

Chapter 16

Affinity Maturation by Phage Display

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Abstract

Antibodies are indispensable tools for research, diagnostics, and therapy. However, sometimes antibodies with the most favourable specificity profile lack sufficient affinity for a desired application. Here, we describe a method to increase the affinity of recombinant scFv antibody fragments based on random mutagenesis and phage display under stringent conditions. Random mutations are inserted by performing several rounds of error-prone PCR. After construction of a mutated antibody gene library, affinity selection is performed by panning with washing conditions optimized for off-rate-dependent selection. Alternatively, panning in solution with competition can be used to enrich binders with improved binding properties.

Key words: Phage display, affinity maturation, error-prone PCR, recombinant antibody, scFv, selection system.

1. Introduction

In the past two decades antibodies have been the fastest growing class of pharmaceutical proteins with a predicted marked volume of US\$ 30 billion in 2010 (1). In vitro antibody selection technologies, most of all antibody phage display, offer advantages when compared to the animal-based generation of antibodies: enhanced throughput by parallelization and miniaturization, the stringent control of selection conditions, and the possibility to use toxic proteins or allosteric conformation variants (2).

Phage display allows the production and screening of large antibody libraries with theoretical complexities of up to 10^{11} different clones (3). However, even complex naïve (non-immune) antibody libraries sometimes result in the selection and amplification

of binders with equilibrium dissociation constant (K_D) ranges >100 nM (4). For many applications in research and therapy, antibodies with affinities in the lower nanomolar or subnanomolar range are preferred.

Consequently, methods for affinity maturation have been developed for the improvement of these low-affinity antibodies. Enhanced K_D values up to two orders of magnitudes are achievable compared to the parental clone (5).

In general, mutations are introduced into the antibody gene, to create a mutated antibody gene library. Several different mutation strategies have been developed. Site-specific mutation of complementarity-determining regions (CDR) by using polymerase chain reaction (PCR) and special randomized primers is one strategy (6). Another strategy is random mutagenesis of the complete phagemid carrying the antibody gene. Here, the use of *E. coli* mutator strains (7) or the use of error-prone TempliPhi DNA amplification (8) are possible strategies. In both strategies, mutations can also occur in the vector backbone, making subsequent recloning of the antibody ORF necessary.

The most common technique to introduce random mutations is the error-prone PCR of the antibody gene (9). It utilizes the natural error-rate of Taq-polymerase (10), which can be increased by suboptimal buffer conditions during DNA amplification. Here, only the gene of interest is affected by mutations and will be subcloned into the phagemid backbone to obtain a mini library.

Subsequent selection of high-affinity mutants is achieved by phage-display panning approaches (11) using many harsh and long washing steps.

A disadvantage is that surface-selection often enriches binders with increased tendency for dimerization, especially when using the scFv format. This problem can be bypassed by panning the antigen in solution (12), for example, by using biotinylated antigen in combination with streptavidin beads. Moreover, it is possible to add unbiotinylated antigen or soluble antibody fragments after antibody phage binding has occurred, to provide competition to improve selection for better off-rates.

Screening of high-affinity variants requires the production of monoclonal soluble antibody fragments and can be performed in microtiter plates (96-well scale) (13). Monoclonal binders can be tested and affinity ranked by using crude cell supernatants in common ELISA techniques. Affinity ranking can be achieved by competitive ELISA (14). Specificity can be validated, for example, by flow cytometry. Affinities are usually determined by kinetic analysis using surface plasmon resonance (SPR) (15).

2. Materials

2.1. Error-prone PCR

1. Template DNA, phagemid containing antibody fragment gene.
2. Up to three site-specific DNA-oligo primer sets.
3. PCR Thermocycler (Biorad PTC 250).
4. GeneMorphII Random Mutagenesis Kit (Stratagene, Amsterdam, Netherlands).
5. PCR clean-up kit (GFX illustra, GE Healthcare, Munich, Germany).
6. Agarose gel.

2.2. Affinity Library Creation

1. Phage display-compatible phagemid (e.g., pHAL14, pSEX81) (16).
2. Restriction enzymes *Nco*I, *Not*I (New England Biolabs, Frankfurt/Main, Germany).
3. Shrimp alkaline phosphatase, SAP (MBI Fermentas, St. Leon Rot, Germany).
4. PCR clean-up kit (GFX illustra, GE Healthcare, Munich, Germany).
5. T4 DNA-Ligase (Promega, Mannheim, Germany).
6. Electrocompetent *E. coli* XL1 Blue MRF' (Stratagene, Amsterdam, Netherlands); Genotype: $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 } supE44 \text{ thi-1 } recA1 \text{ gyrA96 } relA1 \text{ lac [F' } proAB \text{ lacI}^qZ\Delta M15 \text{ Tn10 (Tet}^r\text{)]}$.
7. *SOC medium*: 0.5% (w/v) yeast extract, 2.0% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM MgSO₄, 20 mM glucose.
8. Eppendorf Thermomixer compact.
9. *2 M Mg solution*: 1 M MgCl + 1 M MgSO₄.
10. *SOB medium pH 7.0*: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl; after autoclaving add sterile 1% (v/v) 2 M Mg solution.
11. *SOB-GA*: SOB, containing 100 µg/ml ampicillin, 100 mM glucose, 1.5% (w/v) agar agar.
12. 25 × 25 cm sterile plastic dishes.
13. *2YT medium pH 7.0*: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl.
14. *2YT-GA medium*: 2YT, containing 100 µg/ml ampicillin, 100 mM glucose.
15. 80% (v/v) glycerol solution.
16. 1.8 ml Cryotubes (Nunc, Mannheim, Germany).

2.3. Library Validation

1. 9-cm sterile petri dishes.
2. Phagemid-specific DNA-oligo primer set.
3. RedTaq DNA Polymerase (Sigma, Munich, Germany).
4. PCR clean-up kit (GE Healthcare, GFX illustra)

2.4. Library Packaging

1. *2YT-GA*: 2YT, containing 100 µg/ml ampicillin, 100 mM glucose.
2. 100 ml shake flask.
3. Helperphage M13K07 (Stratagene).
4. *2YT-AK*: 2YT, containing 100 µg/ml ampicillin, 50 µg/ml kanamycin.
5. *Polyethyleneglycol (PEG) solution*: 20% (w/v) PEG 6000, 2.5 M NaCl in water.
6. *Phage dilution buffer*: 10 mM Tris-HCl pH 7.5, 20 mM NaCl, 2 mM EDTA

2.5. Titration

1. *2YT-T*: 2YT, containing 50 µg/ml tetracycline.
2. *2YT-GA*: 2YT, containing 100 µg/ml ampicillin, 100 mM glucose, 1.5% (w/v) agar agar.
3. *E. coli* XL1 Blue MRF' (Stratagene); Genotype: $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac}$ [F' *proAB lacI*^qZ Δ M15 Tn10 (Tet^r)].

2.6. Selection by Panning

1. *Carbonate buffer pH 9.7*: 35 mM NaHCO₃, 15 mM Na₂CO₃.
2. *PBS pH 7.4*: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄*2H₂O, 0.24 g KH₂PO₄ in 1 l water solution.
3. *PBS-T*: PBS + 0.1% Tween 20.
4. *MPBS-T*: 2% skim milk in PBST, prepare fresh.
5. *Panning block solution*: 2% (w/v) skim milk + 2% (w/v) BSA in PBST; prepare fresh.
6. 10 µg/ml trypsin in PBS.
7. *2YT-T*: 2YT, containing 50 µg/ml tetracycline.
8. 15-cm petri dishes.
9. 80% (v/v) glycerol solution.

2.7. Selection in Solution with Competition

1. Soluble antigen.
2. Biotinylated soluble antigen.
3. Streptavidin beads (Dynabeads M-280, Invitrogen, Karlsruhe, Germany).
4. Overhead shaker (Multi Shaker Rotator RS24, G Kisker).
5. Magnet particle concentrator (Dyna).
6. 10 µg/ml trypsin in PBS.

2.8. Production of Soluble Monoclonal AB-Fragments

1. 96-well U-bottom polypropylene (PP) microtiter plates (Greiner BioOne, Frickenhausen, Germany)
2. AeroSeal breathable sealing film (Excel Scientific, USA).
3. 2YT-A containing 50 μ M isopropyl-beta-D-thiogalactopyranoside (IPTG).
4. Thermo Shaker PST-60HL-4 (Lab4You, Berlin, Germany).

2.9. Monoclonal ELISA

1. Maxisorp Plates (Nunc).
2. Mouse α -myc-tag monoclonal antibody (9E10, Sigma-Aldrich, Munich, Germany).
3. Mouse α -His-tag monoclonal antibody (α -Penta His, Qiagen, Hilden, Germany).
4. Goat α -Mouse IgG serum (Fab-specific), HRP conjugated (Sigma-Aldrich, Munich, Germany).
5. *TMB solution A, pH 4.1*: 10 g citric acid solved in 100 ml water, add 9.73 g potassium citrate, add H₂O to make 1 l.
6. *TMB solution B*: 240 mg tetramethylbenzidine, 10 ml acetone, 90 ml ethanol, 907 μ l 30% H₂O₂.
7. 0.5 M H₂SO₄.

3. Methods

All methods not explained in detail are done according to (17).

3.1. Error-Prone PCR

1. Design-specific primer sets for your phagemid (*see Note 1*).
2. Use <1 ng purified plasmid DNA as template for error-prone PCR.
3. Perform PCR by using GeneMorphII Random Mutagenesis Kit (Stratagene) according to manufacturer's instructions, 35 cycles (*see Note 2*).
4. Clean up amplified DNA by using PCR clean-up kit (e.g., GFX illustra PCR clean-up).
5. Photometrically determine DNA concentration after clean-up.
6. Use <1 ng of this DNA for the next error-prone PCR (*see Note 3*).
7. Repeat error-prone PCR up to 6–8 times followed by clean-up (*see Note 4*).
8. Check PCR product on a 1% agarose gel. Use only clean PCR fragment (distinct band, no smear) for **Section 3.2**.

3.2. Affinity Library Creation

1. Digest your PCR product and phage-display vector DNA with suitable restriction enzymes (e.g., *Nco*I, *Not*I in case of pHAL14, pSEX81, pIT). Incubate over night at 37°C. Add 1 µl SAP to the vector preparation and incubate 1 h at 37°C. Inactivate all enzymes by heating at 65°C, 15 min.
2. If necessary separate your digested vector DNA by agarose gel electrophoresis or directly use a DNA clean-up kit for purifying the digested DNA (*see Note 5*).
3. Determine DNA concentration of digested vector DNA and digested PCR product (photometrically or agarose gel).
4. Use approximately 1 µg vector DNA for ligation; molar vector: insert ratio should be 1:3. Perform ligation overnight at 16°C. Inactivate enzymes at 65°C for 10 min.
5. Perform ethanol-precipitation of the DNA and dissolve the pellet in 100 µl H₂O. Split into four aliquots (25 µl each).
6. Mix every ligation aliquot with 25 µl electrocompetent *E. coli* XL1 Blue MRF' (Stratagene) and perform electroporation (BioRad, electroporation unit). *Parameter*: 1.7 kV, 4–5 ms pulse.
7. Resuspend transformed cells in 950 µl prewarmed SOC medium, incubate 1 h at 37°C and 600 rpm (Eppendorf thermomixer).
8. Take 10 µl of the cell suspension and make a dilution series (e.g., 1:100 per step) in SOB medium; up to 10⁸ clones can be expected. Plate an aliquot of each dilution and calculate the transformation efficiency (*see Note 6*).
9. Plate the rest of the cell suspension to 25 × 25 cm SOB-GA agar-plates. Incubate overnight at 37°C.
10. Add 40 ml SOB medium to each plate. Incubate at room temperature on a rocker for 30 min. Carefully scrape the transformed cells from the plate using a spatula.
11. Collect the cell suspension and centrifuge in a 50-ml Falcon tube at 3200 *g* for 10 min. Discard supernatant, and resuspend the pellet in 5 ml 2YT medium.
12. Pool all resuspended pellets into one tube. Prepare glycerol-stocks of your library by mixing 250 µl 80% glycerol and 750 µl cell suspension in 1.8-ml cryotubes. Shock-freeze this tube in liquid nitrogen and store at –80°C.

3.3. Library Validation

1. Pick up to 20 clones from the plates used for determination of transformation efficiency. Plate on fresh 2YT-GA agar plates.
2. Perform colony-PCR (50 µl scale) using a primer set which will amplify the cloned scFv gene.
3. Analyze an aliquot (5 µl) by agarose gel electrophoresis. A band of a defined size (depending on the used primer set,

usually 800–1000 bp for scFv DNA) will be obtained from clones with a “correct” insert. The percentage of these “positive” clones shows the quality of the created library.

4. Clean up the PCR from these clones, by using a PCR clean-up kit (e.g., GFX illustra). Use this purified DNA for sequencing with appropriate primers (*see Note 7*).
5. The average mutation rate can be determined by alignment of the DNA-sequence from the sequenced clones with the parental original sequence of the source clone. Up to 30 point mutations can be expected.

3.4. Library Packaging

1. Gently thaw a library glycerol stock on ice.
2. Inoculate 50 ml 2YT-GA medium with 10–100 μ l cell suspension directly from the glycerol stock to an initial OD₆₀₀ of about 0.1.
3. Grow the cells at 37°C and 250 rpm in a 100-ml Erlenmeyer shake flask to an OD₆₀₀ $\sim$ 0.5.
4. Transfer 20 ml of the culture into a sterile 50-ml polypropylene tube. Add 5×10^{12} cfu M13K07 for infection, mix gently. Incubate 30 min at 37°C, and then incubate another 30 min at 37°C with shaking at 250 rpm.
5. Centrifuge the suspension 10 min at 3200 *g* to pellet the cells. Discard the supernatant to remove the glucose and suspend the pellet in 1 ml fresh 2YT-AK medium (*see Note 8*). Use this suspension for inoculation of 50 ml 2YT-AK in a 100-ml Erlenmeyer shake flask.
6. Incubate cells overnight at 30°C and 250 rpm.
7. Precipitate the phage in the supernatant by adding one-fifths volume PEG solution in 50-ml polypropylene tubes. Incubate for 1 h at 4°C with gentle shaking.
8. Pellet the phage by centrifugation for 1 h at 3200 *g* and 4°C. Put the open tubes upside down on tissue paper and let the viscous PEG solution move out completely. Resuspend the phage pellet in 500 μ l phage dilution buffer. Centrifuge again with 16000 *g* to remove debris and residual cells. Transfer the supernatant into a new tube. Titer the phage preparation and use it for the next panning round. Store the remaining phage at 4°C.

3.5. Titration

1. Inoculate 5 ml 2YT-T medium in a 100-ml Erlenmeyer flask with *E. coli* XL1-Blue MRF' and grow overnight at 37°C and 250 rpm.
2. Inoculate 50 ml 2YT-T medium with 500 μ l overnight culture and grow at 250 rpm at 37°C up to OD₆₀₀ $\sim$ 0.5 (*see Note 9*).
3. Make serial dilutions of the phage solution in PBS. The number of eluted phage during panning depends on several parameters

(e.g., antigen, library, panning round, washing stringency, etc.). The phage titer can vary between 10^2 cfu and 10^7 cfu. The phage preparation after re-amplification of the eluted phage has a titer of about 10^{12} – 10^{13} cfu/ml. Infect 50- μ l bacteria with 10 μ l phage dilution and incubate 30 min at 37°C.

4. Plate the 60- μ l infected bacteria on 2YT-GA agar plates (9-cm petri dishes).
5. Incubate the plates overnight at 37°C.
6. Count the colonies and calculate the colony-forming units (cfus) titer according to the dilution.
7. Library can be used for selection of high-affinity mutants (*see Section 3.6* or *3.7*).

3.6. Selection by Panning

1. Coat antigen in different amounts (1–50 ng/well) in a Maxi-sorp stripe (Nunc), use carbonate buffer (pH 9.7), coat overnight at 4°C.
2. Block microtiter plate stripe with 350 μ l MPBS-T for 1–3 h at room temperature.
3. Wash three times with PBS-T (*see Note 10*).
4. Incubate 10^{11} – 10^{12} cfu of antibody phage from the library in 150 μ l panning block solution and add this solution to the antigen-coated wells. Incubate 3 h at room temperature.
5. After binding of antibody phage, wash three times with PBS-T (*see Note 11*).
6. Put the complete stripe in 2 l PBS, incubate under soft stirring for 1 week at 4°C.
7. Repeat Steps 5 + 6 one time weekly (washing 3–4 weeks recommended).
8. Elute with 200 μ l trypsin solution for 60 min at 37°C (*see Note 12*).
9. Use 10 μ l of the eluted phage for titration (*see Section 3.5*).
10. Inoculate 50 ml 2YT-T with an overnight culture of *E. coli* XL1-Blue MRF' in 100-ml Erlenmeyer flasks and grow at 250 rpm and 37°C.
11. Infect exponential growing 20-ml *E. coli* XL1-Blue MRF' culture ($OD_{600} \sim 0.5$, after 2–3 h) with the remaining 190 μ l of the eluted phage. Incubate 30 min at 37°C without shaking and the following 30 min with shaking at 250 rpm.
12. Harvest the infected bacteria by centrifugation for 10 min at 3200 g in 50-ml polypropylene tubes. Resolve the pellet in 250 μ l SOB-GA and plate the bacterial suspension on SOB-GA agar plates (15-cm petri dish). Grow overnight at 37°C.
13. Pick clone colonies from these plates and perform “Production of soluble monoclonal antibody-fragments” (*see Section 3.8*).

3.7. Selection in Solution

1. For all selection processes “in solution” it is obligatory that the antigen is available unlabeled and biotin-labeled (*see Note 13*).
2. The selection process can be performed by systematically reducing the antigen concentration (A) or by competition with free antigen or soluble antibody-fragments (B).
3. Block 50–100 μl streptavidin beads M-280 ($3.25\text{--}6.5 \times 10^8$ beads) with 1.5-ml panning block for 1 h at room temperature. Use magnet particle concentrator (Dyna) for bead separation.
4. Use 50- μl blocked streptavidin beads for library preincubation (remove unwanted binders) for 30 min at room temperature. Discard beads afterwards.
5. Use the preincubated library for the selection process.
6. (A) Mix 1×10^{11} phage from your preincubated library with 50 nM biotinylated antigen in panning block solution. Incubate 2 h at room temperature, gently mixing using an overhead shaker (*see Note 14*).
7. (A) Add 100- μl blocked streptavidin beads. Perform capturing by incubating 15 min at room temperature and gently mixing in an overhead shaker.
8. (A) Separate the streptavidin beads carrying captured antibody phage from the solution in the magnetic rack.
9. (A) Discard the supernatant and wash the streptavidin beads with fresh washing buffer for 1-2 min using on overhead shaker and collect streptavidin beads in the magnetic particle concentrator (magnetic rack). According to this procedure perform consecutive washing steps three times with PBS-T, two times with MPBS-T, two times with PBS, one time with MPBS and two times with PBS.
10. (A) Elute captured phage by adding 500 μl trypsin, incubate for 30 min, 37°C. Separate and discard the streptavidin beads, use supernatant for titration (*see Section 3.5*) and the production of new phage (start at **Section 3.4.4** by adding the whole eluted phage to the cells).
11. (A) Start the next round by repeating the complete procedure with the newly produced phage particles. Use reduced amount of biotinylated antigen (e.g., 5 nM). The amount of streptavidin beads can also reduced to 50 μl (3.25×10^8 beads)
12. (A) Perform up to four selection rounds (if desired). Reduce antigen amount round by round (*see Note 15*).
13. (A) Pick colonies from the plates that were used for titration and perform “Production of soluble monoclonal AB-Fragments” (*see Section 3.8*).

14. (B) Perform library and bead blocking as mentioned in Steps 3–5).
15. (B) Mix 1×10^{12} phage particles from the library with 1 nM biotinylated antigen (*see* **Note 15**) in 1.5-ml panning block
16. (B) Incubate 2 h at room temperature, gently mixing in an overhead shaker.
17. (B) Add up to 1 mM (max. factor 1000) of the unbiotinylated antigen *or* the parental antibody fragment.
18. (B) Incubate 1 week at 4°C, gently mixing in an overhead shaker to equilibrate the system.
19. (B) Wash the beads three times with panning block solution and four times with PBS-T (the washing steps can be increased, if necessary).
20. (B) Elute the phage as mentioned in Step 10 (A).
21. (B) Perform titration and pick monoclonals for production of soluble monoclonal antibody fragments (*see* **Section 3.8**).

3.8. Production of Soluble Monoclonal Antibody Fragments

1. Fill each well of a 96-well U-bottom polypropylene microtiter plate with 150 μ l 2YT-GA medium.
2. Pick 96 clones with sterile tips from the desired panning round and inoculate each well (*see* **Note 16**). Seal the plate with a breathable sealing film.
3. Incubate overnight in a microtiter plate shaker (e.g., Thermo Shaker PST-60HL-4, Lab4You, Germany) at 37°C and 1200 rpm.
4. (A) Fill a new 96-well polypropylene microtiter plate with 150 μ l 2YT-GA and add 10 μ l of the overnight cultures. Incubate for 2 h at 37°C and 1200 rpm. (B) Add 30 μ l glycerol solution to the remaining 140 μ l overnight cultures. Mix by pipetting and store this master plate at –80°C.
5. Pellet the bacteria in the microtiter plates by centrifugation for 10 min at 3200 *g* and 4°C. Remove 180 μ l glucose-containing media by carefully pipetting (do not disturb the pellet).
6. Add 180 μ l 2YT-A with 50 μ M IPTG and incubate overnight at 30°C and 1200 rpm (*see* **Note 17**). Seal the plate with a breathable sealing film.
7. Pellet the bacteria by centrifugation for 10 min at 3200 *g* in the microtiter plates. Transfer the antibody fragment containing supernatant to a new polypropylene microtiter plate and store at 4°C.

3.9. ELISA of Soluble Monoclonal Antibody Fragments

1. To analyze the antigen specificity of the soluble monoclonal antibody fragments, coat 100 ng antigen per well in carbonate buffer (pH 9.7) overnight at 4°C in a microtiter plate.

2. Wash the coated microtiter plate wells three times with PBST (*see Note 12*).
3. Block the antigen-coated wells with MPBS-T for 1 h at room temperature. The wells must be completely filled.
4. Fill 90 μl MPBS-T in each well and add 10 μl of antibody solution. Incubate for 1 h at room temperature.
5. Wash the microtiter plate wells three times with PBS-T.
6. Incubate 100 μl α -tag antibody solution for 1 h (appropriate dilution in MPBS-T) at room temperature.
7. Wash the microtiter plate wells three times with PBS-T.
8. Incubate 100 μl goat- α -mouse HRP conjugate (1:10,000 in MPBS-T) 1 h at room temperature.
9. Wash the microtiter plate wells three times with PBS-T.
10. Shortly before use, mix 20 parts TMB solution A and 1 part TMB solution B. Add 100 μl of the prepared TMB solution into each well and incubate for 1–15 min.
11. Stop the color reaction by adding 100 μl 0.5 M sulfuric acid. The color turns from blue to yellow.
12. Measure the extinction at 450 nm in an ELISA reader.
13. Identify positive candidates.
14. DNA sequencing of antibody clones allows to get rid of duplicate clones.

4. Notes



1. To increase the number of error-prone PCR rounds, design up to three primer sets and perform the PCR in a nested approach. Consider that the restriction sites you use for library cloning are part of the inner primer set (otherwise, you may mutate and lose the cloning sites).
2. The advantage of the GeneMorphII kit is the mutational spectra of the MutazymeII DNA polymerase. A,T $\rightarrow$ N 50.7%; G,C $\rightarrow$ N 43.8% and the high mutation frequency (up to 16 mutations/kb and PCR) (Stratagene, kit manual).
3. A total of 3–4 rounds of error-prone PCR can be performed by using the same primer set. After more rounds, the PCR product yield will decrease rapidly. We suggest maximum 2–3 error-prone PCR rounds with one primer set, then switch to the next inner primer set. After six rounds of error-prone PCRs (35 cycles each) around 30 point mutations can be found in a common scFv gene (approx. 800 bp).

4. To get more DNA for the following cloning step, scale-up the last PCR.
5. Vectors containing only a small insert < 100 bp (e.g. cassette with stop codons) between the cloning sites do not require purification by agarose gel electrophoresis. Instead, the vector can be directly purified after digestion and dephosphorylation using a PCR purification kit. Avoiding agarose gel electrophoresis can tremendously improve following cloning steps.
6. The transformation efficiency is a benchmark for the theoretical size of the library. The sum of the four transformation yields is the maximum complexity of the new library.
7. Alternatively, perform plasmid-DNA preparation from the plated clones and use the isolated plasmid-DNA for sequencing.
8. Due to the selection with kanamycin, only M13K07 (Kan^r) infected cells will survive and produce antibody-phage.
9. If the bacteria have reached OD₆₀₀ ~ 0.5 before they are needed, store the culture on ice to maintain the F pili on the *E. coli* cells. Store for max. 60 min.
10. The washing should be performed with an ELISA washer (e.g., TECAN Columbus Plus) for reproducible washing results. To remove antigen or blocking solutions wash three times with PBST (“standard washing protocol” for TECAN washer). If no ELISA washer is available, wash manually three times with PBST.
11. After binding of antibody phage, wash three times with PBST (“stringent bottom washing protocol” in case of TECAN washer). If no ELISA washer is available, wash manually ten times with PBST and ten times with PBS.
12. Phagemids like pSEX81 (18), pHAL1 (19), or pHAL14 (16) have coding sequences for a trypsin-specific cleavage site between the antibody fragment gene and the gIII. Trypsin also cleaves within antibody fragments whereas the phage is very resistant to this procedure. As a result, also vectors without a trypsin cleavage site may be used successfully.
13. The antigen can be manually labeled with biotin using the NHS-S-S Biotin system (Pierce).
14. The initial antigen concentration depends on the equilibrium dissociation constant (K_D) of your parent clone and the K_D you try to achieve by these methods. Variation in the concentration should lead to an optimal result.
15. The final antigen concentration should be a maximum of ten times less than the desired K_D for improved binders (e.g., choose 0.5 nM antigen, when binders with K_D around 5 nM are expected).

16. We recommend to pick only 92 clones. Use the wells H3, H6, H9, and H12 for controls. H3 and H6 are negative controls – these wells will not be inoculated. We inoculate the wells H9 and H12 with the clone containing the phagemid encoding for the “parental” antibody fragment.
17. The appropriate IPTG concentration for induction depends on the vector design. A concentration of 50 μ M was well suited for vectors with a Lac promoter like pSEX81 (18), pIT2 (20), pHENIX (21), and pHAL14 (16, 22). The method for the production of soluble antibodies works with vectors with (e.g., pHAL14) and without (e.g., pSEX81) an amber stop codon between antibody fragment and gIII. If the vector has no amber stop codon, the antibody::pIII fusion protein will be produced and may be used instead successfully (23).

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