Chapter 23

Selection of Human VH Single Domains with Improved Biophysical Properties by Phage Display

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Abstract

Human antibody variable heavy (VH) domains tend to display poor biophysical properties when expressed in isolation. Consequently, the domains are often characterized by low expression levels, high levels of aggregation, and increased "stickiness." Here, we describe methods that allow the engineering of human VH domains with improved biophysical properties by phage display. The engineered domains withstand challenging conditions, such as high temperature and acidic pH. Engineered human single domains are a promising new class of antibody fragments and represent robust research tools and building blocks for the generation of antibody therapeutics.

Key words: Human VH domains, Aggregation, Phage display

1. Introduction

Human heavy chain variable (VH) domains tend to have poor biophysical properties when expressed in the absence of light chain partners (1–3). This includes low expression in bacteria and they readily aggregate upon concentration. They also generally do not unfold reversibly upon heating and have an unfortunate tendency to "stick" to gel-filtration matrices. These features are in marked contrast to single domains from camels and llamas (4) and initially prevented the wider use of human domains as research tools and therapeutics.

More recently, we have described phage display methods for the selection of aggregation-resistant human VH domains. These fully human domains display favorable properties in the absence of any "camelising" mutations. For this purpose, we have utilized geneIII display on filamentous bacteriophage using a multivalent system (2, 5, 6). A key aspect of the method is based on the fact that the phage particle is surprisingly resistant to physical and

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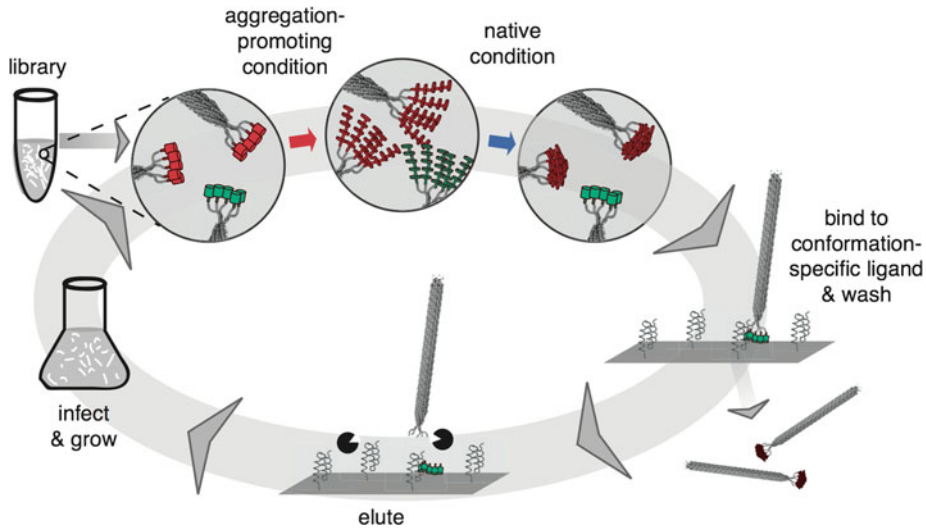

Fig. 1. Selection of VH domains with improved biophysical properties by phage display. Human VH domains are displayed on filamentous bacteriophage in a multivalent format. The domains are first exposed to aggregation-promoting conditions (heat or acid), followed by return to native conditions (room temperature, neutral pH). Human VH domains on phage that resist aggregation are then captured using a conformation-specific ligand (antigen or superantigen). After wash and elution steps, phages are amplified by infection of *E. coli* bacteria.

chemical challenges (such as temperatures exceeding 80°C and low pH) (7, 8). This allows the exposure of the displayed proteins to highly aggregation-promoting conditions, without affecting viability of the phage particle. A second feature of the method relates to the use of conformation-specific superantigens, such as protein A from *Staphylococcus aureus*. Protein A can be used as a probe for folded domains, as it selectively binds to a conformational epitope on folded VH, but does not bind to unfolded or aggregated domains (9). Alternatively, antigen binding can also be used as a selective pressure (2), particularly for VH domains that do not bind to protein A (e.g., domains other than VH3).

The selection process is outlined in Fig. 1 and essentially follows the established phage display selection cycle (10). For selection, a library of human VH domains is displayed on phage in a multivalent phage format. This format (rather than phagemid) is essential to achieve high local concentration of the VH domains on the tip of the bacteriophage. This can be achieved by cloning of VH genes from phagemid libraries into a multivalent phage vector. As the generation of libraries in phage vectors requires high quality DNA preparations, we provide a protocol for the in vitro amplification of phage DNA using Phi29 polymerase (11).

As a source of the domains, single framework synthetic human antibody libraries can be utilized (such as Tomlinson I/J or ETH-2) (12, 13). From these libraries, VH genes can be amplified either from the naïve repertoires or from antigen selections. Alternatively, additional diversity can be introduced through PCR

or oligonucleotide-based mutagenesis methods. Once a repertoire of VH domains on phage has been generated, the displayed domains are exposed to aggregation-promoting conditions. Such conditions include temperatures significantly above the T_m of the domains (such as 80°C), or alternatively acidic conditions (such as pH 3) (14). Human VH domains that resist aggregation are next captured by binding to a conformation-specific ligand (such as antigen or superantigen). Finally, the selected domains are produced in a periplasmic expression vector and their aggregation propensity analyzed by size exclusion chromatography.

2. Materials

2.1. Amplification of VH genes by PCR

1. FdMyc phage vector (derived from FdTet (15), with a *c-Myc* tag introduced between the *Not*I site and geneIII).
2. Thermocycler (BioRad, Foster City, CA, USA).
3. Thermostable DNA polymerase and reaction buffers (e.g., Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany)).
4. dNTPs: deoxynucleoside triphosphates (New England Biolabs, Ipswich MA, USA).
5. 2% Agarose gel: dissolve agarose in TBE buffer (2 g per 100 mL), melt agarose in microwave, and add SYBRSafe (Invitrogen, Carlsbad, CA, USA) or Ethidium Bromide to appropriate working concentration.
6. QIAprep Spin miniprep kit (Qiagen, Hilden, Germany).
7. QIAquick PCR purification kit (Qiagen, Hilden, Germany).
8. Primers used in cloning of VH genes into FdMyc phage vector (restriction sites are shown in italic). These will amplify VH3 domains such as present in the Tomlinson I/J or ETH-2 libraries; the use of other VH domains may require changes to the sequences shown in bold:
   (a) ApaLI_VHFwd: 5′-ACGC *GTGCAC* **AGGTGCA GCTGTTGG**-3′ (anneals in the 5′- region of VH genes and introduces *ApaL*I site).
   (b) VHNotI_Rev: 5′-CTGTTA *GCGGCCGC* **GCTCGAG ACGGTGACCAG**-3′ (anneals in the 3′-region of VH genes and introduces *Not*I restriction site).
9. Restriction enzymes *ApaL*I, *Not*I, and restriction enzyme buffers (New England Biolabs, Ipswich MA, USA).
10. Agarose for gel electrophoresis (Lonza, Basel, Switzerland).
11. TBE buffer: 10.6 g/L Tris base, 5.5 g/L boric acid, 4 mL/L of 0.5 M EDTA, pH 8.0.

12. 100 bp ladder DNA length marker (New England Biolabs, Ipswich MA, USA).
13. PCR purification kit, Plasmid miniprep kit (Qiagen, Hilden, Germany).
14. GoTaq polymerase (Promega, Fitchburg, WI, USA).
15. Water: use sterile, highly pure water such as autoclaved MilliQ (Millipore, Billerica, MA, USA).

2.2. Cloning of VH Genes into FdMyc Phage Display Vector

1. *Phi*29 DNA polymerase and 10× *Phi*29 Buffer (New England Biolabs, Ipswich, MA, USA).
2. Random hexamers (50 μM stock) (Applied Biosystems, Carlsbad, CA, USA).
3. BSA (100×) (New England Biolabs, Ipswich MA, USA).
4. 2× TY medium: 16 g/L bacto-tryptone, 10 g/L yeast extract, 5 g/L NaCl.
5. Amicon Ultra filter unit (10 kDa MWCO) (Millipore, Billerica, MA, USA).
6. Restriction enzymes *ApaL*I, *Not*I, and *Dpn*I and restriction enzyme buffers (New England Biolabs, Ipswich, MA, USA).
7. Sephacryl S-1000 resin (GE Healthcare, Little Chalfont, UK).
8. FLPC system, e.g., AKTA purifier (GE Healthcare, Little Chalfont, UK).
9. T4 DNA ligase and buffer (New England Biolabs, Ipswich, MA, USA).
10. *Escherichia coli* TG1 (Agilent, Santa Clara, CA, USA).
11. Colony PCR buffer: 2% of formamide, 1× *Taq* buffer, 200 μM dNTPs, 400 nM μL of each forward (FdMycFwd4) and reverse (G3Seq6) primers, 1.25 units of GoTaq polymerase.
12. FdMyc sequencing primers:
    (a) FdMycFwd4: 5′-AAATTCACCTCGAAAGCAAGC-3′.
    (b) G3seq6: 5′-CCCTCATAGTTAGCGTAACGA-3′.
13. Agarose for gel electrophoresis (Lonza, Basel, Switzerland).
14. TBE buffer: 10.6 g/L Tris base, 5.5 g/L boric acid, 4 mL/L of 0.5 M EDTA, pH 8.0.
15. 100 bp ladder DNA mass markers (New England Biolabs, Ipswich MA, USA).
16. PCR purification kit, Plasmid miniprep kit (Qiagen, Hilden, Germany).
17. GoTaq polymerase (Promega, Fitchburg, WI, USA).
18. 10 mM Tris–HCl, pH 8.5: 1.21 g/L Tris base, pH 8.5 using 5 M HCl.

2.3. Phage Production and Purification

1. *E. coli* TG1 (Agilent, Santa Clara, CA, USA).
2. TYE/tet agar (15 g/L agarose, 8 g/L NaCl, 10 g/L Bacto-tryptone, 5 g/L Yeast extract (Oxoid, Hampshire, UK) MilliQ water up to 1 L, autoclave), supplemented with 15 μg/mL tetracycline.
3. 2× TY/tet medium: 2× TY medium supplemented with 15 μg/mL tetracycline.
4. 0.45 μm vacuum filter unit (Corning, Midland, MI, USA).
5. 20% polyethylene glycol (PEG 6.000), 2.5 M NaCl.
6. PBS buffer: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 KH_2PO_4, pH 7.4.

2.4. Selection of Heat-Refoldable VH Domains on Phage

1. 96-well MaxiSorp Immunoplate (Nunc, Roskilde, Denmark).
2. Recombinant protein A (Sigma, St Louis, MO, USA).
3. Carbonate buffer: 100 mM $NaHCO_3$, 30 mm Na_2CO_3, pH 9.6.
4. PBST: 0.1% Tween-20 diluted in PBS.
5. 4% MPBS: 4 g/L skim-milk powder diluted in PBS.
6. 2% MPBS: 2 g/L skim-milk powder diluted in PBS.
7. Trypsin (Sigma, St Louis, MO, USA).
8. 2× TY medium.
9. TYE/tet agar: TYE agar supplemented with 15 μg/mL tetracycline.
10. *E. coli* TG1 (Agilent, Santa Clara, CA, USA).

2.5. Selection of Acid-Resistant VH Domains on Phage

1. 96-well MaxiSorp Immunoplate (Nunc, Roskilde, Denmark).
2. Recombinant protein A (Sigma, St Louis, MO, USA).
3. Carbonate buffer: 100 mM $NaHCO_3$, 30 mm Na_2CO_3, pH 9.6.
4. PBST: 0.1% (v/v) Tween-20 diluted in PBS.
5. 4% MPBS.
6. 2% MPBS.
7. HN7: 10 mM *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 10 mM NaCl, pH 7.0.
8. 200 mM Citrate, pH 3.0.
9. 1 M Tris, pH 7.4.
10. Trypsin (Sigma, St Louis, MO, USA).
11. 2× TY medium.
12. TYE/tet agar.
13. *E. coli* TG1 (Agilent, Santa Clara, CA, USA).

2.6. Analysis of Aggregation-Resistance by Phage ELISA

1. Shaking incubator at 30°C.
2. 1.5 mL tubes (Eppendorf, Hamburg, Germany).
3. 2× TY medium.
4. 2× TY/tet medium.
5. 96-well MaxiSorp Immunoplate (Nunc, Roskilde, Denmark).
6. NHS-PEO_4-biotin (Pierce, Rockford, IL, USA).
7. PBS buffer.
8. PBST.
9. 4% MPBS.
10. 1 M Tris–HCl, pH 7.5.
11. 3 M Tris, pH 7.5.
12. 2 M Glycine-HCl, pH 2.5.
13. 2% BSA in PBS: 2 g/L bovine serum albumin (BSA)(Sigma, St Louis, MO, USA) dissolved in PBS buffer.
14. Thermocycler (BioRad, Foster City, CA, USA).
15. PCR strip tubes with strip caps (Axygen, Union City CA, USA).
16. Extravidin-HRP (Sigma, St Louis, MO, USA).
17. TMB substrate solution (BD Biosciences, San Diego CA, USA).
18. 1 M H_2SO_4.
19. ELISA plate washer and plate reader.
20. Platform shaker.

2.7. Cloning of VH Genes into Periplasmic Expression Vector

1. Primers used in cloning of VH genes into pET12a expression vector (restriction sites in italic). These will amplify VH3 domains such as present in the Tomlinson I/J or ETH-2 libraries; the use of other VH domains may require changes to the sequences shown in bold:
   (a) VH_SalIFwd: 5′-ACGC *GTCGAC* **AGTCGACGCA GGTGCAGCTGTTGG**-3′ (anneals in the 5′-region of VH gene and introduces *Sal*I restriction site).
   (b) VH_BamHIRev: 5′-CTGTTA *GGATCC* **GCTCGAGA CGGTGACCAG**-3′ (anneals in the 3′-region of VH gene and introduces *Bam*HI restriction site).
2. Colony PCR buffer: 2% of formamide, 1× *Taq* buffer, 200 μM dNTPs, 400 nM μl of each forward (T7Pro) and reverse (T7Term) primers, 1.25 units of GoTaq polymerase.
3. pET12a sequencing primers:
   (a) T7 Pro: 5′- TAATACGACTCACTATAGG-3′ (anneals approximately 150 bp upstream of insert in pET12a).
   (b) T7 Term: 5′- GCTAGTTATTGCTCAGCGG-3′ (anneals approximately 150 bp downstream of insert in pET12a).

4. Expand High Fidelity PCR kit (Roche Applied Science, Mannheim, Germany).
5. dNTPs (New England Biolabs, Ipswich, MA, USA).
6. pET12a expression vector (Novagen, Gibbstown, NJ, USA).
7. Restriction enzymes *Sal*I, *Bam*HI, and buffers; T4 ligase and buffer (New England Biolabs, Ipswich MA, USA).
8. TYE agar.
9. TYE/amp/tet/glu agar: TYE agar containing 100 μg/mL ampicillin, 15 μg/mL tetracycline, and 4% glucose.
10. *E. coli* BL21Gold (Agilent, Santa Clara, CA, USA).
11. Agarose for gel electrophoresis (Lonza, Basel, Switzerland).
12. TBE buffer.
13. 100 bp ladder DNA length marker (New England Biolabs, Ipswich, MA, USA).
14. PCR purification kit, Plasmid miniprep kit (Qiagen, Hilden, Germany).
15. GoTaq polymerase (Promega, Fitchburg, WI, USA).

2.8. Expression and Purification of Soluble VH Domains

1. *E. coli* BL21Gold (Agilent).
2. TYE/amp/tet/glu agar: TYE agar supplemented with 100 μg/mL ampicillin, 15 μg/mL tetracycline, 4% (v/v) glucose.
3. 2× TY/amp/tet/glu medium: 2× TY medium supplemented with 100 μg/mL ampicillin, 15 μg/mL tetracycline, 4% (v/v) glucose.
4. Sorvall centrifuge and centrifuge bottles (500 mM).
5. IPTG: Isopropyl β-D-1-thiogalactopyranoside (Gold Biotechnology, St. Louis MO, USA).
6. Disposable cuvettes (Eppendorf, Hamburg, Germany).
7. Spectrophotometer (Eppendorf, Hamburg, Germany).
8. 2.5 L baffled flasks.
9. rProtein A sepharose fast flow (GE Healthcare, Little Chalfont, UK).
10. Gravity flow column (BioRad, Foster City, CA, USA).
11. 0.1 M Glycine-HCl, pH 3.0.
12. Amicon Ultra centrifugal filter unit (10 kDa MWCO) (Millipore).
13. PBS buffer.
14. 0.45 μM vacuum filter unit (Corning, Lowell, MA, USA).
15. Periplasmic preparation buffer 1: 100 mM Tris–HCl, pH 8.0, 1 mM EDTA, 20% (w/v) sucrose.
16. Periplasmic preparation buffer 2: 5 mM $MgSO_4$.

17. Complete Mini EDTA free protease inhibitor (Roche Applied Science, Mannheim, Germany).
18. SnakeSkin dialysis tubing (Pierce, Rockford, IL, USA).

2.9. Characterization of Aggregation-Resistant VH Domains by Size Exclusion Chromatography

1. FLPC system, e.g., AKTA purifier (GE Healthcare, Little Chalfont, UK).
2. Superdex 75,10/300 GL gel filtration column (GE Healthcare, Little Chalfont, UK).
3. SpinX centrifuge tube filters 0.22 μm (Corning, Midland, MI, USA).
4. Thermocycler (BioRad, Foster City, CA, USA).

3. Methods

3.1. Amplification of VH Genes by PCR

1. Prepare the template DNA of VH (in phagemid vector or other) using a suitable DNA purification method such as a QIAprep Spin Miniprep kit and as specified by manufacturer.
2. Amplify DNA encoding the VH domains of interest, introducing *ApaLI* and Not*I* restriction enzyme sites for cloning into FdMyc vector. This can be achieved by PCR using the primers ApaLI_VHFwd and VHNotI_Rev. For this purpose, set up a PCR reaction using a suitable system such as the Expand High Fidelity PCR kit (Roche). Following the manufacturer's instructions, PCR reactions are performed in a total volume of 100 μL containing 1× buffer 2 (includes 1.5 mM $MgCl_2$), 200 μM dNTPs, 400 nM of each primer, 200 ng of template DNA, and 5 units of polymerase (Roche kit).
3. Heat reaction mix to 94°C for 5 min. Proceed with 25 cycles at 94°C (1 min), 55°C (1 min), and 72°C (1 min). Incubate at 65°C for 10 min for final elongation.
4. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm correct size of PCR product (approximately 500 bp).
5. Purify amplified DNA fragments using a QIAquick PCR purification kit, eluting purified DNA in 30 μL of EB buffer.
6. Digest the PCR fragments overnight with *ApaLI* and *Not*I restriction enzymes, using restriction enzyme buffer NEB2 supplemented with BSA, overnight at 37°C.
7. Purify the digested fragments using a QIAquick PCR purification kit, eluting in 30 μL of EB buffer.

3.2. Cloning of VH Genes into FdMyc Phage Display Vector

1. Set up a *Phi*29 DNA amplification reaction containing 100 μL of 10× *Phi*29 buffer, 500 μM dNTPs, 50 μM random hexamers, and 1 μg of FdMyc template DNA (see Note 1), adjust volume

to 1 mL with sterile MilliQ water. Divide this reaction mixture into 5 × 200 µL aliquots in thin-walled PCR tubes and heat to 72°C for 5 min, before cooling to 30°C. Combine the samples into one tube.

2. Add 10 µL of BSA (100×) and 2.5 µL of *Phi*29 DNA polymerase. Incubate at 30°C for 6 h. Heat-inactivate polymerase by heating to 70°C for 20 min, cool to 4°C (the sample should be highly viscous at this stage).
3. Digest the amplified DNA by adding 300 µL of NEB2 buffer (10×), 30 µL of BSA (100×), 200 units of *ApaLI* and 400 units *Not*I restriction enzymes, and 30 units of *Dpn*I restriction enzyme. Adjust volume to 3 mL with sterile MilliQ water. Incubate overnight at 37°C, shaking at 250 rpm.
4. Concentrate the digested DNA to a final volume of 500 µL using an Amicon Ultra centrifugal filter unit (10 kDa MWCO), while rinsing the unit with 500 µL of 10 mM Tris–HCl, pH 8.5 on several occasions throughout the process.
5. Purify digested DNA using Sephacryl S-1000 column and a FLPC machine (e.g., AKTA purifier) in using running buffer (10 mM Tris, 100 mM NaCl, pH 7.4). Combine the main peak fractions and concentrate to approximately 50 ng/µL using an Amicon Ultra centrifugal filter unit (10 kDa MWCO).
6. Clone digested PCR products (see Subheading 3.1, step 7) into *ApaLI*/*Not*I digested FdMyc phage vector. Use a molar ratio of insert to vector of approximately 3:1 in a 10 µL reaction, using 400 units of T4 DNA ligase and 1× T4 DNA ligase buffer. Incubate at 23°C for 1 h and purify using a QIAquick PCR purification kit, eluting with 20 µL of 20% EB buffer diluted in sterile MilliQ water. Transform DNA into *E. coli* TG1 and grow single colonies overnight on a TYE/tet agar plate.
7. Screen for positive clones by PCR. For this, set up a 50 µL PCR reaction containing 48 µL of colony PCR buffer containing 400 nM each of FdMycFwd4 and G3Seq6 primers and 2 µL of bacterial overnight culture.
8. Heat reaction mixes to 94°C for 10 min. Proceed with 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (1 min). Incubate at 65°C for 10 min for final elongation.
9. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm correct size of PCR product. Positive clones yield a PCR product of around 650 bp.
10. Confirm correct sequence of clones by DNA sequencing.

3.3. Phage Production and Purification

1. Inoculate 5 mL of 2× TY/tet medium with transformed TG1 bacteria and grow at 37°C, shaking at 250 rpm, until $OD_{600\ nm}$ reaches >1.

2. Inoculate 100 mL of 2× TY/tet medium in a conical flask with overnight culture to an $OD_{600\ nm}$ 0.1. Incubate overnight at 30°C (16–20 h), shaking at 250 rpm.
3. Centrifuge at 3,220 × *g* for 30 min at 4°C.
4. Filter the supernatant by passing through a 0.45 μm filter unit (do not use a 0.22 μm filter at this step).
5. Add 1/5 volume of 20% (w/v) polyethylene glycol (PEG), 2.5 M NaCl to supernatant. Incubate for at least 1 h on ice.
6. Centrifuge at 3,220 × *g* for 30 min at 4°C (phage in pellet). Resuspend each pellet in 4 mL of PBS.
7. Repeat PEG precipitation by adding 1 mL of 20% PEG, 2.5 M NaCl to phage solution. Incubate for 15 min on ice.
8. Centrifuge 3,220 × *g*, 30 min (phage in pellet).
9. Resuspend phage pellet in 1 mL of PBS or a smaller volume if appropriate.
10. Measure $OD_{260\ nm}$ (dilute 1/100) to estimate the number of phage using the following formula:

$$\text{Phage/mL} = OD_{260\ nm} \times 100 \times 22.14 \times 10^{10}.$$

11. Store phage at 4°C until further use.

3.4. Selection of Heat-Refoldable VH Domains on Phage

1. Coat an Immunotube or 96-well MaxiSorp Immunoplate plate with 50 μL/well of protein A at a concentration of 10 μg/mL, diluted in carbonate buffer pH 9.6. Incubate overnight (approximately16 h) at room temperature (23°C). Wash plate once with PBST and block the plate with 4% MPBS for more than 2 h. Wash the plate three times with PBST before use.
2. Dilute 10^{10} phages into 10 μL of PBS (1×10^{12} TU/mL). Heat phage to 80°C for 10 min then cool to 4°C for 10 min. Dilute phage to 800 μL in 2% MPBS and capture heat-refoldable domains on the protein A-coated plate (50 μL/well) by incubating at room temperature for 2 h, shaking gently.
3. Wash wells ten times with PBS and elute protein A-bound phage in 1 mg/mL trypsin in PBS for 10 min at room temperature.
4. Use the eluted phage to infect exponentially growing *E. coli* TG1 by incubation at 37°C for 30 min. Pellet bacteria by centrifugation at 3,220 × *g* for 20 min at 4°C. Resuspend cells in 1 mL of 2× TY medium and plate onto TYE/tet agar. Grow cells overnight at 37°C.
5. Select individual colonies, grow phage, and perform the heat-refoldable phage ELISA screen as described in Subheading 3.6 to determine the heat-refoldability of the clone.

3.5. Selection of Acid-Resistant VH Domains on Phage

1. Coat an Immunotube or 96-well MaxiSorp Immunoplate plate with 50 μL/well of protein A at a concentration of 10 μg/mL, diluted in carbonate buffer pH 9.6. Incubate overnight

(approximately16 h) at room temperature (23°C). Wash plate once with PBST and block the plate with 4% MPBS for more than 2 h. Wash the plate three times with PBST before use.

2. Dilute approximately 1×10^{10} phages in 5 μL of HN7 (2×10^{12} TU/mL) and add equal volumes of 200 mM citrate, pH 3.0 (final pH is 3.2). Incubate phage at 37°C for 2 h and neutralize reaction by adding equal volumes of 1 M Tris–HCl, pH 7.4. Dilute phage to 800 μL in 2% MPBS and capture acid-resistant domains on the protein A-coated plate (50 μL/well) by incubating at room temperature for 2 h, shaking gently.
3. Proceed as described earlier (see Subheading 3.4, steps 3–5) above, following the protocol for the acid-resistant phage ELISA screen as described below (see Subheading 3.6).

3.6. Analysis of Aggregation-Resistance by Phage ELISA

1. Coat a 96-well MaxiSorp Immunoplate plate with 50 μL/well of protein A or antigen at a concentration of 5–10 μg/mL diluted in carbonate buffer, pH 9.6. Incubate overnight (approximately16 h) at room temperature (23°C). Wash plate once with PBST and block the plate with 4% MPBS for more than 2 h. Wash the plate three times with PBST before use.
2. Inoculate 3 mL of 2× TY/tet medium in a 15 mL round bottom tube with VH-FdMyc clone (transformed in *E. coli* TG1) and incubate overnight at 30°C, shaking at 260 rpm (see Note 2).
3. The following day, transfer 1 mL of bacterial culture to a 1.5 mL tube and centrifuge at 16,000 × *g* for 10 min at 4°C.
4. Transfer supernatant, containing phages, into a fresh 1.5 mL tube and add NHS-PEO_4-biotin to a final concentration of 50 μM and incubate for 2 h at room temperature (see Note 3). Quench biotinylation reaction by adding 1 M Tris–HCl, pH 7.5 to a final concentration of 100 mM for 1 h at room temperature. Incubate for 1 h at room temperature.
5. Subject the VH domains to the desired denaturing conditions, as follows:
   (a) *Heat-refoldability*: Transfer half of the biotinylated phages into PCR strip tubes (100 μL per tube) and incubate tubes in a PCR machine preheated at 80°C for 10 min. Remove tubes and incubate them at 4°C for 10 min. Allow samples to equilibrate to room temperature for 15 min before adding to antigen/protein A-coated plate.
   (b) *Acid-resistance*: Add 250 μL of biotinylated phage samples to tubes containing 50 μL of 2 M Glycine-HCl, pH 2.5. This will result in a final pH of approximately 3.0. Incubate for 3 h at 37°C and neutralize by adding 200 μL of 3 M Tris, pH 7.5.
6. Add treated and untreated biotinylated phages to protein A/antigen-coated plates at 50 μL/well. Incubate for 1 h at room temperature on platform shaker.

7. Wash ELISA plate three times with PBST and incubate with 50 μL/well of extravidin-HRP (diluted 1:2000 in 2% BSA in PBST). Incubate for 30 min at room temperature on platform shaker.
8. Wash ELISA plate three times with PBST and develop with 50 μL/well TMB substrate. Incubate at room temperature for 30 min or until sufficient color has developed.
9. Stop reaction by adding 50 μL/well of 1 M H_2SO_4.
10. Read ELISA plate at 450 nm (reference at 630 nm).
11. Determine the retained protein A binding after treatment by expressing the absorbance of the treated sample as a percentage of the untreated sample.

3.7. Cloning of VH Genes into Periplasmic Expression Vector

1. Prepare the template DNA of VH in FdMyc using a suitable DNA purification method such as a QIAprep Spin Miniprep kit.
2. PCR-amplify the DNA encoding the VH genes of interest, introducing *Sal*I and *BamH*I restriction enzyme sites for cloning into the pET12a vector. This can be achieved by PCR using the primers VH_*Sal*IFwd and VH_*Bam*HIRev. For this purpose, set up a PCR reaction using a suitable system such as the Expand High Fidelity PCR kit (Roche). Following the manufacturer's instructions, PCR reactions are performed in a total volume of 100 μL containing 1× buffer 2 (includes 1.5 mM $MgCl_2$), 200 μM dNTPs, 400 nM primers each, 200 ng of template DNA, and 5 units of polymerase (Roche kit).
3. Heat reaction mix to 94°C for 5 min. Proceed with 25 cycles at 94°C (1 min), 55°C (1 min), and 72°C (1 min). Incubate at 65°C for 10 min for final elongation.
4. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm correct size of PCR product (approximately 500 bp).
5. Purify amplified DNA fragments using a QIAquick PCR purification kit, eluting purified DNA in 30 μL of EB buffer.
6. Digest the PCR fragments overnight with *Sal*I and *BamH*I restriction enzymes, using restriction enzyme buffer NEB3 supplemented with BSA, overnight at 37°C.
7. Purify the digested fragments using a QIAquick PCR purification kit, eluting in 30 μL of EB buffer.
8. Clone digested PCR products into *Sal*I/*BamH*I-digested pET12a vector. Use a molar ratio of insert to vector of approximately 3:1 in a 10 μL reaction, using 400 units of T4 DNA ligase and 1× T4 DNA ligase buffer. Incubate at 23°C for 1 h and purify using a QIAquick PCR purification kit, eluting with 20 μL of water. Transform DNA into *E. coli* BL21Gold and grow single colonies overnight on a TYE/amp/tet/glu agar plate.
9. Screen for positive clones by PCR. For this, set up a 50 μL PCR reaction containing 48 μL of colony PCR buffer containing

400 nM each of T7Pro and T7Term primers and 2 μL of bacterial overnight culture.

10. Heat reaction mixes to 94°C for 10 min. Proceed with 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (1 min). Incubate at 65°C for 10 min for final elongation.
11. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm correct size of PCR product. Positive clones yield a PCR product of around 650 bp.
12. Confirm sequence of clones by DNA sequencing.

3.8. Expression and Purification of Soluble VH Domains

1. Freshly transform DNA preparation of VH in pET12a into *E. coli* BL21Gold, plate on TYE/amp/tet/glu agar, and incubate overnight at 37°C.
2. Inoculate 5 mL of 2× TY/amp/tet/glu medium with a single colony and incubate overnight at 37°C, shaking at 260 rpm.
3. Inoculate 1 L 2× TY/amp/tet/glu medium in a 2.5 L baffled flask with overnight culture to an $OD_{600\ nm}$ 0.1 and incubate at 37°C, 260 rpm until $OD_{600\ nm}$ 0.5–0.7.
4. Transfer culture to 2×500 mL centrifuge bottles and spin down at 3,220×*g* for 20 min at 4°C.
5. Discard supernatant and resuspend bacterial pellet in 1 L 2× TY/amp/tet (no glucose) medium containing 1 mM IPTG.
6. Incubate at 30°C for 24–42 h. If expressing for 42 h, re-induce after 24 h by adding IPTG (1 mM final) and ampicillin (100 μg/mL final) after 24 h of growth.
7. Spin down bacteria at 6,400×*g* for 15 min at 4°C.
8. Filter supernatant through a 0.45 μm vacuum filter (see Note 4).
9. Add PBS-washed rProtein A sepharose to the filtered supernatant (5 mL of sepharose per liter of culture) and incubate overnight at 4°C on a roller.
10. Transfer beads to a gravity flow column then run the supernatant by gravity.
11. Wash beads twice with 10 mL of PBS.
12. Elute the antibody by adding 15–20 mL of 0.1 M Glycine – HCl, pH 3.0 and collecting 1 mL fractions. Neutralize by adding 250 μL of 1 M Tris–HCl, pH 8.0 to each fraction.
13. Measure $OD_{280\ nm}$ of eluted fractions and combine those containing protein.
14. Concentrate antibody and change buffer to PBS using an Amicon Ultra centrifugal filter unit (10 kDa MWCO).
15. Measure final concentration of purified protein by measuring $OD_{280\ nm}$.
16. Store purified VH protein at 4°C if used within days to weeks, otherwise snap freeze in liquid nitrogen and store at –20°C.

3.9. Characterization of Aggregation-Resistance VH Domains by Size Exclusion Chromatography

1. Prepare 1 mL of purified VH protein at 10 μM (see Note 5) (diluted into PBS).
2. Aliquot 5 × 100 μL into PCR tubes and heat at 80°C for 10 min, 4°C for 10 min. Combine the aliquots and transfer to a 1.5 mL tube. Centrifuge both the heated and unheated samples at 16,000 × *g* for 10 min at 4°C.
3. Remove the supernatant, transfer to a 0.22 μm SpinX filter unit and briefly centrifuge at 16,000 × *g* for 2 min at 4°C.
4. Run 500 μL of the unheated and heated sample sequentially on a Superdex-G75 gel filtration column, equilibrated with PBS, at a flow rate of 0.5 mL/min.
5. Calculate the recovery of the VH by measuring the area under the curve of the heated sample, expressed as a percentage of the unheated sample.

4. Notes

1. Best results are obtained using FdMyc ssDNA as a template for Phi29 amplification. Grow TG1 cells transformed with FdMyc overnight (approximately 16 h) at 30°C in 2× TY medium supplemented with 15 μg/mL tetracycline. Remove cells by centrifugation and PEG precipitate phage as described previously (see Subheading 3.3, steps 4–7). Once purified phages are obtained, isolate ssDNA using QIAprep Spin M13 Kit according to the manufacturer's instructions.
2. Leaving a sterile tip in the 15 mL tube after inoculation improves aeration.
3. Biotinylate 2× TY medium as a negative control.
4. Optional step—Periplasmic extraction of VH protein:
   (a) Resuspend bacterial pellet in 50 mL of periplasmic preparation buffer 1, add complete Mini EDTA free protease inhibitor tablet and incubate on ice for 1 h.
   (b) Centrifuge at 17,000 × *g* for 15 min at 4°C and transfer supernatant into 50 mL tube.
   (c) Resuspend pellet in 50 mL of periplasmic preparation buffer 2 and incubated on ice for 30 min.
   (d) Centrifuge at 17,000 × *g* for 15 min at 4°C and transfer supernatant into 50 mL tube.
   (e) Dialyse extractions 1 and 2 against PBS in SnakeSkin dialysis tubing overnight at 4°C, using a magnetic stirrer (change dialysis buffer multiple times).

(f) Combine dialysed extractions 1 and 2 and filter through 0.45 μm filter unit.

(g) Purify VH protein as described in Subheading 3.8, steps 9–16.

5. Guideline only. Protein concentration may have to be adapted depending on VH domain.

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