Chapter 2

Construction of a scFv Library with Synthetic, Non-combinatorial CDR Diversity

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Abstract

Many large synthetic antibody libraries have been designed, constructed, and successfully generated high-quality antibodies suitable for various demanding applications. While synthetic antibody libraries have many advantages such as optimized framework sequences and a broader sequence landscape than natural antibodies, their sequence diversities typically are generated by random combinatorial synthetic processes which cause the incorporation of many undesired CDR sequences. Here, we describe the construction of a synthetic scFv library using oligonucleotide mixtures that contain predefined, non-combinatorially synthesized CDR sequences. Each CDR is first inserted to a master scFv framework sequence and the resulting single-CDR libraries are subjected to a round of proofread panning. The proofread CDR sequences are assembled to produce the final scFv library with six diversified CDRs.

Key words Antibody library, scFv, Phage display, Non-combinatorial CDR diversity, Synthetic CDR diversity, Synthetic antibody library

1 Introduction

Synthetic antibody libraries are a powerful and versatile tool for the generation of target-specific human monoclonal antibodies suitable for therapeutic and other demanding applications. Since the construction of the first synthetic antibody library using degenerate random oligonucleotides [1], significant technological advancements in library designs and synthetic methodologies have been made, and many highly sophisticated antibody libraries with synthetic diversity have been successfully constructed and utilized to generate therapeutic antibodies in various stages of preclinical and clinical development.

A current focus of the synthetic library design is the optimization of the antibody sequences for such aspects as lower immunogenicity, higher levels of expression and stability, lower aggregation propensity, and the minimization of undesirable posttranslational modifications (PTM) [2–4]. For example, the heavy and light

Thomas Tiller (ed.), *Synthetic Antibodies: Methods and Protocols*, Methods in Molecular Biology, vol. 1575,
DOI 10.1007/978-1-4939-6857-2_2,

chain framework variable gene segments for the library construction can be selected and/or designed based on their favorable biophysical properties, either individually [2] or as pairs [3]. The complementarity determining regions (CDRs), upon which most of the library's sequence diversity is concentrated, are typically designed to mimic the sequences and amino acid usage of natural human antibodies, and synthesized by the random concatenation of mononucleotide or trinucleotide units. The codon-based random combinatorial syntheses of CDR sequences [3, 5] enable the precise implementation of intricate CDR sequence designs, resulting in the antibody libraries with a high degree of humanness. However, the intrinsically random nature of the synthetic CDR diversity generation inevitably produces a significant number of sequences that are problematic or unnatural, i.e., the emulation of the natural amino acid frequency at each position does not always produce a natural amino acid *sequence*, and sequences that contain undesirable PTM motifs can also be produced.

A novel approach to eliminate or reduce these limitations of the random synthetic sequence diversity has recently been reported by our laboratory [6]. The *SCIEN* (*S*imulation of *C*DRs *I*nspired by and *E*mulating *N*ature) principle is based on the simulation of natural rearranged and hypermutated CDRs and the parallel synthesis of thousands of oligonucleotides encoding the predefined CDR sequences. Because the CDR sequence diversity is predefined and synthesized without relying on random combinatorial events, the incorporation of undesired sequences to the library can be prevented in the design stage. Although the diversity of each of the six CDRs is low because of the non-combinatorial synthetic approach (500 ~ 8000 unique sequences per CDR in the current example; *see* ref. 6), the combination of six such regions provides a total diversity that is large enough to construct a highly functional antibody library.

In this article, we will provide a detailed method for the generation of the antibody library based on the *SCIEN* principle, starting with the oligonucleotide mixtures that contain the simulated CDR sequences prepared by array synthesis. While this chapter describes the construction of an antibody library with non-combinatorial synthetic CDR diversity, the protocol may also be applied to the preparation of synthetic antibody libraries with other methods of CDR diversification such as random degenerate oligonucleotides [1] or trinucleotide-directed mutagenesis (TRIM) [2, 5, 7].

2 Materials

Reagents and equipment suggested here can be substituted with equivalent products from different vendors. If not listed below, standard molecular biology laboratory equipment and molecular biology grade chemicals/reagents can be used.

1. Nuclease-free water.
2. Oligonucleotide mixtures: Thousands of predefined oligonucleotide sequences can be synthesized in parallel on a microarray chip and chemically cleaved into solution. OligoMix® (LC Sciences, Houston, TX, USA), for example, provides up to 3918 individual oligonucleotide sequences of up to 100-mer length in pools.
3. scFv framework gene: The human germline variable gene segments DP47, DPK22, and DPL3 were used as the frameworks for library construction. The template scFv genes (DP47-linker-DPK22 and DP47-linker-DPL3) were codon-optimized and synthesized by GenScript Inc, (Pascataway, NJ, USA) (*see* **Note 1**), with the $(GGGGS)_3$ linker sequence and two asymmetric *Sfi*I restriction sites for cloning into pComb3X phagemid vector [8]. For the fourth framework regions (FR4) of V_H, V_λ, and V_κ, JH1, JL2, and JK1 were used, respectively, and a short dummy CDR-H3 sequence (CARNKLWFDY) was used for V_H. The scFv constructs were cloned and supplied in pUC57 vector. The master framework scFv sequences are shown in Fig. 1.
4. Oligonucleotide primers for polymerase chain reaction: *see* Table 1.
5. DNA polymerases: Taq polymerase (New England Biolabs, Ipswich, MA, USA) and Pfu polymerase (Promega, Madison, WI, USA) with vendor-provided reaction buffers.
6. dNTP mixture (New England Biolabs).
7. T4 DNA ligase (Invitrogen, Carlsbad, CA, USA).
8. *Sfi*I (New England Biolabs).
9. pComb3XTT phagemid vector (Addgene, Cambridge, MA, USA. Plasmid #63891).
10. DH5α *E. coli* chemically competent cells: Prepared according to Inoue method [9].
11. LB medium: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L water. Autoclave and store at room temperature.
12. SB medium: Dissolve 20 g yeast extract, 30 g tryptone, and 10 g 3-(N-morpholino) propanesulfonic acid (MOPS) in 1 L water. Adjust pH to 7.0 and autoclave. Store at room temperature.
13. QIAprep spin miniprep kit (QIAGEN, Hilden, Germany).
14. LBAG (LB-ampicillin-glucose) agar plates: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L water. Add 18 g bacteriological agar and autoclave. When the autoclaved solution cools down below 50 °C, add 1 mL of filter-sterilized

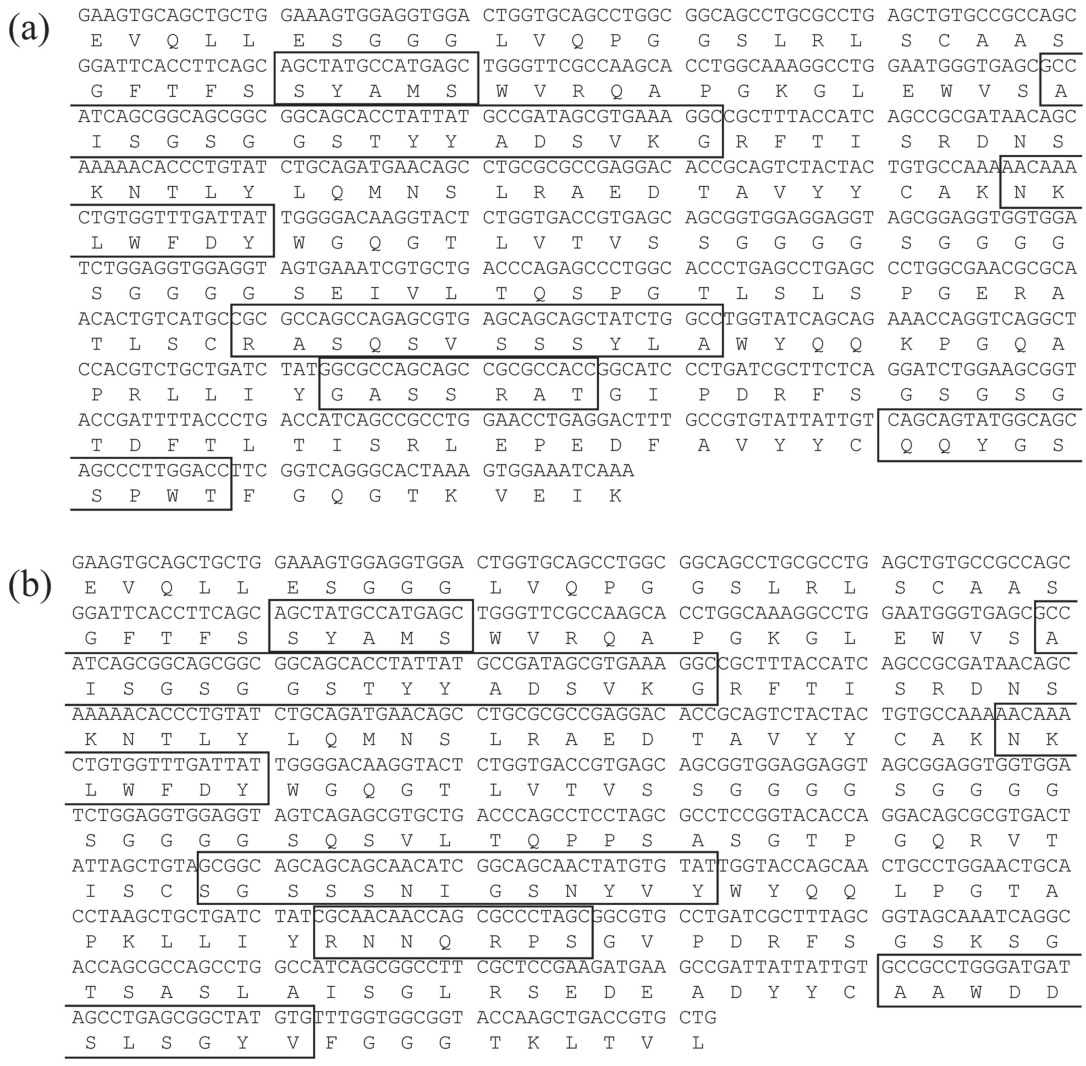

Fig. 1 The master scFv framework sequences for (**a**) VH-linker-VK (DP47 and DPK22 for VH and VK, respectively), and (**b**) VH-linker-VL (DP47 and DPL3 for VH and VL, respectively)

ampicillin (100 mg/mL) and 50 mL of 40% (w/v) filter-sterilized glucose. Mix evenly with gently stirring and pour on 100 mm diameter polystyrene petri dishes (20 mL per dish). Cool down at room temperature until agar solidifies, and keep the plates at 4 °C.

15. Agarose electrophoresis gel: For 1% agarose gel, use 1 g of agarose and 100 mL TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). Change the amount of agarose as needed to make 1.5 or 2% gels.
16. QIAquick Gel Extraction Kit (QIAGEN).
17. Minimal media agar plate: Add 5.6 g 5 × M9 salt and 15 g agar to 500 mL of deionized water and autoclave. When cooled to

Table 1

List of primers used in this protocol

Name*	Sequence
H1-f (H1-rc-b)	CAGCGGATTCACCTTCAGC
H1-b (H1-rc-f)	AGGTGCTTGGCGAACCCA
H2-f (H2-rc-b)	GGCCTGGAATGGGTGAGC
H2-b (H2-rc-f)	GCGGCTGATGGTAAAGCG
H3-f (H3-rc-b)	GGACACCGCAGTCTACTACT
H3-b (H3-rc-f)	CACCAGAGTACCTTGTCCC
K1-f (K1-rc-b)	CGCGCAACACTGTCATGC
K1-b (K1-rc-f)	TGGAGCCTGACCTGGTTTC
K2-f (K2-rc-b)	CCAGGTCAGGCTCCACGT
K2-b (K2-rc-f)	ACCGCTTCCAGATCCTGAG
K3-f (K3-rc-b)	CTGGAACCTGAGGACTTTG
K3-b (K3-rc-f)	ACTTTAGTGCCCTGACCG
L1-f (L1-rc-b)	GCGCGTGACTATTAGCTGT
L1-b (L1-rc-f)	AGGTGCAGTTCCAGGCAGT
L2-f (L2-rc-b)	GCCTGGAACTGCACCTAAG
L2-b (L2-rc-f)	GCCTGATTTGCTACCGCTA
L3-f (L3-rc-b)	CTTCGCTCCGAAGATGAAG
L3-b (L3-rc-f)	GTCAGCTTGGTACCGCCA
FR4 (FR4-b)	CTGGTGACCGTGAGCAGC
kFR1-f (kFR1-b)	GAAATCGTGCTGACCCAG
lFR3-f (lFR3-b)	CTGGCCATCAGCGGCCTTC
pC3X-f	GCACGACAGGTTTCCCGAC
pC3X-b	AACCATCGATAGCAGCACCG
pUC57-b	TTC GCC ATT CAG GCT GCG
pC3-seq	GTGAGCGGATAACAATTGA
dp-seq	AGAAGCGTAGTCCGGAACG

*Reverse-complement (rc) sequences of these primers were also prepared and used. Names of the reverse-complement primers are given in parentheses

~45 °C, add 1 mL of 1 M $MgSO_4$ (autoclaved), 0.1 mL of 1 M $CaSO_4$ (autoclaved), 5 mL of 40% glucose (filter-sterilized), and 0.25 mL of 1% thiamine HCl (filter-sterilized). Mix evenly with gently stirring and pour on 100 mm diameter

polystyrene petri dishes (20 mL per dish). Cool down at room temperature until agar solidifies, and keep the plates at 4 °C.

18. Electrocompetent TG1 *E. coli* cells (Lucigen, Middleton, WI, USA). Recovery medium is supplied with the competent cells. Also streak 1 μL of the electrocompetent TG1 *E. coli* cells on a minimal media plate for panning experiments.
19. Phosphate Buffered Saline (PBS): Dissolve 80 g NaCl, 2.0 g KCl, 17 g Na_2HPO_4, and 1.63 g KH_2PO_4 in 0.95 L deionized water. Set pH to 7.4 with HCl, and add water to 1 L.
20. PBS-Tween-20 (PBST): Add 0.05% (v/v) Tween 20 to PBS. Mix well.
21. 3% milk-PBST (mPBST): Dissolve 3% (w/v) nonfat dried milk in PBST.
22. Electroporation cuvette: 1 mm gap (Bio-Rad, Hercules, CA, USA).
23. Electroporator (Micropulser™, Bio-Rad).
24. Immunotube (Thermo Scientific, Waltham, MA, USA).
25. 5× polyethyleneglycol (PEG) precipitation buffer: 20% PEG-8000 (w/v) and 15% NaCl (w/v) in deionized water.
26. 100 mM triethylamine (TEA) solution: Mix 140 μL of TEA in 10 mL of deionized water.
27. VCSM13 helper phage (Agilent Technologies, Santa Clara, CA, USA).

3 Methods

3.1 Amplification of Oligonucleotides by Polymerase Chain Reaction

1. Dissolve the CDR oligonucleotide mixtures in 25 μL of nuclease-free water. The amount of DNA from the array synthesis is typically small, and the solution will contain a few ng/μL of DNA.
2. Add the followings to nuclease-free water to make a final volume of 100 μL in a PCR tube on ice: 2 ng of template DNA (CDR oligonucleotide mixture), 0.6 μM final concentration each of forward and backward primers, dNTP mixture (0.2 mM final concentration of each dNTP), 10 μL of Pfu polymerase buffer, 1 μL of Taq polymerase (5 units), and 0.2 μL of Pfu polymerase (0.6 units). Primer sequences are shown in Table 1, and primer pairs for the amplification of CDRs are shown in Table 2.
3. Perform PCR with following thermal cycle: initial melting at 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C, 7 min.

Table 2

Primer pairs for the amplification of CDRs from the oligonucleotide mixture

CDR	Forward primer	Backward primer
H1	H1-f	H1-b
H2	H2-f	H2-b
H3	H3-f	H3-b
K1 (kappa L1)	K1-f	K1-b
K2 (kappa L2)	K2-f	K2-b
K3 (kappa L3)	K3-f	K3-b
L1 (lambda L1)	L1-f	L1-b
L2 (lambda L2)	L2-f	L2-b
L3 (lambda L3)	L3-f	L3-b

4. PCR products are loaded onto 2% agarose gel and electrophoresed, and the gel is inspected under UV light. Gel bands near ~100 bp length are excised, and the amplified DNA fragments are extracted from the agarose gel using the DNA gel extraction kit, according to the manufacturer's protocol.

3.2 Construction of Single-CDR scFv Libraries

1. Amplify parts of the master framework sequences (DP47-linker-DPK22 [κscFv] and DP47-linker-DPL3 [λscFv]) in pUC57 by PCR according to Table 3, using primers shown in Table 1. Use the following PCR mixture: 200 ng of template DNA, 0.6 μM of the forward and backward primers, 0.2 mM each of dNTP, 10 μL of Pfu polymerase buffer, 0.5 μL of Taq polymerase (2.5 units), 0.2 μL of Pfu polymerase (0.6 units), and nuclease-free water added to 100 μL. The amplification thermal cycle is: initial melting at 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1.5 min; final extension at 72 °C, 7 min. Purify the amplified PCR products by 1.5% agarose gel electrophoresis as described above.
2. Assemble single-CDR scFv libraries by overlap extension PCR, according to the scheme shown in Table 4. Use the same reaction mixture and thermal cycle as in Subheading 3.2, **step 1**, and purify the PCR products by 1% agarose gel electrophoresis as described above.
3. Digest the PCR products and pComb3X vector with *Sfi*I (*see* **Note 2**), and purify the digested DNA by 1% agarose gel electrophoresis as described above.
4. Ligate the *Sfi*I-digested single-CDR scFv libraries and pComb3X vector at room temperature for ~16 h by mixing 1 μg each of the scFv and vector DNA, 5 μL of T4 DNA ligase

Table 3

PCR scheme for the amplification of framework regions for single-CDR scFv library construction

Template	Forward primer	Reverse primer	Product name
λscFv-pUC57	pUC57-b	H1-rc-b	H1-N
		H2-rc-b	H2-N
		H3-rc-b	H3-N
		L1-rc-b	L1-N
		L2-rc-b	L2-N
		L3-rc-b	L3-N
	H1-rc-f	pC3X-f	H1-C
	H2-rc-f		H2-C
	H3-rc-f		H3-C
	L1-rc-f		L1-C
	L2-rc-f		L2-C
	L3-rc-f		L3-C
κscFv-pUC57	pUC57-b	K1-rc-b	K1-N
		K2-rc-b	K2-N
		K3-rc-b	K3-N
	K1-rc-f	pC3X-f	K1-C
	K2-rc-f		K2-C
	K3-rc-f		K3-C

buffer (10×), and 2 μL of T4 DNA ligase (800 units) in 50 μL final volume adjusted with nuclease-free water.

5. After ligation, precipitate DNA by adding 5 μL of 3 M sodium acetate (pH 5.2) and 128 μL of absolute ethanol to the ligation mixture and incubating it at −20 °C for >2 h (70% final ethanol concentration by volume). Subsequently, the precipitated DNA was spun down (14,000 × *g*, 15 min at 4 °C) and washed twice with cold 70% ethanol. The DNA pellets were briefly dried and dissolved in 10 μL of 10% glycerol solution.
6. Mix the ligated DNA (10 μL) with 50 μL of electrocompetent TG1 *E. coli* cells, and add the mixture to an electroporation cuvette (1 mm gap). Incubate the cuvette on ice for 1 min, and transform the bacteria by electroporation (a single 2.50 kV pulse).
7. After electroporation, immediately add 1 mL of warm (37 °C) recovery medium to the cuvette and pipet up and down several times to resuspend the cells. Repeat the procedure once again,

Table 4

Assembly of single-CDR scFv libraries by overlap extension PCR

Library	Template 1[a]	Template 2[b]	Template 3[a]	Fwd primer	Rev primer
H1-scFv	H1-N	H1	H1-C	pUC57-b	pC3X-f
H2-scFv	H2-N	H2	H2-C		
H3-scFv	H3-N	H3	H3-C		
L1-scFv	L1-N	L1	L1-C		
L2-scFv	L2-N	L2	L2-C		
L3-scFv	L3-N	L3	L3-C		
K1-scFv	K1-N	K1	K1-C		
K2-scFv	K2-N	K2	K2-C		
K3-scFv	K3-N	K3	K3-C		

[a]Templates 1 and 3 are PCR products shown in Table 3
[b]Templates 2 are amplified synthetic CDR oligonucleotide mixtures shown in Table 2

and combine the bacterial suspensions (2 mL). Incubate the transformed cells at 37 °C for 1 h with shaking at 250 rpm.

8. To estimate the transformation titer, plate 100 µL of 10^{-3} and 10^{-4} dilutions of the cells on LBAG agar plates (*see* **Note 3**). Centrifuge the remaining cells (2000 × *g*, 15 min), and resuspend the pellet in 200 µL of LB medium. Plate the resuspended bacteria on a 150 mm diameter LBAG agar plate and incubate overnight at 37 °C.
9. Next morning, add 5 mL of SB medium to the 150 mm diameter agar plates, and scrape the bacterial growth using flame-sterilized glass spreader. Add 0.5 volume of sterile 50% glycerol (16.7% final glycerol concentration), mix well, freeze 1 mL aliquots with liquid nitrogen or dry ice–acetone bath, and store at −80 °C.

3.3 Proofreading of Synthetic CDRs

The synthetic CDRs were proofread by one round of panning of the single-CDR scFv libraries against surfaced-immobilized anti-HA monoclonal antibody. Because the scFv constructs have an HA-tag at C-terminus, CDR sequences with stop codons or frameshifts can be selectively depleted. Follow the protocol below for each single-CDR scFv library.

1. Add 50 µL of the frozen single-CDR library *E. coli* stock from Subheading 3.2, **step 9** to 20 mL of SB medium with 100 µg/mL ampicillin, and grow at 37 °C for 2 h with shaking at 200 rpm.

2. When the culture become slightly turbid (OD600 = ~0.5–1), add VCSM13 helper phage (10^{11} pfu) and incubate for 1 h at 37 °C with gentle shaking (120 rpm).
3. Add kanamycin to 70 μg/mL and shake overnight at 30 °C, 200 rpm.
4. On a minimal media plate, streak TG1 *E. coli* cells (*see* **Note 4**). Incubate the plate overnight at 37 °C.
5. Next morning, centrifuge the overnight culture at 14,000 × *g* for 15 min. Take the phage-containing supernatant and add 5 mL of 5× polyethyleneglycol (PEG) precipitation solution. Incubate the mixture on ice for 30 min.
6. While precipitating phage, coat an immunotube with anti-HA monoclonal antibody. Dilute the antibody in 1 mL PBS at 1 μg/mL concentration and add the solution to an immunotube. Incubate the tube at 37 °C for 1 h. Also inoculate 10 mL of SB medium with a single colony of TG1 *E. coli*.
7. Centrifuge the precipitated phage at 14,000 × *g* at 4 °C for 20 min. Discard the supernatant and dissolve the phage pellet in 300 μL PBS. Centrifuge the phage suspension again at 14,000 × *g* for 15 min to clear cell debris.
8. Take the phage-containing supernatant and add 700 μL of mPBST to block the phage for 1 h at room temperature.
9. Remove the coating solution from the immunotube after 1 h, and rinse the tube with deionized water three times. Block the tube by adding mPBST to the brim and incubating at room temperature for 1 h.
10. Remove the blocking solution (mPBST), and add the blocked phage to the tube. Incubate the tube at 37 °C for 1.5 h with shaking (200 rpm). Save a few microliters of the blocked phage for input titration (*see* below).
11. Discard the phage solution and wash unbound phages with PBST. Add 1 mL PBST to the tube, vortex briefly, further fill the tube with PBST to the brim, discard the wash solution, and rinse with deionized water. Repeat the wash cycle three times.
12. Elute captured phages by adding 1 mL of 100 mM triethylamine solution and incubating at room temperature for 10 min (*see* **Note 5**). Vortex briefly, and transfer the eluted phage to a conical tube. Add 0.5 mL of 1 M Tris (pH 7.0) to neutralize the solution.
13. Add 8.5 mL of mid-log phase (OD600 = ~0.5–1.0) TG1 *E. coli* cells. Gently shake the culture (120 rpm) at 37 °C for 1 h to allow phage infection. Also prepare a 10^{-7} dilution (in SB medium) of the input phage from Subheading 3.3, **step 10**.

Mix 1 μL of the diluted phage with 50 μL of the mid-log phase TG1 and incubate at room temperature for 1 h for input titration.

14. After 1 h, take 1 μL of the infected culture and dilute it in 1 mL SB medium. Plate 10 and 100 μL of the diluted bacteria on LB-ampicillin agar plates for output titration. Also plate the input titration mixture from Subheading 3.3, **step 13** on an LB-ampicillin agar plate. Incubate the plates overnight at 37 °C (*see* **Note 6**).
15. Centrifuge the remaining infected bacteria at 3000 × *g* for 15 min. Discard the supernatant and resuspend the pellet in 500 μL of SB medium. Plate the resuspended bacteria on a 150 mm diameter LBAG plate. Incubate the plate overnight at 37 °C.
16. Next morning, collect bacteria from the 150 mm plate and stock 500 μL frozen aliquots as described in Subheading 3.2, **step 9**.

3.4 Construction of the Final scFv Library

Perform the following protocol for each of the sub-libraries (in this example, scFv libraries with a kappa or a lambda light chain repertoire are constructed, amplified, and rescued separately).

1. Take out one 500 μL frozen stock of each proofread single-CDR scFv library from Subheading 3.3, **step 16**. Centrifuge the bacteria at 14,000 × *g* for 15 min.

Table 5

Amplification of the proofread CDR and adjoining framework regions by PCR

Template	Forward primer	Reverse primer	Product Name
H1-scFv (proofread)	pC3X-f	H2-rc-b	H1-PR
H2-scFv (proofread)	H1-rc-f	H3-rc-b	H2-PR
H3-scFv (proofread)	H2-rc-f	FR4-b	H3-PR
L1-scFv (proofread)	L1-f	L2-rc-b	L1-PR
L2-scFv (proofread)	L1-rc-f	lFR3-b	L2-PR
L3-scFv (proofread)	lFR3-f	pC3X-b	L3-PR
K1-scFv (proofread)	kFR1-f	K2-rc-b	K1-PR
K2-scFv (proofread)	K1-rc-f	K3-rc-b	K2-PR
K3-scFv (proofread)	K2-rc-f	pC3X-b	K3-PR
λscFv-pUC57	FR4-f	L1-rc-b	Lambda linker
κscFv-pUC57	FR4-f	kFR1-b	Kappa linker

2. Discard supernatant, and extract plasmid DNA from the pellets by Miniprep following the manufacturer's protocol.
3. Using primers shown in Table 1 and PCR scheme shown in Table 5, amplify the proofread CDR and adjoining framework regions (initial melting at 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C, 7 min). Use the PCR mixture shown in Subheading 3.2, **step 1**.
4. Separate and extract DNA bands from 1% agarose gel using the DNA gel extraction kit, following the manufacturer's protocol.
5. Assemble V_H and linker-V_L fragments, each with three diversified and proofread CDRs, by overlap extension PCR following the scheme shown in Table 6 (initial melting at 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; final extension at 72 °C, 7 min). Use the PCR mixture shown in Subheading 3.2, **step 1** except the amounts of the template DNA. Separate and extract DNA bands from 1% agarose gel using the DNA gel extraction kit, following the manufacturer's protocol.
6. Assemble the final scFv repertoires with six diversified and proofread CDRs by overlap-extension PCR of V_H and linker-V_L fragments using pC3X-f and pC3X-b primers. Use the same thermal cycle and reaction mixture as in Subheading 3.2, **step 1**, except the amounts of the template DNA (50 ng each of V_H and linker-V_L fragments). Perform eight 100-μL reactions in parallel.
7. Pool the PCR products (800 μL total) and add 80 μL of 3 M sodium acetate (pH 5.2) and 2.0 mL of absolute ethanol. Mix well and incubate at −20 °C for >2 h. Centrifuge the mixture at 14,000 × *g* for 15 min, and dissolve the precipitate DNA

Table 6

Assembly of VH, linker-VL, and scFv by overlap extension PCR

Template 1	Template 2	Template 3 (if any)	Fwd primer	Rev primer	Product name
H1-PR	H2-PR	H3-PR	pC3X-f	FR4-b	VH
L1-PR	H2-PR	L3-PR	L1-f	pC3X-b	VL
K1-PR	K2-PR	K3-PR	kFR1-f	pC3X-b	VK
VL	Lambda linker		FR4-f	pC3X-b	Linker-VL
VK	Kappa linker		FR4-f	pC3X-b	Linker-VK
VH	Linker-VL		pC3X-f	pC3-seq	λ library
VH	Linker-VK		pC3X-f	dp-seq	κ library

pellet in 50 μL of nuclease-free water. Separate and extract DNA bands from 1% agarose gel using the DNA gel extraction kit, following the manufacturer's protocol.

8. Digest the purified PCR product and pComb3X vector with *Sfi*I restriction endonuclease. Incubate 10 μg of DNA with supplied buffer and 40 units of *Sfi*I in 50 μL reaction volume at 50 °C for 16 h. Separate and extract DNA bands from 1% agarose gel using the DNA gel extraction kit, following the manufacturer's protocol.
9. Set up ligation reaction. Mix 2 μg each of the *Sfi*I-digested PCR product and vector, 5 μL of 10× T4 ligase buffer, 2 μL of T4 DNA ligase (800 units), and nuclease-free water to 50 μL final volume. Incubate the reaction mixture overnight at room temperature.
10. Next morning, inactivate the ligase by incubating the reaction mixture for 5 min at 70 °C, then precipitate the ligated DNA and dissolve the pellet in 10 μL of 10% glycerol, as described in Subheading 3.2, **step 5**.
11. Transform electrocompetent TG1 *E. coli* cells with the ligated DNA as described in Subheading 3.2 **steps 6** and **7**.
12. For transformation titration, plate 100 μL of 10^{-3} and 10^{-4} dilutions of the cells on LBAG agar plates (*see* **Note 3**). Add the remaining culture to 400 mL of SB medium supplemented with 100 μg/mL ampicillin and 2% glucose. Incubate the culture overnight at 37 °C with shaking (200 rpm).
13. Next morning, harvest the cells by centrifugation (3000 × *g*, 15 min), resuspend the pellet in 10 mL SB medium, and add 0.5 volume of 50% glycerol (16.7% final glycerol concentration). Mix well, freeze 1 mL aliquots with liquid nitrogen or dry ice–acetone bath, and store at −80 °C.
14. Phage antibody library can be rescued from the frozen *E. coli* stocks and used for the generation of antigen-binding clones, following the protocol described in [6].

4 Notes

1. For specific amplification of CDRs, some nucleotides in the codon-optimized master framework sequences may need to be changed to minimize cross-priming while maintaining the same amino acid sequences. Antibody variable gene segments have relatively high sequence similarities with one another, and careful inspection of the primer sequences for possible nonspecific priming or primer dimer formation is necessary.

2. Incubation of the reaction mixture at 50 °C in a water bath or a dry block heater may cause evaporation and condensation of water droplets on the cap of the microcentrifuge tube, which changes the concentrations of reaction components and affects the digestion efficiency and/or accuracy. To minimize this, a heater with heated lid can be used. Alternatively, the reaction tubes can be put in a 50 mL conical tube, which is then submerged using a flask weight in a water bath set at 50 °C.
3. The transformation titer is calculated as:
   [No. of colonies × 2 (mL recovery medium culture) × 1000 (μL/mL)]/[100 (μL plated) × dilution fold (10^{-3} or 10^{-4})].
4. TG1 *E. coli* has a genotype of:
   K-12 *glnV44 thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK– mK–)* F′ [*traD36 proAB+ lacIq lacZΔM15*].
   Growth of TG1 cells on a minimal media agar plate (with thiamine and without proline) selects for bacteria with F' factor which harbors *proA* and *proB* genes required for proline biosynthesis. Also the amber stop codon (UAG) is suppressed by the *glnV44* mutation, which is required for the display of a foreign protein on the surface of M13 phage when using pComb3X or some other phagemid vectors that carry an amber codon at the 5' end of *gIII*.
5. The pH of 100 mM triethylamine is about 11.5. At pH 11, M13 bacteriophage retains the infectivity for at least 20 min [10], suggesting that the elution time of 10 min would probably not adversely affect the outcome of panning.
6. The input titer is calculated as:

[No. of colonies × 1000 (μL blocked phage)]/[dilution fold (10^{-7}) × 1 (μL of diluted phage added to TG1)].

The output titer is calculated as:

[No. of colonies × 10 (mL infected culture) × 1000 (μL/mL)]/[dilution fold (10^{-3}) × plated volume in μL (10 or 100 μL)].

Acknowledgment

This work was supported by the National Research Foundation of Korea (NRF) grant for Medical Bioconvergence Research Center (NRF-2013M3A6A4044991) and the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (NRF-2015M3A9B6029138) to H.S.

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http://www.springer.com/978-1-4939-6855-8

Synthetic Antibodies
Methods and Protocols
Tiller, Th. (Ed.)
2017, XIV, 420 p. 102 illus., 74 illus. in color., Hardcover
ISBN: 978-1-4939-6855-8
A product of Humana Press