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Exploiting Phage Display for Development of Novel Cellular Targeting Strategies

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Abstract

Targeting strategies for drug delivery applications rely on targeting moieties (i.e., peptide, antibody) specific to the desired cell surface receptor or protein of interest. However, current targeting strategies are limited to previously identified epitopes/ligand pairs. The field of phage display opens up the targeting moiety options whereby new epitope/ligand pairs may be discovered through well-designed biopanning assays for the target cell population of interest. Here, we provide a detailed protocol to perform phage biopanning assays on adherent cell cultures. The methods described here may be modified to user-specific targeting interests.

Keywords

Phage display; Single chain antibody fragment (scFv); Domain antibody (dAb); Astrocytes

1 Introduction

A critical component of an active targeted drug delivery system is the actual targeting motif, typically selected based on unique characteristics of the target cell/tissue system. For example, tumor angiogenesis is commonly marked by high levels of vascular endothelial cell growth factor receptor (VEGFR) motivating the development of VEGFR-based targeting strategies (*see review* [1]). However, many disease pathologies are more complex than the upregulation of a single receptor or cell surface protein. Therefore, epitope/ligand discovery tools such as phage display are essential for identifying new targeting motifs with high specificity to complex pathologies. Phage display is a molecular biology technique that exploits the ease of genetic manipulation of bacteriophage (phage) to generate large combinatorial phage libraries that present motifs on the outer coat proteins (i.e., short peptide sequences, single-domain antibody (sdAb), fragment antigen-binding region of

¹⁴ Depending on the number of active phage, each dilution will give a range of CFUs (1–2 colonies to an uncountable lawn of bacteria). Use the intermediary dilutions with distinct colonies to calculate CFUs (i.e., ~20–80 colonies).

¹⁵ The stock library may have a high percentage of wild type phage that do not display an scFv/dAb fragment. Negative screens do not remove the wild type phage as efficiently as positive screens. After several rounds of positive screens, the prevalence of inserts should greatly increase.

monoclonal antibody (Fab), single chain antibody fragments (scFvs), and nucleic acid sequences) [2, 3]. Subsequent biopanning screens with phage libraries against a target of interest (e.g., tumor biopsy) facilitate discovery of a unique motif with high affinity and specificity.

Particular consideration should be given when selecting the type of targeting motif as a variety of options are available including monoclonal antibodies (full length), Fab, sdAb, scFv, nucleic acids, aptamers, short peptide sequences, and small molecules (*see review* [4]). Peptide and small molecules afford small size; however, these systems typically have an order of magnitude higher equilibrium binding dissociation constants (K_D) compared to antibody-based systems [5, 6]. Whereas approaches that employ full length antibody and even Fab systems trade affinity for larger size. ScFv and sdAb systems are unique as they are truncated antibodies composed of the variable heavy (VH) and/or variable light (VL) chains containing the critical epitope recognition regions (complementary determining regions; CDRs) thus maintaining high affinity without the size tradeoff [3].

Here, in this chapter, we outline in vitro biopanning phage display against viable, adherent cell cultures. We specifically describe methods with commercially available Domain antibody library and the Tomlinson I + J. Phage production, purification, biopanning, and scFv/sdAb production and purification outlined in this chapter are based on a compilation of previous publications and accompanying product documentation [3, 7, 8]. However, to our knowledge, we have uniquely modified the protocol to perform biopanning on viable, adherent cell cultures to identify subtle alterations between quiescent and activated cellular phenotypes. We use primary astrocyte cultures as our model system and developed scFv/sdAbs with high specificity and affinity to reactive astrocytes. This protocol may be modified to suit each user's experimental goals.

2 Materials

2.1 Bacteriophage Production and Purification

1. Phage library (*see Note 2*).
2. 100 mm × 15 mm petri dishes.
3. 245 mm square bioassay dishes (Alternatively, four (*See Note 1*) 100 mm × 15 mm petri dishes can be used as a substitute).
4. Bacterial cell spreader.
5. 2xTY medium: For 1 L stock, dissolve 16 g of bacto-tryptone, 10 g of yeast extract, and 5 g of NaCl 500 mL deionized water, bring final volume up to 1 L with deionized water, autoclave, let it cool to room temperature (RT; 25 °C), store at RT or 4 °C (*see Note 3*).

².Domain antibody library (dAb) or Tomlinson I + J single-chain variable fragment (scFv) antibody libraries, *Escherichia coli* TG1 TR strain, *E. coli* HB2151 strain, positive control clone (β-galactosidase-specific), negative control clone (phagemid) and KM13 helper phage (Source Bioscience, Nottingham, UK).

¹.Bacterial work (reagent preparation and subsequent phage biopanning steps) is best performed using aseptic techniques within 6–10 in. of a burning alcohol lamp. *See reference for a more thorough description of standard bacterial and aseptic techniques* [9, 10].

6. 20% Glucose solution: 200 g of glucose per 1 L of deionized water, sterile filter (0.2 μ m filter), store at 4 °C.
7. Ampicillin stock (1000 $\times$): 100 mg/mL ampicillin in deionized water, sterile filter, and store in 1 mL aliquots at –20 °C. Recommend preparing 50–100 mL at a time. Ampicillin is light sensitive and should be stored in a dark container to avoid degradation.
8. Tryptone Yeast Extract (TYE) agar plates: For 1 L stock, dissolve 8 g of NaCl, 10 g of bacto-tryptone, and 5 g of yeast extract in 800 mL of deionized water, add 15 g of agar and bring final volume up to 1 L with deionized water. Autoclave and then cool down to 50 °C (*see Note 4*). Pour 20 mL of solution into 100 mm $\times$ 15 mm petri dishes. Place lid on dishes, cool for 1 h, store at 4 °C for up to 4 weeks.
9. TYE ampicillin glucose agar (TAG) plates: For 1 L stock, dissolve 8 g of NaCl, 10 g of bacto-tryptone and 5 g of yeast extract in 600 mL of deionized water, add 15 g of agar and bring final volume up to 800 mL with deionized water. Autoclave and then cool down to 50 °C (*see Note 4*), add 1 mL of ampicillin solution (light sensitive) and 200 mL of 20% glucose solution. Pour 20 mL of solution into 100 mm $\times$ 15 mm petri dishes. Place lid on dishes, cool for 1 h, store at 4 °C for up to 4 weeks. A separate batch of TAG should be made to prepare larger TAG plates (245 mm square bioassay dishes) for use in the screening process (Subheading 3.2).
10. Kanamycin stock (1000 $\times$): 50 mg/mL kanamycin dissolved in deionized water, sterile filter, and store in 1 mL aliquots at –20 °C. Recommend preparing 50–100 mL at a time. Kanamycin is light sensitive and should be stored in a dark container to avoid degradation.
11. 1 $\times$ Phosphate buffer (PBS; pH 7.4): For 1 L stock, dissolve 8 g of NaCl (137 mM), 1.44 g of Na₂HPO₄ (10 mM), 0.2 g of KCl (1.8 mM), and 0.24 g of KH₂PO₄ (10 mM) in 900 mL of deionized water, adjust pH to 7.4, bring final volume to 1 L and autoclave. Store at RT.
12. 1 $\times$ Tris Buffer (TBS; pH 7.4): For 1 L stock, dissolve 1.5 g of Trizma base (10 mM), 8 g of NaCl (137 mM) and 0.15 g CaCl₂ (1 mM) in 900 mL of liter of deionized water, adjust pH to 7.4, bring final volume to 1 L and autoclave. Store at RT.
13. 25% PEG 6000, 2.5 M NaCl solution: For 500 mL stock, dissolve 125 g of PEG-6000 and 73 g of NaCl in 500 mL of deionized water (final volume). Autoclave, then stir continuously while cooling to RT. Store at RT.

³. 2 $\times$ TY medium is prone to contamination. Inspect medium visually before each use to verify the medium is clear and free of contamination.

⁴. Place the autoclaved agar solution in a water bath to 50 °C. Allow solution to equilibrate to 50 °C prior to pouring into petri dishes. However, if the temperature drops below 50 °C then agar will begin to gel prior to pouring into the petri dishes.

14. Trypsin stock (100×): 10 mg/mL trypsin at 10 mg/mL in TBS, sterile filter, store in 100 µL aliquots at −20 °C. Recommend preparing 10 mL at a time (100× stock concentration).
15. 0.005 M EDTA, 0.1 mg/mL BSA in PBS (PBS/EDTA/BSA): Dissolve 4 mg of bovine serum albumin in 40 mL of 1× PBS supplemented with 5 mM EDTA, sterile filter and store at 4 °C.
16. 50% Glycerol: Dilute equal parts 100% glycerol with deionized water to obtain an end concentration of 50% glycerol solution.
17. Centrifuge that can house 250 mL centrifuge bottles and reach speeds up to 12,000 × *g*.
 - (a) Example: Beckman Coulter Allegra 25R.
18. Bacterial shaker incubator.
19. 250 mL polypropylene centrifuge bottles.

2.1.1 Biopanning

1. 96-well polystyrene, flat, sterile tissue culture plates.
2. 96-well polystyrene, round bottom, untreated sterile plates.
3. Anti-M13 Antibody Biotinylated.
4. Streptavidin with HRP conjugate.
5. 1-Step Ultra-TMB-ELISA.

2.1.2 Cell Specific

1. Adherent cell line of interest.
2. Cell culture media.

2.1.3 Genetic Analysis PCRss

1. GelRed™ Nucleic Acid Gel Stain.
2. 100 bp PCR molecular ladder.
3. GoTaq® PCR Core System I.
4. TAE Buffer: Prepare a 10× stock buffer by dissolving 48.4 g of Tris-base, 11.4 mL of glacial acetic acid (17.4 M) and 3.7 g of EDTA disodium salt in 900 mL of deionized water. Bring final volume to 1 L with deionized water; store at RT. When needed, dilute 100 mL of 10× TAE stock with 900 mL of deionized water to achieve a final working solution of 40 mM Tris, 20 mM acetic acid and 1 mM EDTA.
5. Primers (Custom order *see* Table 1).

2.1.4 DNA Sequencing

1. QIAprep Miniprep Kit.
2. Primers (Life Technologies, Custom order *see* Table 1).

2.1.5 scFv/dAb Production and Purification

1. 1 M Isopropyl- β -D-thiogalactopyranoside (IPTG): Dissolve 2.38 g of IPTG in 8.5 mL of deionized water. Bring final volume up to 10 mL, sterile filter, and store in 1 mL aliquots at -20°C .
2. Pierce Protease Inhibitor Mini Tablets, EDTA-free.
3. Lysozyme.
4. Triton X-100.
5. 100 U/mL DNase I stock: Dissolve DNase I in the appropriate volume of 10 mM Tris-HCl + 2 mM CaCl_2 . Aliquot into 5 mL portions in 15 mL polypropylene centrifuge tubes and store at -20°C .
6. Probe-based sonicator (for lysing cells).
7. FPLC or alternative protein purification method.
8. Protein-A or AG affinity FPLC column.
9. 0.2% NaN_3 stock solution: Dissolve 200 mg of NaN_3 in 100 mL of $1\times$ PBS. Caution NaN_3 is *very toxic* and possesses explosive properties when exposed to metals. Use extreme caution when handling this product (even when dissolved at 0.2%). Collect all waste and dispose of via a proper chemical waste mechanism.

3 Methods

3.1 Bacteriophage Production and Purification (Modified from [3])

3.1.1 Production and Purification

1. Thaw an aliquot of frozen antibody stock library (dAb or scFv library) on ice.
2. Add phage library 1 mL aliquot to a sterile 2 L Erlenmeyer flask containing 500 mL 2xTY medium supplemented with 4% (wt/vol) glucose and 100 $\mu\text{g/mL}$ of ampicillin (Use appropriate glucose and ampicillin stock solutions; *see* Notes 5 and 6).

⁵The optimal media volume for the Domain antibody library is 500 mL while Tomlinson I + J requires only 200 mL. Adjust the volume depending on which library is being used.

⁶The presence of 4% glucose allowed the effective suppression of antibody expression during bacterial growth by preventing lactose permease production, thereby inhibiting the uptake of the disaccharide lactose by the host *E. coli* from the yeast extract present in TYE. Remaining intracellular lactose would be digested by the production of β -galactosidase produced by the LacZ gene. Without the presence of lactose, the Lac repressor successfully inhibits the LacO operon, thus suppressing expression of our scFv/dAb insert. Ampicillin kills off any bacterium which did not contain the synthetic library vector. *See* Genetic Analysis for insert detection and efficacy testing.

3. Place the bacteria in a bacterial shaker incubator and culture at 37 °C and 250 rpm until reaching an optical density reading at 600 nm of 0.1 (OD₆₀₀; *see* Note 7).
4. Transfer 250 mL of the bacterial culture to a new sterile 1 L Erlenmeyer flask (*see* Note 8).
5. Add 1×10^{12} KM13 helper phage to the 250 mL culture and incubate in a water bath at 37 °C for 30–45 min (*see* Note 8).
6. After incubation, spin cultures down at $3200 \times g$ for 10 min at 4 °C in 250 mL autoclaved centrifuge bottles. Load a maximum of 200 mL per bottle.
7. Discard the supernatant.
8. Resuspend bacterial pellets in 500 mL of 2xTY medium supplemented with 0.1% (wt/vol) glucose, 100 µg/mL of ampicillin, and 50 µg/mL of kanamycin in a 2 L Erlenmeyer flask (Use appropriate prepared stock solutions; *see* Notes 9 and 10).
9. Grow culture overnight for 16–20 h at 25 °C and 250 rpm.

3.1.2 Phage PEG Purification

1. Spin down overnight cultures for 15 min at $10,800 \times g$ at 4 °C in sterile 250 mL polypropylene centrifuge bottles.
2. Collect the supernatant and add 15% by volume of the 25% PEG 6000, 2.5 M NaCl solution.
3. Divide the supernatant/PEG solution equally between two to three autoclaved 250 mL polypropylene centrifuge bottles. Mix well by inverting bottles 50 times. Incubate for 2 h at 4 °C.
4. Spin down precipitated phage at $6000 \times g$ for 45 min.
5. Discard the supernatant.
6. Resuspend the phage pellet in 15 mL of PBS (*see* Note 11). To aid resuspension, place the phage solution on a rocker at 4 °C for 30 min to 1 h at 50 rpm. If a rocker is unavailable, incubate the phage pellet at 4 °C for 1 h and then manually swirl the solution to resuspend the pellet. Do not vortex nor aspirate.
7. Combine phage solutions into a single 250 mL centrifuge bottle or 50 mL polypropylene centrifuge tube.

⁷·Growth of the culture to reach an OD₆₀₀ of 0.1 typically takes about 1.5–2 h at 37 °C and 250 rpm.

⁸·The suggested volume and helper phage concentration is suggested for Domain Antibody Library. For the Tomlinson I + J, incubate 50 mL of the bacterial culture with 2×10^{11} KM13 helper phage in a water bath at 37 °C for 30–45 min.

⁹·For Tomlinson I + J, resuspend cell pellet in 100 mL of 2xTY supplemented with 0.1% glucose 100 µg/mL of ampicillin and 50 µg/mL of kanamycin.

¹⁰·The 0.1% glucose provides a food source for the overnight growth of bacteria without arresting phage expansion.

¹¹·Carefully examine the bottle after centrifugation. The phage pellet will appear as a small white thin layer.

8. Add 15% by volume of the 25% PEG 6000, 2.5 M NaCl solution. Invert 50 times. Incubate at 4 °C overnight.
9. Spin down overnight incubation for 45 min at $6000 \times g$ and 4 °C.
10. Discard the supernatant and resuspend phage pellet in 5 mL of PBS/EDTA/BSA solution (*see* Note 12).
11. Transfer suspension to a new 15 mL polypropylene centrifuge tube. Spin at $10,800 \times g$ for 10 min to remove any remaining biological debris.
12. Transfer to a new 15 mL polypropylene centrifuge tube and store the supernatant at 4 °C. Use phage within 7 days.

3.1.3 Quantification of Phage Concentration (Colony Forming Units; CFU)

1. Streak out stock TG1 bacteria on TYE plates and culture at 37 °C overnight (*see* Note 1).
2. Transfer TG1 plates to 4 °C for storage. Use within 1 month. Prepare new plates as needed for experimental preparation.
3. Prepare an overnight, starved TG1 culture by inoculating 5 mL of 2xTY medium with a single TG1 colony pulled from the TG1 plate. Use vented capped tubes or loosely tape the cap onto a standard 50 mL centrifuge tube. Incubate in bacterial shaker overnight at 250 rpm and 37 °C.
4. The next day, prepare 100-fold dilution of overnight-starved bacteria with 10 mL of fresh 2xTY media. Incubate at 250 rpm and 37 °C until OD600 of 0.5 has reached (~1.5–2.5 h). Culture may be stored at 4 °C for up to 8 h until ready to complete the assay.
5. Prepare serial dilutions from purified phage sample to achieve the following dilutions: 10^{-5} , 10^{-7} , 10^{-9} , 10^{-11} in PBS. The most effective means of performing this series is to generate 10 mL of a 10^{-3} dilution (10 μ L of phage stock + 10 mL of PBS) followed by 100-fold dilutions in PBS (10 μ L + 990 μ L of PBS) (*see* Note 13).
6. Using the appropriate number of 0.5 mL microcentrifuge tubes, transfer 90 μ L of the starved TG1 culture each tube. Transfer 10 μ L of the phage serial dilutions to each tube to achieve an end dilution set of 10^{-4} , 10^{-6} , 10^{-8} , 10^{-10} , 10^{-12} . Include a PBS only +TG1 cells control.
7. Incubate the inoculated phage+TG1 solution in a water bath set at 37 °C for 30–45 min.
8. During this incubation, place two to three TAG plates in the bacterial incubator for 15–30 min (37 °C). This step serves to dry any condensation that may have formed on the TAG plate. If numerous bubbles are observed in the agar after

¹²The EDTA acts as a chelating agent, most likely toward the remaining PEG. The BSA also acts as a protein preservative/protectant.

¹³Recommend using 2 mL round bottom centrifuge tubes for the phage dilutions. After diluting each sample, close the cap and invert 3–5 $\times$ to ensure complete mixing of each dilution.

warming in an incubator, allow plates to slowly warm to RT before placing in an incubator.

9. On the back of the TAG plate, draw two lines to divide the plate into quarters with three small circles in each lane (*see* Fig. 1). It is recommended that each dilution is spotted in triplicates, but the pattern drawn on the plates can be modified to suit each individual's needs.
10. Pipette 10 μ L of each dilution onto the TAG plate in triplicate (Fig. 1). Use the pre-drawn lanes and circles as guides. Place the lid on the plate once the plate is full.
11. Once the solution has absorbed into the TAG plates, seal the plate with a thin layer of Parafilm.
12. Place the plate on a stationary shelf upside down in a bacterial incubator or oven set to 37 °C for 9–16 h. Alternatively, incubate at 30 °C for 16–24 h.
13. After an appropriate incubation time frame, remove the plates, count and record the total number of colonies for each dilution (*see* Fig. 1 and Note 14).
14. Determine colony forming unit (CFU) concentration for phage sample with the following equation:

$$\text{CFU / mL} = (\text{Average colony count})/(\text{Dilution} \times 0.01 \text{ mL}).$$

3.2 Biopanning on Adherent Cell Cultures

All cell culture steps prior to biopanning steps listed below should be performed using the standard tissue culture sterile technique. Once the biopanning steps begin and the phage particles are introduced to the cell cultures, do not return the cell cultures to the standard tissue culture working areas. Perform the remainder of the screen steps using aseptic techniques standard for bacterial work.

3.2.1 Basal Cell Culture

1. Culture cell line of interest according to the specified standard culture protocol in a T25 tissue culture flask. Prepare at least three flasks for the negative screen and one flask for the positive screens.
 - (a) Example: Primary astrocytes cultured in 10% FBS by volume of DMEM plated at 4×10^5 cells/cm² density and allowed to adhere and proliferate for 24–48 h.
2. If needed, apply appropriate signaling agents to induce targeted cell behavior for positive screens.
 - (a) Example: Use TGF- β to induce phenotypic switch from quiescent to reactive astrocyte phenotype. Astrocytes are subjected to serum starvation for 12 h (DMEM without FBS). After 12 h of starvation, astrocytes are treated with standard medium supplemented with 10

ng/mL of TGF- β for 48 h. After 48 h of TGF- β supplementation, remove TGF- β supplemented media and return to basal media.

3. Perform the biopanning screen immediately after cells are ready.

3.2.2 Negative Screens—A negative screen against potential basal adherent cells or background factors (i.e., tissue culture polystyrene, adsorbed FBS, etc.) reduces the phage population with high affinity and specificity to such substrates. This step aids in eliminating phage clones with overlapping recognition to the cell type and/or phenotype of interests.

1. The evening prior to performing negative screen, prepare an overnight, starved TG1 culture by inoculating 5 mL of 2xTY medium with a single TG1 colony pulled from the stock TG1 plate. Use vented capped tubes or loosely tape the cap onto a standard 50 mL centrifuge tube. Incubate in bacterial shaker overnight at 250 rpm and 37 °C.
2. The following day (day of negative screen), prepare 100-fold dilution of overnight-starved bacteria and by transferring 1 mL into 99 mL of fresh 2xTY media. Incubate at 250 rpm and 37 °C until OD600 of 0.5 is reached (~1.5–2.5 h). Culture may be stored at 4 °C for up to 8 h until completion of the negative screen.
3. Prepare a 5×10^{12} phage particle solution by diluting stock phage (prepared in Subheading 3.1) in 5 mL of standard culture medium.
4. Take one T25 flask containing basal cells and aspirate the medium.
5. Place the phage solution (5×10^{12} phage 5 mL of culture medium) in the flask and incubate for 1 h at 50 rpm and 30 °C (use bacterial incubator).
6. After 1 h, remove the supernatant and transfer into a second flask of basal cells. Incubate for 1 h at 50 rpm and 30 °C (use a bacterial incubator).
7. Repeat **step 4** for a third basal culture.
8. At the end of the third and final negative screen, mix the final supernatant with 30 mL of starved TG1 bacterial cells prepared in **steps 1** and **2**. Recommend using a 50 mL polypropylene centrifuge tube.
9. Incubate for 30 min at 50 rpm at 37 °C (bacterial incubator).
10. During the same incubation, set one large TAG 245 mm square bioassay dish (or 4 $\times$ TAG petri dishes) in bacterial incubator stationary rack or oven set at 37 °C to dry the plate.
11. Spin down incubation at $3400 \times g$ for 15 min.
12. Remove the supernatant and discard.
13. Resuspend pellet in 1 mL of 2xTY media.
14. Use a 1 mL pipette to evenly distribute the 1 mL cell suspension over the dried TAG 245 mm square bioassay dish (or 4 $\times$ TAG petri dishes). Spread the cell

suspension gently across the entire surface of the dish with a sterile bacterial cell spreader. Place the lid on the dish.

15. Wait approximately 30 min for the cell solution to absorb into the TAG plate. Wrap dish in parafilm.
16. Incubate TAG dish at 37 °C overnight (16–24 h; bacterial incubator shelf or oven).
17. The following day, remove TAG dish/plates from the incubator. A lawn of bacteria should be observed.
18. Transfer 20 mL of fresh 2xTY media to the TAG dish and quickly use a sterile bacterial cell scraper to gently dislodge the bacteria. Collect the bacteria dense media and transfer into a 50 mL polypropylene centrifuge tube. Final volume will be ~10–13 mL as some of the media will adsorb into the TAG gel.
19. Add 15% by volume glycerol to bacteria dense media and store in 1 mL aliquots at –80 °C. These aliquots represent the negative screen stock and are used in the subsequent biopanning round instead of the stock library. Keep aliquots from each stage of the screen in case a screen needs to be repeated or modified.

3.2.3 Checking for dAb/scFv Inserts—A critical check point after each screen stage of phage biopanning is to randomly check for the over growth of wild type phage or mutations of the genetic coding for scFv/dAb inserts (e.g., frame shifts) (*see* Note 15). Two methods are recommended to monitor the stability of the scFv/dAb inserts, (1) Evaluate insert length (PCR and electrophoresis), and (2) DNA sequencing. Each of these methods is briefly outlined below and relies on a prior knowledge of PCR, electrophoresis, and DNA purification. Table 1 lists the recommended primers for each technique.

1. Dry four TAG plates in a bacterial incubator (37 °C) for 30 min.
2. Use a cotton swab or inoculation loop to obtain a small sample of frozen TG1 cells from the screen of interest. For example, if probing for alterations after the negative screen, one would streak out TG1 cells from the frozen aliquots obtained at the end of the negative screen.
3. Immediately streak out TG1 sample onto the four TAG plates.
4. Incubate TAG plates overnight upside down at 37 °C (~16 h).
5. Remove plates from the incubator and use immediately or place in 4 °C for storage up to 1 month.

Evaluate Insert Length (PCR and Electrophoresis)

1. Prepare enough working PCR solution for five 15 µL PCR reactions according to PCR Core and recommended forward and reverse primers (Table 1).
 - (a) Recommended pairing for Tomlinson I + J library: LMB3 and pHEN. If scFv inserts are present, bands will appear around 900–950 bp. If no scFv inserts are present, bands will appear around 300–350 bp.

- (b) Recommended pairing for domain antibody library: LMB3 and dAb reverse. If dAb inserts are present, bands will appear around 600–700 bp. If no dAb inserts are present, bands will appear around 200–300 bp.
2. Aliquot 10 µL of working PCR solution into five PCR tubes.
3. Using a 10 µL pipettor with matching pipette tip, select one unique clone from the streaked plate and transfer to one PCR tube via titration.
4. Repeat **step 3** for the four remaining PCR samples.
5. Spin samples for ~10 s on a small pulse benchtop centrifuge.
6. Load samples into a thermocycler and run with the following PCR amplification settings:
 - (a) Pre-denaturation: 9 min at 94 °C.
 - (b) Denaturation, Annealing, and Extension: 30 cycles of 45 s at 95 °C, 45 s at 55 °C, 1 min at 72 °C; then 5 min at 72 °C. For Tomlinson I + J, set annealing for 1 min and extension for 2 min.
 - (c) Hold at 4 °C (will maintain sample integrity until removed from the thermocycler).
7. During amplification, prepare a 3% agarose gel in 1× TAE buffer supplemented with Gel Red Nucleic Acid Stain.
8. After PCR amplification, load 10 µL of PCR samples in 3% agarose gel across five different lanes. Include an appropriate base-pair ladder for size reference. Run gel according to the manufacturer's directions.
9. Use a UV box or imager to evaluate the length of the inserts. If the scFv/dAb insert is lost in more than 60% of the samples (i.e., three out of five), then the previous screen step may need to be repeated.

Plasmid Purification and Sequencing

1. Prepare five bacterial culture tubes with 5 mL of 2xTY media.
2. Inoculate each tube with a single TG1 colony pulled from the previously streaked out plate from the screen of interest.
3. Incubate cultures in a bacterial shaker overnight at 250 rpm and 37 °C overnight (~16 h). Use vented capped tubes or loosely tape the cap onto a standard 50 mL centrifuge tube.
4. The following morning, use a Qiagen Miniprep Kit to purify plasmids from the overnight cultures according to the manufacturer's protocol.
5. Submit purified plasmid DNA to a DNA sequencing center for the analysis of the specific clones (*see* Table 1 for suggested primers).
6. Multiple software programs may be used to analyze the sequencing results. The freeware A plasmid Editor (ApE; University of Utah—M. Wayne Davis) is a

useful program to quickly analyze results and determine presence or absence of inserts and mutations.

(a) Tomlinson I + J libraries:

- Open. seq file in ApE program.
- Under “Enzyme” tab, use “Enzyme Selector” tool to highlight SfiI, SmlI, and NotI sites.
- If SfiI is not present, this may indicate that you do not have an insert present. Sometimes, the NotI is not present due to a short or poor read.
- Usually need to perform forward (LMB3 primer) and reverse (pHEN primer) reading to sequence whole insert.
- Highlight sequence beginning right after the SfiI site (ATGG...) and ending about 120 nucleotides after NotI site.
- Translate—Under “ORFs” tab, use “Translate” tool. Specify 10 AA per line and translate selection only.
- Log sequence into Excel spreadsheet.

(b) Domain antibody library:

- Open. seq file in ApE program.
- Under “Enzyme” tab, use “Enzyme Selector” tool to highlight SfiI and NotI sites.
- If SfiI is not present, this may indicate that you do not have an insert present. Sometimes, the NotI is not present due to a short or poor read.
- Highlight sequence beginning right after the SfiI site (ATGG...) and ending about 120 nucleotides after NotI site.
- Translate—Under “ORFs” tab, use “Translate” tool. Specify 10 AA per line and translate selection only.
- Log sequence into Excel spreadsheet.

3.2.4 Positive Screens—Positive screens encompass successive biopanning rounds to generate an enriched phage population of high affinity to the target of interest. Here, an example of a reactive astrocyte phenotype is used as the target adherent cell type. It is recommended that at least three rounds of positive screens are performed. Additional rounds may be required if specificity and high affinity to the target of interest is not achieved.

1. The critical first step of a positive screen is coordination of the phage particle and target sample preparation. Samples and phage need to be prepared in parallel.

- (a) Phage particles obtained from the infected TG1 cells from the previous round need to be produced, purified, and quantified according to the

steps outlined in Subheading 3.1. For instance, the first positive screen will use phage particles produced from a TG1 aliquot from the negative screen. Note that this process will take a minimum of 3 days; account for this time in experimental planning and preparation.

- (b) Prepare one adherent cell target of interest in a T25 flask. Be sure to account for sample maturation/treatment time-lines in experimental planning and preparation. For example, a minimum of four days is required to generate a reactive astrocyte culture.
2. The evening prior to performing positive screen, prepare an overnight, starved TG1 culture by inoculating 5 mL of 2xTY medium with a single TG1 colony pulled from the stock TG1 plate. Incubate in a bacterial shaker overnight at 250 rpm and 37 °C.
 3. The following day (day of screen), prepare 100-fold dilution of overnight-starved bacteria by transferring 1 mL into 99 mL of fresh 2xTY media. Incubate at 250 rpm and 37 °C until OD600 of 0.5 is reached (about 1.5–2.5 h). Culture may be stored at 4 °C for up to 8 h until completion of the screen.
 4. After preparing phage particles and adherent cell target of interest, prepare a 5×10^{12} phage particle solution by diluting concentrated phage in 5 mL of standard culture medium.
 5. Aspirate culture media from T25 flask containing target cells of interest and place the 5 mL of phage/culture media solution onto the cells. Keep 200 μ L of media/phage solution in a 1.5 mL microcentrifuge tube to verify total number of phage at the start of the screen.
 6. Incubate the phage with the positive screen adherent cells for 1 h at 30 °C with agitation (~50 rpm) in a bacterial incubator.
 7. Remove media and keep media to assess the number of unbound phage particles.
 8. Rinse with 5 mL of PBS for 5 min with agitation (~50 rpm) three times. Each time collect the rinse to assess the number of phage particles removed during the rinse process.
 9. After the three rinses, elute the phage by applying 2 mL of trypsin solution. Incubate at 30 °C for 15 min at 50 rpm (*see* Note 16).
 10. Transfer 2 mL trypsin phage elution to a clean 15 mL centrifuge tube and spin down at $1120 \times g$ for 5 min.
 11. Transfer the supernatant (discard pellet) to 30 mL culture of overnight-starved TG1 cells (use 50 mL centrifuge tube) and incubate for 30 min at 37 °C and 50 rpm.
 - (a) Dry out one large TAG bioassay dish (or 4 $\times$ TAG petri dishes) in the bacterial incubator or oven during this incubation.

¹⁶Bound phage are eluted from the target cell cells via trypsin cleavage site between the phage body and scFv/dAb fragment.

12. Spin down the bacterial culture at $3400 \times g$ for 15 min.
13. Discard the supernatant and resuspend the TG1 pellet in 1 mL of 2xTY media.
14. Use a 1 mL pipette to evenly distribute the 1 mL cell suspension over the dried TAG 245 mm square bioassay dish. Spread the cell suspension gently across the entire surface of the dish with a sterile bacterial cell spreader. Place the lid on the dish.
15. Wait approximately 30 min for the cell solution to absorb into the TAG gel. Wrap dish in parafilm.
16. Incubate TAG dish at 37 °C overnight upside down (16–24 h; bacterial incubator shelf or oven).
17. The following day, remove dish from the incubator. A lawn of bacteria should be observed.
18. Transfer 20 mL of fresh 2xTY media to the TAG dish and quickly use a sterile bacterial cell scraper to gently dislodge the bacteria. Collect the bacteria dense media (10–13 mL as some of the media will adsorb into the TAG gel) and transfer to a 50 mL polypropylene centrifuge tube.
19. Add 15% by volume glycerol to bacteria dense media and store in 1 mL aliquots at –80 °C. These aliquots now represent a positive screen stock for your screen. Keep aliquots from each stage of the screen in case a screen needs to be repeated or modified.
20. It is recommended to check for presence of scFv/dAb inserts after each round. Follow the steps outlined in Subheading 3.2.3. If inserts are still present, continue with next screen (i.e., repeat steps outline in this section with TG1 cells from the round that was just completed). After completing three rounds of positive screens, continue onto Subheading 3.3.

3.3 Identification of Potential High Affinity scFv/dAb via ELISA

The next phase entails identifying specific phage clones with high affinity and specificity to target of interest. To accomplish this goal, single clones will be produced in a high-throughput 96-well plate manner to evaluate single clone affinity via a modified enzyme-linked immunosorbent assay (ELISA).

3.3.1 Phage Clone Preparation (96-well plate format)

1. Dry eight TAG plates in a bacterial incubator (37 °C) for 30 min.
2. Use a cotton swab or inoculation loop to obtain a small sample of frozen TG1 cells from the third positive screen.
3. Immediately streak out TG1 sample onto four TAG plates.
4. Using a fresh cotton swab or inoculation loop, repeat **steps 2 and 3** on two TAG plates for the initial scFv/dAb stock library (required for negative control clones).

5. Using a fresh cotton swab or inoculation loop, repeat **steps 2 and 3** on the remaining two TAG plates for the anti-ubiquitin vial supplied by the phage library kit (required for positive control clones).
6. After parafilming, incubate TAG plates overnight upside down at 37 °C (~16 h).
7. Remove plates from the incubator and use immediately or place in 4 °C for storage up to 1 month.
8. Prep a 96-well round bottom plate with 200 µL of 2xTY supplemented with 4% glucose + 100 µg/mL ampicillin in each well (recommend preparing master solution of supplemented media and then dispensing 200 µL into each well).
9. Using the streaked out plates from the third positive screen, inoculate each well of the 96-well plate with a single colony picked with a 10 µL pipette tip (supplemented with the media). It is recommended to pick at least 88 clones (11 columns). The remaining column (eight wells) should be inoculated with colonies from the original scFv/dAb library stock to serve as random controls and anti-ubiquitin stock to provide a positive control (four wells per). Place the lid on the plate when complete and parafilm the lid to the base plate.
10. Place the 96-well plate into 96-well plate racks in bacterial incubator. Grow overnight at 37 °C and 250 rpm.
11. After the overnight growth, prepare a fresh 96-well round bottom plate containing 200 µL of 2xTY + 4% glucose + 100 µg/mL ampicillin.
12. Transfer 5 µL of the overnight culture from each well to the matching corresponding well in the fresh 96-well plate; it is helpful to use a multi-channel pipettor for this process but be sure to use a clean pipette tip for each well.
 - (a) Do not discard remainder of overnight 96-well plate. Add 55 µL of a 50% glycerol (100% glycerol diluted with 2xTY) to each well.
 - (b) Place the lid on the plate and secure with Parafilm. Store at –80 °C. This plate serves as the single clone stock for the phage biopanning (*see* Subheading 3.4.3).
13. Secure the freshly inoculated plates in the bacterial incubator and incubate at 37 °C and 250 rpm for 4 h.
14. Add 50 µL of 2xTY containing 4×10^8 KM13 helper phage (8×10^9 phage/mL) to each well.
15. Secure the plate in a bacterial incubator and incubate at 37 °C and 50 rpm for 45 min.
16. Spin plate down at $3200 \times g$ for 10 min.
17. Discard the supernatant by quickly inverting the plate over a wide-mouth collection bin.

18. Resuspend cell pellets in the bottom of each well in 2xTY + 0.1% glucose + 100 µg/mL ampicillin + 50 µg/mL kanamycin.
19. Secure the plate in a bacterial incubator and incubate overnight at 25 °C and 250 rpm for 16–24 h.
20. Spin down the plate at $3200 \times g$ for 10 min.
21. Carefully transfer the supernatant to a new 96-well round bottom plate and store at 4 °C for use within 1 week. This supernatant contains the phage particles for the modified ELISA.

3.4 Modified ELISA Protocol

3.4.1 Adherent Cell Plate Prep—Prepare two 96-well plates for the modified ELISA. One plate consists of the basal non-targeted cell population prepared for the negative screen. The other plate will be prepared according to the target cell plating protocol used during the phage biopanning screens. The example outlined here details specifics for generating basal and reactive astrocytes.

1. Seed two 96-well cell culture plates with 40,000 cells/cm² and allow to grow for a minimum of 48 h in 10% FBS DMEM media.
2. After 48 h, serum-starve one plate (DMEM without FBS) for 12 h in preparation for treating the astrocytes with 10 ng/mL of TGF-β. The second plate will remain in DMEM +10% FBS.
3. After the 12 h starvation period, aspirate the starvation media and treat the cells with 10 ng/mL of TGF-β for a minimum of 48 h

3.4.2 Modified ELISA

1. Prepare diluted phage clone solutions in a new 96-well round bottom plate.
 - (a) Dispense 185.5 µL of working astrocyte media (DMEM + 10% FBS) into each well.
 - (b) Transfer 62.5 µL of phage supernatant acquired in Subheading 3.3.1 to each corresponding/matching well.
2. Remove media from the two 96-well adherent cultures (one basal and one reactive plate) by gently inverting the plate over a wide-mouth collection container.
3. Transfer 100 µL of diluted phage into each corresponding well of the adherent cell plates (one basal and one reactive plate).
4. Secure plates in bacterial incubator and incubate at 30 °C for 1 h at ~50 rpm.
5. Discard the supernatant by gently inverting plate over a wide-mouth collection container.
6. Wash the wells three times with PBS.

7. Add 100 μ L of 1:2000 HRP-anti-M13 conjugate (diluted in working adherent culture media) to each well of both ELISA plates.
8. Secure plates in bacterial incubator and incubate at 30 °C and ~50 rpm.
9. Discard the supernatant by gently inverting the plate over a wide-mouth collection container.
10. Wash the wells three times again with PBS.
11. Add 65 μ L of 1-Step Ultra-TMB-ELISA to each well.
12. Allow the solution to develop for approximately 30 min with gentle agitation on an orbital shaker at room temperature. A deep blue color should develop in the positive wells.
13. Stop the TMB-HRP reaction with 40 μ L of 2 N sulfuric acid. The solution should turn yellow and enzymatic breakdown of TMB should cease.
14. Read the absorbance at 450 nm in the plate reader with reference wavelength of 560 nm.
15. Analyze the absorbance readings.
 - (a) Subtract average blank absorbance readings (i.e., cell + no antibody phage + HRP-anti-M13 conjugate) from all wells on each plate.
 - (b) Take note of the baseline response for each control random clone for each plate. This cell-based ELISA may generate very noisy results. Absorbance readings above the random clone baseline indicate preferential binding to the specific cell population.
 - (c) Generate a ratio of active/basal absorbance for each phage clone.
 - (d) Use bar graph to visually compare absorbance ratios.
 - (e) Identify top ten clones with highest ratio of active/basal response.
16. Sequence the top ten clones using the methods outlined in Subheading “Plasmid Purification and Sequencing”.

3.4.3 Concentration-Dependent Modified ELISA—The initial modified ELISA provides a high-throughput method for identifying clones of interest to characterize further. Here, the modified ELISA will be performed with a controlled concentration of phage particles for the top four to five clones identified in Subheading 3.4. In addition to four to five clones, it is recommended to also test one random initial stock control clone as a negative control and anti-ubiquitin clone as a positive control.

1. Generate stock aliquots of phage clones of interest by using frozen infected TG1 cells from the 96-well plate prepared in Subheading 3.3.1 **step 12**.
2. Inoculate 20 mL of 2xTY + 4% glucose +100 μ g/mL of ampicillin with phage clone of interest pulled from stock 96-well plate (50 mL tube; loosely tape lid). Grow culture at 37 °C and 250 rpm for 3–4 h.

3. Add 2×10^{11} helper phage to 20 mL culture. Incubate in 37 °C water bath for 60 min.
4. Spin culture at $3200 \times g$ for 10 min.
5. Discard the supernatant and resuspend pellet in 50 mL of 2xTY + 0.1% glucose + 100 µg/mL of ampicillin + 50 µg/mL kanamycin (250 mL Erlenmeyer flask).
6. Grow culture at 250 rpm and 30 °C for ~20 h.
7. Follow the standard PEG purification protocol as outlined in Subheading 3.1.2 and determine total phage CFU (Subheading 3.1.3).
8. **Steps 1–7** may be performed in parallel for each clone of interest.
9. Prepare 96-well plates with basal and target cells as outlined in Subheading 3.3 (Plan according to prepare prior to running ELISA).
10. On the day of the ELISA, prepare diluted stock phage solutions for each clone in the appropriate cell culture medium to achieve a concentration of 1×10^{11} CFU/mL (5 mL).
11. Prepare serial dilutions with concentrations of 10^{11} , 10^9 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^1 in the cell culture medium (1.5 mL each).
12. Obtain the prepared 96-well cell cultures. Aspirate medium out of wells.
13. Transfer 200 µL of phage dilution solution to three wells in the basal and targeted cell plates. Continue until all dilutions (including a non-phage control sample well) are dispensed on the plates.
14. Follow **steps 4–14** in Subheading 3.4 to complete the ELISA.
15. Analysis for the concentration-dependent ELISA entails generating a modified Klotz plot whereby the absorbance is plotted versus the phage CFU for each individual clone (*see* Fig. 2 for representative plot). This analysis enables relative comparisons of affinity characteristics across different clones.

4 Production of scFv/dAb

4.1 Generating HB2151 Stocks

The TG1 strain is a suppressor strain of bacteria that recognizes an amber stop codon as a glutamine residue, thus facilitating phage endowing an scFv/dAb fusion protein on the outer coat protein. However, non-suppressor strains such as HB2151 recognize the amber stop codon and enable the production of the scFv/dAb portion alone. Here, the basic outline for generating HB2151 stocks and basic production and purification of the scFv/dAb are provided (*see* Note 17).

1. Streak out HB2151 bacteria on TYE plates and culture at 37 °C overnight (*see* Note 1).

¹⁷HB2151 cells are not an optimal production line and therefore alternative expression/production lines will be required for large scale protein production.

2. Transfer HB2151 plates to 4 °C for storage. Use within 1 month. Prepare new plates as needed for experimental preparation.
3. Generate phage for clones of interest by using frozen infected TG1 cells from the 96-well plate prepared in Subheading 3.3.1 **step 12**.
4. Inoculate 20 mL of 2xTY + 4% glucose + 100 µg/mL of ampicillin with phage clone of interest pulled from stock 96-well plate (50 mL tube; loosely tape lid). Grow culture at 37 °C and 250 rpm for 3–4 h.
5. Add 2×10^{11} helper phage to 20 mL culture. Incubate in 37 °C water bath for 60 min.
6. Spin culture at $3200 \times g$ for 10 min.
7. Discard the supernatant and resuspend the pellet in 50 mL of 2xTY + 0.1% glucose + 100 µg/mL of ampicillin + 50 µg/mL kanamycin (250 mL Erlenmeyer flask).
8. Grow culture at 250 rpm and 30 °C for ~20 h.
9. Follow the standard PEG purification protocol as outlined in Subheading 3.1.2 and determine total phage CFU (Subheading 3.1.3).
10. **Steps 1–9** may be performed in parallel for each clone of interest.
11. Prepare an overnight, starved HB2151 culture by inoculating 5 mL of 2xTY medium with a single HB2151 colony pulled from the stock HB2151 plate. Incubate in a bacterial shaker overnight at 250 rpm and 37 °C.
12. The following day, prepare 100-fold dilution of overnight-starved bacteria and by transferring 100 µL of starved culture in 10 mL of 2xTY (use 50 mL tube loosely tape lid to prevent it from unscrewing in the incubator). Incubate at 250 rpm and 37 °C until OD600 of 0.5 is reached (about 1.5–2.5 h). Prepare one 10 mL culture per clone.
13. At the same time, set one large bioassay TAG plate and one TAG petri dish in a bacterial incubator or oven to dry (one plate and dish per clone).
14. Add 100 µL of one phage clone to each 10 mL HB2151 culture and incubate in a water bath at 37 °C for 30 min.
15. Spin culture at $3200 \times g$ for 5 min.
16. Discard the supernatant and resuspend the pellet in 0.5 mL of 2xTY.
17. Plate 450 µL of the concentrated cell solution one TAG bioassay dish. Spread evenly across plate surface with bacterial cell spreader. Grow overnight at 30–37 °C. Titer remaining cells at 10^2 , 10^3 , 10^4 , 10^5 dilutions on the TAG petri dish.
18. Repeat **step 17** for all clones.
19. After overnight growth, add 10 mL of 2xTY medium per large biodish and gently dislodge cells with bacterial cell scraper/spreader. Use serological pipette to transfer cell suspension to a 50 mL centrifuge tube.

20. Add 100% glycerol to generated 15% glycerol final volume and aliquot into 1 mL aliquots. Freeze and store at -80°C . Store the titer plate at 4°C .
21. It is recommended to use four to six colonies from the titer plate to generate cultures for plasmid DNA extraction to send out for sequencing in order to verify clone homogeneity (*See Subheading “Plasmid Purification and Sequencing”*).

4.2 Production of scFv/dAb

The final step in this process is to produce and purify the scFv/dAb for further characterization and eventual use for the desired application. Below are basic outlines for protein purification from the cell lysates and cell media, however, it is noted that as with any recombinant proteins, production rates and amounts vary from clone to clone. Optimal methods for purification may need to be performed for each clone.

1. Add 1 mL of HB2151 infected stock to 500 mL of 2xTY + 0.1% glucose + 100 $\mu\text{g/mL}$ ampicillin (use 2 L Erlenmeyer flask). Grow at 37°C and 250 rpm ~4 h.
2. Add 0.5 mL of 1 M IPTG to culture (induce scFv/dAb production) and grow overnight at 30°C and 250 rpm.
3. Spin overnight culture at $8000 \times g$ for 10 min. Recommend using a single 250 mL centrifuge bottle to collect the entire cell pellet. This may require multiple rounds of centrifugation using the same collection bottle.
4. Aspirate the supernatant and store in 500 mL bottle and keep cell pellet.

4.3 Purification of scFv/dAb: Cell Lysate

1. Prepare 45 mL of 1xPBS + one tablet of Protease Inhibitor Mini Tablet.
2. Use the 45 mL of PBS + Protease inhibitor to resuspend cell pellet.
3. Prepare 5–10 mL of 10 mg/mL lysozyme in 25 mM Tris-HCl, pH 8.0.
4. Add 5 mL of lysozyme solution to cell solution.
5. Use a probe sonicator to further lyse the cells (on ice). Suggested sonication sequence is 3–4 cycles of 20 s sonication at 40–50% maximum power and then off for 30 s. No foam should appear. Do not over sonicate.
6. Immediately add Triton X-100 and 100 U/mL of DNase I to the cell lysate and bring the final concentration to 1% Triton X-100 and 10 U/mL of DNase I.
7. Place on an orbital shaker to gently mix for 30 min.
8. Freeze lysate -20°C for minimum of 6 h. The lysate may be stored at -20°C for longer if lysate will not be purified immediately.
9. The day of purification, thaw lysate out at RT.
10. Spin the sample down for 15 min at $12,000 \times g$.
11. Filter the supernatant through a 0.22 μm pore filter and add 0.1% NaN_3 to generate a final concentration of 0.02% NaN_3 .

12. Use preferred method of protein purification to extract scFv/dAb. The recommend method is Fast-Performance Liquid Chromatography (FPLC) with a Protein-A affinity column. In order to achieve optimal protein purification, it may be necessary to use alternative resin designed to capture specific types of antibody fragments.

4.4 Purification of scFv/dAb: Culture Supernatant

Some clones will readily produce the scFv/dAb as soluble proteins in the bacterial culture medium. The culture medium may be desalted and concentrated using a tangential flow filtration system. The resulting concentrated solution may then be purified using the same FPLC with a Protein-A affinity column recommended above in Subheading 4.3.

4.5 Further Characterization

After production and purification of scFv/dAb for clones of interest, perform concentration-dependent ELISAs to validate results obtained from the phage-based assays. Further characterization assays to include surface Plasmon resonance (SPR) and immunocytochemistry to determine dynamic binding constants and spatial location of epitopes.

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Fig. 1.

Bacterial colony forming unit assay. **(a)** Schematic of TAG plate to determine the number of functional phage as measured by bacterial colony forming unit (CFU) assay. Each quadrant of the TAG plate represents various potential outcomes for this assay, lawn formation, colony overpopulation, ideal colony count, and low colony population. **(b)** Schematic of colony progression over time. If the incubation runs over the recommended 12–16 h time, distinct individual colonies will be difficult to identify

Fig. 2.

Modified Klotz plot sample results. Concentration-dependent binding curves for three scFv clones to basal or activated cell targets. (a) scFv/dAb clone with affinity to activated cells. (b) scFv/dAb clone with comparable high affinity to both basal and activated cell targets. (c) Negative control scFv/dAb clone with no discernable affinity to either basal or activated cell targets

Table 1

Recommended primers for analyzing ScFv/dAb insert length (PCR + Electrophoresis) and sequence

Primer	Sequence	Use	References
LMB3	5'-CAGGAAACAGCTATG AC-3'	Forward primer for Tomlinson I + J and dAb libraries	
pHEN	5'-CTATGCGGCCCCATTCA-3'	Reverse primer for Tomlinson I + J libraries	
dAb Reverse	5'-GTTTTGTCGTCTTTCCAGACG-3'	Reverse primer for dAb library	Dudgeon et al. [7]