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Construction of synthetic phage displayed Fab library with tailored diversity

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Nov 28, 2017

Dr. Mohammad Alaghemandi
Review Editor
JoVE

Dear Dr. Alaghemandi,

Please find the revised manuscript “**Construction of synthetic phage displayed Fab library with tailored diversity**” we have submitted online for publication as a **protocol** in “**JoVE**”. Based on the valuable and critical comments from reviewers and editors, substantial revisions have been made and we believe that the manuscript is now well qualified for publication and this protocol will undoubtedly guide the novices in the field of antibody engineering to construct phage displayed antibody libraries step by step. We confirm that this manuscript has not been submitted for publication elsewhere.

As our lab is moving house during the next several months and we expect that moving will be complete till early Feb of 2018, we ask that this article will be in press on 15/Feb/2018 together with the video shooting. Hope you can understand. Sorry for the inconvenience caused.

I am the corresponding authors for this paper. (**Dr. Donghui Wu**, Shanghai Institute for Advanced Immunochemical Studies, ShanghaiTech University, Shanghai, China 201210. email: wudh@shanghaitech.edu.cn; Tel: 86-21-20685138.).

I thank you for your kind attention and look forward to hearing from you in due course.

Yours Sincerely,

Donghui Wu, PhD
Co-Principal Investigator
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TITLE:

Construction of Synthetic Phage Displayed Fab Library with Tailored Diversity

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KEYWORDS:

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SUMMARY:

This protocol describes a detailed procedure for the construction of a phage-displayed synthetic antibody library with tailored diversity. Synthetic antibodies have broad applications from basic research to disease diagnostics and therapeutics.

ABSTRACT:

Demand for monoclonal antibodies (mAbs) in basic research and medicine is increasing yearly. Hybridoma technology has been the dominant method for mAb development since its first report in 1975. As an alternative technology, phage display methods for mAb development are increasingly attractive since Humira, the first phage-derived antibody and one of the best-selling mAbs, was approved for clinical treatment of rheumatoid arthritis in 2002. As a non-animal based mAb development technology, phage display bypasses antigen immunogenicity, humanization, and animal maintenance that are required from traditional hybridoma technology based antibody development. In this protocol, we describe a method for

construction of synthetic phage-displayed Fab libraries with diversities of 10^9 – 10^{10} obtainable with a single electroporation. This protocol consists of: 1) high-efficiency electro-competent cell preparation; 2) extraction of uracil-containing single-stranded DNA (dU-ssDNA); 3) Kunkel's method based oligonucleotide-directed mutagenesis; 4) electroporation and calculation of library size; 5) protein A/L-based enzyme-linked immunosorbent assay (ELISA) for folding and functional diversity evaluation; and 6) DNA sequence analysis of diversity.

INTRODUCTION:

mAbs have broad applications ranging from basic research to disease diagnostics and therapeutics. As of 2016, more than 60 mAbs have been approved by the United States Food and Drug Administration (USFDA) for clinical treatment of autoimmune diseases, cancer, and infectious diseases^{1,2}.

In 1975, Kohler and Milstein reported a technique for the continuous generation of antibodies of a single clonal specificity from a cellular source referred to as 'hybridomas' and this technique has subsequently become a cornerstone in medicine and industry^{3,4}. Generation of mAbs by this method requires various steps including antigen production, mouse immunization, extraction of B lymphocytes, fusion of B cells with myeloma cells to form immortal hybridoma cells, clone selection, and for therapeutic applications, humanization is required to avoid human anti-mouse antibody (HAMA)^{4,5}. However, for this technology, antigens including toxins, pathogens, and highly conserved proteins are relatively ineffective in triggering an *in vivo* immune response for mAb production⁵.

In 1978, Hutchison *et al.* reported the use of an oligonucleotide to direct mutagenesis of a residue in a single-stranded bacteriophage virus⁶. In 1985, Smith reported that foreign gene fragments can be fused in frame with the gene encoding phage coat protein III and can thus be displayed on the phage surface without compromising its infectivity⁷. These pioneering works laid a foundation for the subsequent construction of phage-displayed antibody libraries in immune, naïve, and synthetic forms with the formats of single-chain variable fragment (scFv) and antigen-binding fragment (Fab) for therapeutic mAb development^{8,9}. From the technical point of view, phage display-based antibody development offers a complementary approach to hybridoma-based mAb development that can help to circumvent the limitations some antigens can pose and the humanization process that hybridoma-derived antibodies often require⁵. As of 2016, 6 phage display-derived mAbs have been approved in the market including Humira, one of the most successful mAbs used for treatment of rheumatoid arthritis, and many phage display-derived antibody candidates are currently at various stages of clinical investigation¹⁰.

For immune and naïve phage antibody libraries, the diversity of complementarity-determining regions (CDRs) in light and heavy chain is derived from the natural immune repertoire (*i.e.*, from B cells). In contrast, the diversity of CDRs in synthetic phage antibody libraries is entirely artificial. Synthetic approaches to library construction provide precise control over the design of sequence diversity and offer opportunities for mechanistic studies of antibody structure and function^{11,12}. Moreover, the framework for synthetic libraries can be optimized before library construction to facilitate downstream, large-scale industrial development^{11,12}.

In 1985, Kunkel reported a single-stranded DNA (ssDNA) template-based mutagenesis approach to introduce site-directed mutations into M13 bacteriophage efficiently¹³. This approach was subsequently used widely for construction of phage-displayed libraries. Chemically synthesized DNA oligonucleotides designed to introduce diversity into Fab CDRs are incorporated into a phagemid with an antibody backbone template. In this process, the phagemid is expressed as a uracil-containing ssDNA (dU-ssDNA) and the oligonucleotides are annealed onto the CDRs and extended to synthesize double-stranded DNA (dsDNA) in the presence of T7 DNA polymerase and T4 DNA ligase. Finally, generated ds-DNA can be introduced into *Escherichia coli* by electroporation.

For high diversity, phage-displayed library construction, high-voltage electroporation of a two-component mixture of electro-competent cells and covalently closed circular dsDNA (CCC-dsDNA) should be prepared carefully. Sidhu *et al.* modified the preparation of electro-competent cells and DNA from traditional methods and greatly improved library diversity¹⁴.

In this protocol, we describe a method for construction of synthetic phage-displayed Fab libraries with diversities of 10^9 – 10^{10} obtainable with a single electroporation. **Figure 1** shows an overview of library construction including: 1) high-efficiency electro-competent cell preparation; 2) extraction of dU-ssDNA; 3) Kunkel's method based oligonucleotide-directed mutagenesis; 4) electroporation and calculation of library size; 5) protein A/L-based ELISA for folding and functional diversity evaluation; and 6) DNA sequence analysis of diversity. All the reagents, strains and equipment are listed in the **Material's Table**. **Table 1** shows the reagent setup.

PROTOCOL:

Note: Filter sterile tips must be used throughout when dealing with phage to avoid contamination to pipette gun and surrounding area. Aseptic area or hood must be used when handling with bacteria and phage experiments. Phage experiment area must be cleaned up using 2% sodium dodecyl sulfate (SDS) followed by 70% ethanol to avoid phage contamination. For making serial dilutions in this protocol, new tips should be used for each dilution.

1 *E. Coli* SS320 Electro-competent Cell Preparation

1.1. Pre-warm LB/tet agar plate (prepared and stored at 4 °C for less than 1-week old) at 37 °C incubator for 1 h. Use a sterile inoculation loop or sterile tip to streak out a glycerol stock of *E. coli* SS320 cells onto the pre-warmed LB/tet agar plate in a zig-zag direction gently along the surface of the plate. Incubate the plate at 37 °C incubator overnight for around 12–15 h.

1.2. The following day, use a sterile inoculation loop or sterile tip to pick a well-separated single colony along the zig-zag line and inoculate into 10 mL of 2YT/tet medium in a 50-mL round bottom tube by dipping the loop into the medium and stirring briefly.

1.3. Incubate at 37 °C with shaking at 200 rpm for around 3–4 h until OD600 is reached at

around 0.4–0.8 (log phase growth). Monitor OD600 at 2 h. During this period, pre-warm 5 LB/tet agar plates (prepared and stored at 4 °C for less than 1-week old) in a 37 °C incubator for at least 1 h.

1.4. Add 20 µL M13KO7 helper phage from lab stock with titer around 1×10^{13} colony forming unit (cfu)/mL into 180 µL of sterile 1X PBS in autoclaved 1.5-mL tubes to prepare 10 ten-fold serial dilutions.

1.5. Aliquot 4 mL autoclaved and liquid 2YT top agar into 5 X 14 mL round bottom tubes, label each tube with one dilution from the fifth to the ninth of the ten-fold dilution, respectively, and incubate in a 65 °C incubator to maintain the 2YT top agar in the liquid state.

1.6. Mix 500 µL of log phase *E. coli* SS320 cells into the M13KO7 dilution tube (choose the fifth ten-fold dilution to the ninth ten-fold dilution) and incubate in a 37 °C incubator for 5–10 min.

1.7. During the incubation of step 1.6, transfer 2YT top agar from step 1.5 to room temperature (RT) to cool down for around 5 min. Use inner wrist to test temperature of 2YT top agar; it should remain as liquid. Transfer each dilution mixture from step 1.5 to each corresponding 2YT top agar tube. Tighten the lid of each tube and mix by turning upside down several times gently and briefly to avoid bubble generation.

1.8. Carefully pour each mixture along the edge of a pre-warm LB/tet agar plate (from step 1.3) and slant the plate slightly to fully and evenly fill the plate with the mixture while avoiding the introduction of bubbles. Keep the plates at RT for around 5–10 min to solidify the top agar within each plate and incubate the 2YT top agar plates at 37 °C overnight for around 15–18 h.

1.9. Choose the dilution plate after overnight growth from step 1.8 with around 100–200 average-sized, single, well-separated plaques for plaque inoculation. Use one hand to hold the agar plate against a light source, and the other hand to hold a pipette gun loaded with a long sterile pipette tip to vertically stab into the top agar, and collect a single and well-separated plaque.

1.9.1. Slant the pipette tip slightly to separate top agar with plaque. Pipette up and down several times to dislodge the agar with plaque into a 14-mL round bottom culture tube pre-loaded with 1 mL of 2YT/kan/tet medium (see **Table 1**).

1.9.2. Repeat this procedure to pick 3–5 plaques in total. The purpose of picking several plaques is to ensure successful inoculation, as a plaque can be faint and relatively small when compared with a bacteria colony. Grow the plaques for 8 h at 37 °C with shaking at 200 rpm.

1.10. Transfer a tube of growing culture into 50 mL of 2YT/kan/tet medium in a 250-mL baffled flask. Grow at 37 °C with shaking at 200 rpm overnight for around 12 h.

- 1.11. Inoculate three 2-L baffled flasks containing 900 mL of superbrotth/tet/kan medium each with 5 mL of the overnight culture. Incubate at 37 °C with shaking at 200 rpm for 6–7 h to OD600 around 0.6–0.8.
- 1.12. Chill the three flasks of bacterial culture in an ice bath for 5 min with gentle constant swirling by hand. The following steps should be done in a cold room, on ice, with prechilled solutions and equipment.
- 1.13. Use absorbance tissue towel to dry the outer glass of each flask; use one hand to slant the 1-L autoclaved centrifuge bottle on bench and the other hand to pour the medium gently from each flask into each centrifuge bottle.
- 1.14. Spin at 5,000 × g and 4 °C for 10 min to pellet the bacteria.
- 1.15. Following centrifugation, gently decant the supernatant into a 5-L autoclaved waste beaker.
- 1.16. Fill each bottle with 100 mL of sterile-filtered 1.0 mM HEPES, pH 7.4, and add an autoclaved magnetic stir bar to each bottle to aid in pellet resuspension at 200 rpm. Swirl and dislodge the entire pellet from the bottle wall every several minutes during the magnetic stirring. Once the pellet is dissolved, fill each bottle with an additional 400 mL of sterile-filtered 1.0 mM HEPES, pH 7.4.
- 1.17. Centrifuge at 5,000 × g and 4 °C for 10 min. Decant the supernatant, retaining the stir bar in the bottle.
- 1.18. Repeat steps 1.16 to 1.17 once.
- 1.19. Resuspend each pellet in 150 mL of sterile-filtered, 10% ultrapure glycerol with the aid of the stir bars. Swirl and dislodge the entire pellet from the bottle wall every several minutes during the magnetic stirring. Once the pellet is dissolved, fill each bottle with an additional 400 mL of sterile-filtered 1.0 mM HEPES, pH 7.4. Centrifuge and decant as in step 1.17.
- 1.20. Repeat step 1.19 once. Use long-arm autoclaved forceps to remove the stir bar.
- 1.21. Centrifuge at 5,000 × g and 4 °C for 15 min. Decant the supernatant and remove any remaining traces of supernatant from each centrifuge bottle with a pipette.
- 1.22. Add 3.0 mL of 10% ultrapure glycerol to one bottle and gently resuspend the pellet by pipetting. Transfer the suspension to the next bottle and repeat until all of the pellets are resuspended and combined in one bottle. Approximately 6 mL of highly concentrated cells are obtained with a titer of around 3×10^{11} cfu/mL.
- 1.23. Pre-chill 1.5-mL autoclaved microcentrifuge tubes and one 96-well tube storage box

without dividers at -80°C for at least 1 h before this step. Transfer the pre-chilled microcentrifuge tubes to the cold room on ice before pipetting aliquots of the cell pellet suspension. Use a pipette to aliquot 350 μL of cell suspension into each 1.5 mL microcentrifuge tube.

1.24. Transfer the aliquots into a foam box container filled with liquid nitrogen for flash freezing (3–5 min).

1.24.1. Transport the foam box with liquid nitrogen to a -80°C freezer (see step 1.23).

1.24.2. Remove the 1.5-mL microcentrifuge tube storage box from the -80°C freezer (see step 1.23) and put on ice.

1.24.3. Quickly use a metal mesh to sieve the aliquots from liquid nitrogen and place them in the tube storage box. Store at -80°C .

CAUTION: Liquid nitrogen can cause burns and care must be taken for safety protection.

1.25. Use a Fab backbone phagemid to check the efficiency of the prepared electro-competent cells (the Fab backbone phagemid stock should be in ultrapure (MilliQ) water at 400 ng/ μL). Thaw 1 μg of the Fab backbone phagemid in 10 μL ultrapure water and a 350 μL aliquot of electro-competent SS320 on ice, and prechill a 0.2-cm gap electroporation cuvette on ice.

1.26. Add the 350 μL electro-competent SS320 cells to the 10 μL DNA after thawing and mix by pipetting several times while keeping the mixtures on ice. Avoid introducing bubbles.

1.27. Pre-warm 15 mL of SOC medium in a 50-mL tube in a 37°C water bath for at least 30 min before electroporation. Transfer the 360 μL mixture to the cuvette and perform electroporation following the manufacturer's instructions.

1.28. Immediately rescue the cells after electroporation by adding 1 mL of pre-warmed SOC medium (from step 1.27) and pipetting. Transfer the medium from the cuvette to a 125-mL baffled flask pre-loaded with 5 mL of pre-warmed SOC.

1.29. Rinse the cuvette twice, each time with 1 mL of pre-warmed SOC medium, and transfer the medium to the 125-mL baffled flask (see step 1.28). Add pre-warmed SOC medium to a final volume of 10 mL to the 125-mL baffled flask.

1.30. Incubate the 10-mL cell culture for 30 min at 37°C with shaking at 200 rpm.

1.31. Make serial dilutions to determine the transfection efficiency and M13KO7 pre-infection rate.

1.31.1. Use a multi-channel pipette to add 180 μL of 2YT media to each well of a single column

of a 96-microwell plate.

1.31.2. Make 8 tenfold serial dilutions: transfer 20 μ L of culture from step 1.30 to the first well of the plate, mix by pipetting, and transfer 20 μ L of the mixture to the next well. Repeat this step to the end of the serial dilution.

1.31.3. Plate 10 μ L of each serial dilution on the LB/carb, LB/kan, and LB/tet plates in duplicate.

1.31.4. Incubate overnight at 37 °C.

1.31.5. Efficiency calculation formula: Assume that M is the average colony number counted from the diluted fold 10^N (N is from 1–8). The *E. coli* SS320 electro-competent cell transfection efficiency of the Fab backbone phagemid from LB/carb plate is equal to $M \times 10^{N+3}$ cfu/ μ g. The M13KO7 pre-infection rate is estimated from the ratio of the colonies in LB/kan and LB/tet. The percentage of *E. coli* SS320 competent cells transfected with the Fab backbone phagemid is estimated from the ratio of colonies in LB/carb and LB/kan.

2. Preparing Uracil-containing ssDNA (dU-ssDNA) from the Phagemid Template

Note: A previously reported Fab backbone phagemid was used as the template for dU-ssDNA preparation¹⁵. The architecture of the Fab backbone phagemid is shown in **Figure 2**. A plasmid spin kit (QIAprep Spin M13) is used for extraction of dU-ssDNA with slight modifications.

2.1. Pre-warm a LB/cmp plate (prepared and stored at 4 °C for less than 1-week old) in a 37 °C incubator for 1 h. Streak out a glycerol stock of *E. coli* CJ236 cells (or another dut–/ung– strain) with sterile loops or tips and using laminar flow onto the pre-warmed LB/cmp plate. Incubate the plate at 37 °C overnight for around 12–15 h.

2.2. Pick a single colony with a sterile tip and inoculate into 2 mL of 2YT/cmp medium in a 14-mL polypropylene round-bottom tube.

2.3. Incubate the medium at 37 °C with shaking at 200 rpm for 3–4 h until OD600 is reached at around 0.4–0.8 (log phase growth).

2.4. Add 5–10 μ L phage of Fab backbone template into the culture, and incubate at 37 °C with shaking at 200 rpm for 30 min to allow phage infection.

2.5. After incubation, use a sterile tip to streak out 10 μ L of culture onto a pre-warmed LB/carb plate at 37 °C. Incubate at 37 °C overnight for around 12–15 h.

2.6. Pick a single colony of the *E. coli* CJ236 containing Fab backbone phagemid to inoculate a starter culture of 3 mL 2YT/carb/cmp in a 14-mL polypropylene round-bottom tube, and grow at 37 °C with shaking at 200 rpm overnight for around 12 h.

- 2.7. Inoculate the 0.3 mL starter culture into 30 mL 2YT/carb/cmp in a 250-mL baffled bottle.
- 2.8. Incubate the cell culture at 37 °C with shaking at 200 rpm for 3–4 h until OD600 is reached at around 0.4–0.8 (log phase growth).
- 2.9. Add the M13KO7 (lab stock, titer of approximately 1×10^{13} cfu/mL) to the culture from step 2.8 with a multiplicity of infection (MOI) of approximately 10 and the final titer of M13KO7 of approximately 1×10^{10} cfu/mL.
- 2.10. Incubate at 200 rpm and 37 °C for 1 h.
- 2.11. Pellet the culture by centrifugation in a 50-mL round-bottom tube at $5,000 \times g$ and 25 °C for 20 min.
- 2.12. Decant the supernatant and resuspend the pellet with 50 mL of fresh 2YT/carb/kan/uridine. Transfer the resuspension into a new 250-mL baffled bottle.
- 2.13. Incubate at 200 rpm and 25 °C for 22–24 h.
- 2.14. Transfer the culture from step 2.13 into a 50-mL round-bottom tube and centrifuge the culture at $12,000 \times g$ for 20 min to separate the phage supernatant from bacterial cell pellet. Transfer the phage supernatant into a new 50-mL round-bottom tube and add 1/5 the final volume of PEG/NaCl solution to precipitate the phage. Mix well and incubate on ice for 30 min to precipitate the phage particles.
- 2.15. Centrifuge at $12,000 \times g$ and 4 °C for 30 min. Decant the supernatant and centrifuge at $4,000 \times g$ and 4 °C for 2 min. Aspirate the remaining supernatant.
- 2.16. Resuspend the phage pellet in 2 mL of sterile-filtered 1X PBS and transfer to 1.5-mL microcentrifuge tubes. Centrifuge at $12,000 \times g$ for 5 min in a benchtop microcentrifuge to remove any remaining bacterial debris and transfer the phage supernatant to new 1.5-mL microcentrifuge tubes and store at 4 °C.
- 2.17. Check the uracil incorporation efficiency in *E. coli* CJ236 through side by side comparison with *E. coli* SS320.
 - 2.17.1. Add 180 µL of 2YT media to each well of a single row of a 96-well plate.
 - 2.17.2. Make ten 10-fold serial dilutions: transfer 20 µL of the phage supernatant to the first well of the plate, mix by pipetting, and transfer 20 µL of the mixture to the next well. Repeat this step to the end of the serial dilution.
 - 2.17.3. Add 10 µL of phage from last eight serial dilutions to infect 90 µL of *E. coli* CJ236 and *E. coli* SS320 in log phase (OD600 = 0.4–0.8). Incubate at 37 °C for 30 min with gentle shaking.

2.17.4. Plate 10 μ L from the serial dilution of each infection in *E. coli* CJ236 and *E. coli* SS320 on 2YT/Carb plates.

2.17.5. Incubate overnight at 37 °C.

2.17.6. Titer calculation formula: Assume that M is the average colony number counted from the diluted fold 10^N (N is from 1–10). The titer from *E. coli* CJ236 or *E. coli* SS320 is equal to $M \times 10^{N+2}$ cfu/mL. Estimate the efficiency of uracil incorporation from the titer ratio of *E. coli* CJ236 and *E. coli* SS320.

2.18. Add 1/100 volume of the phage precipitation buffer MP into the phage supernatant in 1.5-mL microcentrifuge tubes, and turn upside down gently to mix for several times. Incubate at RT for at least 2 min. Phage particles are precipitated from the culture medium, and thus, a cloudy solution should be visible at this point.

2.19. Apply the sample from step 2.18 to a plasmid spin column (*e.g.*, QIAprep) in a 1.5-mL microcentrifuge tube. The binding capacity of one spin column for ssDNA can reach at least 10 μ g. Centrifuge at $6,000 \times g$ and 25 °C for 30 s in a benchtop microcentrifuge. Discard the flow-through which is in the 1.5-mL microcentrifuge tube. Phage particles remain bound to the column matrix at this stage.

2.20. Add 0.7 mL of the UT-MLB phage lysis and binding buffer (see **Discussion**) to the column. Centrifuge at $6,000 \times g$ and 25 °C for 30 s and discard the flow-through.

2.21. Add another 0.7 mL of the UT-MLB buffer and incubate at RT for at least 1 min.

2.22. Centrifuge at $6,000 \times g$ for 30 s. Discard the flow-through. At this stage, the phage coat protein is separated from the dU-ssDNA, which remains bound to the column matrix.

2.23. Add 0.7 mL of wash buffer PE containing ethanol following the manufacturer's instructions. Centrifuge at $6,000 \times g$ for 30 s and discard the flow-through.

2.24. Repeat step 2.23, then centrifuge once more at $6,000 \times g$ for 30 s to remove residual buffer PE.

2.25. Transfer the column to a new 1.5-mL microcentrifuge tube and add 100 μ L of elution buffer EB (10 mM Tris-CL, pH 8.5) to the center of the column membrane.

2.26. Incubate at RT for 10 min and centrifuge at $6,000 \times g$ for 1 min to elute the dU-ssDNA. Approximately, 1.5–2.5 μ g dU-ssDNA/mL culture can be obtained.

2.27. Analyze the eluted DNA by electrophoresing 1 μ L on a 1% agarose TAE gel. The DNA should appear as a predominantly single band with no smearing.

2.28. Determine the DNA concentration by measuring absorbance on a nanodrop spectrophotometer at 260 nm ($A_{260} = 1.0$ for 33 ng/ μ L of ssDNA). Typical dU-ssDNA concentrations are within 200–500 ng/ μ L.

3. Kunkel's Method Based Oligonucleotide-directed Mutagenesis

Notes: It is advisable to conduct small-scale reactions prior to full scale reactions to ensure the quality of the mutagenic oligonucleotide and reaction components. A cartoon of Kunkel's method based oligonucleotide-directed mutagenesis is shown in **Figure 3**. Various amino acid diversities are introduced into CDRH1, CDRH2, CDRH3, and CDRL3 regions with IMGT numbering nomenclature¹⁶ (**Table 2**). The theoretical amino acid diversity of each CDR, total theoretical amino acid diversity, and oligonucleotide sequences are listed in **Table 2**.

3.1. Oligonucleotide phosphorylation with T4 polynucleotide kinase

3.1.1. Combine 0.6 μ g of mutagenic oligonucleotides, designed to mutate a single CDR, with 2 μ L 10X TM buffer, 2 μ L 10 mM ATP, and 1 μ L 100 mM DTT. Add ultrapure H₂O to a total volume of 18 μ L in a 1.5-mL tube. For the library construction, 4 separate and parallel phosphorylation reactions are set up corresponding to CDRH1, CDRH2, CDRH3, and CDRL3, respectively.

3.1.2. Add 20 U (2 μ L) of T4 polynucleotide kinase to each tube and incubate for 1 h at 37 °C (Reaction 1 in **Table 3**). Use immediately for annealing.

3.2. Annealing of the oligonucleotides to the template

3.2.1. To 20 μ g of dU-ssDNA template, add 25 μ L of 10X TM buffer, 20 μ L of each phosphorylated oligonucleotide solution, and ultrapure H₂O to a final volume of 250 μ L in a 0.5-mL tube. Mix well and transfer into 0.20 mL PCR tubes (50 μ L each) as Reaction 2 in **Table 3**. These DNA quantities provide a molar ratio of 3:1 between oligonucleotide and template, assuming that the length ratio of oligonucleotide and template is 1:100.

3.2.2. Incubate the reaction in a PCR machine at 90 °C for 3 min, 50 °C for 3 min, and 20 °C for 5 min.

3.3. Enzymatic synthesis of CCC-dsDNA

3.3.1. To the 250 μ L annealed oligonucleotide/template mixture, add 10 μ L of 10 mM ATP, 10 μ L of 25 mM dNTP mix, 15 μ L of 100 mM DTT, 30 Weiss units T4 DNA ligase, and 30 U T7 DNA polymerase as Reaction 3 in **Table 3**.

3.3.2. Incubate the reaction in the 1.5-mL tube at 20 °C overnight.

3.3.3. Wash and concentrate the synthesized CCC-dsDNA in a 0.5-mL centrifugal filter device

with a 30 kDa pore size membrane at RT.

3.3.3.1. Transfer the overnight reaction mixture to the filter device and add ultrapure H₂O to 400 µL final volume. Spin at 14,000 × g for 10 min; the volume is less than 50 µL.

3.3.3.2. Discard the flow through, add 400 µL of ultrapure H₂O into the filter, and spin at 14,000 × g for 10 min.

3.3.3.3. Repeat step 3.3.3.2 once more.

3.3.3.4. Place the filter upside down in a clean microcentrifuge tube to recover the CCC-dsDNA. Spin at 1,000 × g for 2 min; the recovered volume is generally around 20–40 µL. The recovered CCC-dsDNA can be used immediately for electroporation of *E. coli* or frozen at -20 °C for later use. Normally 20–40 µg CCC-DNA can be obtained.

3.3.4. Electrophorese 1.0 µL of the eluted reaction product alongside the dU-ssDNA template to visualize the outcome of the reaction.

4. Electroporation and Calculation of the Library Size

4.1. Chill the purified CCC-dsDNA (20 µg in a maximum volume of 50 µL) in a 1.5-mL microcentrifuge tube and a 0.2-cm gap electroporation cuvette on ice.

4.2. Pre-warm 20 mL of SOC media in a 50-mL polypropylene conical centrifuge tube in 37 °C water bath for at least 30 min.

4.3. Thaw a 350 µL aliquot of electro-competent *E. coli* SS320 on ice. Add the cells to the DNA and mix thoroughly by pipetting several times. Avoid introducing bubbles.

4.4. Transfer the mixture to the cuvette and perform electroporation following the manufacturer's instructions. For example, when the BTX ECM-630 electroporation system is used, the relevant settings are 2.5 kV field strength, 125 Ω resistance, and 50 µF capacitance.

4.5. Immediately rescue the electroporated cells by adding 1 mL of pre-warmed SOC medium and transferring into 17 mL of SOC medium in a 125-mL baffled flask. Rinse the cuvette twice with 1 mL of SOC medium and transfer to the same flask (final volume is 20 mL).

4.6. Incubate for 30 min at 37 °C with shaking at 200 rpm.

4.7. Determine the electroporation efficiency.

4.7.1. Add 180 µL of 2YT media to each well of a single column of a 96-microwell plate.

4.7.2. Make 8 ten-fold serial dilutions: transfer 20 µL of the 20-mL culture to the first well of

the plate, mix with pipetting, and transfer 20 μ L of the mixture to the next well. Repeat this step to the end of the serial dilution.

4.7.3. Plate 10 μ L of each of the serial dilutions on one LB/carb plate in duplicate. Plate 100 μ L remaining from each of the serial dilutions onto separate LB/carb plates for colony count cross-check. These plates will also provide single clones for ELISA and sequence analysis (see section 5).

4.7.4. Incubate overnight at 37 °C.

4.7.5. Count the colonies from the 10 μ L duplicates on the LB/carb plate. Assume that M is the average colony number counted on 10^N fold LB/carb plate (N is from 1–8). The total library size is equal to $2M \times 10^{N+3}$ colonies.

4.8. Aliquot the culture from step 4.6 equally into two 2-L baffled flasks, each containing 500 mL of 2YT/carb/kan medium for phage library generation.

4.9. Incubate at 37 °C with shaking at 200 rpm overnight for around 16 h.

4.10. Transfer the culture to two 1-L autoclaved centrifuge bottles and centrifuge for 30 min at $12,000 \times g$ at 4 °C.

4.11. Transfer the supernatant to two new 1-L autoclaved centrifuge bottles and add 1/5 the final volume of PEG/NaCl solution to precipitate the phage. Incubate on ice for 30 min.

4.12. Centrifuge for 30 min at $12,000 \times g$ and 4 °C. Carefully decant the supernatant and avoid disturbing of the phage pellet. Spin for 1 min at $4,000 \times g$ and remove the remaining supernatant with a pipette.

4.13. Resuspend the phage pellet with 20 mL of sterile-filtered 1X PBS buffer and transfer to a new 50-mL tube.

4.14. Pellet the insoluble matter by centrifuging for 5 min at $12,000 \times g$ and 4 °C. Transfer the supernatant to a new 50-mL tube.

4.15. Measure the phage concentration by spectrophotometer ($OD_{268} = 1.0$ for a solution of 5×10^{12} phage/mL).

4.16. Adjust the phage concentration to 5×10^{12} phage/mL in 1X PBS with 10% ultrapure glycerol.

4.17. Aliquot 1 mL of phage solution per 1.5-mL microcentrifuge tube. Use the libraries immediately for panning or store at -80 °C.

5. Quality Assessment by Protein A/L Direct Binding ELISA Assay and Sequencing

5.1. Randomly pick 96 single colonies on LB/carb plate from step 4.7.3 into a 96-deep well culture plate containing 800 μL of 2YT/carb in each well. Incubate for 3–4 h at 37 °C with shaking at 1,000 rpm to $\text{OD}_{600} = 0.4\text{--}0.8$.

5.2. Add 100 μL of M13KO7 (1×10^{11} cfu/mL) to each well of a 96-deep well culture plate with a multichannel pipette. Incubate at 37 °C with shaking at 1,000 rpm for 1 h.

5.3. Add 100 μL of 2YT containing 10X concentration of kanamycin (500 $\mu\text{g/mL}$) to each well with a multichannel pipette. Incubate overnight at 37 °C with shaking at 1,000 rpm.

5.4. Dissolve protein L to 1 $\mu\text{g/mL}$ in 1X PBS. Coat 3/4 of the wells in a 384-well high protein-binding polystyrene plate with 30 $\mu\text{L/well}$ of the protein L solution. Incubate overnight at 4 °C.

5.5. Discard the overnight protein L coating solution from step 5.4. Add 50 μL of M-PBST (the blocking solution) to all of the wells in the 384-well high protein-binding polystyrene plate with a multichannel pipette. Incubate the plates on a microplate shaker for 1 h at RT.

5.6. Spin down the overnight culture from step 5.3 in a deep 96-well culture plate at 3,000 x g for 10 min at 4 °C. The phage is in the supernatant.

5.7. Discard the blocking solution from step 5.5. Add 15 μL of M-PBST and 15 μL of each phage supernatant (step 5.6) to 3 of the protein L coated wells as triplicate, and 1 non-coated well as negative control using a multichannel pipette. Incubate at RT for 1 h with shaking at 200 rpm.

5.8. Discard the phage solution. Wash the plate 6 times with 80 μL of PBST by an automated plate washer.

5.9. Add 15 μL of M-PBST and 15 μL of Protein A-HRP (1:1,500 diluted in 1X PBS) to each well with a multichannel pipette, and incubate at RT for 1 h with shaking at approximately 200 rpm.

5.10. Discard the Protein A-HRP/M-PBST solution. Wash the plate 6 times with 80 μL of PBST.

5.11. Add TMB substrate (30 $\mu\text{L/well}$) with a multichannel pipette and incubate for 2–3 min until the color develops. Stop the reaction with 1.0 M H_3PO_4 (30 $\mu\text{L/well}$).

5.12. Read the plates spectrophotometrically at 450 nm. Positive clones are defined as those that exhibit an average ratio of OD_{450} absorbance of protein L wells to the M-PBST well greater than 3.0.

5.13. In a 96-deep well, infect 50 μL of SS320 in 2YT/tet at log phase with 5 μL of the same phage used for the ELISA (step 5.6) for 30 min at RT.

5.14. Add 950 μ L of 2YT/carb into the 96-deep well from step 5.13 and incubate overnight at 37 °C with shaking at 1,000 rpm.

5.15. Extract the phagemid DNA by mini-prep DNA extraction kit. Use the upstream primers of VL (5'-TCGCTTTGTTTTATTTTAAATGTA-3') and VH (5'-GACTACTAATAACATAAAGTCTACGCCG-3') for sequence analysis to estimate the library sequence diversity.

REPRESENTATIVE RESULTS:

Following the flow chart of the Fab library construction (see **Figure 1**), we prepared M13KO7 helper phage pre-infected *E. coli* SS320 electro-competent cells. The efficiency of these electro-competent cells is estimated as 2×10^9 cfu/ μ g when the Fab phagemid backbone for library construction was used (**Figure 4**).

The uracil incorporation efficiency by comparison of titer in both *E. coli* CJ236 and *E. coli* SS320 cells was checked. The *E. coli* CJ236 and *E. coli* SS320 cells were infected by phage harboring dU-ssDNA for the uracil incorporation efficiency test. *E. coli* SS320 has enzymes (dUTPase and uracil glycosylase) that can degrade uracil-containing DNA, while *E. coli* CJ236 lacks these enzymes and cannot degrade uracil-containing DNA. To achieve an acceptable uracil incorporation efficiency, titers from *E. coli* CJ236 need to be at least 10^4 times higher than those from *E. coli* SS320. Otherwise the wild-type population will increase in the constructed antibody library due to inefficient uracil incorporation. **Figure 5** showed that the titer from *E. coli* CJ236 is approximately 3×10^5 times higher than that from *E. coli* SS320, indicating an efficient uracil incorporation into phage ssDNA.

Next, we prepared and extracted dU-ssDNA. The dU-ssDNA purity is checked by agarose gel electrophoresis (**Figure 6**). Then the oligonucleotide-directed mutagenesis was conducted and the efficiency of the dU-ssDNA conversion to CCC-DNA was evaluated (**Figure 6**). Three products with lower motility than dU-ssDNA can be visualized on the gel including the fastest-running band (CCC-dsDNA), the middle weak band (Nicked band), and the slowest-running band (strand-displaced DNA).

After electroporation into *E. coli* SS320, the library size was estimated from the overnight incubation plate (see step 4.7.5). The average library size was 5×10^9 from duplicate serial dilutions on LB/carb plates (**Figure 7**). However, the estimated size at this step may contain phage that do not display Fabs due to the presence of a frameshift or stop codon, or display misfolded Fabs. Sequencing and ELISA were used to estimate the functional diversity of constructed library. 96 randomly picked single clones were sent for sequencing analysis. **Table 4** shows that 90 out of the 96 randomly picked single clones were successfully sequenced, which contains 70 clones without a premature stop codon (53 clones with at least one CDR mutant and 17 clones with the wild-type sequence) and 20 clones with a premature stop codon at different regions. Within the 70 clones, mutant rates of CDRH1, CDRH2, CDRH3, and CDRL3 are 50%, 57%, 53%, and 56%, respectively, while the mutant rate with at least one CDR is 76%. In the 20 clones with a premature stop codon (90%), the premature stop codon was mainly

derived from the frameshift of oligonucleotide mutagenesis primers, including 45% (CDRH1), 10% (CDRH2), 15% (CDRH3), and 20% (CDRL3).

To detect the display of properly folded Fabs, a protein A/L based ELISA was employed as it is known that protein A and protein L can recognize proper folding of the VH framework and VL framework, respectively^{17,18}. In agreement with the sequencing analysis, the ELISA assay in triplicate (**Figure 8**) showed that the 20 clones with a premature stop codon were all negative while the 17 clones with a wild-type sequence were all positive when the positive ratio was empirically set at 3.0. For the 53 clones with at least one CDR mutant, 43 clones were positive in ELISA while 10 clones were negative; this indicates that most of the clones were well folded while the CDRs from the 10 clones can have detrimental effects on Fab folding. In total, 43 clones out of the 90 clones (48%) were well folded and contained at least one CDR mutant. Thus, the functional amino acid diversity of the constructed library based on protein A/L ELISA and sequence analysis was estimated to be 2.4×10^9 (*i.e.*, 48% of 5×10^9).

FIGURE AND TABLE LEGENDS:

Figure 1. Overview of the phage-displayed Fab library construction. Phage-displayed Fab library construction follows a basic series of steps. It involves preparation of high-efficiency electro-competent bacterial cells, extraction of dU-ssDNA, Kunkel's method based oligonucleotide-directed mutagenesis, electroporation and calculation of phage Fab library diversity, functional evaluation by protein A/L ELISA, and DNA sequence analysis.

Figure 2. Phagemid architecture for the Fab library construction. The basic features of the phagemid backbone consist of origins of single-stranded (f1 ori) and double-stranded (dsDNA ori) DNA replication, and an ampicillin/carbenicillin resistance gene (AmpR). For Fab display, under the control of the alkaline phosphatase promoter (PhoA), the phagemid contains a bicistronic cassette to drive expression and secretion of: light chain (LC) consisting of a secretion signal, VL (variable region of light chain), CL (constant region of light chain), and C-terminal flag tag; and heavy chain (HC) consisting of a secretion signal, VH (variable region of heavy chain), and CH1 (constant region 1 of heavy chain) fused with a p3 phage minor coat protein. Assembly of the light chain and heavy chain into Fab within the *E. coli* periplasm directs the display of Fab on the phage surface.

Figure 3. Schematic of Kunkel's method based oligonucleotide-directed mutagenesis. In this protocol, we used Kunkel's method to prepare dU-ssDNA template. Oligonucleotides for CDRH1, CDRH2, CDRH3, and CDRL3 with designed diversity are phosphorylated, annealed to the template, and used to convert ss-DNA to CCC-dsDNA. Following electroporation into *E. coli* SS320 electro-competent cells, the heteroduplex DNA is repaired to either the wild type or the mutant form; Due to the presence of uracil in the wild type strand, the repair process favors the mutant form, and thus, the mutant form dominates the library.

Figure 4. Estimation of the M13KO7 pre-infected *E. coli* SS320 electro-competent cell efficiency. A phagemid backbone vector was used to check the electroporation efficiency of the competent cells. Formula to calculate the efficiency is as follows: assume that M is the average

colony number counted from the most diluted fold 10^N (N is from 1–8) in duplicate. *E. coli* SS320 efficiency from LB/carb plate is equal to $M \times 10^{N+3}$ cfu/ μ g. The efficiency of the electro-competent cells is around 2×10^9 cfu/ μ g.

Figure 5. Assessment of uracil incorporation into ssDNA by phage infection of *E. coli* CJ236 and *E. coli* SS320 cells. Based on Kunkel's method, the uracil incorporation efficiency is checked by comparison of phage infection titer in both *E. coli* CJ236 and *E. coli* SS320 cells. The titer calculation formula is as follows: assume that M is the average colony number counted from the most diluted fold 10^N (N is from 1–10), and that the titer from *E. coli* CJ236 or *E. coli* SS320 is equal to $M \times 10^{N+2}$ cfu/mL. The efficiency of uracil incorporation can be estimated from the titer ratio of *E. coli* CJ236 and *E. coli* SS320. The titer in *E. coli* CJ236 was 9×10^{12} cfu/mL while the titer in *E. coli* SS320 was 3×10^7 cfu/mL. The titer ratio of *E. coli* CJ236 and *E. coli* SS320 was 3×10^5 .

Figure 6. Conversion of dU-ssDNA to CCC-DNA by oligonucleotide-directed mutagenesis. Following oligonucleotide-directed mutagenesis, the efficiency of dU-ssDNA conversion to CCC-DNA was evaluated. dU-ssDNA was completely converted to dsDNA. The dominant band is CCC-dsDNA while there is a minor portion of nicked dsDNA and strand-displaced DNA.

Figure 7. Phage titration for calculation of library size. After electroporation into *E. coli* SS320, the library size was estimated from serial dilutions on LB/carb plates. The size calculation formula is as follows: assume M is the average colony number counted from the most diluted fold 10^N from a 2YT/Carb plate (N is from 1–8), size is equal to $2M \times 10^{N+3}$.

Figure 8. Protein A/L direct binding phage ELISA. Protein L can recognize the framework of well folded kappa light chain VL and protein A can recognize the framework of well folded heavy chain VH. Binding of Fab with protein L and A indicates proper folding of both heavy chain and light chain. In brief, protein L in triplicate and the negative control M-PBST were coated to the plate, Fab phage supernatants from different clones were incubated with protein L and M-PBST, then after wash, protein A-HRP was used to capture bound Fab phage. Phage ELISA readings showed 90 randomly picked clones with successful sequencing readout. A threshold line representing the clone as positive was empirically defined where the ratio of OD450 absorbance value from protein L (average of triplicate with error bar) versus negative control was more than 3.0. Three groups based on sequencing analysis were shown, corresponding to a mutant without stop codon in red (53 clones), wild type (WT) in blue (17 clones), and mutant with stop codon in green (20 clones).

Table 1. Reagent setup.

Table 2. CDR diversities and mutagenesis primers. The DNA sequences of the CDR regions to be mutated are shown in red; sequences are formatted using the IUPAC nucleotide code. "X" indicates tri-nucleotide from a mixture designed to contain different amino acid sets; "n" indicates different number of X. Five primers with a different number of X were used to

diversify CDRL3 or CDRH3, respectively, to generate variable length of CDRL3 and CDRH3². The residue numbers are defined by the IMGT nomenclature.

Table 3. Procedures and components of Kunkel's method based reaction.

Table 4. Sequence analysis of CDRH1, CDRH2, CDRH3, and CDRL3 from the synthetic Fab library.

DISCUSSION:

To construct high diversity, phage-displayed Fab libraries, quality control check points are needed to monitor various stages of the construction process, including the competency of electro-competent cells, quality of the dU-ssDNA template, efficiency of CCC-dsDNA synthesis, titer after electroporation, Fab folding, and amino acid diversity of CDRs by sequence analysis of Fab-phage clones.

High yield and purity of dU-ssDNA is essential for high mutagenesis rate. In our experience, phage induction at 25 °C overnight can yield more dU-ssDNA than that from phage induction at 37 °C overnight. This is in agreement with a previous report¹⁹. Regarding ssDNA extraction, the initial plasmid Spin kit (QIAprep) contained MLB for phage lysis and binding. Later, MLB was discontinued with unknown reason and replaced by PB. We found that the yield of dU-ssDNA is much lower from PB treatment as compared with that from MLB treatment. In this protocol, we used a reagent named UT-MLB²⁰ to replace MLB and found the yield of dU-ssDNA is similar to that from the initial Spin Kit.

As CDRH3 and CDRL3 are the most diverse regions for antigen recognition²¹, to introduce a tailored diversity with a specific set of amino acid combinations and ratios, and to remove redundancy bias and stop-codons introduced by degenerate codons such as NNK (N, equimolar of A/C/G/T; K, equimolar of G/T), trimer codon phosphoramidite-based oligonucleotides²² with exactly one trimer codon corresponding to one specific amino acid were designed for CDRH3 and CDRL3. Moreover, variable lengths of CDRH3 and CDRL3 oligonucleotides were used to further increase diversities.

After enzymatic synthesis of CCC-dsDNA, generally three bands are observed by agarose gel electrophoresis and the bands should be clear without smear. Among them, the fastest-running band is the CCC-dsDNA that can yield a high mutation rate (around 80%) after electroporation²³. The slowest-running band is the strand-displaced DNA that arises from inherent strand-displacement activity of T7 DNA polymerase and has a low mutation rate (around 20%)²³. The middle weak band is nicked DNA after extension due to insufficient T4 DNA ligase activity or insufficient oligonucleotide phosphorylation.

A small sequencing sample pool was used to estimate the library diversity though not accurate²⁴. To estimate the real diversity accurately, next generation sequencing (NGS) may be a good option in mining the diversity depth of the constructed library²⁵. In practice, due to the current challenges of NGS technology including read length, accuracy, cost, and high

throughput, the sequencing of the Fab phage library used in this protocol with the length of around 950 bp spanning CDRH1, CDRH2, CDRH3, and CDRL3 is not achievable; however, it is possible to estimate the scFv (around 700 bp) library diversity within the range of millions^{24,25}. Another key standard to evaluate the diversity of constructed library is to use the library to pan against many different types of antigens and calculate the positive clones captured since library diversity is directly correlated with the successful rate of antigen panning²⁶. High throughput selection platform is well suited for this purpose and readers can refer to a detailed protocol reported by Miersch *et al.*²⁷

Theoretically, phage-displayed synthetic antibody libraries with tailored diversity can be used to target any antigen and thus have broad applications. Currently, companies including Cambridge Antibody Technology (CAT), MedImmune, Genentech, Dyax, Bioinvent, Pfizer, and MorphoSys rely heavily on phage display platforms for therapeutic antibody development²⁸. Moreover, many phage display core technology patents have expired²⁹. Undoubtedly, this will unleash the maximum potential of phage-displayed antibody technology.

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DISCLOSURES:

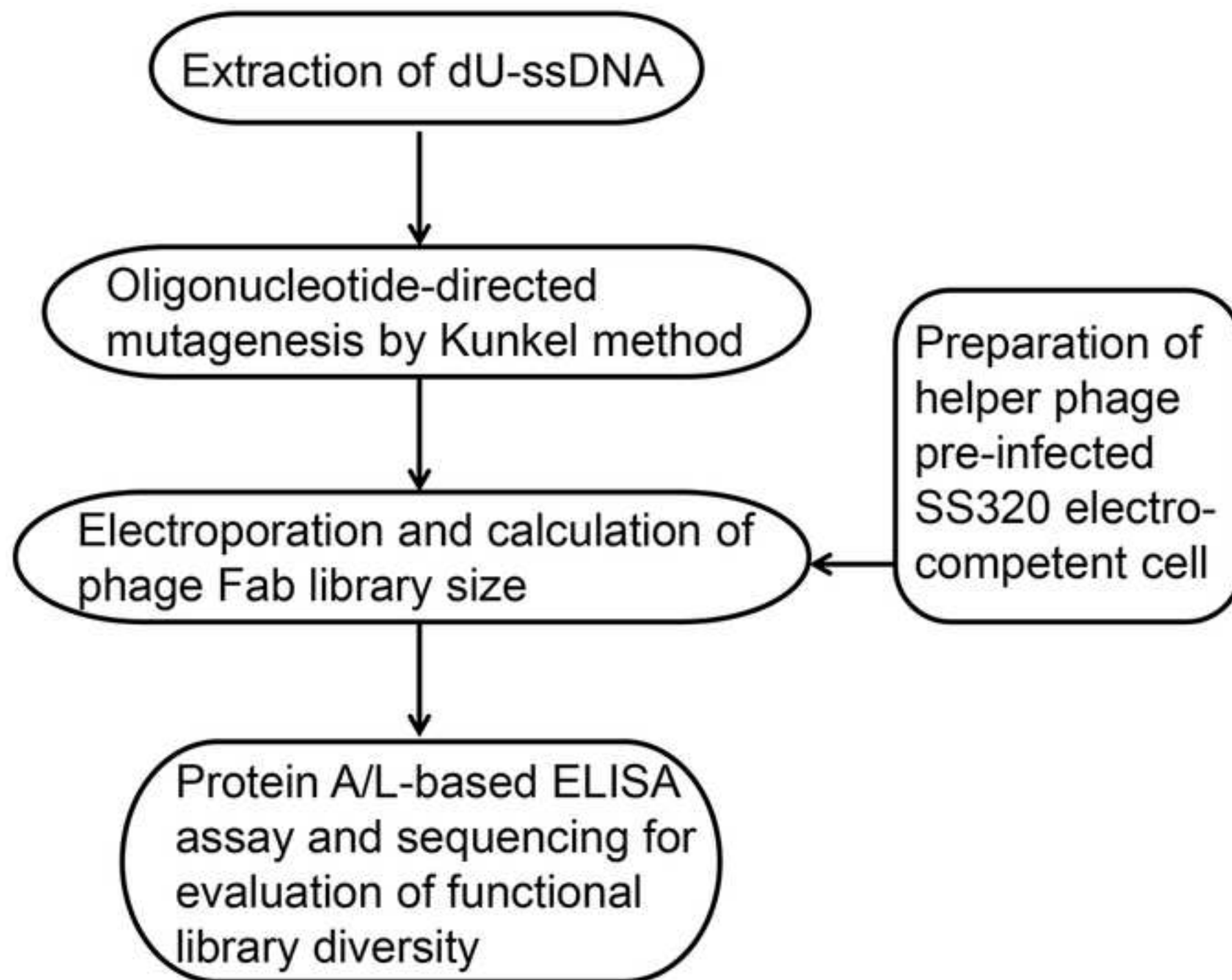
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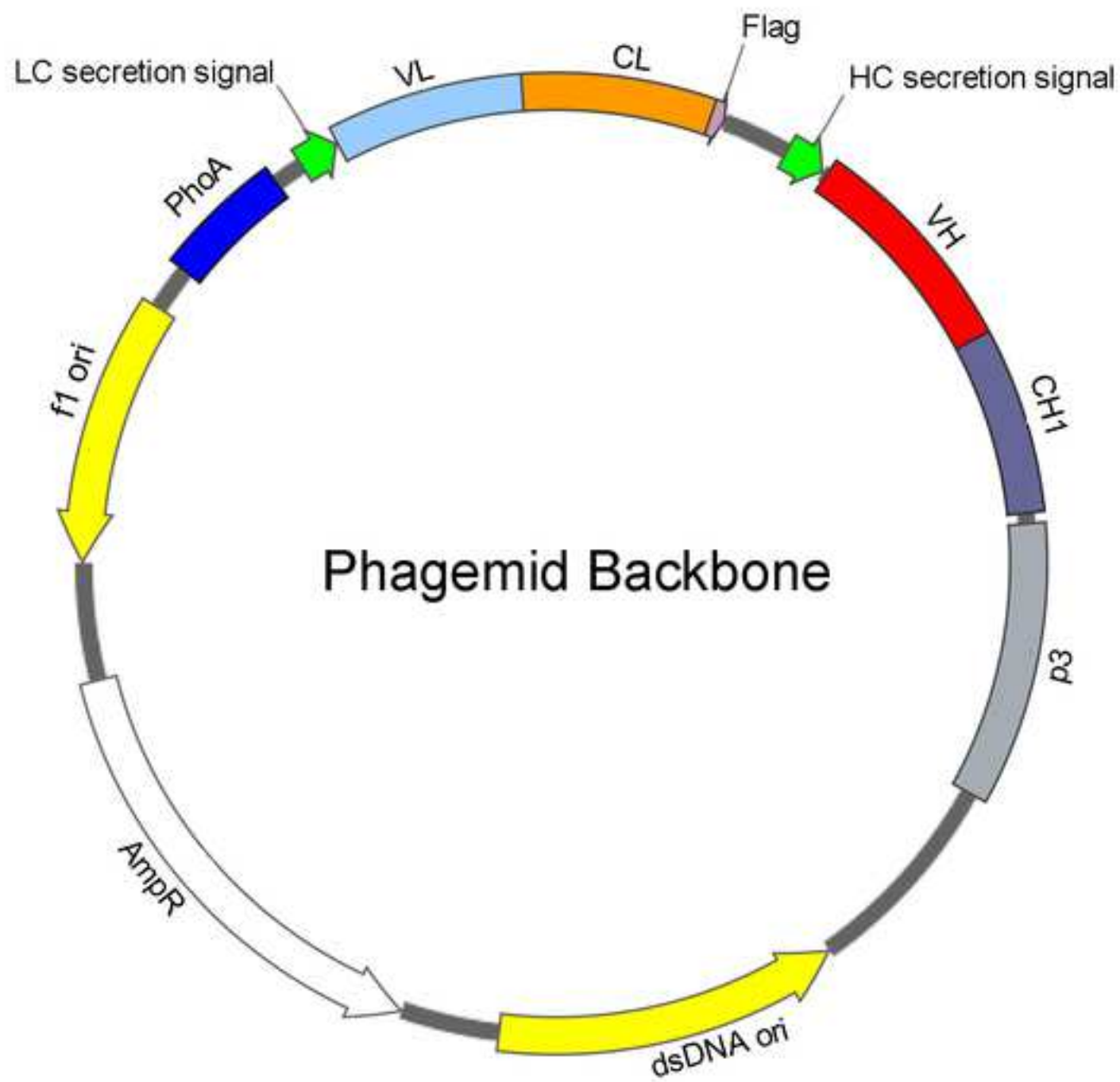
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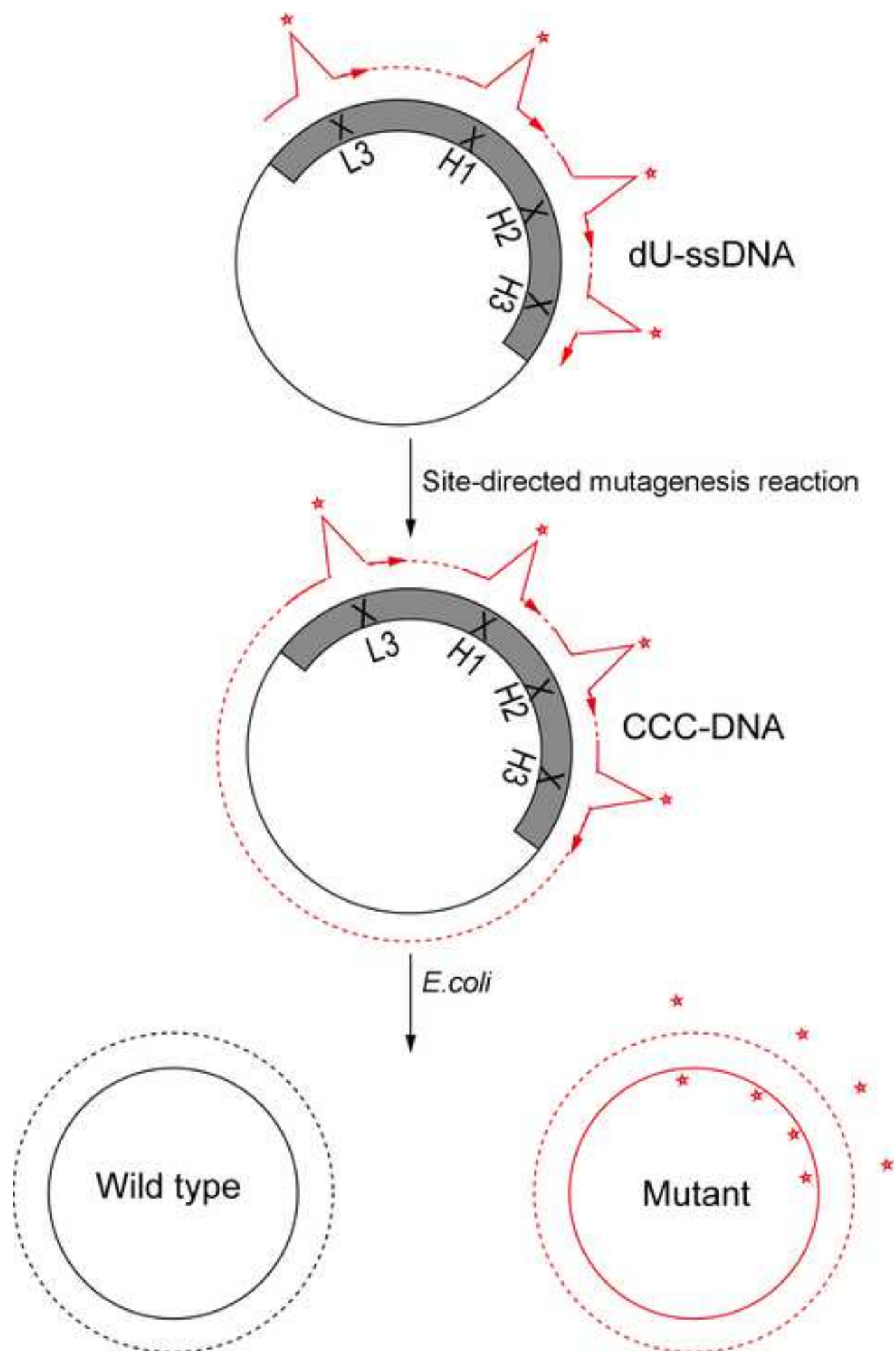
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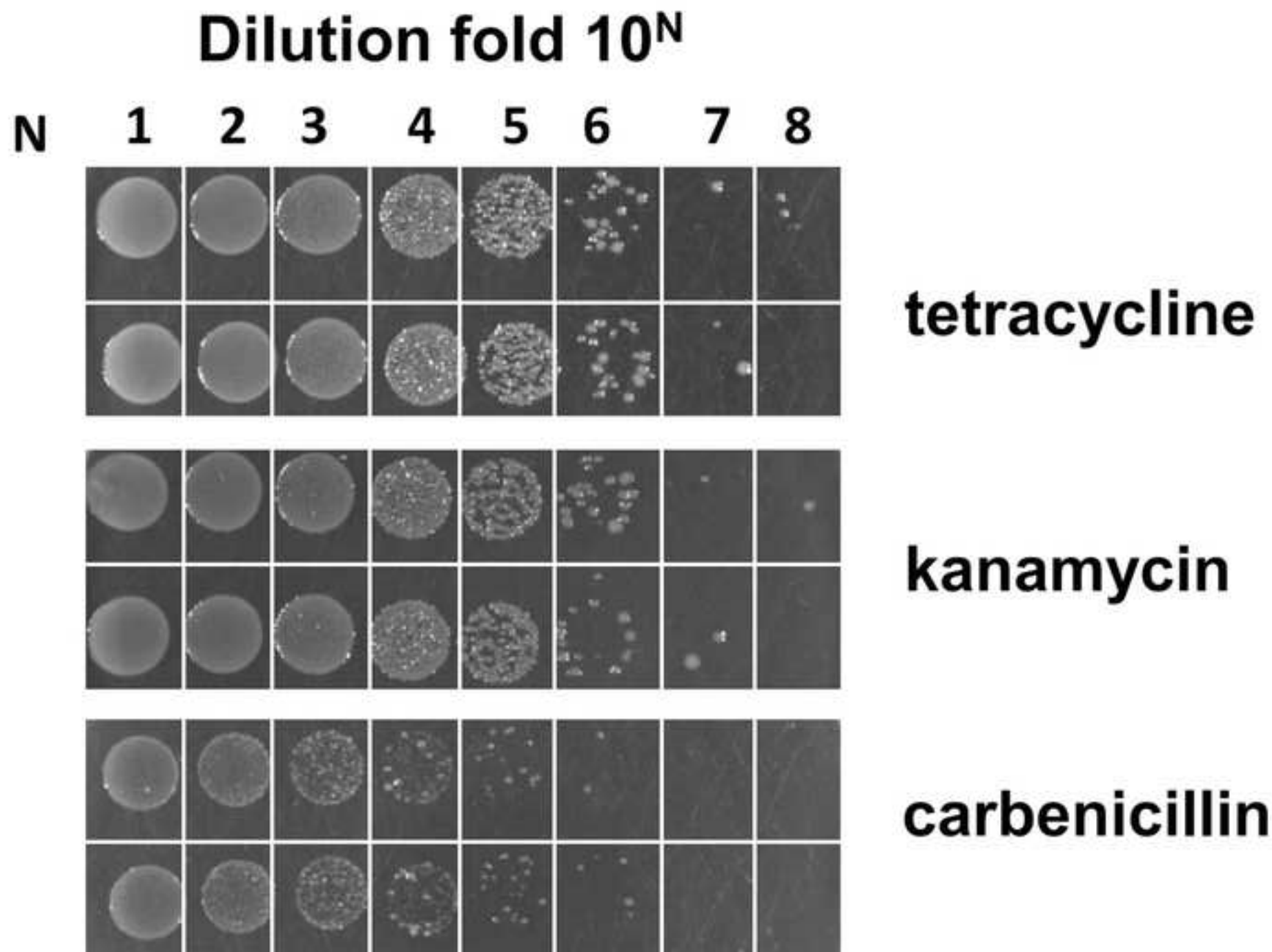
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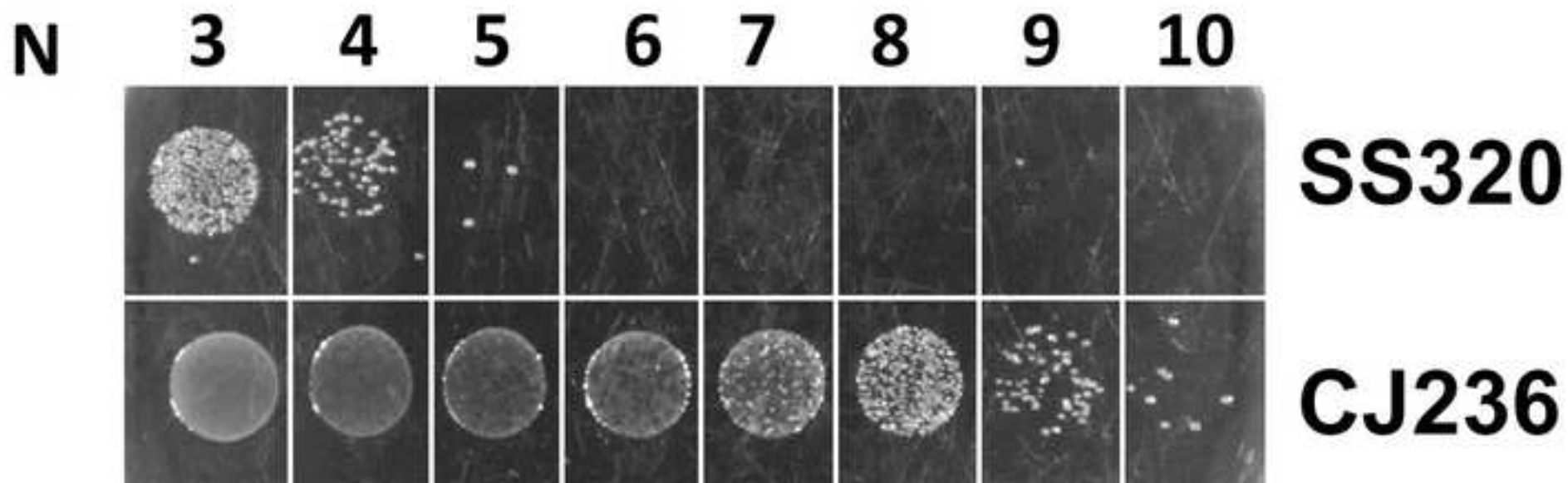


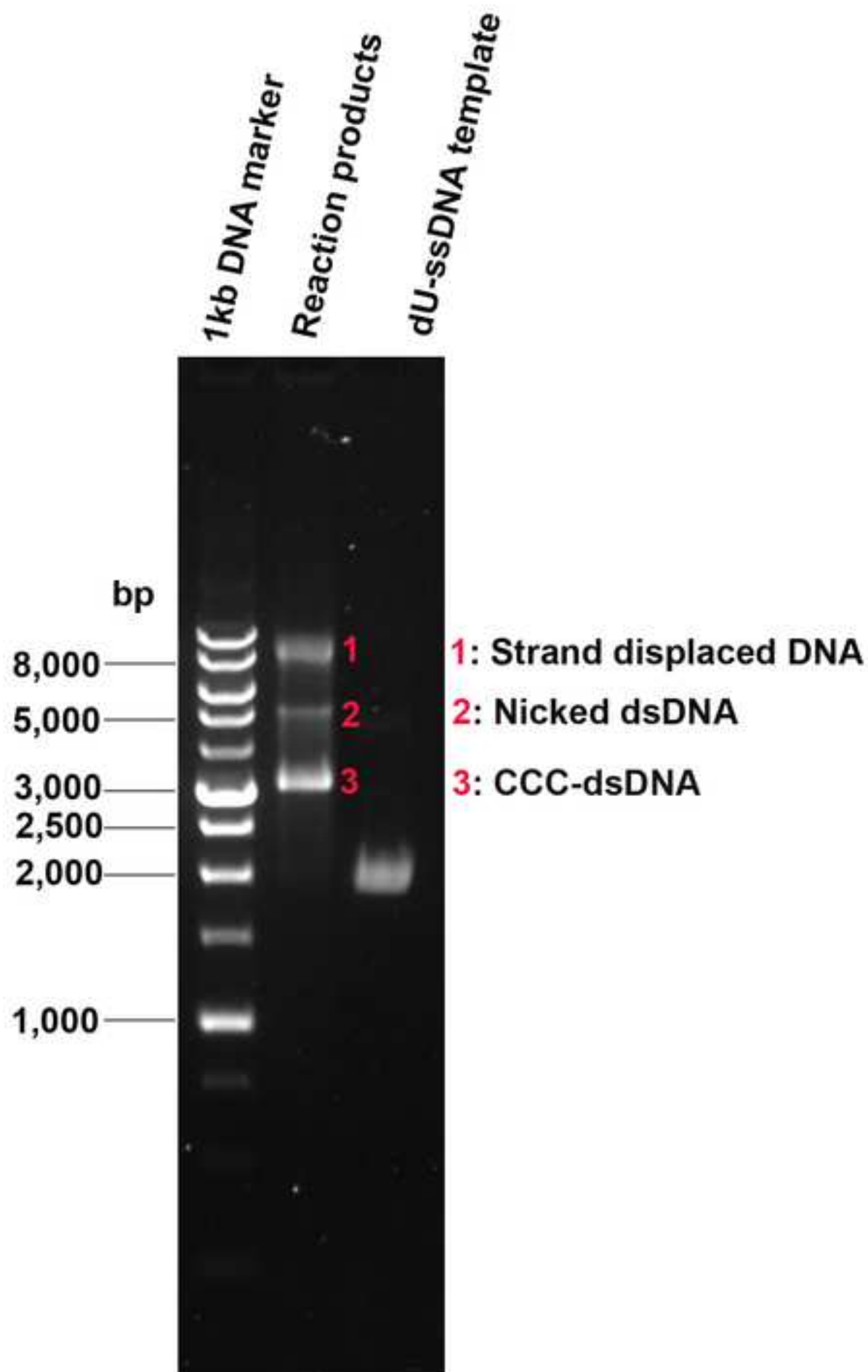


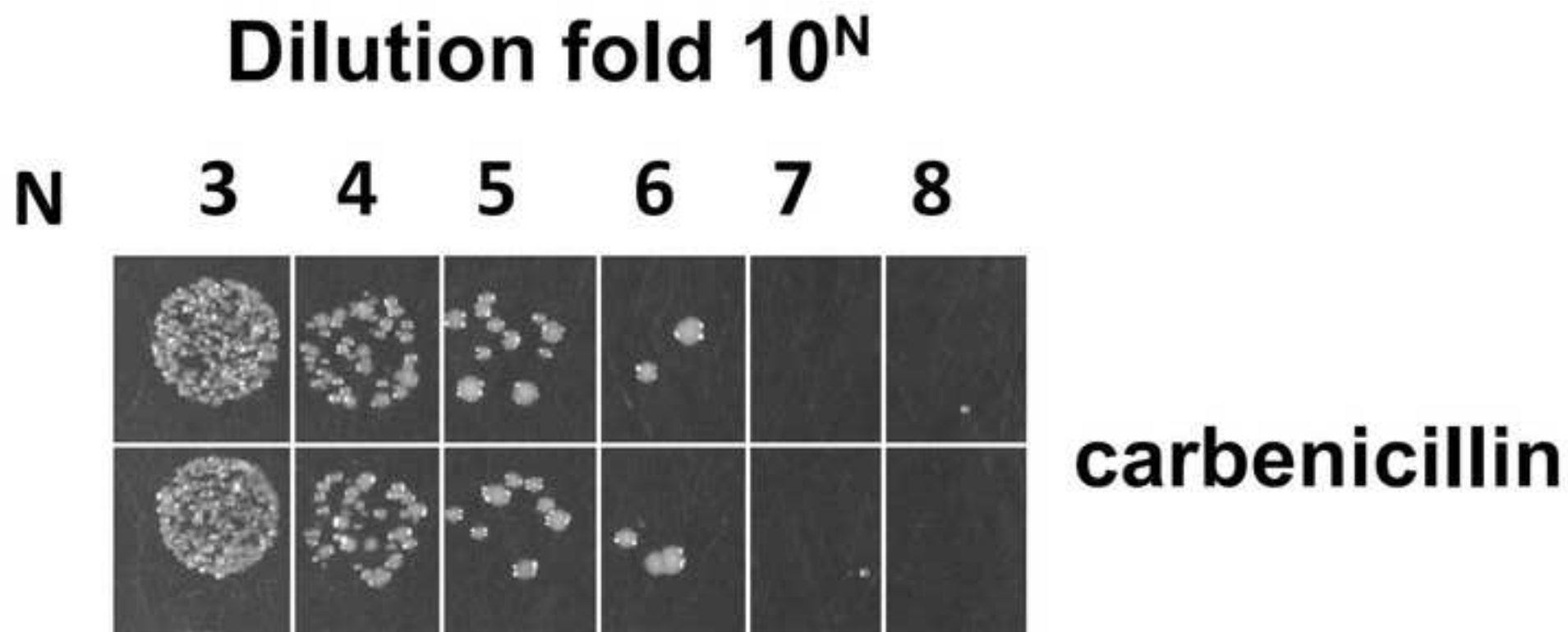


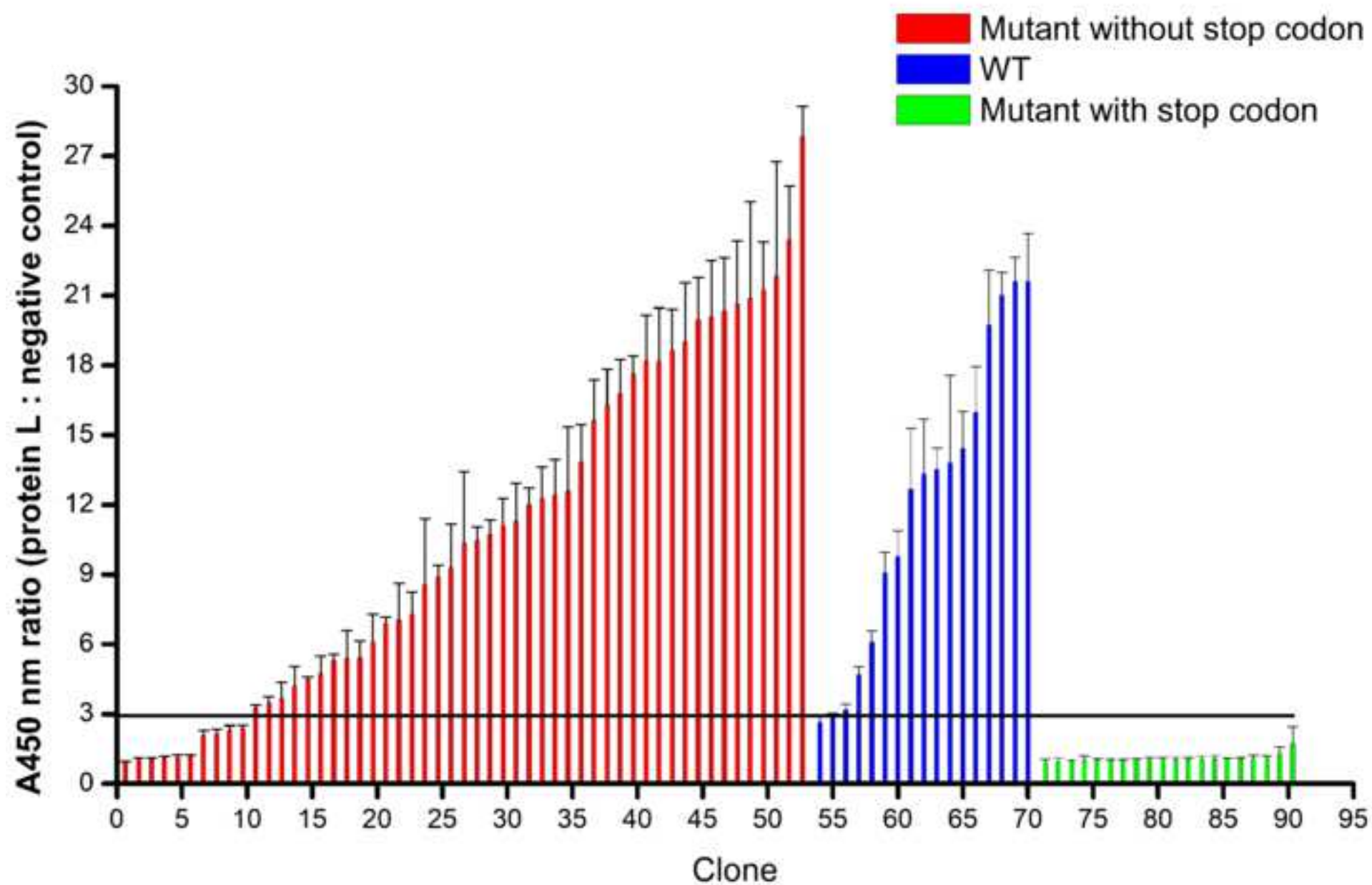


Dilution fold 10^N









Reagent setup	Component	Amount
2YT medium	Yeast extract	10 g
	Tryptone	16 g
	NaCl	5 g
2YT top agar	Yeast extract	10 g
	Tryptone	16 g
	NaCl	5 g
	Granulated agar	7.5 g
2YT/carb/cmp medium	Carbenicillin	100 µg/mL
	Chloramphenicol	10 µg/mL
2YT/carb/kan/uridine medium	Carbenicillin	100 µg/mL
	Kanamycin	50 µg/mL
	Uridine	0.25 µg/mL
2YT/carb/tet medium	Carbenicillin	100 µg/mL
	Tetracycline	10 µg/mL
2YT/carb medium	Carbenicillin	100 µg/mL
2YT/kan medium	Kanamycin	50 µg/mL
2YT/kan/tet medium	Kanamycin	50 µg/mL
	Tetracycline	10 µg/mL
2YT/tet medium	Tetracycline	10 µg/mL
LB medium agar	Yeast extract	5 g
	Tryptone	10 g
	NaCl	10 g
LB/carb plates	LB agar	1L
	Carbenicillin	100 µg/mL
LB/tet plates	LB agar	1 L
	Tetracycline	10 µg/mL
LB/kan plates	Kanamycin	50 µg/mL
SOC medium	Yeast extract	5 g
	Tryptone	20 g
	NaCl	0.5 g
	KCl	0.2 g
	2.0 M MgCl ₂	5.0 mL
	1.0 M glucose	20 mL
Superbroth medium	Tryptone	12 g
	Yeast extract	24 g
	Glycerol	5 mL
Superbroth kan/tet medium	Kanamycin	50 µg/mL
	Tetracycline	10 µg/mL
1X PBS	NaCl	137 mM
	KCl	3 mM
	Na ₂ HPO ₄	8 mM

	KH ₂ PO ₄	1.5 mM
TAE/agarose gel	TAE buffer	
	Agarose	1% (w/v)
	GelRed	1:10000 (v/v)
TMB substrate	TMB	50% (v/v)
	H ₂ O ₂ peroxidase substrate	50% (v/v)
M-PBST buffer	1X PBS	100 ml
	Tween-20	0.05% (v/v)
	NON-Fat Powdered Milk	5% (v/v)
5X PEG/NaCl	PEG-8000	20% (w/v)
	NaCl	2.5 M
PBST buffer	1X PBS	1 L
	Tween-20	0.05% (v/v)
10X TM buffer	MgCl ₂	0.1 M
	Tris	0.5 M
1.0 mM HEPES, pH 7.4	1.0 M HEPES	4.0 mL
	Ultrapure water	4.0 L
10% (v/v) ultrapure glycerol	Ultrapure glycerol	100 ml
	Ultrapure water	900 mL
Ultrapure water	H ₂ O	

comments/description
Add ultrapure water to make up the volume to 1.0 L, adjust pH to 7.0, autoclave.
Add ultrapure water to make up the volume to 1.0 L and adjust pH to 7.0, heat to dissolve, autoclave.
Add ultrapure water to make up the volume to 1.0 L, adjust pH to 7.0, autoclave. For LB agar, add 20 g of granulated agar, autoclave.
Add ultrapure water to make up the volume to 1.0 L and adjust pH to 7.0, autoclave.
Add ultrapure water to 900 mL, autoclave, add 100 mL of autoclaved 0.17 M KH_2PO_4 , 0.72 M K_2HPO_4 .
Adjust pH to 7.2, autoclave.

Add ultrapure water to make up the volume to 1L, and autoclave.

0.22 μm filter-sterilize.

Adjust pH to 7.5.

0.22 μm filter-sterilize.

0.22 μm filter-sterilize.

Dnase-free, Rnase-free, Pyrogen-free.

Target CDRs	Primer	Sequences ¹
CDRH1	Template	5'-GCAGCTTCTGGCTTC AACTTTTCTTCTTCTAT CACTGGGTGCGTCAG-3'
	H101	5'-GCAGCTTCTGGCTTCGATNTCWCTWCTKMTTRGTATSCACTGGGTGCGTCAG-3'
	H102	5'-GCAGCTTCTGGCTTCGATNTCWCTWCTKMTGMTATSCACTGGGTGCGTCAG-3'
	H103	5'-GCAGCTTCTGGCTTCGATNTCWCTWCTKMTTACATSCACTGGGTGCGTCAG-3'
	H104	5'-GCAGCTTCTGGCTTCGATNTCWCTWCTKMTTGGATSCACTGGGTGCGTCAG-3'
	H105	5'-GCAGCTTCTGGCTTCGATNTCWCTGACKMTRGTATSCACTGGGTGCGTCAG-3'
	H106	5'-GCAGCTTCTGGCTTCGATNTCWCTGACKMTGMTATSCACTGGGTGCGTCAG-3'
	H107	5'-GCAGCTTCTGGCTTCGATNTCWCTGACKMTTACATSCACTGGGTGCGTCAG-3'
	H108	5'-GCAGCTTCTGGCTTCGATNTCWCTGACKMTTGGATSCACTGGGTGCGTCAG-3'
CDRH2	Template	5'-GGCCTGGAATGGGTTGCAT CTATTTCTTCTTATGGCTA TACTTATTATGCCGATAGCGTC-3'
	H201	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTTCTRGTRGTAVTACTCGTTATGCCGATAGCGTC-3'
	H202	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTTCTRGTRGTAVTACTDATTATGCCGATAGCGTC-3'
	H203	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTTCTRGTRGTDATACTCGTTATGCCGATAGCGTC-3'
	H204	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTTCTRGTRGTDATACTDATTATGCCGATAGCGTC-3'
	H205	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTNATRGTRGTAVTACTCGTTATGCCGATAGCGTC-3'
	H206	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTNATRGTRGTAVTACTDATTATGCCGATAGCGTC-3'
	H207	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTNATRGTRGTDATACTCGTTATGCCGATAGCGTC-3'
	H208	5'-GGCCTGGAATGGGTTGCATMTATTTMCYMTNATRGTRGTDATACTDATTATGCCGATAGCGTC-3'
	H209	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTTCTRGTRGTAVTACTCGTTATGCCGATAGCGTC-3'
	H210	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTTCTRGTRGTAVTACTDATTATGCCGATAGCGTC-3'
	H211	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTTCTRGTRGTDATACTCGTTATGCCGATAGCGTC-3'
	H212	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTTCTRGTRGTDATACTDATTATGCCGATAGCGTC-3'
	H213	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTNATRGTRGTAVTACTCGTTATGCCGATAGCGTC-3'
	H214	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTNATRGTRGTAVTACTDATTATGCCGATAGCGTC-3'
	H215	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTNATRGTRGTDATACTCGTTATGCCGATAGCGTC-3'

	H216	5'-GGCCTGGAATGGGTTGCABGGATTTMCYMTNATRGTRGTDATACTDATTATGCCGATAGCGTC-3'
CDRH3	Template	5'-CGTCTATTATTGTGCTCGC ACTGTTCTGGATCCAAAAACCGTACTTCTCTGGTTGGGCTATG GACTACTGGGGTCAAG-3'
	H301-5	5'-CGTCTATTATTGTGCTCGC(X)nGSTWTKGACTACTGGGGTCAAG-3'
CDRL3	Template	5'-GCAACTTATTACTGTCAGCAAT CTTCTATTCTCTGATC ACGTTCCGGACAGGG-3'
	L301-5	5'-GCAACTTATTACTGTCAGCAA(X)nCYGWTCACGTTCCGGACAGGG-3'

Mutagenized residues ²								Theoretical amino acid diversity of CDR	Total theoretical amino acid diversity
30	35	36	37	38	39			1.15E+03	1.41E+20
FILV	ST	STD	YSAD	GSADYW	IM				
55	57	58	59	62	63	64	66	1.60E+04	
YSWRG	YS	PYSH	SYHND	GS	GS	STNDY	RDNY		

107-113	114	115						1.95E+07	
(X)n	AG	FILM							
107-113	115	116						3.91E+05	
(X)n	PL	FI							

Kunkel's method based mutagenesis	
Reaction 1. Oligonucleotide phosphorylation with T4 polynucleotide kinase	
Component	Amount
mutagenic oligonucleotides	
10X TM buffer	2 µL
10 mM ATP	2 µL
100 mM DTT	1 µL
T4 polynucleotide kinase (10 U/µL)	2 µL
Ultrapure H ₂ O	Up to 20 µL
Reaction setting	
Step 1.	37 °C for 1 h
Reaction 2. Annealing of the oligonucleotides to the template	
Component	Amount
dU-ssDNA template	20 µg
10× TM buffer	25 µL
phosphorylated CDRH1 oligonucleotides	20 µL
phosphorylated CDRH2 oligonucleotides	20 µL
phosphorylated CDRH3 oligonucleotides	20 µL
phosphorylated CDRL3 oligonucleotides	20 µL
Ultrapure H ₂ O	Up to 250 µL
Reaction setting	
Step 1.	90 °C for 3 min
Step 2.	50 °C for 5 min
Step 3.	20 °C for 5 min
Reaction 3. Enzymatic synthesis of CCC-dsDNA	
Component	Amount
annealed oligonucleotides/template mixtures	250 µL
10 mM ATP	10 µL
dNTP mix (25 mM of each nucleotide)	10 µL
100 mM DTT	15 µL
T4 DNA ligase	1 µL
T7 DNA polymerase	3 µL
Reaction setting	
Step 1.	20 °C for overnight

ase
Final
0.6 µg
1X
1 mM
5 mM
20 U
Final
20 µg
1x
0.6 µg
0.6 µg
0.6 µg
0.6 µg
Final
346 µM of each nucleotide
865 µM of each nucleotide
5 mM
30 Weiss units
30 U
t

CDR sequence summary	
	Percentage
CDRH1 mutation	62%
CDRH2 mutation	71%
CDRH3 mutation	67%
CDRL3 mutation	67%
Clones with at least one CDR mutation	86%
Correctly folded clones	79%

Group	Clone number	Region	Percentage
No premature stop codon	70	CDRH1 mutation	50% (35/70)
		CDRH2 mutation	57% (40/70)
		CDRH3 mutation	53% (37/70)
		CDRL3 mutation	56% (39/70)
		At least one CDR mutation	76% (53/70)
Premature stop codon	20	CDRH1 defect	45% (9/20)
		CDRH2 defect	10% (2/20)
		CDRH3 defect	15% (3/20)
		CDRL3 defect	20% (4/20)
		Other defect	10% (2/20)

Reagents	Company
1.0 M H ₃ PO ₄	Fisher
1.0 M Tris, pH 8.0	Invitrogen
10 mM ATP	Invitrogen
100 mM dithiothreitol	Fisher
100 mM dNTP mix	GE Healthcare
3,3',5,5'-tetramethylbenzidine (TMB)	Kirkegaard & Perry Laboratories Inc
50X TAE	Invitrogen
Agarose	Fisher
Carbenicillin, carb	Sigma
Chloramphenicol, cmp	Sigma
EDTA 0.5 M, pH 8.0	Invitrogen
Granulated agar	VWR
H ₂ O ₂ peroxidase substrate	Kirkegaard & Perry Laboratories Inc
K ₂ HPO ₄	Sigma
Kanamycin, kan	Fisher
KH ₂ PO ₄	Sigma
Na ₂ HPO ₄	Sigma
NaCl	Alfa Aesar
Nanodrop	Fisher
NaOH	Fisher
NON-Fat Powdered Milk	Sangon Biotech
PEG-8000	Fisher
Protein A-HRP conjugate	Invitrogen
QIAprep Spin M13 Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Recombinant Protein L	Fisher
T4 DNA polymerase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs
T7 DNA polymerase	New England Biolabs
Tetracycline, tet	Sigma
Tryptone	Fisher
Tween-20	Sigma
Ultrapure glycerol	Invitrogen
Uridine	Sigma
Yeast extract	VWR
Strains	Company
<i>E.coli</i> CJ236	New England Biolabs
<i>E.coli</i> SS320	Lucigen
M13KO7	New England Biolabs

Equipment	Company
0.2-cm gap electroporation cuvette	BTX
96-well 2mL Deep-well plates	Fisher
96-well Maxisorp immunoplates	Nunc
Baffled flasks	Corning
Benchtop centrifuge	Eppendorf
Centrifuge bottles	Nalgene
ECM-630 electroporator	BTX
Magnetic stir bars	Nalgene
Thermo Fisher centrifuge	Fisher
High speed shaker	TAITEK
Microplate shaker	QILINBEIER
Liquid handler for 96 and 384 wells	RAININ
Mutil-channel pipette	RAININ
Amicon concentrator	Merck

Catalog Number
AC29570
15568-025
18330-019
BP172
28-4065-60
50-76-02
24710030
BP160
C1389
C0378
AM9620G
J637-500G
50-65-02
795488
AC61129
P2222
94046
U19C015
ND2000C
SS256
A600669
BP233
101123
22704
28706
28104
77679
M0203S
M0201S
M0274S
T7660
0123-07-5
P2287
15514-011
U3750
DF0127-08

Catalog Number
E4141
60512
N0315S

Catalog Number
278743
151759
5811000096
MBR-034P
QB-9002
E4XLS
UFC803096

Comments/Description
solution containing 25 mM each of dATP, dCTP, dGTP and dTTP.
100 mg/mL in water, 0.22 µm filter-sterilize, work concentration: 100 µg/mL.
100 mg/mL in ethanol, 0.22 µm filter-sterilize, work concentration: 10 µg/mL.
50 mg/mL in water, 0.22 µm filter-sterilize, work concentration: 50 µg/mL.
! CAUTION NaOH causes burns.
50 mg/mL in water, 0.22 µm filter-sterilize, work concentration: 10 µg/mL.
25 mg/mL in ethanol, work concentration: 0.25 µg/mL.
Comments/Description
Genotype: <i>dut⁻ ung⁻ thi-1 relA1 spoT1 mcrA/pCJ105 (F' cam^r)</i> . Used for preparation of dU-ssDNA.
Genotype: [<i>F'proAB+lacIq lacZΔM15 Tn10 (tet^r)</i>] <i>hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galUgalK rpsL thi</i> . Optimized for high-efficiency electroporation and filamentous bacteriophage production.



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Author(s):

Donghui Wu

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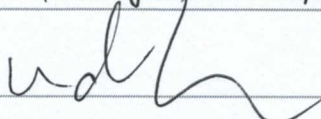
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Article Title:

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We addressed the points that were raised by editors and reviewer with details as below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please provide an email address for each author on the first page.
3. Please define all abbreviations before use.
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5. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
6. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
7. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

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9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

12. Protocol: 1.1: “Streak out”? How? Using what? How is the plate warmed? At which temperature? If it should be kept for one day, what is the keeping conditions? Please describe the step clearly or refer to an appropriate protocol or reference.

13. Protocol: 1.2: How to pick a single colony? Using what? “inoculate”,

How?

14. Protocol: 1.3: Shake for how long? How long is needed to reach mid-log phase? Please provide a quantitative measure.

15. Protocol: 1.4: How to prepare? In which container? Please describe clearly or refer to an appropriate protocol or reference.

16. Protocol: 1.5: How is mixing done? In which container? Incubate at which temperature?

17. Protocol: 1.6: How to mix? Incubate for how long?

18. Protocol: 1.7: "Pour", How? Using what?

19. Protocol: 1.8: Please revise the step to make it clear that there is only one plaques in each tube.

20. Protocol: 1.10: How that is done? For how long is that incubated?

21. Protocol: 1.11: Swirling by hand?

22. Protocol: 1.12: How is the transfer done?

23. Protocol: 1.14: How? In which container?

24. Protocol: 1.15: "gently stir" by hand? Otherwise, what is the speed?

25. Protocol: 1.17: "without combining the pellets", how?

26. Protocol: 1.18: How to remove the stir bar?

27. Protocol: 1.19: Are all accumulated in one bottle at the end of this

step?

28. Protocol: 1.20: Using pipette?

29. Protocol: 1.21: How?

30. Protocol: 1.22: Please use proper numbering if next steps are sub-part of this step, i.e. 1.22.1, 1.22.2, etc.

31. Protocol: 1.23: How the step is done?

32. Protocol: 1.25: Please avoid using commercial language, please use generic terms instead.

33. Protocol: 1.26: Please define pre-warmed. What is the temperature? "Rinse", how?

34. Protocol: 1.27: Incubate what?

35. Protocol: 1.28: Please use Jove style for numbering the sub-steps.

36. Protocol: 2: If the text is a Note, please indicate it as a Note. Otherwise, please move it to Discussion.

37. Protocol: 2.1: Please revise the step and make it clear of how to do the step.

38. Protocol: 2.3: How? In what?

39. Protocol: 2.4: Please describe the step clearly.

40. Protocol: 2.5: How? Please describe the step clearly.

41. Protocol: 2.11: "centrifuge briefly", please provide a quantitative measure.
42. Protocol: 2.13: Please use Jove style for numbering the sub-steps. Please ensure you answer the "how" question, i.e., how is the step performed?
43. Protocol: Please revise the numbering in 2.
44. Protocol: 2.13 (second 13): "Add", "mix", How?
45. Protocol: 2.11 (second 11): Please avoid using commercial language, please use generic terms instead.
Centrifuge at which temperature? "Discard", how?
46. Protocol: 2.12 (second 12): How? Please describe the step clearly.
47. Protocol: 3: Please move the discussion to the Discussion section.
48. Protocol: 3.1.1: Please use the imperative tense.
49. Protocol: 3.1.2: Incubate what?
50. Protocol: 3.2.1: Please use the imperative tense. Steps should include an action.
51. Protocol: 3.2.2: Please describe the step clearly.
52. Protocol: 3.3.1: Please revise the step. If referring to a Table, please clearly mentioned the Table number. Please do not embed any Table in the Manuscript. Please follow JoVE policy.
53. Protocol: 3.3.2: Incubate what? In which container?

54. Protocol: 3.3.3: Please avoid using commercial language, please use generic terms instead.

55. Protocol: 3.3.4: How? Please describe the step clearly.

56. Protocol: 3.3.5: Please avoid using commercial language, please use generic terms instead. Please describe the step clearly.

57. Protocol: 3.3.8: How is the transfer is done? Using what?

58. Protocol: 4: Please revise the numbering.

59. Protocol: 4.1: How?

60. Protocol: 4.2: How?

61. Protocol: 4.2 (second 2): How?

62. Protocol: 4.6: Please use JoVE style for numbering the sub-steps. Please describe the steps clearly.

63. Protocol: 4.14: How?

64. Protocol: 4:15: Please describe the step clearly.

65. Protocol: 4:15 (second 15): How?

66. Protocol: 5: Please move the discussion to the Discussion section.

67. Protocol: 5:3: Add with what? Pipette?

68. Protocol: 5.4: Please describe the step clearly.

69. Protocol: 5.6: Please describe the step clearly.

70. Protocol: 5.7: How?

71. Protocol: 5.8: "Remove", How?

72. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

73. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows reprints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

74. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. Please combine all panels of one figure into a single image file.

75. Please revise the Figures numbering. Currently, Figure 4 is mentioned after Figure 6.

76. Figure 2: Please describe clearly the Figure in the legend. What is each panel? Please describe each row. What each circle is presenting? Please add a scale bar.

77. Figure 5: Please describe clearly the Figure in the legend. Please add a scale bar.

78. Figure 7: Please describe clearly the Figure in the legend. What is each row? Please add a scale bar.

79. Figure 8: Please remove panel (B) and change it to a Table and number it accordingly.

80. Please revise the Representative Results as well as Discussion. Some parts of current Discussion section can be presented in the Representative Results.

81. Representative Results: Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

82. Representative Results: Please explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

83. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

84. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Response:

Based on editor's valuable comments, we modified our manuscript to address each point as listed above in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This provides an overview protocol for the creation of synthetic phage antibody libraries.

Major Concerns:

This protocol paper reiterates much that has been previously published while missing essential details that are to be found in other papers. In particular, nothing is said about the antibody scaffold used, the strategy behind antibody library creation (e.g. which sites are to be mutated, with what diversity and why) and the sequences of mutagenic oligonucleotides. As it is this publication is very superficial.

Response:

Based on the valuable comments, we added a lot of essential details of experiment. Sites to be mutated and sequences of mutagenic oligonucleotides are provided in Table 3.

Reviewer #2:

Manuscript Summary:

The most part of this manuscript is very clear, the protocol is reasonably easy to read. There are a few things to be fixed.

Minor Concerns:

1. The steps numbering in the section 2 is problematic, it is 11 again after 13

Response:

Corrected.

2. This is an experiment protocol type of paper, it would be better if the authors can list the expected yield of each step. For instance, normally how much ss DNA from section 2, how much ccc DNA from section 3.

Response:

Yields of ss DNA, ccc DNA and titer of expected electro-competent cells are provided in corresponding sections.

3. In section 3, the authors use "trimer codon phosphoramidite-based oligonucleotides". However, it is not clear that how this is designed. It would be helpful to illustrate this design to justify the "advantage" over NNK.

Response:

Sites to be mutated and sequences of mutagenic oligonucleotides are provided in Table 3. Design of diversity is a combination of our experience and published literature. The advantage of trimer codon phosphoramidite-based oligonucleotides over NNK is cited in the text.

4. Again in section 4, what is the normal efficiency to expect? To generate a reasonable sized library, how many transformations are needed? Are they all going to be inoculated for shaking overnight?

Response:

The normal efficiency per one electroporation is from $1-10 \times 10^9$. Depending on the expected library size and theoretical library diversity, time of electroporation can be increased and thus library size will be increased correspondingly. For phage library generation, in our experience, it is needed to be shaking overnight.

5. The phagemid backbone seems to have only a regular promoter. In section 4, shaking the transformant directly overnight may cause certain bias. How do the authors address this issue?

Response:

The normal efficiency per one electroporation is from $1-10 \times 10^9$. Depending on the theoretical library diversity and expected library size, time of electroporation can be increased and thus library size will be increased correspondingly. For phage library generation, in our experience, it is needed to be shaking overnight, otherwise phage cannot be generated efficiently. It is possible to cause bias from overnight culture. However, sometimes, the bias may favor the selection of Fab phage clones that have good expression, good solubility and correct folding.

Reviewer #3:

Comments and Suggestions

1. The first report of use of an oligonucleotide to direct mutagenesis of a residue in a single-stranded bacteriophage virus is Hutchison CA, Phillips S, Edgell MH, Gillam S, Jahnke P & Smith M (1978) Mutagenesis at a specific position in a DNA sequence. J Biol Chem 253, 6551-6560.

Response:

The citation is added in introduction section.

2. The first report of using more than one oligonucleotide to direct mutagenesis of multiple DNA sequences in bacteriophage is Weiss GA, Watanabe CK, Zhong A, Goddard A & Sidhu SS (2000) Rapid mapping of protein functional epitopes by combinatorial alanine scanning. Proc Natl Acad Sci U S A 97, 8950-8954.

Response:

The citation is added in introduction section.

3. High-efficiency electrocompetent SS320 cells are now commercially available (<http://www.lucigen.com/Phage-Display-Competent-Cells/#subcat-tabs1>).

Response:

Yes, the high-efficiency electro-competent SS320 cells are commercially available. However, in this protocol, we prepared M13KO7 pre-infected electro-competent SS320, which is not commercially available.

4. Please list the genotype of SS320 bacteria.

Response:

The genotypes of SS320 and CJ236 are added in Table 1.

5. Why start 8 cultures and only use one (i.e., 112-115)?

Response:

Corrected and addressed in the text (See lines 145-147).

6. Line 115: "in to" should be "into"

Response:

Corrected (See line 149).

7. Why grow 115 mL of cells (line 115), when only 15 mL will be used in the next step (line 117)?

Response:

Corrected (See lines 149-152).

8. We find that DNA that has been spot dialyzed gives more transformants in electroporation.

Response:

Thanks for sharing the experience. In a similar way, we found that the yield and transformants in electroporation can be increased by using centrifugal filter device for dialysis and extraction of Kunkel reaction products (See lines 345-350).

9. Is uracil added to the culture medium for CJ236 cells?

Response:

Yes, uracil is added to the culture medium of CJ236 for generation of dU-ssDNA.

10. What are the recipes of MP (line 208) and PBS (line 337)?

Response:

The recipe of MP is unknown and is from a commercial kit. The recipe of PBS is added in Table 2.

11. Do the authors encourage users to bias CDRs in favor of certain amino acids?

Response:

Yes, we prefer to bias CDRs in favor of certain amino acids. This may increase the Fab solubility and thermostability. This also depends on the purpose of synthetic phage library.

12. We monitor conversion of single-stranded into CCC DNA during in vitro replication by agarose gel electrophoresis.

Response:

Yes, conversion of single-stranded into CCC DNA during in vitro replication is monitored by agarose gel electrophoresis (See Figure 6 and Line 303 and 354).

13. One can also use template DNA than contains stop codons in the CDR coding regions to eliminate expression of non-recombinants in the library.

Response:

Yes, stop codons in the CDR coding regions can avoid the wild-type display in the library. But we found that the template without stop codon can keep the clones with 1, 2, or 3 CDRs replaced by the mutagenesis primers, which may be also functional against some antigens in phage panning.

14. What is the benefits of creating an immunosandwich with proteins A and L, versus using just one and anti-Flag Ab (assuming the Flag epitope is part of the Fab)?

Response:

Protein A and protein L can bind to the correctly folded light chain and heavy chain of the antibody template we used. Therefore, using proteins A/L ELISA can ensure the folding of light chain and heavy chain of Fab displayed. Anti-Flag can also be used as Flag epitope is part of the Fab vector. However, Flag epitope is located at the C-terminal of light chain and it is possible that the Fab is not well folded but it still be recognized by anti-Flag antibody and may cause false positive.

15. Why not have the box for electrocompetent cell preparation on the side of Figure 1?

Response:

Corrected in Figure 1.

16. Figures 2, 5, and 7 are not obvious without cartoons or text accompanying them.

Corrected in Figure 4, 5 and 7. Legends are provided in the text.

17. What is meant by "strand displaced DNA" in Figure 6?

Response:

Strand displaced DNA is an inherent activity of T7 DNA polymerase and citation is added in text (lines 612-614).

18. It is hard to see what components are in what buffer without separating lines.

Response:

Corrected in Table 2.

19. Please provide more information regarding the mutagenic oligonucleotides (i.e., length, sequence, T_m , purity, etc.).

Response:

Sequences of mutagenic oligonucleotides are added in Table 3.

Reviewer #4:

This manuscript entitled "Construction of synthetic phage displayed Fab library with tailored diversity", Huang et al. described a protocol for preparing phage-displayed synthetic antibody library with tailored diversity. The authors achieved Fab diversity of 5×10^9 .

Overall, the manuscript is well written and the experiments were carefully designed. A good amount of work was done in describing the protocol in depth. The manuscript fits within the scope of the journal. However, a minor revision is necessary to improve the overall quality of the manuscript. Specific comments and suggestions are enumerated

below:

1. Lane 104, indicate estimated time to reach OD=0.8, for example - 5-7 hrs, etc.

Response:

Added in line 117.

2. Lane 115, did you mean 2YT/kan/tet medium?

Response:

Corrected in line 149.

3. In the 'prepared solution' section 'superbroth medium salt' is redundant and can be deleted.

Response:

Corrected in Table 2.

4. Lane 115, would you estimate 'fastest-growing culture' by OD or eye-balling?

Response:

Corrected in line 149. Growing is visualized by eye that the culture will become turbid. OD monitor is not used in this step.

5. Starting lane 141, add a subheading (such as evaluation of electrocompetent cells)

Response:

Based on the journal format, modification is made from lines 207-221.

6. Electrocompetent cell preparation: A high aeration is preferred (~ 250

rpm) whereas the authors used 200 rpm. Secondly, single use aliquots of cells (in 50-100ul) are preferred than what was used in the study.

Response:

200 rpm is routinely used in our preparation. Thanks for sharing the experience. We found that 250 rpm sometimes can cause noise within the shaker. To get good aeration, baffled flasks in our experience have to be used. 350 µL per aliquot of competent cell are used to for electroporation within 0.2-cm gap electroporation cuvette. Small volume of electroporation cells (50-100 µL) can be used within 0.1-cm gap electroporation cuvette.

7. Lane 147, indicate the volume of cell-DNA mixture to be transferred to cuvette.

Response:

The information is added in line 197.

8. Lane 185. The centrifugation speed of 28880xg seems too high to pellet only the bacterial cells.

Response:

Corrected in line 257 to 12,000 × g.

9. It was not clear how the oligonucleotides were designed.

Response:

Sequences of mutagenic oligonucleotides are added in Table 3.

10. Lane 318. Centrifugation speed of 27000xg seems too high to pellet insoluble matter.

Response:

Corrected in line 393 to 12,000 × g.

11. The authors used the highest dilution plate for counting CFU which may not yield representative colonies. Ideally, from the serial dilution plates, the one should be chosen that has countable colonies (generally 30-300 colonies) and used for CFU calculation.

Response:

Corrected in corresponding position of text.

12. A small number of clones were sequenced to determine the diversity which was then linked to 'functional' diversity. A citation is needed.

Response:

We increased sequencing clone number. Meanwhile, relevant citation is added in discussion section.

13. For sequence analysis, was the whole phagemid DNA sequenced?

Response:

The variable heavy and light chains were sequenced.