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Molecules and Cells

Construction of a Large Synthetic Human scFv Library with Six Diversified CDRs and High Functional Diversity

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Antibody phage display provides a powerful and efficient tool for the discovery and development of monoclonal antibodies for therapeutic and other applications. Antibody clones from synthetic libraries with optimized design features have several distinct advantages that include high stability, high levels of expression, and ease of downstream optimization and engineering. In this study, a fully synthetic human scFv library with six diversified CDRs was constructed by polymerase chain reaction assembly of overlapping oligonucleotides. In order to maximize the functional diversity of the library, a β-lactamase selection strategy was employed in which the assembled scFv gene repertoire was fused to the 5′-end of the β-lactamase gene, and in-frame scFv clones were enriched by carbenicillin selection. A final library with an estimated total diversity of 7.6×10^9, greater than 70% functional diversity, and diversification of all six CDRs was obtained after insertion of fully randomized CDR-H3 sequences into this proofread repertoire. The performance of the library was validated using a number of target antigens, against which multiple unique scFv sequences with dissociation constants in the nanomolar range were isolated.

INTRODUCTION

Phage display technology has been successfully employed in the selection of antigen-specific clones from antibody fragment libraries (Hoogenboom and Chames, 2000; Rader and Barbas, 1997; Winter et al., 1994). Antibody discovery by phage display from a large, naïve library is especially useful because it greatly facilitates the discovery and development process by circumventing the lengthy immunization stage, and it also enables the development of fully human antibodies for therapeutic applications (Kim et al., 2005). Target-specific monoclonal antibodies can be identified within weeks by phage display, compared with several months required for the conventional hybridoma technology. However, the construction of large (> 10^9) antibody libraries takes a lot of time and resources and is a major obstacle that impedes the wider applicability of this technology.

Diversity of antibody libraries can be obtained from natural (i.e. B-cells from humans or lab animals) or synthetic sources. Natural libraries consist of antibody clones that are proofread (during B-cell development) and thus encode functional proteins; the sequence diversity is concentrated in the complementarity-determining regions (CDRs) but the framework regions are also highly diverse (de Haard et al., 1999; Vaughan et al., 1996). The framework diversity of the natural libraries may be advantageous in antibody function and stability because the CDRs that form the antigen combining site exist within the context of their natural framework partners, but it also complicates downstream optimization and engineering of the selected clones. Synthetic libraries usually have a single or limited number of synthetic frameworks upon which the CDR diversity from either synthetic or (in case of semi-synthetic libraries) natural sources is introduced (Knappik et al., 2000; Sidhu and Fellouse, 2006; Silacci et al., 2005; Soderlind et al., 2000). The limited framework diversity makes it easier to standardize the engineering and optimization process of the selected clones; however, the structural compatibility between the CDR loops and the framework regions cannot be guaranteed.

The functional diversity of a synthetic antibody library is significantly affected by the design and construction strategy. Because the CDR diversity is typically introduced by a polymerase chain reaction (PCR) using degenerate oligonucleotides, errors during oligonucleotide synthesis may result in frameshifts and premature stop codons that reduce the functional diversity of the library. Indeed, it was reported that the introduction of a long synthetic CDR-H3 loop significantly reduced the proportion of full-length single chain variable fragment (scFv) clones in synthetic libraries (Lee et al., 2004). Obviously, this problem can be alleviated by restricting the number of CDRs that are diversified (de Wildt et al., 2000; Lee et al., 2004; Pini et al., 1998; Silacci et al., 2005); however, it is likely that this will negatively affect the performance of the resulting library. Synthetic or semi-synthetic antibody libraries with full diversification of all six CDRs have also been reported; for example, the introduction of the natural CDR diversity to a synthetic scaffold (Jirholt et al., 1998; Soderlind et al., 2000) produced semi-synthetic libraries with high functional diversity. While libraries with high quality

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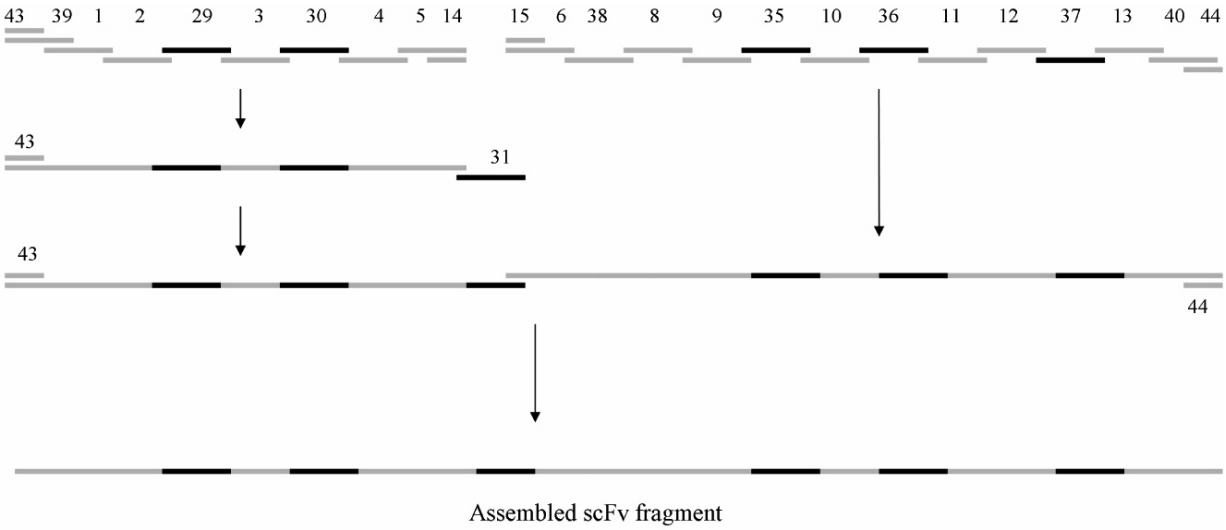

Fig. 1. Assembly of oligonucleotides into scFv by PCR. Framework regions (gray) and complementarity-determining regions (black) are indicated. See "Materials and Methods" for experimental detail and Table 1 for the sequence of each oligonucleotide.

and functional diversity can be obtained by using the proofread CDR repertoires from natural sources, the availability of a large number of human B-cells from multiple individual donors required for the construction of a large naïve human antibody library may be a challenge for many in the field. Libraries can also be subjected to an affinity selection process for the enrichment of functional clones; for example, the functional diversity of an scFv phage library with kappa light chain could be enhanced by protein L selection (Loset et al., 2005). Yet another strategy to improve the functional diversity is through genetic selection (Rothe et al., 2008; Seehaus et al., 1992), in which the fusion of the antibody fragments with β-lactamase leads to the selection of in-frame clones via antibiotic selection.

In this study, we designed and constructed a synthetic human antibody library with a high functional diversity and selected multiple target-specific antibody clones against a number of antigens from this library using phage display technology. This scFv library is based on a single scaffold and, like other single-scaffold libraries, will facilitate the standardization of the engineering of selected clones such as reformatting to IgG or affinity maturation. In order to maximize the functional diversity (i.e., the proportion of the clones that encode full-length scFv proteins), the CDRs, except the highly diverse CDR-H3, were simultaneously subjected to a β-lactamase proofreading process. This resulted in a highly functional, fully synthetic human scFv library with six diversified CDRs that yielded multiple unique, target-specific clones when challenged with various antigens.

MATERIALS AND METHODS

Materials

Polyacrylamide gel electrophoresis (PAGE)-purified DNA oligonucleotides were supplied by Genotech (Korea). PCR was performed using the Expand High Fidelity PCR system (Roche Applied Science). The SfiI restriction enzyme and T4 ligase were also purchased from Roche Applied Science. Human peroxiredoxins 1 and 2 (hPrxI and hPrxII) and tumor necrosis factor-alpha (TNF-α) were obtained from AbFrontier (Korea). Rat sulfiredoxin-glutathione S-transferase fusion protein (rSrx-GST) was a gift from professor Sue Goo Rhee (Ewha Womans University). Human, rabbit, and bovine serum albumins and fluorescein isothiocyanate (FITC) were purchased from Sigma (USA). Fluoresceine-BSA was prepared by reacting FITC with BSA following the manufacturer's protocol. The β-lactam antibiotics carbenicillin and ampicillin can be (and were) used interchangeably.

Assembly of V gene repertoires

V_H and J_H-linker-V_L sequences were assembled by PCR using 10 and 14 overlapping PAGE-purified oligonucleotides, respectively (Table 1, Fig. 1). Briefly, 0.1 μM each of the internal oligonucleotides and 6 μM each of the amplification primers were mixed with 0.8 mM dNTPs, 1x PCR buffer, and 3.5 units of DNA polymerase in 100 μl, and PCR (94°C, 2 min; 30 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1.5 min at 72°C; 7 min at 72°C) was performed. The products (~300 and ~400 bps, respectively) were purified from agarose gel bands. CDR-H3 sequences were appended to V_H using degenerate oligonucleotides (Table 1) by PCR (V_H-short-f and CDR-H3-9b, -14b, -20b, or -20SSb primers; PCR program of: 2 min at 94°C; 30 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1 min at 72°C; 7 min at 72°C). The resulting V_H-CDR-H3 fragments were purified from agarose gel bands and fused to J_H-linker-V_L by PCR using V_H-short-f and V_L-short-b primers to yield scFv PCR products (~750 bps). The products were digested with SfiI and cloned into the pComb3X vector (Scott and Barbas, 2001). After sequencing, clones with the correct framework sequences were selected and used as templates for PCR to construct scFv repertoires with mutation-free framework regions (Table 2) (2 min at 94°C; 25 cycles of 0.5 min at 94°C, 0.5 min at 58°C, and 2 min at 72°C; 7 min at 72°C).

Proofreading of the assembled scFv repertoires

PCR was performed using pET17b as a template and primers bla-f and bla-b (2 min at 94°C; 20 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 3 min at 72°C; 7 min at 72°C). The PCR product was digested with SfiI and ligated with an SfiI-digested scFv gene (used as a stuffer) to yield the plasmid vector pFDV. Plasmid vector pFDV is a modified pET17b plasmid that has two SfiI sites between the beta lactamase signal sequence to the beginning of the beta lactamase open reading frame. Inser-

Table 1. List of oligonucleotides used in this work

Primer name	Number	Sequence
Primers for the framework regions		
FR-H1-f	1	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTG
FR-H1-b	2	GCTAAAGGTGAATCCAGAGGCTGCACAGGAGAGTCTCAGGGACCCCCCAGGCTG
FR-H2-b	3	TGAGACCCACTCCAGCCCCTTCCCTGGAGCCTGGCGGACCCA
FR-H3-b	4	GGCTGTTCATTTGCAGATACAGCGTGTTCTTGGAATTGTCTCTGGAGATGGTGAACCG
FR-H3-f	5	GTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCG
JH-15-f	6	TTCGACTACTGGGGCCAGGGTACACTGGTCACCGTGAGCTCA
JH-6-f	7	ATGGACTGCTGGGGCCAGGGTACACTGGTCACCGTGAGCTCA
FR-L1-f	8	CAGTCTGTGCTGACTCAGCCACCCTCAGCGTCTGGGACCCCC
FR-L1-b	9	ACAAGAGATGGTGACCCTCTGCCCGGGGGTCCCAGACGCTGAG
FR-L2-b	10	ATAGATGAGGAGTTTGGGGGCCGTTCCTGGGAGCTGCTGGTACCAG
FR-L3-b	11	GATGGCCAGGGAGGCTGAGGTGCCAGACTTGGAGCCAGAGAATCGGTCAGGGACCCC
FR-L3-f	12	TCAGCCTCCCTGGCCATCAGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTG
JL-f	13	TATGTCTTCGGCGGAGGCACCAAGCTGACGGTCCTAGGC
FRH3-short-b	14	CGCACAGTAATACACGGCC
JH15-short-f	15	TTCGACTACTGGGGCCAG
JH6-short-f	16	ATGGACGTCTGGGGCCAGGGTACACTG
pC3X-f	17	GCACGACAGGTTTCCCGAC
pC3X-b	18	AACCATCGATAGCAGCACCG
H1-b	19	GCTAAAGGTGAATCCAGAG
H2-f	20	CTGGGTCCGCCAGGCTCCAG
H2-b	21	TGAGACCCACTCCAGCCC
H3-f	22	CGGTTCACCATCTCCAGAG
L1-b	23	CAAGAGATGGTGACCCTC
L2-f	24	CTGGTACCAGCAGCTCCCAG
L2-b	25	ATAGATGAGGAGTTTGGG
L3-f	26	GGGGTCCCTGACCGATTC
L3-b	27	CACAGTAATAATCAGCCTC
JL-short-f	28	TATGTCTTCGGCGGAGGC
Degenerate primers for the complementarity-determining regions		
CDR-H1-f	29	CTCTGGATTCACCTTTAGCRRTTATKMTATGAGCTGGGTCCGCCAGGCTCCAG
CDR-H2-f	30	GGGCTGGAGTGGGTCTCAKBGATCTMTYMTRRTRRTRGTARTAHATATTACGCTGATTCTGTAAA AGGTCGGTTCACCATCTCCAGAG
CDR-H3-9-b	31	CTGGCCCCAGTAGTCGAAMNNMNNMNNMNNMNNTYTCGCACAGTAATACACGGC
CDR-H3-14-b	32	CTGGCCCCAGTAGTCGAAMNNMNNMNNMNNMNNMNNMNNMNNAVSAYCTYTCGCACAGTAATACAC GGC
CDR-H3-20-b	33	CTGGCCCCAGACGTCCATASCATHAKMAKAAKAMNNMNNMNNMNNMNNMNNMNNAMBAVBANV TYTCGCACAGTAATACACGGC
CDR-H3-20SS-b	34	CTGGCCCCAGACGTCCATASCATHAKMAKAAKAACAMNNMNNMNNMNNACAMNNAMBAVBANC TYTCGCACAGTAATACACGGC
CDR-L1-f	35	GAGGGTCACCATCTCTTGTASTGGCTCTTCATCTAATATTGGCARTAATDMTGTCWMCTGGTACC AGCAGCTCCCAG
CDR-L2-f	36	CCCAAACTCCTCATCTATKMTRATARTMAKCGGCCAAGCGGGGTCCCTGACCGATTC
CDR-L3-b	37	GAGGCTGATTATTACTGTGSTDCTTGGGATKMTAGCCTGARTGSTTATGTCTTCGGCGGAGGC
Primer for the V_H-V_L linker		
linker-b	38	CTGAGTCAGCACAGACTGCGATCCGCCACCGCCGGATCCACCTCCGCCTGAACCGCCTCCACC TGAGCTCACGGTGACCAG
Primers for the amplification of the scFv gene		
VH-f	39	AATTCGGCCCAGGCGGCCGAGGTGCAGCTGTTGGAG
VL-b	40	TTAAGGGCCGGCCTGGCCTAGGACCGTCAGCTTG
pET-bla-f	41	AAATGTGCGCGGAACCCC
pET-bla-b	42	CCCACTCGTGCACCCAAC
VH-short-f	43	AATTCGGCCCAGGCGGCC
VL-short-b	44	TTAAGGGCCGGCCTGGCC
Primers for the introduction of SfiI sites in pET17b vector		
bla-f	45	GAGGAGGAGGAGGGCCGCCTGGGCCAGGCAAAATGCCGCAAAAAAGG
bla-b	46	GAGGAGGAGGAGGGCCAGGCCGGCCAGCACCCAGAAACGCTGGTG
Sequencing primer		
ompseq	47	AAGACAGCTATCGCGATTGCAG

Table 2. Polymerase chain reaction (PCR) assembly of mutation-free framework regions with complementarity determining regions

PCR template	Forward primer	Reverse primer	product
M-*	20	18	#1
M-	22	18	#2
M-	24	18	#3
M-	26	18	#4
M-	28	18	#5
M-	17	19	a
M-	17	21	b
M-	17	23	c
#1	29	18	d
#2	30	18	e
#3	35	18	f
#4	36	18	g
#5	37	18	h
a + d	17	21	ad
b + f	17	25	bf
c + f	17	25	cf
ad + e	17	14	ade
ade	17	31	A
ade	17	32	B
ade	17	33	C
ade	17	34	D
bf + g	17	27	bfg
cf + g	17	27	cfg
bfg + h	15	18	E
cfg + h	16	18	F
A + E	17	18	AE
B + E	17	18	BE
C + F	17	18	CF
D + F	17	18	DF

*M- denotes scFv clones from initial assembly PCR (Fig. 1) that have mutation-free framework regions.

tion of intact open reading frames between those two SfiI sites results in a sequence encoding a functional beta-lactamase fusion protein that confers ampicillin resistance to carrying bacteria. This vector and the scFv PCR products described above were digested with SfiI, ligated, and transformed into the TOP10F′ *E. coli* strain. The transformed bacteria were plated on 15 cm LB-carbenicillin agar plates and incubated at 37°C for 12 h. Colonies were scraped from the agar plate and the plasmid DNA was isolated (Maxiprep kit, QIAGEN). The V_H region was amplified using pET-bla-f and FR-H3-short-b primers, and the J_H-linker-V_L region was amplified using J_H15-short-f or J_H6-short-f and pET-bla-b primers (50 ng plasmid DNA as template; 2 min at 94°C; 25 cycles of 0.5 min at 94°C, 0.5 min at 58°C, and 1 min at 72°C; 7 min at 72°C). CDR-H3 sequences were appended to the V_H PCR product as described above. The final assembly of scFv sequences was done in a two-step PCR, performed separately for each of the four CDR-H3 variants. First, 60 ng of the V_H-CDRH3 fragment and 90 ng of the J_H-linker-V_L fragment were mixed with 1× PCR buffer and 0.8 mM dNTPs in 100 μl, and 3.5 units of DNA polymerase were added. The mixtures were subjected to a PCR program of: 2 min at 94°C; three cycles of 0.5 min at 94°C, 0.5 min at 58°C, and 1 min at 72°C; 2 min at 72°C. After the reaction, 6 μM of each primer, pET-bla-f and pET-bla-b, and 3.5 units of DNA polymerase were added to each mixture, and the second PCR was performed: 2 min at 94°C; 25 cycles of 0.5 min at 94°C, 0.5 min at 58°C, and 2 min at 72°C; 7 min at 72°C.

Library ligation and transformation

SfiI-digested scFv fragments (1.3 μg) and the SfiI-digested pComb3X vector (4 μg) were ligated overnight as previously described (Andris-Widhopf et al., 2001). The reaction mixture was purified using a PCR purification kit (QIAGEN), eluted with 30 μl of 10% glycerol in nuclease-free water, and transformed into 2 × 300 μl of freshly prepared electrocompetent ER2537 *E. coli* cells. The transformed bacteria were incubated in 6 ml SOC media for one hour at 37°C. 150 μl each of 4×10^{-5}, 4×10^{-6}, and 4×10^{-7} dilutions were plated on LB-carbenicillin agar plates to measure the transformation titer. The remaining cells were added to 1 L of Super broth (SB) media (3% bactotryptone, 2% yeast extract, and 1% 3-(N-morpholino)propanesulfonic acid [MOPS], pH 7.0), supplemented with 2% glucose and 100 μg/mL carbenicillin, and cultured overnight at 37°C with shaking (250 rpm). Cells were harvested the next morning and resuspended in 10 ml SB media. A half volume of 50% sterile glycerol (v/v) was subsequently added, and the resulting glycerol stock was divided into 1 ml aliquots and kept at -80°C.

Library rescue

One aliquot (1 ml) of each of the frozen library stocks was thawed and added to 1 L of SB-carbenicillin media supplemented with 2% (w/v) glucose. Cells were allowed to grow at 37°C with shaking at 250 rpm until the optical density at 600 nm reached 0.5, at which point the culture was centrifuged (3,000 rpm, 15 min) and the cell pellet was resuspended in 1 L of SB-carbenicillin without glucose. VCSM13 helper phage (10^{12} plaque forming units [PFU]) was added, and the culture was incubated at 37°C for 1 hour with slow agitation (100 rpm). After helper phage infection, kanamycin was added to a final concentration of 70 μg/mL and the culture was grown 12 to 16 hours at 30°C with shaking at 250 rpm. The next morning, the culture was centrifuged and 250 mL of cold 5× polyethylene glycol (PEG) precipitation buffer (20% [w/v] PEG8000 and 15% [w/v] NaCl) was added to the phage-containing supernatant, and the mixture was incubated on ice for 30 minutes. Precipitated phages were harvested by centrifugation and resuspended in 20 ml phosphate buffered saline (PBS). After a clearing centrifugation at 10,000 rpm for 30 min to remove remaining *E. coli* cells and cell debris, the phages were PEG-precipitated again as described above and finally resuspended in 4 ml of PBS to which 2 ml of 50% glycerol was added. 1 μl each of 10^{-7}, 10^{-8}, and 10^{-9} dilutions of the phage library preparations were used to infect 50 μl of mid-log phase ER2537, which was then plated on an LB-carbenicillin agar plate to estimate the phage titer. The four sub-libraries were mixed to yield the final scFv library in such a way that the resulting library had an equal number of colony forming units (CFUs) from each of the four sub-libraries. The final library was divided into aliquots of 10^{12} CFUs and was kept frozen at -80°C.

Characterization of the library

Dot-blot analysis was performed using the induced culture supernatants and the periplasmic fractions of randomly chosen library clones. From each of the four sub-libraries, 24 clones were randomly picked (96 total) and grown in SB-ampicillin

(200 μl/clone in a 96-well plate). After isopropyl β-D-thiogalactopyranoside (IPTG) induction and overnight growth, the plate was centrifuged and the supernatants were transferred to a new 96-well plate. Periplasmic fractions were prepared from the remaining bacterial pellet by osmotic shock using 40 μl of 1× Tris-EDTA-sucrose buffer (TES; 20% sucrose, 50 mM Tris, and 1 mM EDTA, pH 8.0) and 60 μl of 0.2× TES buffer (total of 100 μl periplasmic extract). One microliter each of the supernatant and the periplasmic extract was applied to a nitrocellulose membrane. The membrane was dried at room temperature for 1 h, blocked with 5% milk-tris buffered saline (TBS)-0.1% tween 20 (mTBST), and detected with an anti-HA-horseradish peroxidase (HRP) conjugate.

In order to estimate the efficiency of scFv display on the phage, phage library preparations (4×10^8 - 1.6×10^9 cfu) along with VCSM13 helper phage (1.25×10^8 - 2×10^9 pfu) were analyzed by immunoblot using a monoclonal anti-pIII antibody (MoBiTec, Goettingen, Germany; used at 1/1,000 dilution). The percentage of phage particles displaying scFv was estimated by comparing the intensities of scFv-pIII and pIII bands with those of the control bands (wild-type pIII from VCSM13).

Selection from the library

For the first round of panning, 1 ml of antigen solution (1-10 μg/ml in PBS) was added to an immunotube and incubated overnight at 4°C or at 37°C for 1 h. The solution was decanted and the antigen-coated tube was rinsed twice with running water. Blocking of the tube surface was done with 3% BSA-PBS or 3% skim milk-PBS (mPBS) at room temperature for one hour. One library equivalent (10^{12} cfu) in 1 ml of 3% mPBS (or BSA-PBS) was added to the tube and incubated for one hour. In the case of rSrx-GST, the fusion partner GST was also added at 100 μg/ml concentration as a competitor. After the one-hour binding period, the tube was washed three times with TBS-0.1% Tween 20 (TBST) to remove unbound phages, and the bound phage particles were eluted with 1 ml of 100 mM triethylamine (30 min at room temperature) and neutralized with 0.5 mL of 1 M Tris buffer (pH 7.4). The neutralized phage solution (1.5 ml) was mixed with 8.5 ml of mid-log phase ER2537 *E. coli* cells. The infection was performed at 37°C with gentle shaking (100 rpm), and then 1 and 10 μl of the infected bacteria were plated onto Luria-Bertani (LB)-carbenicillin agar plates for output titering. The remaining cells were centrifuged and the cell pellet was resuspended in 0.2 ml of SB. The cells were then plated on a 15 cm agar plate containing SB-carbenicillin-2% glucose and grown overnight at 37°C. The next day, 5 ml of SB media was added to the plate and the cells were scraped off and thoroughly resuspended. A half volume of 50% glycerol (sterile) was added and mixed, and 1 ml aliquots were stored at -80°C. Output phages from the first round were rescued following the same protocol as described above, except that 20 μl of the *E. coli* stock was used to inoculate 20 ml of SB-carbenicillin-2% glucose and that 1 ml of the rescued phage library (culture supernatant) was used in the subsequent rounds of panning without PEG precipitation. Input titering was also performed in the second and subsequent rounds of panning (see above).

Screening of the selected clones

Single colonies from the output agar plate were inoculated into 200 μl of SB-carbenicillin in the wells of a 96-well microtiter culture plate and grown at 37°C with shaking (400 rpm) until turbid. IPTG (1 mM final concentration) or VCSM13 helper phage (10^9 pfu) was added to each well. IPTG-induced cultures were incubated at 30°C with shaking for 12-16 h; VCSM13-infected cultures were incubated stationary at 37°C for one hour, kanamycin was added (70 μg/ml final concentration) and allowed to grow overnight at 30°C with shaking. The next day, the plate was centrifuged and either phage-containing supernatants or scFv-containing periplasmic extracts (prepared by osmotic shock using TES buffer) were used in an enzyme-linked immunosorbent assay (ELISA) to screen for the target-binding clones. An anti-HA-HRP antibody (clone 3F10, Roche Applied Science) or anti-M13-HRP (GE Healthcare) antibody was used for the colorimetric detection of bound clones using the tetramethylbenzimidine (TMB) substrate.

Expression and purification of scFv clones

A single colony of ER2537 or TOP10F′ harboring a scFv clone in the pComb3X vector was grown overnight in 3 ml of SB-carbenicillin. This starter culture was added to 400 ml of SB-carbenicillin and grown at 37°C with shaking until the absorbance at 600 nm was 0.5. IPTG was added to the mid-log phase culture (1 mM final concentration) and the induced cells were grown overnight at 30°C with shaking. After centrifugation, periplasmic extract was obtained using the osmotic shock method. Briefly, the cell pellet was resuspended in 10 ml of ice-cold TES buffer and 15 ml of cold 0.2× TES was subsequently added. After incubation on ice for 30 min, the suspension was centrifuged and the supernatant containing the periplasmic fraction was obtained. The periplasmic extract was incubated with 0.5 mL of Ni-NTA agarose (Novagen) for 1 h at 4°C with slow rotation. The mixture was poured onto a Polyprep column (BioRad, USA), and the agarose beads were washed twice with 10 mL of wash buffer (PBS with 5 mM imidazole, pH 7.4). ScFv was eluted with three 0.5 mL fractions of elution buffer (PBS with 200 mM imidazole, pH 7.4), and analyzed by SDS-PAGE, ELISA, and surface plasmon resonance (SPR).

Affinity determination of purified scFv clones

ScFv proteins purified by immobilized metal ion affinity chromatography (IMAC) as described above were further purified by size exclusion chromatography (Superdex 75 column, GE Biosciences) to isolate monomeric scFv. Dissociation constants of these clones were determined by surface plasmon resonance using a BIAcore 3000 instrument and antigens immobilized on a carboxymethylated dextran surface (BIAcore's CM5 chip) at the density of 500-2000 RU. The typical measurement condition was: 10-2,000 nM analyte (scFv) in degassed PBS, 40 μl/min flow rate, an association phase for 120 s, and a dissociation phase for 600 s.

RESULTS

Design of the library

A synthetic human scFv library with a single scaffold (human variable heavy chain V_H3-23 [DP47] and human variable light chain V_λ1g [DPL3] joined by a 15-amino acid linker $[GGGGS]_3$) and six randomized CDRs was designed (Fig. 2). The CDR diversities in CDR-H1, H2, L1, L2, and L3 were introduced by using partially degenerate codons that mimic the natural diversity of human CDRs (Lee et al., 2004). The CDR-H3 has a much greater diversity in both the amino acid composition and the length of the loop than the other CDRs; therefore, four sub-libraries (AE, BE, CF, and DF) with fully randomized CDR-H3 of different lengths were designed and constructed. Of known human immunoglobulin sequences, a CDR-H3 length of 12 amino acids (Kabat definition) is the most common (Zemlin et al., 2003), therefore the sub-library BE was designed to have 12 CDR-H3 residues. Sub-libraries with shorter (7 amino acids, sub-library AE) and longer (18 amino acids, DF) CDR-H3

A

EVQLLESGGGLVQPGGSLRLSCAASGFTFS[XYXMS]WVRQAPGKGLEWVS[XIXXXXXXX]
YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA[XXXXXXXX]WGQGTLVTVSS
GGGGSGGGGSGGGGSQSVLTQPPSASGTPGQRVTISC[XGSSSNIGXNXVX]WYQQLPGT
APKLLIY[XXXX]RPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYC[XXWDXSLXX]YVF
GGGTKLTVLG

B

CDR-H1: (NSDG)-Y-(ADSY)-M-S

CDR-H2: (AGVSWL)-I-(YS)-(HPYS)-(NSDG)-(NSDG)-(SG)-(NS)-(KIT)

CDR-H3-AE: (RK)-(Xaa)$_4$-F-D-Y

-BE: (RK)-(DG)-(LPRVAG)-(Xaa)$_7$-F-D-Y

-CF: (RK)-(FSYCLPHRVADG)-(LPRITSVAG)-(LRISVG)-(Xaa)$_7$-(YS)-(YS)-(ADSY)-(YND)-(GA)-M-D-V

-DF: (RK)-(VADG)-(LPRITSVAG)-(LRISVG)-Xaa-C-(Xaa)$_4$-C-(YS)-(YS)-(ADSY)-(YND)-(GA)-M-D-V

CDR-L1: (ST)-G-S-S-S-N-I-G-(NS)-N-(NTDAYS)-V-(NTYS)

CDR-L2: (ADSY)-(ND)-(NS)-(KNQH)

CDR-L3: (GA)-(AST)-W-D-(ADSY)-S-L-(NS)-(GA)

Fig. 2. (A) Amino acid sequence of the constructed scFv library. The library is in V_H-linker-V_L format. Randomized parts of the CDRs are shown in boxes; the linker part is underlined. (B) Amino acid sequences of the CDRs (boxed residues in (A)). Any of the amino acids shown in parenthesis can occur in that position. Xaa: any of the 20 amino acids, plus a stop codon (TAG).

lengths were also designed. Lastly, the sub-library DF has an intraloop disulfide bond in its long CDR-H3 (18 amino acids). The intra-CDR-H3 disulfide bond encoded by the human D_H2 gene family is relatively common in human antibodies (Zemlin et al., 2003), and it is thought that the presence of the disulfide bond stabilizes the conformation of a long CDR-H3 loop.

Assembly of the scFv gene

As the first step in constructing the library, twenty-five overlapping oligonucleotides were assembled by PCR to yield a ~750 bp scFv DNA fragment (Table 1, Fig. 1), which was cloned into the pComb3X phagemid vector (Andris-Widhopf et al., 2001). Not surprisingly, sequencing analysis of the clones showed that the assembled sequences contained many mutations and frameshifts that made these initial transformants unsuitable for library construction. Mutation-free stretches from these sequences, which encoded the germline framework regions of V_H3-23 and V_λ1g, together with the degenerate oligonucleotides for complementarity-determining regions (Table 1) were assembled into scFv gene repertoires through a series of PCRs (Table 2) and were again ligated into the pComb3X vector. After transformation into ER2537, phage ELISA of individual clones was performed to evaluate the functional diversity of the assembled scFv repertoires. A positive ELISA signal against a surface-coated anti-HA antibody indicates the display of the phagemid-encoded HA tag and the scFv to which the tag is C-terminally fused on the phage surface. Thirty-six percent of the tested clones (35/96) showed positive phage ELISA signals.

Selection of functional scFv sequences and library construction

The ratio of the functional scFv clones after the second PCR assembly (36%) was still significantly lower than many other previously reported libraries. In order to further improve the functional diversity of the library, the scFv gene repertoires described above were ligated into the pFDV vector, a modified version of the pET17b vector that has two SfiI sites between the β-lactamase signal sequence and the beginning of the mature β-lactamase sequence (Fig. 3). The ligated DNA was transformed into TOP10F′ *E. coli* cells (a non-suppressor strain), which then were grown on a carbenicillin-containing LB agar plate so that only functional scFv clones without premature stop codons could survive and grow. Plasmid DNA from ~10^7 transformants thus obtained was used as a template for PCR to amplify V_H (from framework region 1 [FR1] through FR3) and J_H - linker - V_L regions. These fragments were assembled with CDR-H3 oligonucleotides into full-length scFv DNA in order to maximize the diversity of the antigen combining site. After ligation into the pComb3X phagemid vector and transformation into ER2537, a library with 7.6×10^9 total diversity was obtained (Table 3).

Characterization of the library

More than 70% of the clones encoded full-length, functional scFv molecules as determined by dot-blot and sequencing analyses. In the dot-blot analysis, the presence of scFv in the induced culture supernatants and the periplasmic fractions was detected using anti-HA-HRP conjugate (Fig. 4). Out of 96 clones tested, 71 showed a positive signal (74%); the periplasmic fraction in general contained more scFvs than the culture supernatant. For sequence analysis, 8 randomly chosen clones from each sub-library were sequenced (32 total sequencing reactions). Of these, two sequences were unreadable and 25 of the remaining 30 readable sequences encoded correct scFv genes (83%; sequence alignment in the supplementary material). Sequence analysis revealed that, with the exception of a small number of positions, each degenerate position contained the expected partial or full randomization of amino acids in roughly equal ratios. Of 23 randomized, non-CDR-H3 positions, four show marked deviations from the expected equal distribution (H31, H54, L32, and L95a; see supplementary material for the amino acid distribution of the CDRs). All 20 amino acids were represented in the twenty-three fully randomized positions of CDR-H3 (4 residues in the sub-library AE, 5 residues in DF, 7 residues each in BE and CF), at roughly the expected frequencies. The efficiency of the display was evaluated by the immunoblot analysis of the phage library using an anti-pIII antibody (Fig. 5). It is estimated that roughly 20 to 30 percent of

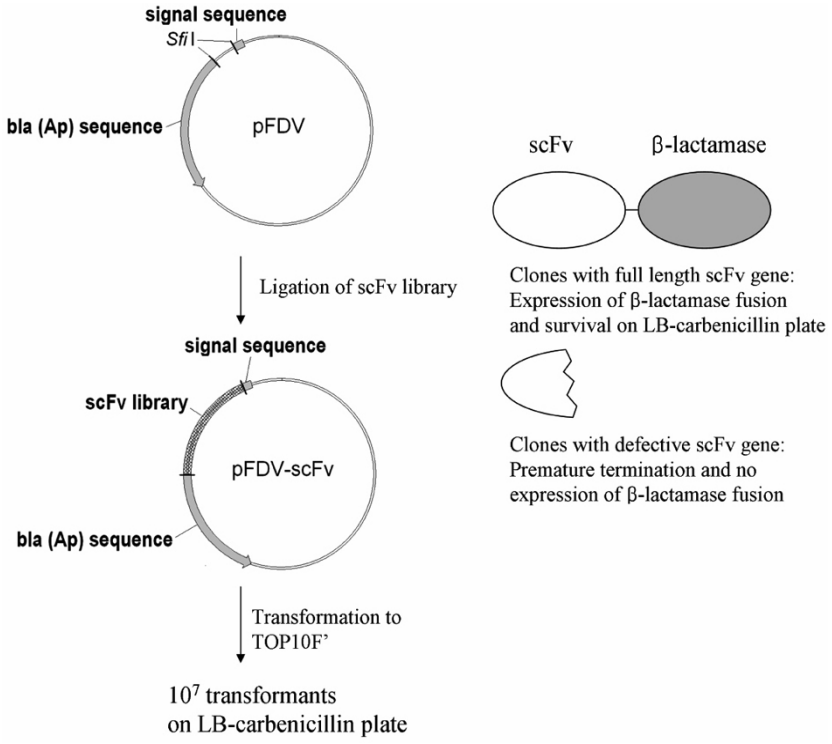

Fig. 3. Proofreading of the assembled scFv sequences using the pFDV vector. The assembled scFv repertoire was inserted into the pFDV vector between the β-lactamase signal sequence and the beginning of the mature β-lactamase sequence. In a non-suppressor *E. coli* strain such as TOP10F′, clones with stop codon (s) introduced by mutations and/or frameshift cannot produce a β-lactamase fusion protein and hence cannot grow on carbenicillin-containing media plate; the result is an enrichment of functional scFv clones in the repertoire.

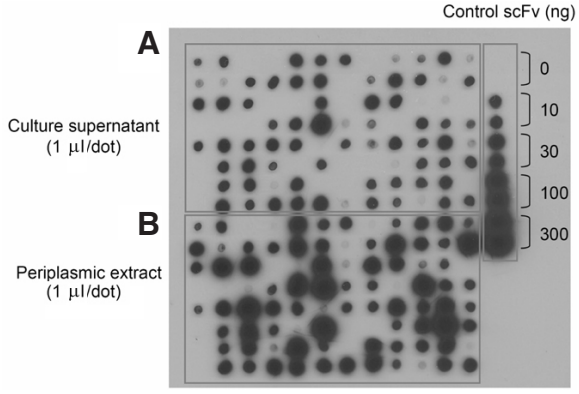

Fig. 4. Dot-blot analysis of 96 randomly selected clones from the four sub-libraries. (A) Induced culture supernatants and (B) periplasmic fractions (1 μl /dot each on nitrocellulose membrane) were detected by an anti-HA-HRP conjugate. Known amounts of purified scFv were used as a control. For each clone, total volume of the extracted periplasmic fraction (100 μl) was a half of the total culture volume (200 μl); therefore for a given clone, comparable dot intensities in (A) and (B) indicate twice more scFv was secreted to the culture media than remained in the periplasm.

phage particles display scFv on the surface, assuming 5 copies of pIII per phage particle (Sidhu, 2001).

Selection and characterization of antigen-specific scFv clones from the library

A number of antigens were used to validate the constructed library, as summarized in Table 4. Human targets such as human peroxiredoxins 1 and 2 (hPrxI and hPrxII; 91% homologous and 78% identical to each other), human serum albumin (HSA), insulin, and human tumor necrosis factor-α, as well as non-human proteins like rabbit serum albumin (RSA, 85% homologous and 75% identical to HSA), rat sulfiredoxin-GST fusion protein (rSrx-GST), hen egg white lysozyme (HEL), and a hapten (fluoresceine conjugated to BSA) were tested as panning antigens.

Table 3. Estimated size of the library

Sub-library	Size
AE	2.1×10^9
BE	2.7×10^9
CF	1.1×10^9
DF	1.7×10^9
Total	7.6×10^9

Panning the library against surface-immobilized antigens yielded multiple target-specific scFv clones. After three to four selection rounds, up to 94 output clones were screened by ELISA for their binding to the target antigen. Notably, anti-hPrxI clones were not (or very weakly) cross-reactive towards hPrxII, and vice versa, while one clone each of the RSA- and HSA-binders also bound HSA and RSA, respectively (data not shown). No GST binder was isolated from the rSrx-GST panning, in which GST was used as a competitor. Similarly, with BSA as a blocking agent, only one out of 94 clones screened from the fluorescein-BSA panning bound BSA. Sequence analysis of some of the ELISA-positive clones revealed that all of the sequenced clones encoded full-length scFv's; however, a small number of them contained amber stop codons (TAG), which are suppressed in the ER2537 strain. Convergence of CDR sequences (especially of V_H chain) was observed in some of the clones against certain targets (Table 5). For example, two of the unique anti-hPrxI clones share exactly the same

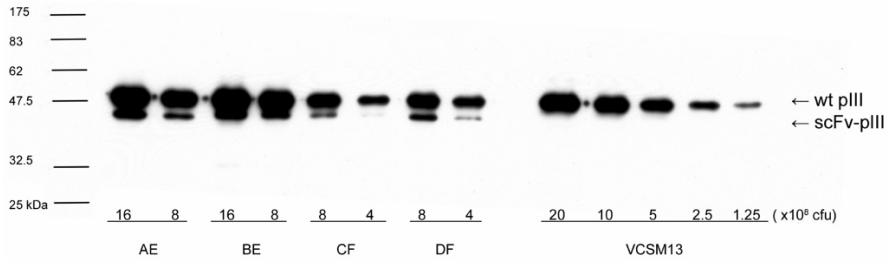

Fig. 5. Immunoblot of the phage-displayed scFv sub-libraries AE, BE, CF and DF detected with an anti-pIII antibody. Various amounts of VCSM13 helper phage (1.25×10^8 to 2×10^9 pfu) were used as control in order to estimate the efficiency of display. Since the pComb3X vector has a truncated gene III (amino acids 230 to 406), the scFv-pIII fusion and the wild-type pIII from VCSM13 have similar molecular weights (~46 kDa).

Table 4. Summary of library selection campaigns on target antigens

Antigen	Selection rounds	ELISA positive/ screened	Unique sequences/ analyzed
hPrxI	3	11/94	4/8
hPrxII	3	28/31	5/28
TNF-α	4	66/94	6/7
insulin	3	72/90	4/9
HSA	4	9/47	6/6
RSA	4	4/47	2/4
rSrx	3	27/31	4/4
HEL	4	21/94	1/4
fluorescein	3	92/94	12/12

CDR-H1 and CDR-H2, while they differed considerably in other CDRs including CDR-H3. The co-selection of the clones with identical or highly similar CDR sequences exemplifies the importance of these CDRs in the antibody-antigen binding interaction.

Up to 1 mg/liter of scFv could be obtained from a shaker flask culture of ER2537 or TOP10F′ cells after purification by immobilized metal ion affinity chromatography (IMAC) and size exclusion chromatography (Figs. 6A and 6B). Dissociation constants of the purified scFv clones against fluorescein, hPrxII, and TNF-α were in the nanomolar range, as determined by surface plasmon resonance measurement using a BIAcore 3000 instrument (Fig. 6C, Table 6).

DISCUSSION

Within the human genome, 51 V_H, 30 V_λ, and 40 V_κ gene segments exist (Pini et al., 1998). While they are all capable of producing functional variable domains, their usage, level of expression, and the stability of their gene products are not equal. Also, most of the antigen-binding interaction is attributed to the residues in the six complementarity-determining regions (CDRs); as is evident in humanized antibodies made by CDR grafting (Jones et al., 1986; Riechmann et al., 1988; Verhoeyen et al., 1988), the antigen specificity of the CDRs is largely conserved when they are grafted onto a different framework scaffold. In light of these observations, many antibody libraries have been successfully constructed using a single framework scaffold. Using a single scaffold for synthetic antibody library construction has several advantages. Library construction becomes easier, since all the diversity is concentrated in the six CDRs. The scaffold is generally chosen for the level of expression in various hosts, stability, and immunogenicity, therefore the selected clones from the library are likely to have favorable characteristics for downstream applications. Human heavy

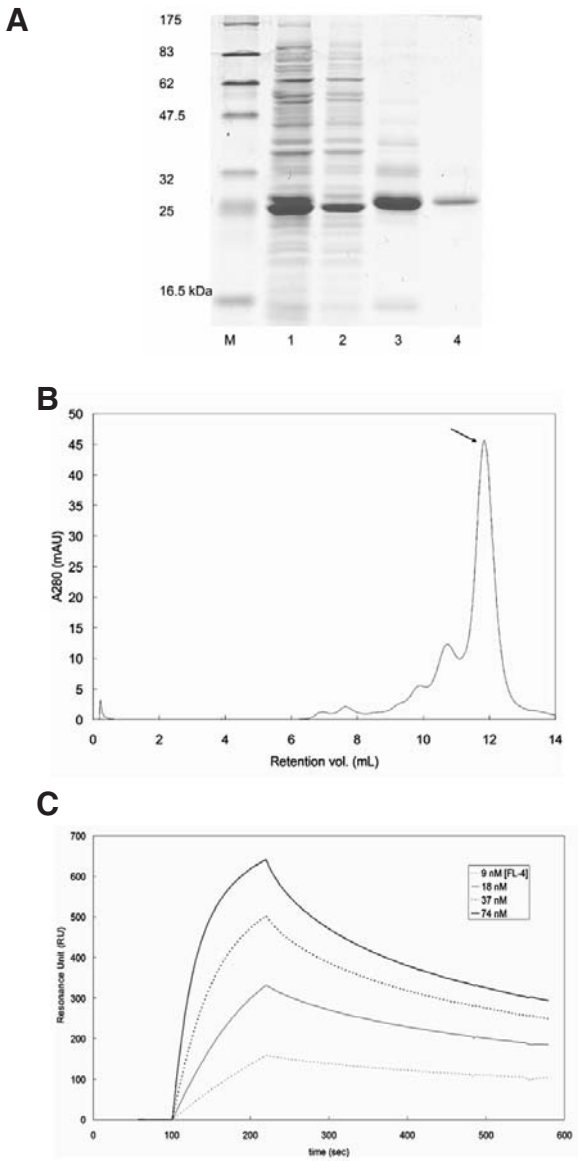

Fig. 6. (A) SDS-PAGE analysis of scFv purification (anti-fluorescein clone FL-4). M, marker; 1, periplasmic extract; 2, IMAC flow-through; 3, IMAC elution; 4, Size-exclusion chromatography (SEC)-purified scFv. (B) Purification of FL-4 scFv by SEC. Monomeric scFv peak is indicated by an arrow. (C) SPR sensogram of FL-4 binding to fluorescein-BSA conjugate immobilized on carboxymethyl dextran surface (CM5 chip).

Table 5. Convergence of CDR sequences after multiple rounds of binding selection. Randomized positions are underscored and the sequence convergence is shown in gray. Isolated occurrences of a single amino acid at positions where only two or three amino acids were allowed to occur (e.g. position L89 of CDR-L3) were not considered as convergence. IN, insulin; FL, fluorescein.

Clone		H1	H2	H3	L1	L2	L3
PrxI	1	SYSMS	AISPDDGSK	TDSP	SGSSSNIGSNSVT	ADSQ	GAWDASLSA
	2	SYSMS	AISPDDGSK	TYLV	TGSSSNIGNNYVN	ADNN	GTWDDSLSG
IN	1	NYSMS	AIYPSGSNT	GKRL	SGSSSNIGNNAVN	ANSH	GSWHSSLSA
	2	NYSMS	SIYPNGSSK	NKLW	SGSSSNIGSNSVS	ANSH	GTWDYSLSA
	3	NYSMS	LIYPDGSST	NKRV	TGSSSNIGNNSVT	YNSH	GAWDASLNG
FL	2	NYSMS	GISSSSSSK	GRTRVWGRR	TGSSSNIGNNYVY	YNSH	ASWDSSLNG
	3	NYAMS	GISHSSGSK	RRVPATDPMRSYANG	TGSSSNIGNNSVY	ANNQ	GAWDASLSG
	5	NYSMS	GIHSSSGSK	LRRRNALRMGYYSYA	SGSSSNIGNNYVT	ANSH	GAWDASLNG
	6	DYSMS	GISSSSGSK	RGHLKMRPT	TGSSSNIGNNNVS	ANNN	GAWDSSLNA

Table 6. Dissociation constants of selected target-specific clones determined by surface plasmon resonance. FL, fluorescein; Prx, human peroxiredoxin 2; TNF, human tumor necrosis factor alpha.

Clone	K_D (nM)
FL-1	390
FL-2	29
FL-3	240
FL-4	9
Prx-1	35
Prx-3	52
Prx-4	120
TNF-1	610

chain variable segment V_H3-23 and lambda light chain variable segment V_λ1g are known to exhibit high levels of expression and stability (Hoet et al., 2005; Silacci et al., 2005; Soderlind et al., 2000) and were chosen for the scaffold of the library construction in this study.

The initial PCR-assembled scFv repertoires contained an unacceptably high occurrence of defective clones even after mutation-free stretches of framework regions were selected and re-assembled, presumably due to the errors during oligonucleotide synthesis. The β-lactamase proofreading of the library (Faix et al., 2004; Gerth et al., 2004; Lutz et al., 2002; Rothe et al., 2008; Zacchi et al., 2003) was performed by cloning the library into the pFDV vector. With the library in the pFDV vector transformed into a non-suppressor strain such as TOP10F′, β-lactamase is expressed as a scFv C-terminal fusion only if the scFv gene does not contain premature stop codons (including the amber codon) introduced by mutations or frameshifts. When the transformants were allowed to grow on a carbenicillin-containing media plate, the result is an enrichment of the clones that are capable of expressing full-length scFv's. Through this proofreading, a scFv repertoire with a greatly enhanced functional diversity could be obtained. The introduction of artificial diversity to a synthetic antibody library, in general, reduces the functional diversity of the library because of the mutations, additions, and deletions that occur during the synthesis of the oligonucleotides used in the library construction. This becomes more problematic in libraries with longer CDRs (and hence longer oligonucleotides) and/or with an increasing number of CDRs that are diversified. The β-lactamase selection has recently been described to eliminate out-of-frame DNA sequences from a synthetic Fab antibody library (Rothe et al., 2008; Seehaus et al., 1992). Our study reconfirms the effectiveness of this strategy in antibody library construction and suggests that the selection process does not seem to significantly affect the randomness of the resulting library (but see below).

The number of pFDV transformants (~10^7) was sufficiently large to fully cover the combined maximum diversities of CDR-L1, L2, and L3 (5.9×10^5) or CDR-H1 and H2 (1.5×10^5), but not enough to cover the CDR-H3 diversity that makes a disproportionately large contribution to the antigen specificity and affinity. Therefore, the CDR-H3 sequences were subsequently grafted to the proofread scFv repertoire to maximize the total diversity of the library. While this inevitably introduced frameshifts and amber stop codons (from NNK randomization) in less than 20% of the final library clones, the resulting > 100-fold increase in the library diversity more than compensated for the decrease in the percentage of functional clones.

The final library has a total diversity of 7.6×10^9, and > 70% of the clones encode functional, full-length scFv as determined by the dot-blot and sequence analyses. Even after proofreading by the scFv-β-lactamase fusion genetic selection, some clones contain stop codons or frameshifts in regions outside of CDR-H3. It is possible that some defective clones survived and grew as satellite colonies on the densely grown LB-carbenicillin agar plate. However, a majority of the defective sequences, as expected, had frameshifts in the CDR-H3 that was grafted, after the proofreading step, for the maximization of the library diversity. That most of these errors occur in the sub-libraries CF and DF, which have longer CDR-H3 and hence should be more susceptible to oligonucleotide synthesis errors, also supports this result. The composition of the randomized CDR residues does not seem to have been affected by the proofreading process in general; although, there are a few positions in which the randomness is clearly biased. Whether this is due to the effect of the interaction between the scFv and its fusion partner (β-lactamase) on the activity of the latter, bias introduced during oligonucleotide synthesis, or the effect of the amino acid residue on the stability and expression level of the scFv is not clear at this stage.

Multiple target-specific scFv clones were isolated after three to four rounds of panning on various surface-immobilized, human and non-human antigens of varying size (from 67 kDa for serum albumins to 5.8 kDa for insulin and 332 Da for fluorescein). Highly specific binders that can distinguish their target

from a homologous protein (e.g. hPrxI and hPrxII binders), as well as cross-reactive clones that bind both their intended target and its homolog (e.g. RSA/HSA binders), were identified. The specificity/cross-reactivity profile of antibodies is especially important in the development of therapeutic antibody candidates, which need to specifically bind the disease target and not its paralogs on normal tissues, but at the same time should also cross-react with the mouse ortholog for preclinical evaluation. With adequate selection strategies, it may generally be possible to isolate clones with a desired specificity profile from this library. Dissociation constants were measured for some of the target-specific clones and were determined to be in the nanomolar range, which are comparable to those of many hybridoma-derived mouse monoclonal antibodies or of scFv and Fab clones obtained from other large, naïve phage-display libraries. The convergence of CDR sequences of the selected clones is indicative of the significant contribution made by these CDRs to the binding interaction, and justifies the efforts to diversify all six CDRs during the construction of the antibody library.

Only a limited number of fully synthetic antibody libraries with six diversified CDRs have been reported to date (Rothe et al., 2008; Sidhu and Fellouse, 2006; Soderlind et al., 2000) despite the potential advantages of such libraries, due in part to the difficulties of introducing diversity to multiple regions while maintaining the functionality of the library. This report describes a relatively simple method of constructing a large, fully synthetic, single-scaffold human scFv phage library with a high functional diversity and the diversification of all six CDRs. With the capability to yield multiple, moderate- to high-affinity binders against a broad range of target antigens, this library is expected to provide a useful source of human monoclonal antibodies for research, diagnostic, and clinical applications.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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