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Isolation of isoform-specific binding proteins (Affimers) by phage display using negative selection

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One-Sentence Summary: XXXXXXX

Abstract

Some thirty years after its discovery, phage display remains one of the most widely used in vitro selection methods. Initially developed to revolutionise the generation of therapeutic antibodies, phage display is now the first choice for screening artificial binding proteins. Artificial binding proteins can be used as reagents to study protein-protein interactions, to target posttranslational modifications, to distinguish between homologous proteins, as research and affinity reagents, for diagnostics, and as therapeutics. However, the ability to identify isoform-specific reagents remains highly challenging. Here we describe an adapted phage display protocol using an artificial binding protein (Affimer) for the selection of isoform-selective binding proteins.

Introduction

There is a critical need for highly selective antibodies in biological research, diagnostics, and therapy. However, there is a widespread lack of reproducibility and selectivity of antibodies and a growing drive to use methods that directly yield recombinant binding reagents that can be sequenced and easily produced in a reproducible manner (1, 2). These include artificial binding proteins such as DARPins (3), monobodies (4), and Affimers (formerly referred to as Adhirons, (5-7), which offer the advantages of recombinant bacterial production as well as the ability to be expressed in mammalian cells to study protein function. The Adhiron scaffold, depicted in Fig. 1, was derived from a consensus sequence of phytocystatins and was designed to constrain two separate variable peptide sequences for molecular recognition (5). It is related in structure to a previously reported scaffold engineered from human Stefin

A (8). Non-antibody binding proteins derived from these two scaffolds are now referred to collectively as Affimers, and the utility of Affimer technology as affinity reagents has been demonstrated (7).

Despite the growing number of methods for isolating protein-binding reagents, such as ribosomal display (9), cis-display (10) and mRNA display (11), phage display remains the most widely used approach. Phage display was developed over thirty years ago (12) and has since been adapted for presenting large libraries of antibody fragments, peptides, and artificial binding proteins. Phage display is performed by mixing highly complex libraries of phage that display the binding moiety with purified target molecules normally presented as immobilised antigens on solid surfaces. This immobilisation is commonly accomplished by direct adsorption to a solid surface by or chemical modifcation of the antigen using biotin linkers to immobilize them on streptavidin-coated surfaces. After three to five panning rounds, monoclonal phage displaying the selected binding reagents are tested to confirm their ability to bind to the target antigen. These clones are sequenced, and the coding regions are sub-cloned into expression vectors. This process typically takes two to three weeks and can yield reagents with binding affinities suitable for many applications.

Here we describe a phage display strategy performed with an Affimer phage display library that uses relatively low amounts of target and non-target homologous antigens, making it suitable for proteins that are expensive or difficult to express (5). This method (Fig. 2) allows selection of highly specific Affimer clones that are able to discriminate between protein isoforms with very similar three-dimensional structures. This method includes a process of negative selection wherein small amounts of the homologous proteins, for which you wish to deselect against, are premixed with the phage library from the second round of panning onward, both as immobilised proteins and as proteins in solution. In theory, this process allows capture or blocking of the cross-reactive Affimer-displaying phage, thereby allowing the highly selective Affimers to be enriched and isolated throughout the subsequent panning

rounds. This protocol could potentially be used for any phage display library when screening to achieve similarly high selectivity and would be particularly useful when developing reagents to to study signalling cascades by blocking protein-protein interactions, such as those mediated by Src homology domains, for which current small molecule inhibitors lack specificity.

Materials

Biotinylation of the Target Protein

Target protein dissolved in an amine-free buffer such as phosphate-buffered saline between 0.5-20 mg/ml

EZ-Link™ NHS-Biotin (Thermo Scientific, Cat. No. 20217)

Dimethyl sulfoxide (DMSO)

Phosphate-buffered saline (PBS) (137 mM NaCl; 2.7 mM KCl; 12 mM Na_2HPO_4; 1.2 mM KH_2PO_4; pH 7.4) or other amine-free buffer at pH 7.0-8.0

Zeba Spin Desalting Columns, 7K MWCO, 0.5 ml (Thermo Scientific, Cat. No. 89882)

80% Glycerol stock (in sterile dH_2O)

ELISA to Check Biotinylation

Nunc-Immuno™ MaxiSorp™ strips (Thermo Scientific, Cat. No. 469949)

Phosphate-buffered saline (PBS)

PBST [PBS with 0.1% (v/v) Tween-20]

10x Casein Blocking Buffer (Sigma, Cat. No. B6429)

2x Blocking Buffer (10x Casein Blocking Buffer diluted in PBST)

Pierce™ High Sensitivity Streptavidin-HRP (Thermo Scientific, Cat. No. 21130)

SeramunBlau® fast TMB/substrate solution (TMB) (Seramun, Cat. No. S-001-TMB)

Affimer Selection by Phage Display

Biotinylated target protein at 4.2 μM

Non-biotinylated protein for deselection of isoform-specific binders (10-100 μM)

E. coli ER2738 cells (Lucigen)

2TY media [1.6% (w/v) tryptone; 1% (w/v) yeast extract; 0.5% (w/v) NaCl]

Tetracycline hydrochloride (1000x stock: 12 mg/ml in 70% ethanol)

Streptavidin-Coated (HBC) 8-well strips (Thermo Scientific, Cat. No. 15501)

0.2M glycine, pH 2.2

1M Tris-HCl, pH 9.1

Triethylamine (Sigma-Aldrich, #T0886)

1M Tris-HCl, pH 7

Carbenicillin (500x stock: 50 mg/ml in ddH_2O)

LB agar plates with 100 μg/ml carbenicillin

M13K07 helper phage (titre ca. 10^{14}/ml) (New England Biolabs)

Kanamycin (500x stock: 25 mg/ml in ddH_2O)

PEG-NaCl precipitation solution [20% (w/v) PEG 8000; 2.5M NaCl]

Tris-EDTA buffer (TE buffer) (10 mM Tris; 1 mM EDTA; pH 8.0)

80% glycerol stock (in sterile dH_2O)

Eppendorf® LoBind microcentrifuge tubes (Eppendorf, Cat. No. 0030 108.116)

Dynabeads™ MyOne™ Streptavidin T1, 10 mg/ml (Thermo Scientific, Cat. No. 65601 or 65602)

Deep well 96 plate (Thermo Scientific, Cat. No. 95040450)

KingFisher (200 μl) 96 plates (Thermo Scientific, Cat. No. 97002540)

NeutrAvidin-Coated (HBC) 8-well strips (Thermo Scientific, Cat. No. 15508)

Halt Protease Inhibitor Cocktail (100X) (Thermo Scientific, Cat. No. 87786)

Equipment

Standard molecular biology equipment, including micropipettes and tips, microcentrifuge tubes, etc.

Microcentrifuge

Microplate strip washer (for example, TECAN HydroFlex™)

Incubator set at 37°C

Vibrating platform shaker (for example, Heidolph VIBRAMAX 100)

Microplate reader for measuring absorbance (for example, Multiskan Ascent)

Shaking incubator set at 37°C (for 50 ml conical centrifuge tubes)

Tube rotator (for example, Stuart SB2 fixed speed rotator)

KingFisher™ Flex magnetic particle processor (ThermoFisher Scientific Cat. No. 5400630)

Recipes

Recipe 1: Phosphate-buffered saline (PBS) and PBST

Prepare 10x stock by dissolving 80 g of NaCl (MW = 58.44), 2.0 g of KCl (MW = 74.55), 17.0 g of Na_2HPO_4 (MW = 141.96), and 1.63 g of KH_2PO_4 (MW = 136.09) in 800 ml of dH_2O. Adjust the pH to 7.4 with HCl, and then add dH_2O to 1 L. Autoclave to sterilise. Store at room temperature.

For a 1x stock of PBS, dilute the 10x stock with dH_2O and adjust to pH 7.4 with HCl.

For PBST, dilute the 10x stock with dH_2O plus 0.1% (v/v) Tween-20 and adjust to pH 7.4 with HCl.

Recipe 2: 2x Blocking Buffer

Dilute 10 x Blocking Buffer (Sigma, Cat. No. B6429) 5-fold in PBS.

Recipe 3: 2TY media Dissolve 16 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 5 g NaCl (MW = 58.44) in 900 ml ofd H_2O. Adjust the pH to 7.0 with NaOH, and then add dH_2O to 1 L. Autoclave to sterilise. Store at room temperature.

Recipe 4: Tetracycline hydrochloride (1000x stock)

Dissolve 12 mg/ml in 70% ethanol and store at -20°C in the dark. Working concentration is 12 µg/ml.

Recipe 5: 0.2 M Glycine, pH 2.2

Dissolve 1.5 g of glycine (MW = 75.07) in 80 ml of dH_2O. Adjust the pH to 2.2 with HCl, and then add H_2O to 100 ml. Autoclave to sterilise. Store at room temperature.

Recipe 6: Triethylamine

Mix 14 µl of triethylamine (Sigma-Aldrich, Cat. No. T0886) with 986 µl PBS immediately before use.

Recipe 7: 1 M Tris-HCl Stocks

Dissolve 121.14 g of Tris base (MW = 121.14) in 800 ml of dH_2O. Adjust pH to the desired value with concentrated HCl at room temperature. The pH of Tris buffers varies with temperature and concentration. Adjust the volume of the solution to 1 L with dH_2O. Autoclave to sterilise. Store at room temperature.

Recipe 8: Carbenicillin (500x stock)

Dissolve 50 mg/ml in ddH_2O. Filter sterilise and store at -20°C. Working concentration is 100 μg/ml.

Recipe 9: LB agar plates with 100 μg/ml carbenicillin

Add 32.0 g of LB agar (Lennox L agar) (ThermoFisher Scientific #22700041) per L of dH_2O. Autoclave to sterilise. Cool to ~50-55°C. Add carbenicillin to a final concentration of 100 μg/ml. Pour into petri dishes and allow to solidify. Store at 4°C for up to 2 months.

Alternatively, the formulation for 1 L of Lennox L agar is as follows: 10 g SELECT Peptone 140, 5 g SELECT Yeast Extract, 5 g Sodium Chloride, 12 g SELECT Agar. Swirl to mix and autoclave to sterilise.

Recipe 10: Kanamycin (500x stock)

Dissolve 25 mg/ml kanamycin in ddH2O. Filter sterilise and store at -20°C. Working concentration is 50 μg/ml.

Recipe 11: PEG-NaCl precipitation solution

Dissolve 200 g PEG 8000 and 146.1 g NaCl (MW = 58.44) in dH_2O to a total volume of 1 L. Autoclave to sterilise. Store at room temperature.

Recipe 12: Tris-EDTA buffer (TE buffer)

Prepare stocks of 1 M Tris-HCl, pH 8.0 (Recipe 5), and 0.5 M EDTA, pH 8.0. To prepare 0.5 M EDTA, pH 8.0, add 93.06 g of EDTA disodium salt (MW = 372.24) to 400 ml of dH_2O. Adjust the pH to 8.0 with NaOH; the disodium salt of EDTA will not dissolve until the pH of the solution is adjusted to pH 8.0. Once dissolved, adjust volume to 500 ml with dH_2O. Autoclave to sterilise. Store at room temperature.

For 1 L of 1x TE Buffer, mix 10 ml of 1M Tris-HCl, pH 8.0 and 2 ml of 0.5 M EDTA, pH 8.0 with 988 ml of ddH_2O. Store at room temperature.

Instructions

Biotinylation of the Target Protein

The selection procedure is performed against a target protein that is biotinylated and captured on streptavidin- or NeutrAvidin-coated surfaces. Biotinylation reagents that utilise various conjugation chemistries are commercially available. We used the EZ-Link™ NHS-Biotin reagent, which reacts with primary amines such as the amino termini of polypeptides or the side chain amine of lysine residues.

Note: When screening against biologically active proteins, ensure that the biotinylation does not affect the protein's activity. See the Troubleshooting *section for alternative methods of biotinylation.*

1. Equilibrate the vial of EZ-Link™ NHS-Biotin to room temperature before opening.
2. Prepare the target protein in an amine-free buffer at 1 mg/ml.
3. Immediately before use, prepare a 10 mM solution of NHS-Biotin in DMSO by dissolving 2.0 mg of NHS-Biotin in 590 µl of DMSO.
4. Add a 10-fold molar excess of NHS-Biotin solution to the target protein. For example, add 0.8 µl of 10 mM NHS-Biotin to 10 µl of a 1 mg/ml 12 kDa protein in a total volume of 100 µl PBS.
5. Incubate at room temperature for 30 min.
6. Desalt to remove any remaining biotin using Zeba Spin Desalting Columns, 7K MWCO according to the manufacturer's instructions.
7. Mix with an equal volume of 80% glycerol and store at -20°C.

ELISA to check biotinylation

1. Aliquot 50 µl per well of PBS to Nunc-Immuno™ MaxiSorp™ strips, and add 1 µl of biotinylated protein to each well. Incubate overnight at 4°C.
2. Wash 3x with 300 µl PBST per well with a microplate strip washer (e.g. TECAN HydroFlex™).
3. Add 200 µl 2x Blocking Buffer per well and incubate at 37°C for 2-3 hours.
4. Wash 3x with 300 µl PBST per well on a microplate strip washer.
5. Dilute High Sensitivity Streptavidin-HRP 1:1000 in 2x Blocking Buffer and add 50 µl per well.
6. Incubate for 1 hour at room temperature on a vibrating platform shaker, such as a Heidolph VIBRAMAX 100 set to 300 rpm.
7. Wash 6x with 300 µl PBST per well on a microplate strip washer.
8. Aliquot 50 µl TMB per well and allow to develop. Note the amount of time the plate is allowed to develop; usually, 2-3 minutes is sufficient.
9. Measure absorbance at 620 nm on a microplate reader.

Affimer Selection by Phage Display – First Panning Round

DAY 1

1. Pick a colony of ER2738 *E. coli* cells into 5 ml 2TY media plus 12 µg/ml tetracycline and incubate overnight in an orbital incubator at 37°C, 230 rpm.
2. Aliquot 300 µl 2x Blocking Buffer per well into Streptavidin-coated (HBC) 8-well strips and incubate overnight at 37°C. Set up 4 wells in total for each target (3 wells for pre-panning the phage and 1 well for binding the target and panning with phage).

DAY 2

3. Wash 3x with 300 µl PBST per well on a microplate strip washer.
4. Aliquot 100 µl 2x Blocking Buffer per well into all wells.

5. Pre-pan the phage: To the first pre-pan well add 5 µl of phage library (approximately 10^{12} cfu). Mix and incubate on a vibrating platform shaker for 40 min. Remove buffer from the 2nd pre-pan well and transfer the buffer containing the phage from the first pre-pan well to the 2nd pre-pan well. Incubate for 40 min, and then repeat for the 3rd pre-pan well.
6. Whilst pre-panning the phage: Add 20 µl (up to 1 µg) of the biotinylated target to the wells to be used for panning. Incubate for 1-2 hours at room temperature on the vibrating platform shaker.
7. Wash the wells containing the target 3x with 300 µl PBST per well.
8. Transfer the phage from the pre-pan wells to the wells containing the target and incubate for 2 hours at room temperature on the vibrating platform shaker).
9. In the meantime, set up a fresh culture of ER2738 cells (8 ml per target) by diluting the overnight culture to an OD600 of ~0.2 and incubating for approximately 1 hour at 37°C, 230 rpm until OD600 reaches ~0.6.
10. Wash the panning well 27x in 300 µl PBST per well on the microplate strip washer.
11. Elute the phage by adding 100 µl of 0.2 M glycine, pH 2.2, and incubating for 10 min at room temperature.
12. Neutralise by adding 15 µl of 1 M Tris-HCl, pH 9.1. Mix and add immediately to an 8 ml aliquot of the ER2738 cells in a 50 ml falcon tube.
13. Dilute 14 µl of Triethylamine with 986 µl of PBS.
14. Elute any remaining phage by adding 100 µl of the diluted Triethylamine and incubating for 6 min at room temperature.
15. Neutralise by adding 50 µl of 1 M Tris-HCl, pH 7. Mix and add immediately to the ER2738 cells.
16. Incubate the cells for 1 hour at 37°C (no shaking or shake at low speed, 90 rpm maximum). Mix at least once during the incubation period.

17. Plate 1 µl of the phage-infected ER2738 cells onto LB-carb plates (LB agar plates containing 100 µg/ml carbenicillin) – incubate overnight at 37°C.
18. Centrifuge the remaining cells at 3,000 x g for 5 min and resuspend in a smaller volume and plate onto LB carb plates – incubate overnight at 37°C.

DAY 3

19. Count the colonies on the plates containing 1 µl of cells to estimate the phage titre – multiply by 8,000 to determine the total number per 8 ml of cells (usually between 0.1 – $2x10^6$).
20. Scrape the cells from the remaining plates. To do this, add 5 ml of 2TY carb (2TY media containing 100 µg/ml carbenicillin) to the plate, scrape using a disposable plastic spreader, transfer to a 50 ml falcon tube and mix. Add an additional 2 ml of 2TY carb to scrape off any remaining cells.
21. Measure the absorbance at 600 nm of a 1:10 dilution to determine the dilution required for an 8 ml culture at an OD600 of 0.2.
22. Dilute the cells in 2TY carb in 50 ml falcon tubes to achieve an OD600 of 0.2.
23. Incubate at 37°C, 230 rpm, approximately 1 hour until an OD600 of 0.6.
24. Add 3.2 µl of M13K07 helper phage (titre ca. 10^{13}/ml, MOI ~30) and incubate at 37°C, 90 rpm, for 30 min.
25. Add 16 µl of kanamycin (25 mg/ml) and incubate overnight in an orbital incubator at 25°C, 170 rpm.

DAY 4

26. Centrifuge the phage-infected cultures at 3,500 x g for 10 min.
27. Transfer the phage-containing supernatant to fresh tubes.
28. Remove the required volume of phage-containing supernatant for the second panning round (see below).
29. Add 2 ml of PEG-NaCl precipitation solution (20% (w/v) PEG 8000, 2.5 M NaCl) to the remaining supernatant. Incubate for at least 1 hour or overnight at 4°C.

30. Centrifuge at 4,800 x g for 30 min to pellet the phage.
31. Pour off the supernatant (blotting the tube on tissue paper to remove all of the supernatant) and resuspend the pellet in 320 µl of TE.
32. Transfer to microcentrifuge tubes and centrifuge at 16,000 x g for 10 min. The supernatant contains the phage. Phage can be stored for several weeks at 4°C. For long-term storage, add an equal volume of 80% glycerol, mix thoroughly, and store at -80°C.

Affimer Selection by Phage Display – Second Panning Round

DAY 1

1. Resuspend the Dynabeads™ MyOne™ Streptavidin T1 magnetic beads in the vial (vortex) and transfer 20 µl per target into Eppendorf® LoBind microcentrifuge tubes.
2. Wash the Streptavidin beads by resuspending in 500 µl PBST. Place the tube on a magnetic separation rack and discard the supernatant.
3. Resuspend the Streptavidin beads in 300 µl of 2x Blocking Buffer and incubate overnight at room temperature on a tube rotator.

DAY 2

4. Pre-block plates for the KingFisher™ Flex magnetic particle processor for a minimum of 2 hours at 37°C:
    a. Pre-block one well per target in a deep well 96 plate with 1 ml per well of 2x Blocking Buffer. These wells will be used for panning.
    b. Pre-block one well per target in a KingFisher (200 µl) 96 plates with 300 µl per well of 2x Blocking Buffer. These wells will be used to recover the Streptavidin beads containing the bound target and selected phage.

5. Prepare one well per target in 4x deep well 96 plates with 950 µl 2x Blocking Buffer per well of. These wells will be used for the wash steps in the KingFisher protocol.
6. Centrifuge the pre-blocked Streptavidin beads at 800 x g for 1 min. Place the tube on the magnetic separation rack and discard the blocking buffer.
7. Replace with fresh 2x Blocking Buffer, resuspending in 100 µl per 20 µl of Streptavidin beads.
8. Pre-pan the phage: Mix 125 µl of phage-containing supernatant from the first panning round with 125 µl of 2x Blocking Buffer and add 25 µl of the pre-blocked Streptavidin beads. For the negative selection, add the non-biotinylated homologous protein at a concentration of 10-100 µM. Incubate for 1 hour at room temperature on the rotator.
9. Centrifuge at 800 x g for 1 min and place the tube on the magnetic separation rack. Transfer the supernatant containing the phage to a fresh tube and add another 25 µl of the pre-blocked Streptavidin beads. Incubate for 1 hour at room temperature on the tube rotator.
10. Bind the target to the Streptavidin beads: Add 15 µl (up to 750 ng) of biotinylated target to 200 µl of 2x Blocking Buffer and 50 µl of the pre-blocked Streptavidin beads. Incubate for 1 hour at room temperature on the tube rotator.
11. Remove buffer from the pre-blocked deep well 96 plate (step 4a) and the pre-blocked KingFisher (200 µl) 96 plate (step 4b). Aliquot 100 µl per well of PBS into the pre-blocked KingFisher (200 µl) 96 plate.
12. Centrifuge the tubes containing the biotinylated target at 800 *xg* for 1 min and place on the magnetic separation rack.
13. Wash the beads containing the biotinylated target 3 times in 500 µl of 2x Blocking Buffer.
14. Centrifuge the tubes containing the pre-panned phage at 800 x g for 1 min and place on the magnetic separation rack for 1 min.

15. Transfer the supernatant containing the pre-panned phage to the beads containing the biotinylated target and resuspend. Transfer to the pre-blocked deep well 96 plate (step 4a).
16. Setup the KingFisher Flex to run the protocol "Phage_Display_Competition" (see Table 1). The total run time is 1 hour 30 min.
17. The protocol will release the beads into the 100 µl PBS. Transfer the beads into Eppendorf® LoBind microcentrifuge tubes and add the following:

60 µl	10x Blocking Buffer
60 µl	80% Glycerol
3 µl	Halt Protease Inhibitor Cocktail (100X)
2.5 µg	Non-biotinylated protein for deselection
	PBS to a total volume of 300 µl

16. Incubate at room temperature on the tube rotator up to 24 hours.
17. Pick a colony of ER2738 *E.coli* cells into 5 ml of 2TY media with 12 µg/ml tetracycline and incubate overnight at 37°C, 230 rpm.

DAY 3

18. Pre-block plates for the KingFisher Flex magnetic particle processor for a minimum of 2 hours at 37°C:
    a. Pre-block one well per target in a deep well 96 plate with 1 ml per well of 2x Blocking Buffer. The competitively panned samples will be returned to this plate for washing and elution.
    b. Pre-block one well per target two KingFisher (200 µl) 96 plates with 300 µl per well of 2x Blocking Buffer. One plate will be used for eluting with glycine, the other for eluting with Triethylamine.

19. Prepare one well per target in a 4x deep well 96 plates with 950 µl per well of 2x Blocking Buffer. This plate will be used for the wash steps in the KingFisher protocol.
20. Remove buffer from the pre-blocked elution plates (step 18b). Aliquot 100 µl per well of 0.2M glycine, pH 2.2, into one plate. Aliquot 100 µl per well Triethylamine into the other plate. Remove buffer from the pre-blocked deep well 96 plate (step 18a).
21. An hour before required, set up a fresh culture of ER2738 cells (8 ml per target) by diluting the overnight culture from step 17 to an OD600 of ~0.2 and incubating for approximately 1 hour at 37°C, 230 rpm until OD600 reaches ~0.6.
22. After 24 hours of competitive binding, centrifuge the samples at 800 x g for 30 sec, resuspend, and transfer to the pre-blocked deep well 96 plate.
23. Set up the KingFisher Flex to run the protocol "Phage_Display_Wash_Elute" (see Table 2).
24. The protocol is set up to incubate the beads in glycine for 10 min to elute the phage. As soon as this is finished, neutralise by adding 15 µl of 1 M Tris-HCl, pH 9.1. Mix and add to the 8 ml aliquots of ER2738 cells.
25. The beads are transferred into triethylamine for 6 min to elute any remaining phage. As soon as this is finished, neutralise by adding 50 µl of 1 M Tris-HCl, pH 7.0. Mix and add to the ER2738 cells.
26. Incubate the cells for 1 hour at 37°C (no shaking or shake at low speed, 90 rpm maximum). Mix at least once during the incubation period.
27. Centrifuge the cells at 3,000 x g for 5 min to resuspend in a smaller volume and plate onto LB carb plates. Incubate overnight at 37°C.

DAY 4

28. Prepare phage as described for the first panning round, Affimer Selection by Phage Display – First Panning Round steps 20-32.

Table 1: KingFisher Flex Protocol "Phage_Display_Competition"

Protocol Step	**Plate**	**Volume (µl)**	**Settings**
Tipcomb	96 DW tip comb		
Pick-Up: Tipcomb	KingFisher 96 KF plate		
Collect Beads	Plate: Binding Microtiter DW 96 plate		Collect count 1 Collect time (s) 1
Binding	Plate: Binding Microtiter DW 96 plate	300	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:00:10 Speed: fast Mix time [hh:mm:ss]: 01:00:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 1	Plate: Wash 1 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 2	Plate: Wash 2 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step**

			Collect beads, count: 5 Collect time (s): 30
Wash 3	Plate: Wash 3 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 4	Plate: Wash 4 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Particle Release	Plate: pH elution KingFisher 96 KF plate	100	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:10 Speed: Fast
Leave: Tipcomb	96 DW tip comb		

Table 2: KingFisher Flex Protocol "Phage_Display_Wash_Elute"

Protocol Step	**Plate**	**Volume (µl)**	**Settings**
Tipcomb	96 DW tip comb		
Pick-Up: Tipcomb	KingFisher 96 KF plate		
Collect Beads	Plate: Binding Microtiter DW 96 plate		Collect count 1 Collect time (s) 1
Binding	Plate: Binding Microtiter DW 96 plate	300	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00

			Mixing/Heating Parameters Mix time [hh:mm:ss]: 00:00:10 Speed: fast **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 1	Plate: Wash 1 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 2	Plate: Wash 2 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 3	Plate: Wash 3 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
Wash 4	Plate: Wash 4 Microtiter DW 96 plate	950	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters**

			Mix time [hh:mm:ss]: 00:01:00 Speed: slow **End of step** Collect beads, count: 5 Collect time (s): 30
pH Elution	Plate: pH elution KingFisher 96 KF plate	100	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:07:30 Speed: slow Postmix[hh:mm:ss]: 00:00:05 Speed: Bottom mix **End of step** Collect beads, count: 5 Collect time (s): 30
Triethylamine Elution	Plate: Triethylamine KingFisher 96 KF plate	100	**Beginning of Step** Release beads [hh:mm:ss]: 00:00:00 **Mixing/Heating Parameters** Mix time [hh:mm:ss]: 00:03:30 Speed: slow Postmix[hh:mm:ss]: 00:00:05 Speed: Bottom mix **End of step** Collect beads, count: 5 Collect time (s): 30
Leave: Tipcomb	96 DW tip comb		

Affimer Selection by Phage Display – Third Panning Round

DAY 1

1. Aliquot 300 µl per well of 2x Blocking Buffer into NeutrAvidin-coated (HBC) 8-well strips and incubate overnight at 37°C. Set up 6 wells in total for each target (4 wells for pre-panning the phage, one for panning against the target, and a negative control for panning against the deselected protein).

DAY 2

2. Wash 3x with 300 µl PBST per well on a microplate strip washer.
3. Aliquot 200 µl 2x Blocking Buffer per well to the wells to be used for panning and 100 µl 2x Blocking Buffer per well to the wells to be used for pre-panning. To the pre-panning wells, add 0.1-1.0 µM of the biotinylated homologous protein. Incubate for 1 hour at room temperature on a vibrating platform shaker.
4. Pre-pan the phage: Wash the first pre-pan well 3x with 250 µl of PBST (manually, using a pipette). Add 20 µl of 10x Blocking Buffer and 200 µl of phage-containing supernatant from the 2nd panning round. Incubate for 1 hour at room temperature on the vibrating platform shaker
5. Wash the second pre-pan well 3x with 250 µl of PBST and transfer the contents of the first pre-panning well to the second pre-panning well. Incubate for 1 hour and repeat for the third and fourth pre-panning wells.
6. Remove buffer from the wells to be used for panning against the target and negative control. Aliquot 100 µl per well of 2x Blocking Buffer and add 10 µl (500 ng) of the biotinylated target or deselected protein. Incubate for 1 hour at room temperature on the vibrating platform shaker.
7. Wash the wells containing the target and the negative control 3x in PBST.
8. Transfer 100 µl per well of phage from the pre-pan wells to the wells containing the target and the negative control. Incubate for 45 min at room temperature on the vibrating platform shaker.
10. Wash the panning wells 27x with 300 µl per well of PBST on the microplate strip washer.

11. Add the following: 80 μl of 2x Blocking Buffer; 20 μl of 80% glycerol; 1 μl Halt Protease Inhibitor Cocktail (100X); 2.5 – 5 μg of the non-biotinylated protein for deselection.
12. Incubate for 24 h at room temperature on the vibrating platform shaker.
13. Pick a colony of ER2738 *E.coli* cells into 5 ml of 2TY media with 12 μg/ml tetracycline and incubate overnight at 37°C, 230 rpm.

DAY 3

14. Setup a fresh culture of ER2738 cells (5 ml per target and 5 ml per negative control) by diluting the overnight culture to an OD600 of ~0.2 and incubating for approximately 1 hour at 37°C, 230 rpm until OD600 reaches ~0.6.
15. Wash the panning wells 27x with 300 μl per well of PBST on the microplate strip washer.
16. Elute the phage by adding 100 μl of 0.2 M glycine, pH 2.2, and incubating for 10 min at room temperature.
17. Neutralise by adding 15 μl of 1 M Tris-HCl, pH 9.1. Mix and add immediately to a 5 ml aliquot of the ER2738 cells in a 50 ml falcon tube.
18. Elute any remaining phage by adding 100 μl of the diluted triethylamine and incubating for 6 min at room temperature.
19. Neutralise by adding 50 μl of 1 M Tris-HCl, pH 7.0. Mix and add immediately to the ER2738 cells.
20. Incubate for 1 hour at 37°C (no shaking or shake at low speed,.90 rpm maximum). Mix at least once during the incubation.
21. Plate a range of volumes (for example 0.01, 0.1, 1, 10 and 100 μl) onto LB carb plates. Also centrifuge and plate remaining cells as described for the first panning round (Affimer Selection by Phage Display – First Panning Round, step 18). For the negative controls, just select one volume to plate (usually 10 μl). Incubate overnight at 37°C.

Troubleshooting

Biotinylation Blocks the Active Site of the Target Protein

The biotinylation reagent (EZ-Link™ NHS-Biotin) reacts with primary amines such as the amino termini of polypeptides or the amine of the side chain of lysine residues. Therefore, if there are lysine residues present in a functional region of a target protein, biotinylation of these residues could block access for binding in the selection process. Other biotinylation reagents that target different functional groups or residues are available. For example, the EZ-Link™ HPDP-Biotin reagent (Thermo Scientific, Cat. No. 21341) reacts with sulfhydryls, such as the side-chain of cysteine residues.

An alternative to chemical conjugation methods of biotinylation is enzymatic biotinylation. This is most often achieved by fusing the protein of interest with a Biotin Acceptor Peptide (BAP). The 15 amino acid peptide tag (GLNDIFEAQKIEWHE) serves as a highly specific recognition site for *E. coli* biotin ligase (BirA), which covalently attaches a single biotin molecule. Enzymatic biotinylation can be carried out in vitro but can also be achieved in vivo in the appropriate expression strains of *E. coli.*

The Selection Procedure is Too Stringent

Occasionally, our negative selection method has failed to select binders. This may happen when sequence homology between the two isoforms is especially high. This can result in the selection of weaker binders, which may be lost during the stringent deselection process. To decrease stringency, the 24 h incubation step during the second and third panning round can be removed and the phage eluted immediately after washing.

Notes and Remarks

A *Nature Protocols* paper described an in-depth protocol for a related technique, the selection of human antibody fragments by phage display (13). This method could of course be adapted for other phage display libraries, including the Affimer library; however this method does not describe a strategy to deselect against homologous proteins. Furthermore, our protocol uses lower amounts of target in each round of selection, which is advantageous for expensive or poorly expressed targets.

Our method was originally developed to select isoform-specific inhibitors of SUMO-dependent protein-protein interactions (REF). Gilbreth *et al.* (14) described an alternative method, also utilising phage display for selection, and were successful in isolating isoform-specific monobody inhibitors of yeast SUMO (ySUMO) and the human SUMO-1 isoform (hSUMO-1). The authors expressed the targets (ySUMO, hSUMO-1 and hSUMO-2) as C-terminal fusions to a GST variant engineered to be devoid of cysteine residues except for a single cysteine for the purpose of biotinylation using the EZ-Link™ HPDP-Biotin reagent. Monobody-displaying phage particles were pre-incubated with non-biotinylated GST from round 2 of selection to remove binders to GST. They did not employ this method of deselection to remove binders against the different isoforms of SUMO. They first identified monobody binders to ySUMO, then using crystal structure analysis of the binding mechanism, designed a SUMO-targeted monobody library that was independently screened against the different isoforms. Whilst this method isolated specific binders to hSUMO-1, it failed to isolate hSUMO-2–specific binders.

Our method has been successful in generating specific binders to numerous proteins and protein domains. The advantages of using the deselection method described here are that it requires only small amounts of target proteins, the process is relatively quick, and selection can be done from a large naïve randomised library. The stringency can also be adjusted by altering incubation times and wash steps.

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Figure Legends

Fig. 1. The Adhiron scaffold as determined by X-ray crystallography at 2.25 Å (PDB: 4N6T). Highlighted in dark blue are the interaction loop residues (VVAG in loop 1 and PWE in loop 2) that were replaced with nine randomized amino acids (excluding cysteine residues) per loop in the library design (5).

Fig. 2. Affimer phage display selection conducted over 3 panning rounds. In each round of panning, the immobilised target (red dots) is incubated with a population of phage (green bars). After washing away any unbound phage, bound phage is eluted and propagated for subsequent rounds of selection. From Pan 2 onwards, the population of phage is first incubated with proteins homologous to the target protein to remove any phage that bind to these closely related homologs, before incubating with the intended target.