# Fusion BioLabs Complete Protocol for Phage display Library Panning and Candidate Validation by Monoclonal Phage ELISA

## 1. Materials

1. anti-M13-HRP: anti-M13 monoclonal antibody, horse radish peroxidase conjugate.
2. 0.2 M Glycine-HCl: adjust pH to 2.5 and filter sterilize (0.2 µm).
3. 1 M Tris–HCl: adjust pH to 8 (with HCl) and filter sterilize (0.2 µm).
4. 1.5 mL microcentrifuge tubes.
5. 2 M $H_2SO_4$.
6. 25 mM Tris–HCl, 2 mM EDTA.
7. 5% (w/v) BSA: 5% (w/v) bovine serum albumin in PBS. Mix and filter sterilize (0.2 µm).
8. 96-well deep-well plates (1 mL).
9. Bovine serum albumin (BSA).
10. Carbonate buffer: 0.03 M $Na_2CO_3$, 0.07 M $NaHCO_3$. Mix, adjust pH to 9.6 with HCl/NaOH and filter sterilize (0.2 µm).
11. Cooled centrifuge and centrifuge bottles (variable size).
12. Clear Flat-Bottom Immuno Nonsterile 96-Well Plates (Thermo Fisher Scientific, Rockford, IL, USA).
13. D-glucose.
14. DMSO: dimethyl sulfoxide.
15. Dynabeads M-280 Streptavidin (Thermo Fisher Scientific).
16. Freeze dryer.
17. Heating block.
18. Magnetic rack.
19. Microplate reader (for A450 nm).
20. PBS: phosphate-buffered saline, 150 mM NaCl, 8 mM $Na_2HPO_4$, 2 mM $NaH_2PO_4{\cdot}H_2O$. Adjust pH to 7.4 with HCl, filter sterilize (0.2 µm), and autoclave.
21. PBST: PBS, 0.05% (v/v) Tween 20. Adjust pH to 7.4 with HCl, filter sterilize (0.2 µm) and autoclave.
22. PEG/NaCl: 20% (w/v) PEG8000, 15% (w/v) NaCl. Mix and filter sterilize (0.2 µm).
23. Petri dishes.
24. End-over-end (eoe) Rotamixer.
25. SDS-PAGE gels and reducing SDS-PAGE sample loading buffer.
26. Incubator shaker at 37 °C.
27. Spectrophotometer
28. TMB substrate (TMB Substrate Kit, Thermo Fisher Scientific).
29. 2xYT broth medium: 16 g Peptone, 10 g yeast extract, 5 g NaCl. Add water to 1 L and autoclave.
30. 2xYT/carb medium: 2xYT, 100 µg/mL carbenicillin.

31. 2xYT/carb/kan medium: 2xYT, 100 µg/mL carbenicillin, 25 µg/mL kanamycin.
32. 2xYT/carb/glucose medium: 2xYT, 100 µg/mL carbenicillin, 4% (w/v) glucose.
33. 2xYT/carb/IPTG: 2xYT, 100 µg/mL carbenicillin, 1 mM isopropyl β-D-1 thiogalactopyranoside.

## 2. Panning Protocol using biotin labelled antigen target

### 2.1 1st round panning

1. The day before the panning project, prepare a TG1 culture to be used later for infection with the phage eluate: Inoculate 5 mL 2xYT medium with an inoculated colony of *E. coli* TG1 (includes in the phage kit). Grow at 37 °C at 250 rpm overnight.

2. During the panning day, inoculate the *E. coli* TG1 overnight culture from step 1, 1:100 to 50 mL 2xYT medium. Grow at 37 °C at 250 rpm to mid-log phase (OD600 = 0.6–0.8, around 3 h) and use for phage output titration (step 16) and phage output amplification (step 30).

3. Take phage library phage tube out from the kit and thaw it by put on ice for 10 minutes.

4. Take a low protein binding microcentrifuge tube (2.0 ml) out; add 0.25 ml of PEG/NaCl, then add 1.0 ml phage library from the kit into the tube, mix by vortex, and incubate for 30 min on ice to precipitate phage.

5. Spin for 20 min at 12,000× g at 4 °C to pellet precipitated phage. Carefully decant the supernatants.

6. Add 0.2 ml cold PBST with 2% BSA (w/v), vortex to suspend the phage pellets. This is the phage source of the 1st round panning.

7. Prepare beads (Dynabeads M-280 Streptavidin, Thermo Fisher Scientific) by washing 3 times with 1 ml PBS using a magnetic rack. Use an amount of beads (~ 200 µL) to immobilize the desired amount of biotin-labeled target antigen.

8. Immobilize target antigen onto the beads by incubating biotin-labeled target antigen with the beads in PBST to a final volume of 250 µL for 1 h at room temperature (RT) with rotation. Use an amount of target antigen that in the final selection volume corresponds to the desired antigen concentration for the particular selection round for your desired affinity KD (for example need 20 nM $K_D$, we suggest using antigen concentration around 20 nM for the 1st round, 2 nM for the 2nd round, and 1-0.2 nM for the 3rd round….).

9. Remove the supernatant and add 1 mL 2% (w/v) BSA in PBST to the beads for 30 min at RT with rotation.

10. Remove the blocking solution and wash the blocked beads once with PBST to remove excess BSA.

11. Add phage library stock from Step 6 (1st round panning phage source), to the washed and blocked beads in a final selection volume of 1 mL, with a final concentration of 2% (w/v) BSA. Incubate for 1-3 h at RT with rotation.

12. Place the selection tube in a magnetic rack and remove the supernatant after 10 min.

13. Wash the beads with 1 mL PBST for a total wash of 10 times (in later cycles, the washing time may be extended significantly to increase the selection stringency, such as 15-20 times).

14. Transfer the solution containing both beads and wash solution to a new low protein binding microcentrifuge tube in a magnetic rack. Remove and discard the supernatant.

15. To elute phage, incubate the beads with 500 µL 0.2 M Glycine-HCl, pH 2.5 for 10 min with occasional hand shaking after which the supernatant (containing the enriched and eluted phages) is transferred to a new blocked tube containing 500 µL 1 M Tris–HCl (pH 8.0) for neutralization.

16. Evaluate the selection eluate using titration on *E. coli* TG1 cells: determine the phage concentration by infecting log-phase *E. coli* TG1 cells with serial dilutions of the phage eluate. Dilute 10 µL of eluate in 90 µL PBS and prepare 12 such tenfold dilutions. Transfer 10 µL of each dilution to a 96-well round bottom plate, and add 100 µL of log-phase *E. coli* TG1 cells. Incubate still for 30 min at 37 °C and plate 5 µL of each dilution on 2xYT/carb plates.

17. Infect 50 mL log-phase *E. coli* TG1 cells with the whole (neutralized) phage eluate volume (rinse the tube carefully). In later panning rounds, 0.8 mL eluated phage volume can be used, and keep 0.2 ml phage in case of failure later.

18. Swirl gently and incubate at 37 °C by shaking at 250 rpm for 1 h.

19. Add 50 ml 2xYT/carb/glucose medium by continual shaking at 250 rpm for 1 h.

20. Add M13K07 at an MOI of 10 (around 40 µL phage in the kit) to the culture, and continual shaking at 250 rpm for 0.5 h.

21. Pellet infected TG1 by centrifuging at 3500× g for 15 min at RT.

22. Resuspend the pellet in 100 mL 2xYT/carb/kan and shake at 250 rpm overnight at 37 °C.

23. After overnight culture (ca. 16–18 h), transfer the phage producing bacterial culture to a centrifuge bottle, and pellet bacteria by centrifugation for 15 min at 8000× g at 4 °C.

24. Transfer the supernatant to a fresh centrifuge bottle containing 25 mL cold PEG/NaCl solution to precipitate phage and Incubate 1 h on ice.

25. Spin for 45 min at 18,000× g at 4 °C to pellet precipitated phage. Decant the supernatant.

26. Resuspend the phage pellet in 2 mL cold PBS.

27. Pellet residual bacteria by centrifuging at 18,000× g for 15 min at 4 °C.

28. Transfer the phage-containing supernatants to new low protein binding microcentrifuge tube containing 1/5 final

volume of PEG/NaCl, and incubate for 0.5 h on ice to precipitate phage.

29. Use a pipette to resuspend the phage pellets in 0.2 ml cold PBST with 2% BSA (w/v), vortex to suspend the phage pellets. This is the phage output from 1st panning round for the 2nd panning round.

30. Determine phage concentration by infecting log-phase *E. coli* TG1 cells with serial dilutions of phage as described in step 16.

31. Inoculate 5 mL 2xYT medium with inoculated colonies of *E. coli* TG1 (includes in the phage kit). Grow at 37 °C and 250 rpm overnight for 2nd round panning.

### 2.2 2nd round panning and later

32. The day of 2nd round, inoculate the *E. coli* TG1 overnight culture 1:100 to 50 mL 2xYT medium. Grow at 37 °C at 250 rpm to mid-log phase (OD600 = 0.6–0.8. around 3 h), and use it for phage output titration and phage output amplification.

33. Following steps 7–30 for 2nd round panning.

34. Following steps 31, 7–30 for 3rd round, 4th round panning.

Generally 3-4 rounds panning should be enough to get your candidates with the $K_D$ you preferred.

## 3. Phage-ELISA Screening

An initial binding screen to identify candidate affibody binders is done by performing a monoclonal phage-ELISA, where phage particles are produced from individual colonies generated from the eluate obtained after the final selection cycle. Each monoclonal phage sample produced is assayed for binding to a set of proteins, including the target antigen (positive), and relevant control (negative control, such as the same tagged antigen as the targeted antigen). A similar procedure can be applied also on polyclonal phage samples to monitor the enrichment of target binding phage populations throughout the biopanning progress.

1. Coat ELISA plates (Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, Thermo Fisher Scientific) with 100 µL target antigen at 1–10 µg/mL or control protein(s) at 1–10 µg/mL per well in 0.1 M carbonate buffer. Cover the plates, spin down briefly, and incubate still or with gentle shaking at 4 °C overnight.

2. Wash the ELISA plate 3 times with PBST and block with 60 µL 1% (w/v) BSA in PBS per well for 1 h at RT with gentle shaking, then wash the ELISA plate 3 times with PBST.

3. Inoculate individually grown *E. coli* colonies containing the affibody phagemid to 500 µL 2xYT/carb/glucose

medium in a 96-well deep-well plate. Cover with a breathable sealing film and incubate overnight at 250 rpm at 37 °C.

4. Inoculate 50 µL of each overnight culture to 450 µL 2xYT/carb/glucose medium in a new 96-well deep-well plate for 2 h at 250 rpm at 37 °C.

5. Superinfect the grown bacteria cultures with M13K07 helper phage at an MOI of 10 by adding M13K07 diluted in 100 µL 2xYT/carb medium/4% glucose. Incubate without shaking for 30 min at 37 °C and shaking at 250 rpm for 30 min at 37C.

6. Spin down the infected E coli TG1 cells at 3,200× g for 15 min at 4 °C. Decant the supernatant.

7. Resuspend the cell pellet in 2xYT/carb/kan medium, and cover with a breathable sealing film and incubate overnight at 250 rpm at 37 °C.

8. After overnight culture (ca. 16–18 h), transfer the 96-well deep-well plates to a swing buckets centrifuge, and centrifuge for 20 min at 3200× g at 4 °C.

9. Blocking new 96-well deep-well plate by addition 20 µL 4% (w/v) BSA in PBST to each well, and transferring the phage supernatants (~ 400 µL) to the blocked 96-well plate and with gentle shaking at RT for 30 minutes. During the transferring, do not disturb the pellets in overnight culture plate.

10. Add 200 µL above blocked phage supernatant to coated ELISA plates (target and control). Incubate for 2 h at RT with gentle shaking.

11. Wash the ELISA plate four times with PBST.

12. Add 100 µL α-M13-HRP diluted in PBST according to the manufacturer's instructions (typically 1:1000-5000) to each well, and incubate for 30 min at RT with gentle shaking.

13. Wash the ELISA plate four times with PBST.

14. Add 100 µL TMB substrate (TMB Substrate Kit, Thermo Fisher Scientific) to each well.

15. Stop the reaction after 5–30 min by adding 100 µL2M $H_2SO_4$.

16. Measure absorbance at 450 nm using a microplate reader.

A positive ELISA result should show a high absorbance signal against positive control protein(s) and a relatively high absorbance signal against the target antigen compared to negative control protein(s). Monoclonal phage candidate eliciting a positive ELISA result should be retraced to their original bacterial colonies to send for sequencing. It is not necessary to subcloning unique and ELISA positive affibody binders to other expression vector of choice for further screening and characterization of affinity proteins on the protein level. You just need transform the candidate to a non-amber suppressor strain (such as SS320 phage display cell, Cat# PDCS).