Chapter 10

Generation of Human Single Domain Antibody Repertoires by Kunkel Mutagenesis

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Abstract

Human antibody single domains are a promising new class of antibody fragments. Here we describe methods for the cloning of human V_H and V_L genes into phage and phagemid vectors. Furthermore, we provide detailed protocols for the generation of single domain antibody libraries by Kunkel mutagenesis and the analysis of diversity by DNA sequencing and superantigen binding.

Key words: Human antibody domains, Phage display, Kunkel mutagenesis, Protein A, Protein L, Superantigen

1. Introduction

1.1. V_H and V_L Families Suitable for Construction of Human Single Domain Libraries

The main factor limiting the use of human single domains as affinity reagents has been their variable biophysical properties (1, 2). This has also restricted the use of phage display libraries of such domains. Recent studies have generally used protein engineering approaches and selection on phage to further improve the biophysical properties of human single domains (3–8). However, in other cases no further optimization was reported, and soluble binders were obtained from naïve repertoires (9).

The differences in biophysical behavior of human single domains have been studied in detail by Ewert and coworkers (1, 2). They discovered that, while consensus domains from many human families readily aggregated when expressed in isolation, human V_H3 and kappa domains had more favorable properties. This also applied to lambda domains, albeit to a lesser degree. Considering their more favorable properties, human V_H3 and kappa domains have been frequently used as building blocks for the construction of synthetic human antibody phage libraries.

Patrick Chames (ed.), *Antibody Engineering: Methods and Protocols, Second Edition*, Methods in Molecular Biology, vol. 907,
DOI 10.1007/978-1-61779-974-7_10,

This includes scFv (10, 11) and single domain libraries (5, 7, 9). In addition, the use of V_H3 and kappa domains provides the advantage of being able to utilize protein A and protein L superantigen for purification and detection (12, 13). Superantigen interaction also provides an excellent "quality control" mechanism for antibody phage libraries, as binding is dependent on the display of correctly folded domains. This allows for rapid determination of "accessible" diversity in the generated repertoires.

1.2. Generation of Human V_H and V_L Single Domain Libraries

As both phagemid and phage vectors provide easy access to single stranded DNA (by means of isolation from phage particles), single strand mutagenesis provides a straightforward way of introducing diversity into antibody variable regions. We provide a protocol based on the original method developed by Kunkel (14) with minor modifications (15). The use of highly transforming electrocompetent cells is essential for obtaining very large library sizes ($>10^9$) and a detailed protocol is provided for this purpose.

As Kunkel mutagenesis is not dependent on flanking restriction sites, diversity can be introduced at essentially any position within the antibody variable region. Sequential modification of multiple sites is often not necessary, as the frequency of mutation is generally high, even when multiple mutagenic oligonucleotides are used in a single reaction. Additional measures should be taken in those cases where the presence of unmutated template is detrimental (this can be achieved by introduction of stop codons into the template DNA) (15). However, this is often not required for antibody libraries, where the presence of unmutated template is generally of little consequence.

While a more extensive discussion is outside the scope of this article, it is essential to note that the nature and position of diversity introduced into antibody variable domains is of great importance. We refer the reader to earlier work on synthetic human antibody libraries (such as ETH-2 or Tomlinson I/J) (10, 11). Common sites targeted for randomization include 95–100 in V_H CDR3 and 91, 93–96 in V_L CDR3 (numbering according to Kabat) (16). Additional diversity in other CDR regions may be required for generating high affinity binders. Finally, any randomization strategy should aim to limit the number of stop codons in the design.

2. Materials

2.1. Amplification of Antibody Variable Genes (V_H or V_L) by PCR

1. FdMyc phage vector (derived from FdTet (17) or pHEN1 phagemid vector (18)). Both vectors encode a *c-Myc* tag introduced between a *Not*I site and geneIII.
2. Thermocycler.

3. Thermostable DNA polymerase and reaction buffers.
4. dNTPs: deoxynucleoside triphosphates.
5. 2% agarose gel: dissolve agarose in TBE buffer (2 g/100 ml), melt agarose in microwave and add SYBRSafe 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 variable genes into phage display vectors: These will amplify V_H3 and kappa genes such as those present in the Tomlinson I/J or ETH-2 libraries; the use of other domains may require changes to the annealing sequences shown in bold:
   (a) FdMyc V_H forward primer: 5′-ACGCGTGCA**CAGGT GCAGCTGTTGG**-3′ (anneals in the 5′-region of V_H genes and introduces *ApaL*I site).
   (b) FdMyc V_H reverse primer: 5′- CTGTTAGCGGCCGC**GCT CGAGACGGTGACCAG**-3′ (anneals in the 3′-region of V_H genes and introduces *Not*I restriction site).
   (c) FdMyc V_L forward primer: 5′-TCTCACAGTGCA**CA GATCCAGATGACCCAG**-3′ (anneals in the 5′- region of V_L genes and introduces *ApaL*I site).
   (d) FdMyc V_L reverse primer: 5′-TTCTGCGGCCGC**CCGTT TGATTTCCACCTTGGTC**-3′ (anneals in the 3′-region of V_L genes and introduces *Not*I restriction site).
   (e) pHEN1 V_H forward primer: 5′-AGCCGGCCATGG CC**CAGGTGCAGCTGTTGG**-3′ (anneals in the 5′-region of V_H genes and introduces *Nco*I site).
   (f) pHEN1 V_H reverse primer: 5′-CTGTTAGCGGCCGC**GC TCGAGACGGTGACCAG**-3′ (anneals in the 3′-region of V_H genes and introduces *Not*I site).
   (g) pHEN1 V_L forward primer: 5′-AGCCGGCCATGGCC **GACATCCAGATGACCCAG**-3′ (anneals in the 5′-region of V_L genes and introduces *Nco*I site).
   (h) pHEN1 V_L reverse primer: 5′-TTCTGCGGCCGC**CCG TTTGATTTCCACCTTGGTC**-3′ (anneals in the 3′-region of V_L genes and introduces *Not*I restriction site).
9. Restriction enzymes *ApaL*I, *Not*I, *Nco*I and restriction enzyme buffers.
10. NEBuffers 2 and 3 (10×) (New England Biolabs, Ipswich MA, USA).
11. BSA (100×).
12. Agarose for gel electrophoresis.
13. TBE buffer.

14. 100 bp ladder DNA length marker.
15. PCR purification kit, Plasmid miniprep kit.
16. Glycerol.
17. Formamide.
18. GoTaq polymerase (Promega, Fitchburg, WI, USA).

2.2. Cloning of Antibody Variable Genes into Phage Display Vectors

1. Restriction enzymes *ApaL*I, *Not*I, *Nco*I, and *Dpn*I and restriction enzyme buffers.
2. NEBuffers 2 and 3 (10×) (New England Biolabs, Ipswich MA, USA).
3. BSA (100×).
4. 2xTY media: 16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl.
5. 2xTY/Tet: 2xTY media supplemented with 15 μg/ml tetracycline.
6. 2xTY/Amp/Glu: 2xTY media supplemented with 100 μg/ml ampicillin and 4% (w/v) glucose.
7. Amicon Ultra filter unit (10 kDa MWCO) (Millipore, Billerica, MA, USA).
8. Sephacryl S-1000 resin (GE Healthcare, Little Chalfont, UK).
9. FLPC system, e.g. AKTA purifier (GE Healthcare, Little Chalfont, UK).
10. T4 DNA ligase and buffer.
11. *Escherichia coli* TG1.
12. TYE agar: 10 g/l bacto-tryptone, 5 g/l yeast extract, 8 g/l NaCl, 15 g/l agar.
13. TYE/Tet: TYE agar supplemented with 15 μg/ml tetracycline.
14. TYE/Amp/Glu: TYE agar supplemented with 100 μg/ml ampicillin and 4% (w/v) glucose.
15. Sequencing primers:
    (a) FdMyc forward primer: 5′-AAATTCACCTCGAAAGCA AGC-3′;
    (b) FdMyc reverse primer: 5′-CCCTCATAGTTAGCGTAAC GA-3′.
    (c) pHEN1 forward primer: 5′-CAGGAAACAGCTATGA CC-3′;
    (d) pHEN1 reverse primer: 5′-CCCTCATAGTTAGCGTAA CGA-3′.
16. Thermocycler.
17. dNTPs: deoxynucleoside triphosphates.
18. Formamide.

19. GoTaq polymerase (Promega, Fitchburg, WI, USA).
20. Agarose for gel electrophoresis.
21. TBE buffer.
22. 100 bp ladder DNA mass markers.
23. PCR purification kit, Plasmid miniprep kit.
24. Glycerol.

2.3. Preparation of ssDNA Template for Kunkel Mutagenesis

1. *E. coli* CJ236.
2. TYE agar.
3. TYE/Tet/Cam: TYE agar supplemented with 15 μg/ml tetracycline and 10 μg/ml chloramphenicol (CJ236 selection).
4. TYE/Amp/Cam/Glu: TYE agar supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, and 4% (w/v) glucose.
5. 2xTY media.
6. 2xTY/Tet/Cam: 2xTY media supplemented with 15 μg/ml tetracycline and 10 μg/ml chloramphenicol.
7. 2xTY/Amp/Cam/Glu: 2xTY media supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, and 4% (w/v) glucose.
8. 2xTY/Amp/Cam/Kan/Glu 0.1%: 2xTY media supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 0.1% (w/v) glucose.
9. Uridine.
10. 0.45 μm vacuum filter unit.
11. 20% (w/v) polyethylene glycol (MW 6,000), 2.5 M NaCl.
12. PBS buffer.
13. QIAprep Spin M13 Kit (Qiagen, Hilden, Germany).

2.4. Synthesis of dsDNA and Analysis by Agarose Gel Electrophoresis

1. Mutagenic oligonucleotides (see Note 2).
2. dU-ssDNA template (described in Subheading 2.3)
3. TM buffer (10×): 500 mM Tris–HCl, 100 mM $MgCl_2$, pH 7.5.
4. 10 mM ATP.
5. mM dNTPs.
6. 100 mM DTT.
7. T4 DNA ligase.
8. T7 DNA polymerase.
9. QIAquick PCR purification kit.
10. 2% (w/v) agarose for gel electrophoresis.
11. TBE buffer.
12. 100 bp ladder DNA length marker.

2.5. Preparation of Electrocompetent E. coli

1. *E. coli* TG1.
2. 2xTY media.
3. Shaking incubator at 37°C.
4. 1 mM HEPES, pH 7.0.
5. MQ water.
6. Glycerol.

2.6. Transformation of Electrocompetent E. coli

1. Electroporator.
2. Electroporation cuvettes.
3. Water bath at 37°C.
4. Shaking incubator, 37°C
5. TYE agar.
6. TYE/Tet agar.
7. TYE/Amp/Glu agar.
8. 2xTY media.

2.7. Analysis of Single Domain Libraries by DNA Sequencing and ELISA

1. Shaking incubator at 30°C.
2. 96-well round-bottom plate—sterile.
3. 1.5 ml tubes.
4. 2xTY media.
5. 2xTY/Tet media.
6. 2xTY/Amp/Glu media.
7. Thermocycler.
8. PCR strip tubes with strip caps.
9. dNTPs: deoxynucleoside triphosphates.
10. Formamide.
11. GoTaq polymerase (Promega, Fitchburg, WI, USA).
12. 96-well MaxiSorp Immunoplate.
13. Recombinant *Staphylococcus aureus* Protein A.
14. Recombinant *Peptostreptococcus magnus* Protein L.
15. Carbonate buffer: 100 mM $NaHCO_3$, 30 mm Na_2CO_3, pH 9.6.
16. PBS buffer.
17. PBST: 0.1% (v/v) Tween-20 diluted in PBS.
18. MPBS: 4% (w/v) skim-milk powder diluted in PBS.
19. KM13 Helper phage (19).
20. 2xTY/Amp/Kan/Glu 0.1%: 2xTY media supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.1% (w/v) glucose.

21. NHS-PEO_4-biotin.
22. 1 M Tris–HCl, pH 7.5.
23. Extravidin-HRP.
24. TMB substrate solution.
25. 1 M H_2SO_4.
26. Platform shaker (optional).
27. ELISA plate washer and plate reader.

3. Methods

3.1. Amplification of Antibody Variable Genes (V_H or V_L) by PCR

1. Prepare template DNA using a suitable DNA purification method such as a QIAprep Spin Miniprep kit. If suitable DNA templates are unavailable, these can be generated by gene synthesis.
2. Amplify DNA encoding the variable domains of interest, introducing the restriction sites *ApaLI* and *Not*I for cloning into phage vector FdMyc, or *NcoI*I and *Not*I for cloning into the phagemid vector pHEN1. This can be achieved by PCR using appropriate forward and reverse primers (as described in the Subheading 2 above).

   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 forward and reverse primer, 200 ng of template DNA, and 5 units of the provided DNA polymerase (Roche).
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 EB buffer.
6. Digest the PCR fragments overnight with *ApaLI* and *Not*I (FdMyc), or *Nco*I and *Not*I (pHEN1) restriction enzymes using restriction enzyme buffer NEB2 or NEB3 respectively, supplemented with BSA. Incubate overnight at 37°C.
7. Purify the digested fragments using a QIAquick PCR purification kit, eluting in 30 μl EB buffer.

3.2. Cloning of Antibody Variable Genes into Phage Display Vectors

1. Digest 100 μg of vector DNA by adding 300 μl of digestion buffer (NEB2 for *ApaLI*/*Not*I or NEB3 for *Nco*I/*Not*I), 30 μl BSA (100×), 200 units of *ApaLI* and 400 units *Not*I restriction enzymes (FdMyc) or 200 units of *Nco*I and 200 units *Not*I restriction enzymes (pHEN1). Adjust volume to 3,000 μl with MQ water. Incubate overnight at 37°C with gentle agitation. Ideally, the vector DNA should be purified by cesium-chloride purification or alternatively be generated by in vitro amplification using *Phi29* polymerase (see Note 1) (20, 21). This is particularly the case for the low copy number FdMyc phage vector, which has a tendency to yield poor quality preparations.
2. 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.
3. Purify digested DNA using a Sephacryl S-1000 column and a FLPC machine (such as an AKTA purifier (GE Healthcare)) 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).
4. Clone digested PCR products into the digested phage display 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 room temperature for 1 h and purify using a QIAquick PCR purification kit, eluting with 30 μl of MQ water (pH 7–8). Transform DNA into *E. coli* TG1 and grow single colonies overnight on a TYE/Tet agar plate (FdMyc) or TYE/Amp/Glu agar plate (pHEN1).
5. Screen for positive clones by PCR. Inoculate single colonies into wells of a 96-well round-bottom plate containing 200 μl of medium (2xTY/Tet for FdMyc, 2xTY/Amp/Glu for pHEN1) and incubate overnight at 37°C, shaking at 250 rpm. Set up a 50 μl PCR reaction containing formamide (2% final), 1× GoTaq buffer (Promega, Madison, WI, USA), 200 μM dNTPs, 400 nM of each forward and reverse primers (as described previously) and 1.25 units of GoTaq polymerase (Promega). Add 2 μl of bacterial culture directly to the reaction.
6. 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.
7. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm the correct size of PCR product.
8. Confirm correct sequence of clones by DNA sequencing.
9. Generate a dsDNA preparation using a QIAprep Spin Miniprep kit following the manufacturer's instructions.

3.3. Preparation of ssDNA Template for Kunkel Mutagenesis

1. Transform dsDNA preparation into *E. coli* CJ236 and grow single colonies overnight on a TYE/Tet/Cam agar plate (FdMyc) or TYE/Amp/Cam/Glu agar plate (pHEN1).
2. Inoculate 5 ml of 2xTY/Tet/Cam media (FdMyc) or 2xTY/Amp/Cam/Glu media (pHEN1) from a single colony of transformed CJ236 bacteria and grow at 37°C, shaking at 250 rpm, until OD_{600nm} reaches >1.
3. Inoculate 100 ml of 2xTY/Tet/Cam media (FdMyc) or 50 ml of 2xTY/Amp/Cam/Glu media (pHEN1) in a conical flask with overnight culture to an OD_{600nm} 0.1.
4. Helper phage rescue (pHEN1 *only*): Grow to an OD_{600nm} 0.6 at 37°C, shaking at 250 rpm. Add 2×10^{11} KM13 helper phages, mix by gentle agitation and incubate at 37°C for 1 h. Centrifuge at $3{,}200\times g$ for 10 min at 4°C. Discard supernatant and using gentle agitation, resuspend the pellet in 100 ml of 2xTY/Amp/Cam/Kan/Glu 0.1% media.
5. Supplement all media with 0.25 μg/ml uridine (Sigma). Incubate overnight at 30°C (16–20 h), shaking at 250 rpm.
6. Centrifuge at $3{,}220\times g$ for 30 min at 4°C.
7. Filter the supernatant by passing through a 0.45 μm filter unit (do not use a 0.22 μm filter at this step).
8. Precipitate the phage by adding 1/5 volume of 20% (w/v) polyethylene glycol (PEG), 2.5 M NaCl to supernatant. Incubate for at least 1 h on ice.
9. Centrifuge at $3{,}220\times g$ for 30 min at 4°C (phage in pellet). Resuspend each pellet in 4 ml PBS.
10. Repeat PEG precipitation by adding 1 ml of 20% PEG, 2.5 M NaCl to phage solution. Incubate for 15 min on ice.
11. Centrifuge $3{,}220\times g$, 30 min (phage in pellet).
12. Resuspend phage pellet in 1 ml PBS.
13. Measure OD_{260nm} (dilute 1/100) to estimate the number of phage using the following formula:

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

14. Isolate ssDNA using QIAprep Spin M13 Kit (Qiagen) according to the manufacturer's instructions.

3.4. Synthesis of dsDNA and Analysis by Agarose Gel Electrophoresis

1. To anneal the mutant oligonucleotide (see Note 2) to the template, combine the following in an Eppendorf tube: 10 μg of dU-ssDNA template, 12.5 μl of 10× TM buffer. 5′-phosphorylated oligonucleotide should be added at a oligo–template molar ratio of 3:1. Add MQ water to 125 μl.
2. Heat to 90°C for 2 min, 50°C for 3 min, and 20°C for 5 min.

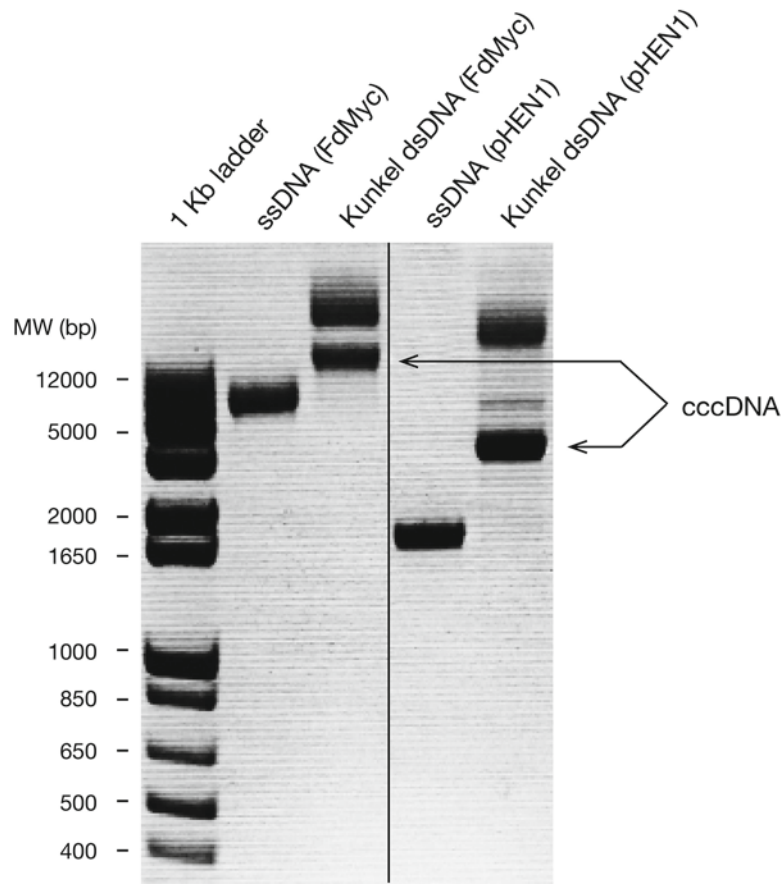

Fig. 1. Generation of dsDNA by Kunkel mutagenesis. Incubation of single strand DNA (ssDNA) with oligonucleotides and polymerase results in the generation of double-stranded DNA (dsDNA). The form that is most efficiently transformed into bacteria is covalently closed circular DNA (cccDNA), while unwanted higher molecular weight products arising from strand-displacement activity of T7 DNA polymerase transform at lower efficiencies (14, 15).

3. Synthesize covalently closed circular DNA (cccDNA) by adding the following to the reaction: 5 µl of 10 mM ATP, 5 µl of 25 mM dNTPs, 7.5 µl of 100 mM DTT, 7.5 µl of T4 DNA ligase (6000 NEB units), and 1.5 µl of T7 DNA polymerase (15 units). Incubate at room temperature (23°C) for at least 3 h.
4. Purify and desalt the DNA using a QIAquick PCR purification kit (Qiagen), eluting in 30 µl of MQ water (pH 7–8).
5. Analyze DNA on a 2% agarose gel in TBE buffer to confirm correct size of products. Expected results are shown in Fig. 1.

3.5. Preparation of Electrocompetent E. coli

1. Grow an overnight culture of TG1 in 2xTY media (2×5 ml).
2. Inoculate 2×500 ml of 2xTY media in 2 L flasks with overnight culture of TG1, diluting to 100:1.

3. Grow at 37°C for 1 h 45 min (or until $OD_{600nm} = 0.5–0.7$).
4. Chill the cells on ice for 1 h.
5. Centrifuge the cells in a prechilled (4°C) rotor at 3,220 × *g* for 30 min.
6. Remove supernatant and resuspend cell pellet in 50 ml of cold 1 mM HEPES pH 7.0, 10% glycerol and transfer to a 50 ml sterile tube (Falcon).
7. Leave on ice for 15 min.
8. Centrifuge the cells at 3,220 × *g* for 15 min at 4°C.
9. Remove supernatant and resuspend cell pellet in 50 ml of cold high purity (MQ) water.
10. Leave on ice for 15 min.
11. Centrifuge the cells at 3,220 × *g* for 15 min at 4°C.
12. Resuspend the cells in 1–2 ml of cold high purity (MQ) water.

3.6. Transformation of Electrocompetent E. coli

1. Chill the purified, mutated dsDNA and electroporation cuvettes (0.2 cm gap; Bio-Rad) on ice.
2. Add a 100 µl aliquot of electrocompetent *E. coli* TG1 to 1–3 µg of DNA and gently mix by pipetting several times.
3. Transfer the mixture to the cuvette and transform using an electroporator (such as Bio-Rad Gene Pulser using the Bacterial EC2 setting (2.5 kV, 25 µF, 200 Ω)). See Note 3.
4. *Phagemid (pHEN1)*: Immediately add 1 ml of 2xTY medium to the cuvette and combine all transformations used for library construction. Plate 1–3 transformations onto a large TYE/Amp/Glu agar plate. Perform serial dilutions in to 2xTY (ten-fold dilution steps) and plate cells onto TYE/Amp/Glu agar plates in order to determine library size.

   Phage (FdMyc): Immediately add 1 ml of 2xTY medium to the cuvette and incubate at 37°C for 60 min, shaking at 250 rpm. Combine all transformations used for library construction and plate as per phagemid transformations above, using TYE/Tet agar plates. Determine library size as described above.
5. Grow the plates overnight at 37°C. Count cells to determine the approximate library size. Expected library sizes are $5–8 \times 10^8$/µg for pHEN1 DNA and $1–2 \times 10^8$/µg for FdMyc DNA.
6. Scrape all the cells from plates using 2xTY medium and combine into one tube on ice. The cells can be frozen in liquid nitrogen and stored at −80°C for future (add sterile glycerol to a final concentration of 20% (v/v)). Alternatively, the cells can be added immediately to 2xTY containing the appropriate antibiotics to generate phage for subsequent antigen selection steps.

3.7. Analysis of Single Domain Libraries by DNA Sequencing and ELISA

1. Select single colonies from library transformations (plates used for library size determination are a good source of single colonies) and grow clones overnight at 37°C in a 96-well round-bottom plate containing 200 µl of medium (2xTY/Tet for FdMyc, 2xTY/Amp/Glu for pHEN1), shaking at 250 rpm.
2. Screen for positive clones by PCR. Set up a 50 µl PCR reaction containing formamide (2% final), 1× GoTaq buffer (Promega), 200 µM dNTPs, 400 nM of each forward and reverse primers (as described previously) and 1.25 units of GoTaq polymerase (Promega). Add 2 µl of bacterial culture directly to the reaction.
3. 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.
4. Analyze PCR products on a 2% agarose gel in TBE buffer to confirm correct size of PCR product.
5. Confirm sequence of clones by DNA sequencing.
6. In addition to DNA sequencing, the display of correctly folded antibody domains on phage can be detected by ELISA using *Staphylococcus aureus* protein A (to detect human V_H3) or *Peptostreptococcus magnus* protein L (to detect human kappa V_L) superantigens (see Note 4 and (22)). Coat a 96-well MaxiSorp Immunoplate (Nunc) with 50 µl/well of Protein A or Protein L at a concentration of 5–10 µg/ml diluted in carbonate buffer pH 9.6. Incubate overnight (~16 h) at room temperature (23°C). Wash the plate once with PBST and block the plate with 4% MPBS for longer than 2 h. Wash the plate three times with PBST before use.
7. Inoculate transformed *E. coli* TG1 into wells of a 96-well round-bottom plate containing 200 µl of medium (2xTY/Tet for FdMyc, 2xTY/Amp/Glu for pHEN1) and incubate overnight at 30°C (FdMyc) or 37°C (pHEN1), shaking at 250 rpm.
8. Helper phage rescue (pHEN1 *only*): The next day, inoculate a second 96-well round-bottom plate with 5 µl of the overnight culture in 200 µl of 2xTY/Amp/Glu. Grow for 3 h at 37°C, shaking at 250 rpm. Add 4×10^8 KM13 helper phages to each well, mix by gentle agitation and incubate at 37°C for 1 h without shaking. Centrifuge at 3,200×*g* for 10 min at 4°C. Discard supernatant by aspiration and using gentle agitation, resuspend the pellets in 200 µl of 2xTY/Amp/Kan/Glu 0.1% media. Grow overnight at 30°C, shaking at 250 rpm.
9. Centrifuge at 3,200×*g* for 10 min at 4°C.
10. Transfer supernatant, containing phage, to a fresh plate and add NHS-PEO_4-biotin to a final concentration of 50 µM and

incubate for 2 h at room temperature (see Note 4). Quench biotinylation reaction by adding Tris–HCl pH 7.5 to a final concentration of 100 mM for 1 h at room temperature.

11. Add biotinylated phages to protein A/L-coated plates at 50 μl/well. Incubate for 1 h at room temperature on platform shaker.
12. Wash ELISA plate three times with PBST and incubate with 50 μl/well of extravidin-HRP (diluted 1:2,000 in 2% BSA in PBST). Incubate for 30 min at room temperature on platform shaker.
13. Wash ELISA plate four times with PBST and develop with 50 μl/well TMB substrate. Incubate at room temperature for 30 min or until sufficient color has developed.
14. Stop reaction by adding 50 μl/well of 1 M H_2SO_4.
15. Read ELISA plate at 450 nm (reference at 620 nm).

4. Notes

1. For details of *Phi*29 amplification *see* refs. (20, 21). In brief:
   (a) Best results are obtained using ssDNA as a template for *Phi*29 amplification. Grow transformed TG1 cells overnight (~16 h) at 30°C in 2xTY/Tet media. Remove cells by centrifugation and PEG precipitate phage as described above in Subheading 3. Once purified phage is obtained, isolate ssDNA using QIAprep Spin M13 Kit according to the manufacturer's instructions.
   (b) Set up the *Phi*29 DNA amplification reaction containing 100 μl of 10× *Phi*29 buffer, 500 μM dNTPs, 50 μM random hexamers and 1 μg of template DNA, adjust volume to 1 ml with MQ 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.
   (c) 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). Expected yields are in the range of 50–100 μg per ml. Continue protocol by digesting the vector DNA. Adjust buffer conditions by diluting reaction into appropriate NEB digestion buffer.
2. Mutagenic oligonucleotides should be 5′-phosphorylated and designed to include around 21 bp of complementary sequence

both upstream and downstream of the randomized area. Quality of DNA synthesis is essential and longer oligonucleotides should be PAGE purified. It is essential to design oligonucleotides that anneal to the ssDNA packaged by the phage particle: this will usually require reverse complementation of the primer sequence.

3. Carefully monitor time constants. For the Bio-Rad Gene Pulser time constants of between 5 and 6 ms should be obtained. Lower time constants indicate the presence of residual salt and require further desalting or reduction of DNA concentration.
4. Direct biotinylation of phage in solution is essential as protein A and protein L bind to most antibody-based secondary reagents usually used in phage ELISA (such as anti-M13-HRP).

Acknowledgments

The protocols are based on methods originally developed in Greg Winter's group at the MRC Laboratory of Molecular Biology and were modified in our laboratory at the Garvan Institute. This work was funded by the Garvan Institute of Medical Research, the Australian National Health and Medical Council, the Australian Research Council, the Cancer Institute NSW, and the United Kingdom Medical Research Council.

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