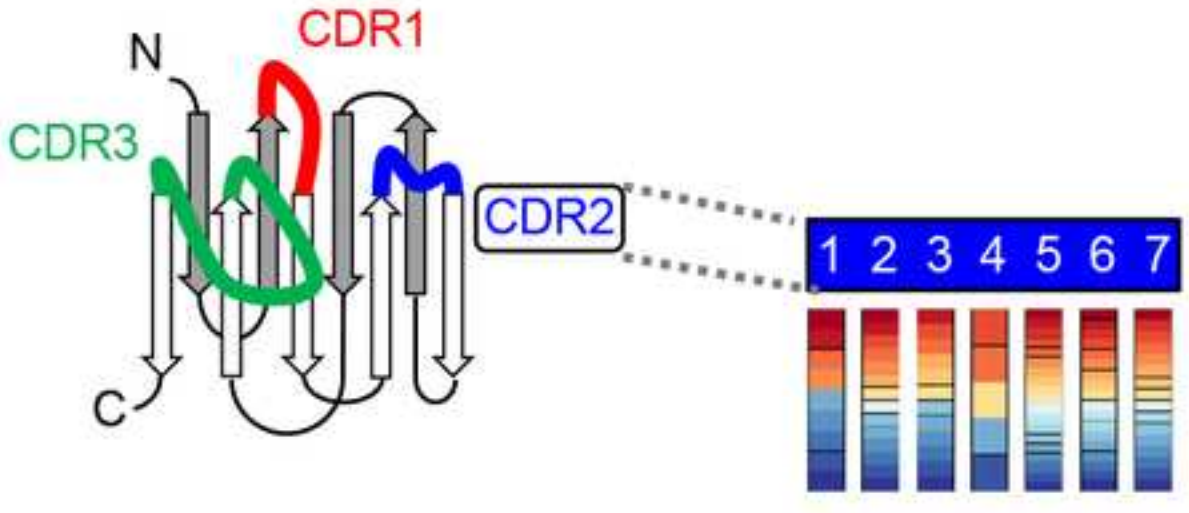
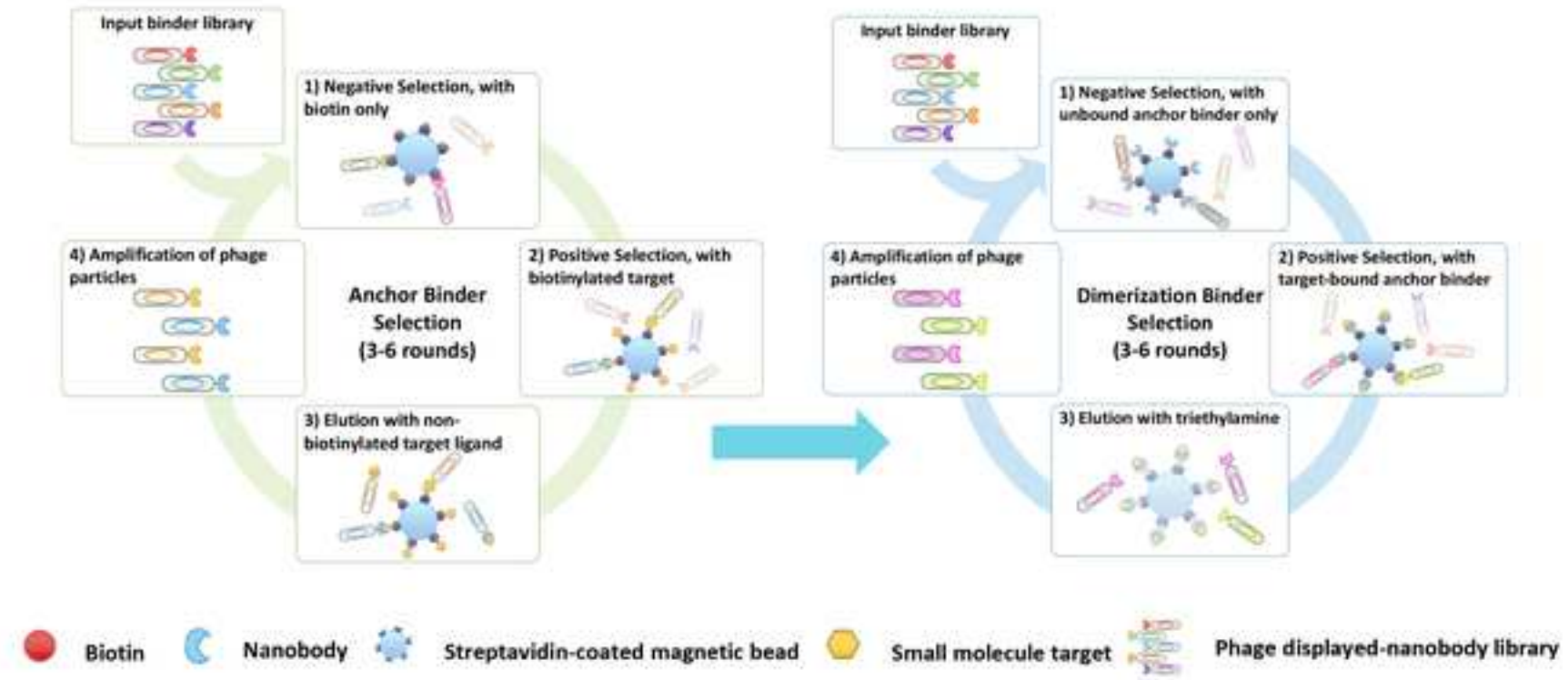
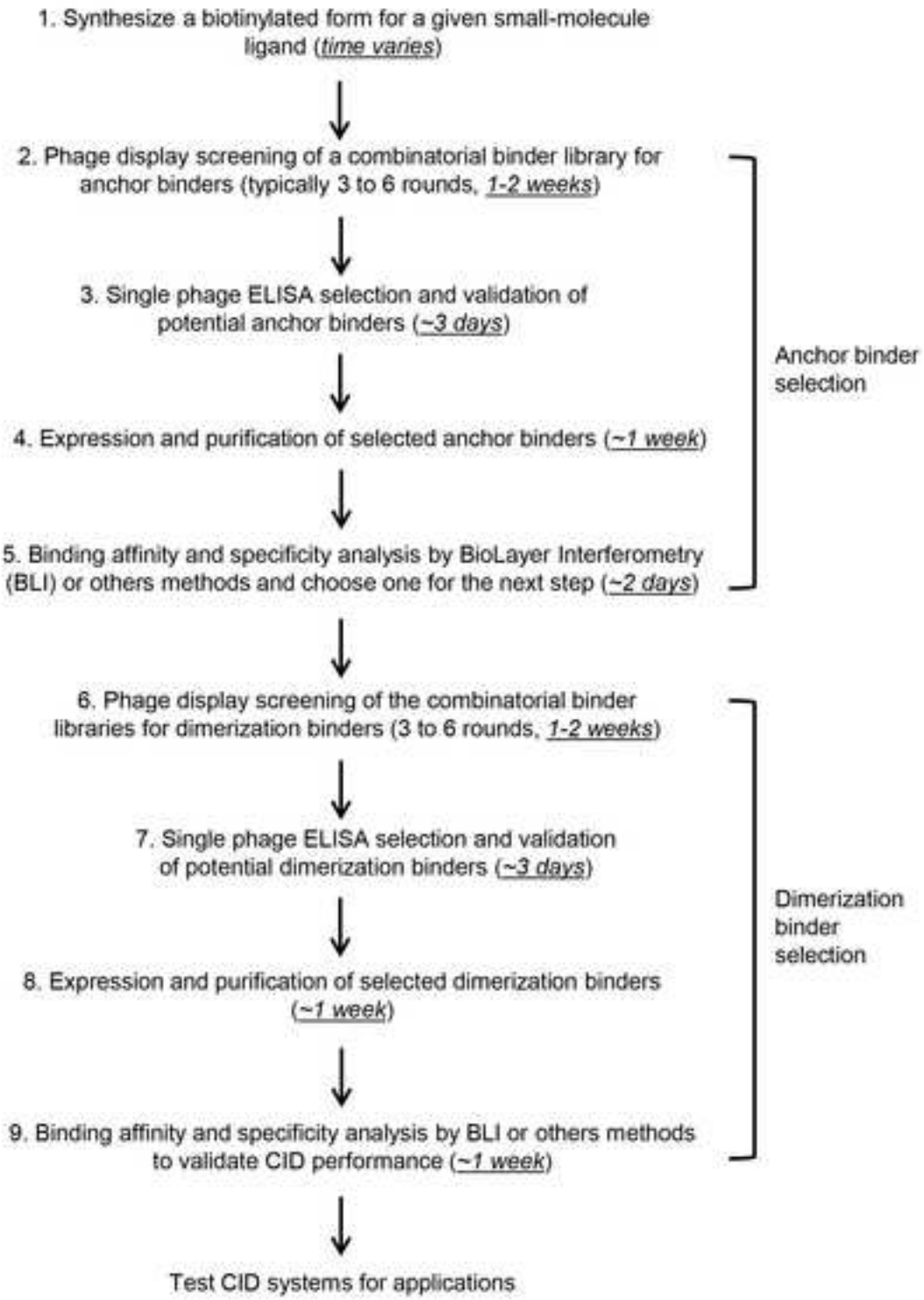
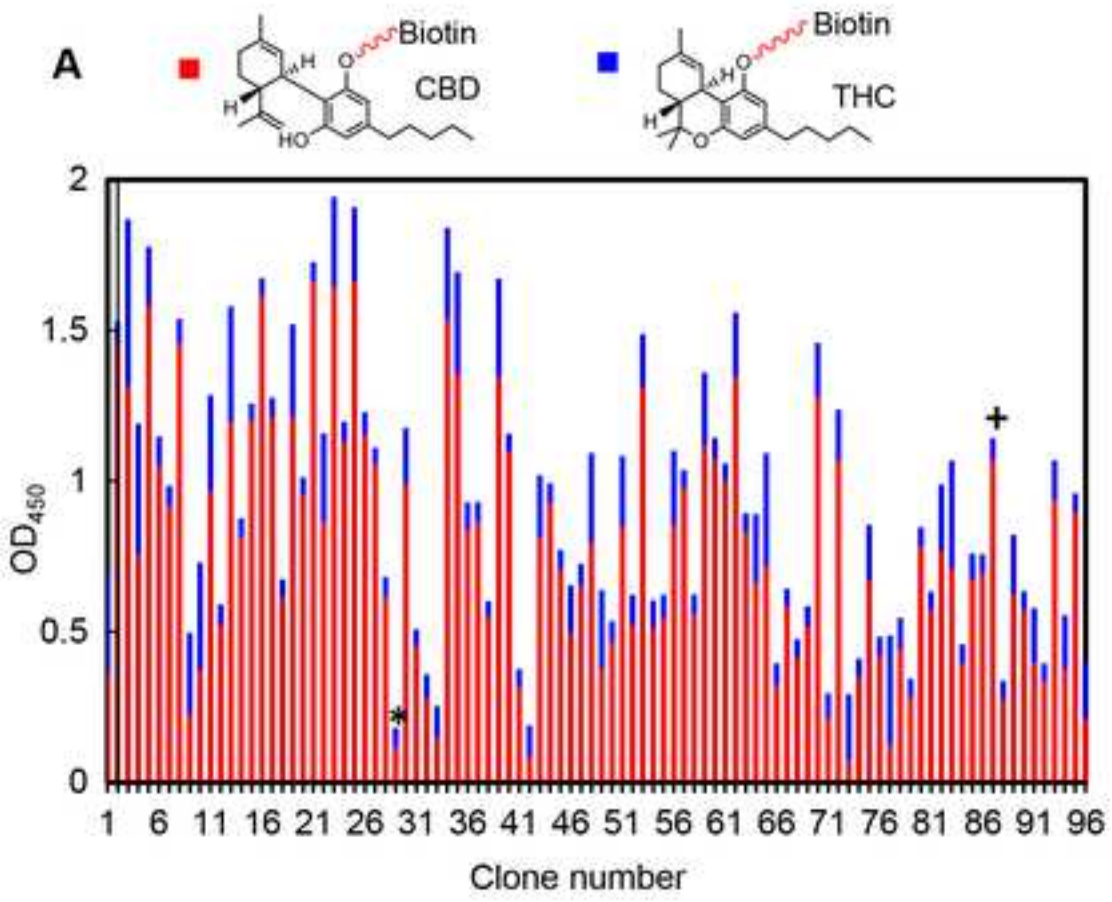


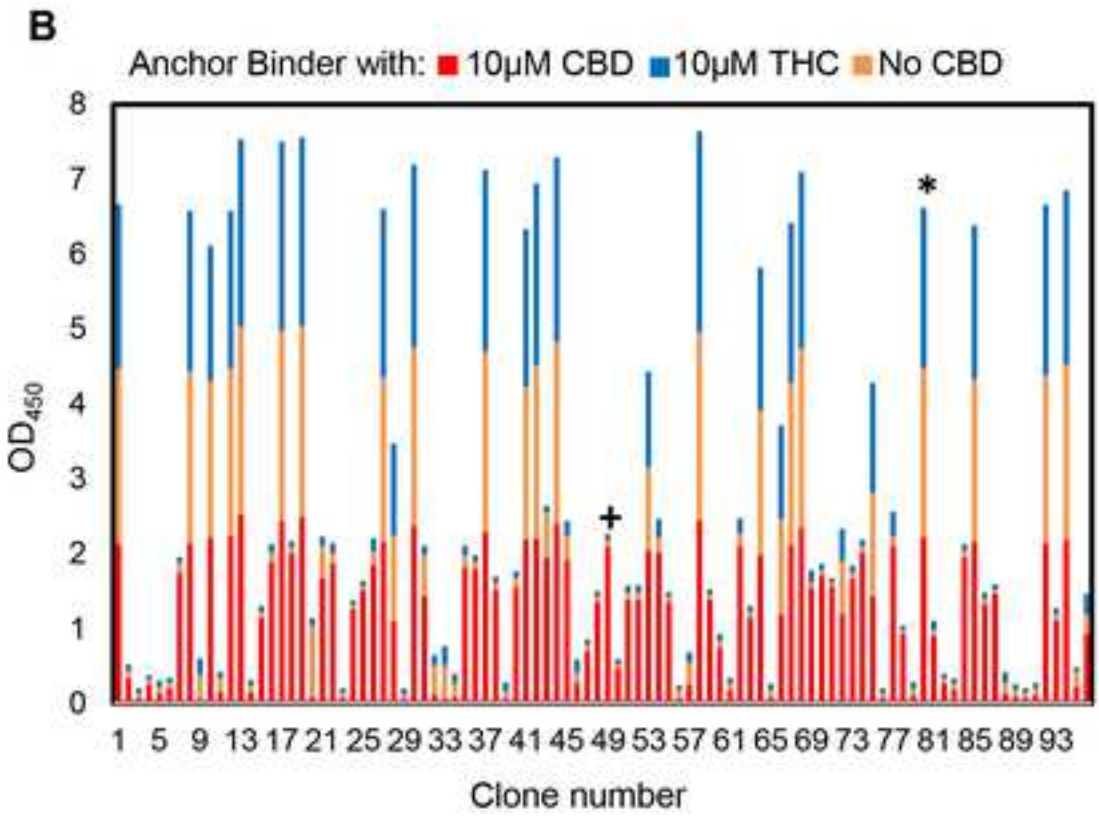


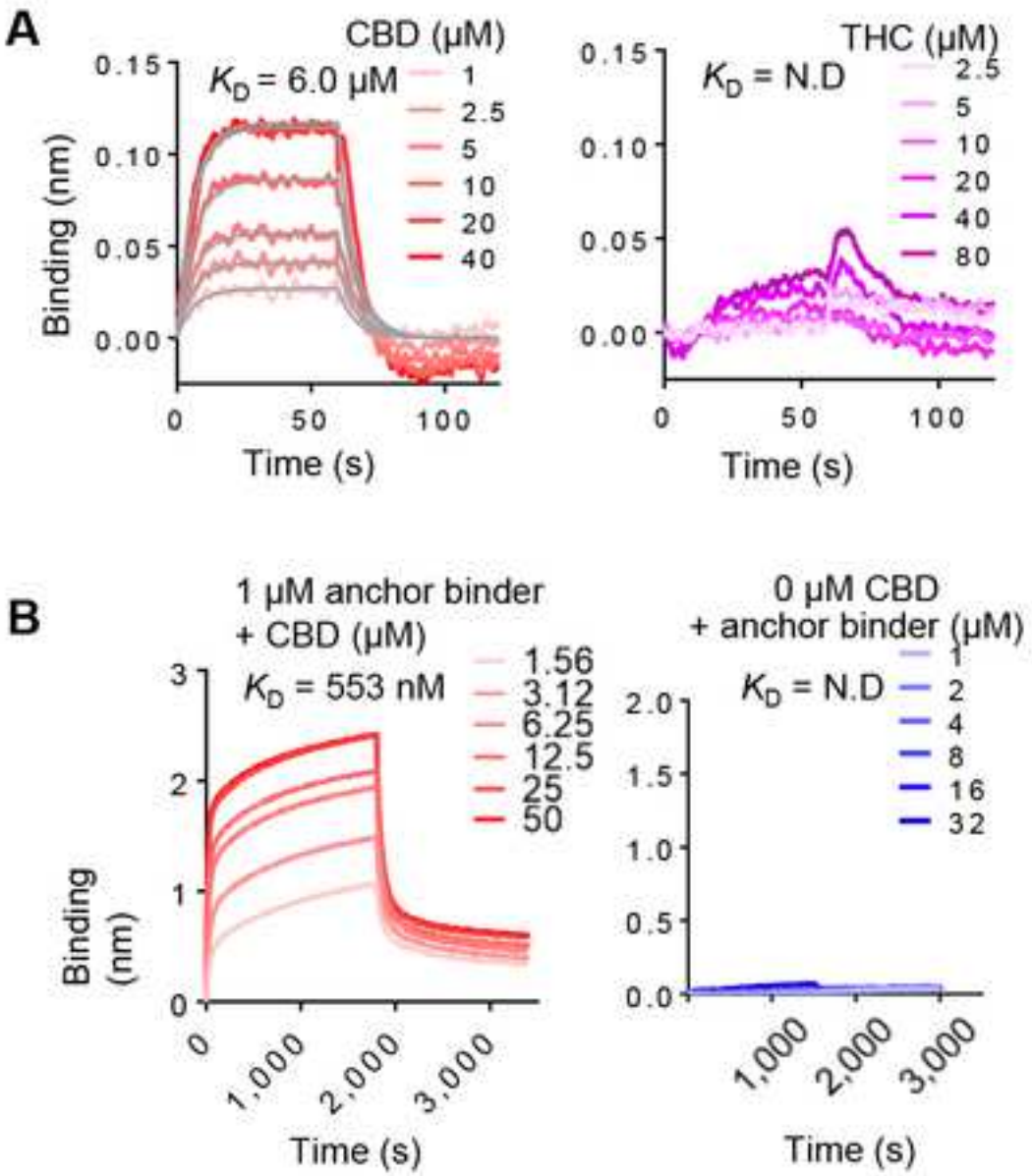




Round	Input phage	Output phage
1	2.4×10^{13}	3.5×10^{5}
2	1.3×10^{12}	4.7×10^{5}
3	1.4×10^{12}	2.3×10^{6}
4	1.5×10^{12}	1.2×10^{7}
5	1.8×10^{12}	1.8×10^{9}
6	2.2×10^{12}	5.2×10^{9}







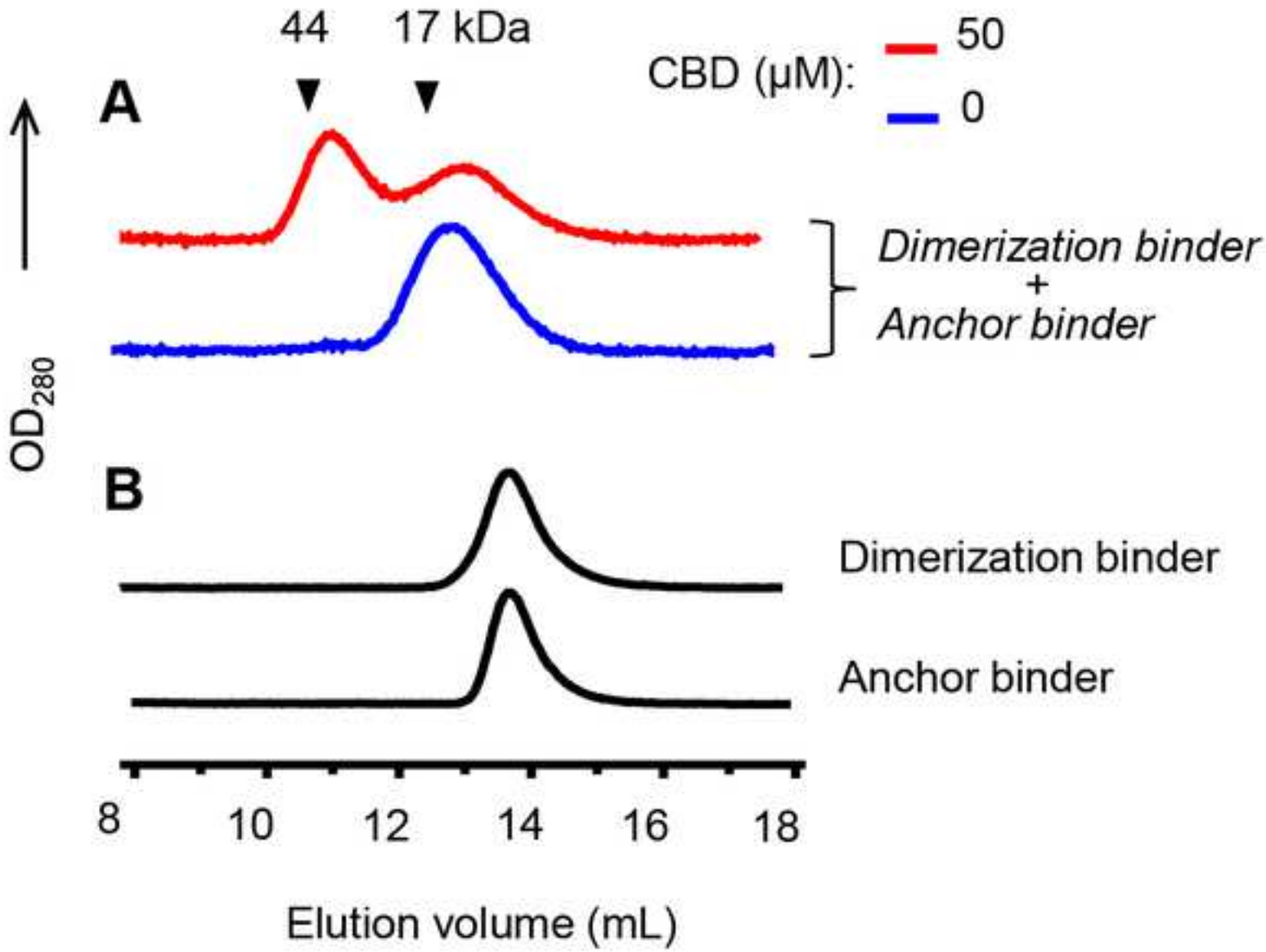


Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Revised.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1-Step Ultra TMB ELISA substrate solution	Thermo Fisher Scientific	34029	
Agar	Thermo Fisher Scientific	BP1423-2	
Amicon Ultra-15 Centrifugal Filter unit (3 kDa cutoff)	Millipore	UFC900324	
Ampicillin	Thermo Fisher Scientific	BP1760-25	
Bio-Rad Protein Assay Kit II	Bio-Rad	5000002	
BirA biotin-protein ligase standard reaction kit	Avidity	BirA500	
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A2153-50G	
Casein	Sigma-Aldrich	C7078-1KG	
CM13 Helper phage	Antibody Design Labs	PH020L	
D-(+)-Glucose monohydrate	Alfa Aesar	A11090	
Dynabeads M-280 Streptavidin	Thermo Fisher Scientific	11205D	
DynaMag-2 Magnet	Thermo Fisher Scientific	12321D	
EDTA	Thermo Fisher Scientific	BP120-1	
Fast DNA Ladder	New England Biolabs	N3238S	
FastDigest BglI	Thermo Fisher Scientific	FD0074	
Glycerol	Thermo Fisher Scientific	BP229-1	
HiLoad 16/600 Superdex 200 pg	GE Healthcare	28989335	
HiPrep 26/10 Desalting Column	GE Healthcare	17508701	
HisTrap-FF-1ml	GE Healthcare	11000458	
Imidazole	Alfa Aesar	161-0718	
IPTG	Thermo Fisher Scientific	34060	
Kanamycin	Thermo Fisher Scientific	BP906-5	
M13 Major Coat Protein Antibody	Santa Cruz Biotechnology	sc-53004	
NaCl	Sigma-Aldrich	S3014-500G	
NanoDrop 2000/2000c Spectrophotometers	Thermo Fisher Scientific	ND-2000	
Nunc 96-Well Polypropylene DeepWell Storage Plates	Thermo Fisher Scientific	260251	
Nunc MaxiSorp	Thermo Fisher Scientific	44-2404-21	
Octet RED96	ForteBio	N/A	
pADL-23c Phagemid Vector	Antibody Design Labs	PD0111	
PEG-6000	Sigma-Aldrich	81260-1KG	
Platinum SuperFi DNA Polymerase	Invitrogen	12351010	
PureLink PCR Purification Kit	Thermo Fisher Scientific	K310001	

QIAprep Spin M13 Kit	Qiagen	27704
Recovery Medium	Lucigen	80026-1
SpectraMax Plus 384	Molecular Devices	N/A
Sucrose	Sigma-Aldrich	S0389-1KG
Super Streptavidin (SSA) Biosensors	ForteBio	18-5057
Superdex 75 increase 10/300 GL Column	GE Healthcare	28-9909-44
T4 DNA Ligase	Thermo Fisher Scientific	15224-025
TG1 Electrocompetent Cells	Lucigen	60502-1
Triethylamine	Sigma-Aldrich	471283-100mL
Trizma Base	Sigma-Aldrich	T1503
Tryptone	Thermo Fisher Scientific	BP9726-5
Tween 20	Thermo Fisher Scientific	BP337-500
Yeast Extract	Thermo Fisher Scientific	BP1422-2
Zeba Spin Desalting Column	Thermo Fisher Scientific	89882

Point-by-Point Responses

We appreciate valuable comments from the editor and reviewers and provide a detailed point-by-point response to each concern.

Editor:

General

1. *"Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues."*

Response 1-1: The manuscript has been proofread.

2. *"Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps."*

Response 1-2: The manuscript has been formatted according to the guidelines.

3. *"Please revise lines 34-37 and 63-72 to avoid textual overlap with previously published work."*

Response 1-3: We revised the text in lines 34-37 and 63-72 (see the revision highlighted pdf).

4. *"JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*
   For example: Nanodrop, Santa Cruz Biotechnology, AviTag, HisTrap, HiPrep, Octet RED96, etc."

Response 1-4: All trademark symbols, registered symbols, and company names have been removed. All commercial products are referenced in the Table of Material and Reagents (see the revision highlighted pdf).

Summary

1. *"Please reduce the length of the summary to 10-50 words."*

Response 1-5: The summary has been shortened.

Protocol

1. *"There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol."*

Response 1-6: 2.75 pages of the Protocol have been highlighted to include the essential steps of the protocol for the video.

2. *"For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps."*

Response 1-7: We have made sure that every step and subset in the protocol answers the "how" question, and references have been added accordingly.

Specific Protocol Steps

1. *"1, 2, 6: Please include references for these procedures."*

Response 1-8: References to steps 1, 2, and 6 have been added referencing published material specifying how these steps are to be performed.

2. *"3.6.7: How exactly is phage concentration determined here (e.g., wavelength used, extinction coefficient)?"*

Response 1-9: The phage concentration is calculated by measuring the absorption at 269 and 320 nm using the following formula:

$$\frac{phages}{ml} = \frac{(A_{269} - A_{320}) \cdot 6 \times 10^{16}}{vector\ size}$$

Step 3.6.7 has been edited to include a description of how phage concentration is measured.

3. *"5.7: Please include more details here if this step is to be filmed."*

Response 1-10: Step 5.7 has been removed from the list of steps to be included in the video.

Figures

1. *"Please use figures of (roughly) uniform size."*

Response 1-11: All figures have been adjusted to one uniform size.

2. *"It's unclear how exactly Figure 2 shows a '[s] chematic of the generation of a synthetic nanobody combinatorial library'; please explain further in the legend."*

Response 1-12: We have revised the Figure 2 legend to explain that the synthetic combinatorial gene library was generated using a single-domain antibody made up of a universal backbone and three variable complimentary-determining regions, that were synthetized by trinucleotide mutagenesis technology.

3. *"Figure 7: Please use 's' instead of 'sec'."*

Response 1-13: Figure 7 has been edited to use 's' instead of 'sec'.

4. *You have included a movie with your submission, although it is not apparently referenced in the manuscript at all-please clarify.*

Response 1-14: The movie we have included is intended to be used in the video introduction.

References

1. *"Please do not abbreviate journal titles."*

Response 1-15: The Reference section has been edited to include all relevant journal titles without any abbreviations.

Table of Materials

1. *"Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol."*

Response 1-16: The Table of Materials has been edited in order to include to all the information on the materials and equipment used and mentioned in the protocol.

2. "*Please remove trademark (™) and registered (®) symbols from the Table of Materials."*

Response 1-17: All trademark and registered symbols have been removed from the Table of Materials.

Reviewer #1:

1. *"Small molecules are generally not very immunogenic and typically have to be haptenized. From the intro and protocol I was not able to determine if the library was an immune or naïve library. Was the llama immunized with a haptenized small ligand prior to the panning?"*

Response 2-1: We agree that small molecules generally are not immunogenic and typically have to be haptenized. The library was constructed by using a universal nanobody scaffold and incorporating designed distributions of amino acids to each randomization position in three complementarity-determining regions by a Trinucleotide Mutagenesis technology. The DNA library was chemically synthesized with a diversity higher than 10^{12}, and then sub-cloned into a phagemid vector for transformation into *Escherichia coli* to produce phage-displayed nanobody. The phage-displayed nanobody library has a diversity of > 10^{9}.

2. *"Step 3 - please mention the device that you are using to remove the magnetic beads."*

Response 2-2: A magnetic rack was used to separate the beads from the buffer used. Once separation occurred, the supernatant was removed without disturbing the magnetic beads.

3. *"Step 3.4.1. - please mention the concentration range of the non-biotinylated small molecule that should be tested."*

Response 2-3: Step 3.4.1 has been edited to state a concentration range of 10 - 50 µM

4. *"Step 7.2 - What are the ball-park Kd values that you can obtain for sdAb that bind small molecule ligands?"*

Response 2-4: Obtained K_D values for small molecule ligands using sdAb can vary from single digit micro molar to double digit micro molar.

5. *"Step 4 - Please describe what the helper phage is doing in this step."*

Response 2-5: Step 4 has been edited to explain that CM13 helper phage provides required phage coat proteins for the assembly of complete phage particles.

6. *"Step 8 - please describe what triethylamine is doing in this step."*

Response 2-6: Step 8 has been edited to explain that the 100 mM trimethylamine solution (pH = 11.5) is used to elute positive clones by disrupting the protein interactions.

7. *"Representative Results - please add a Kd range. For the SEC I am surprised that the small molecule ligand is not removed in this step. Is that a possible result? What is the success rate for this procedure? How often do you not get complex off the column? How often do you not?"*

Response 2-7: Observed K_D ranges have been included in the Representative Results section. During validation using SEC, 5 µM (each) dimerization binder and anchor binder in the presence or absence of CBD were crosslinked by 100 µM bis-*N*-succinimidyl-(pentaethylene glycol) ester for 30 min at room temperature before the analysis. This crosslinking event allowed the complex to form and not disassociate during the SEC assay.

8. *"Discussion, Line 370 - will the phage concentration depend upon the Kd?"*

Response 2-8: No, the phage concentration in the binding assay is not dependent on the K_D values. The K_Ds determine the bound vs. unbound ratios for different phage clones.

9. *"Could this method be extended to covalent drugs or post-translational modifications?"*

Response 2-9: Yes, similar to standard antibodies that can bind to drugs or *post-translational* modifications covalently attached to a protein, nanobodies should also be applicable.

10. *"Figure 7 - Add the Kd values to the figure"*

Response 2-10: K_D values have been added to Figure 7.

Reviewer #2:

1. *"My only concern with respect to presentation of this method in JOVE is that the protocol lacks visual interest. I fear it will merely look like any number of other techniques in general molecular biology. I do not know if this a concern for the editor."*

Response 2-11: Our method is based on standard molecular cloning, phage display, and protein expression techniques, therefore we expect that it can be easily (and widely) used by other labs. This video protocol provides the detailed information to help other labs to reproduce our results or develop their own applications.