Generation of a phagemid mouse recombinant antibody fragment library by multisite-directed mutagenesis

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A nonimmune phagemid recombinant antibody fragment (rFab) library was generated with a nominal diversity of 1.16×10^7 using the QuikChange® Multi Site-Directed Mutagenesis kit. Two degenerate primers spanning the third complementarity-determining region (CDR) loops of the antibody fragment light and heavy chain were mutated such that eight or nine amino acids were randomly changed per CDR loop. Seven proteins were used to evaluate the library quality. Protein-specific rFab antibodies were selected after three panning cycles. From 12% to 64% of the randomly selected colonies produced positive ELISA signals to the phagemid rFabs. Multisite-directed mutagenesis allowed a diverse rFab library to be rapidly constructed while retaining the structural framework of a Fab that had been optimized for production in Escherichia coli.

INTRODUCTION

Phagemid and phage libraries of various types of antibody fragments are being exploited for commercial, medical, and fundamental research purposes (1–14). The successful completion of many genomes has spawned “proteome projects,” where high-throughput methodologies are applied to gain insight into protein structure and function. As an alternative to conventional crystallization techniques used for determining the 3-D structure of proteins, we are developing recombinant antibody fragments (rFabs) as co-crystallization reagents (15,16) that can be used for structural genomics. An important aspect of our use of rFabs is that a combinatorial library must be constructed so that a selection system, in this case phage display technology (17), can be used to select for rFabs recognizing diverse targets. The construction of phage-antibody libraries generally employs fairly laborious approaches that can include multiple cloning steps, cDNA amplification, and recombination. Diversities (effective library size) range from 10^6 to 10^{10} (13). Many of these antibody libraries are initially derived from immune cells (2,18). However, synthetic libraries have been generated (19), which form the basis for our library’s approach.

Fabs contain six complementarity-determining region (CDR) loops, three in the heavy chain and three in the light chain, which recognize diverse antigens. We utilized the QuikChange® Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) (20,21) to randomly mutate the third CDR loops of both the heavy and light chain of an rFab optimized for efficient prokaryotic expression. By using multisite mutagenesis, a complex library was constructed in minimal time without multiple subcloning steps.

MATERIALS AND METHODS

Chemical and Biological Reagents

The *Escherichia coli* XL1-blue strain and *PfuTurbo*® DNA polymerase were purchased from Stratagene (20). A QIAquick® PCR purification kit was purchased from Qiagen (Valencia, CA, USA). T4 DNA ligase and carbenicillin were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-mouse immunoglobulin G (IgG)[F(ab')₂]-alkaline phosphatase (RAM-AP) came from Pierce Biotechnology (Rockford, IL, USA), and phosphatase substrate tablets came from Sigma (St. Louis, MO, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG), 3-(N-morpholino)

propane-sulfonic acid (MOPS) buffer, yeast extract, tryptone, tetracycline, and kanamycin were purchased from Fisher Scientific (Pittsburgh, PA, USA). *Sac*I, *Spe*I, *Xba*I, and *Xho*I restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Super Broth (SB) consisted of 30 g of tryptone plus 20 g yeast extract and 10 g of MOPS per liter titrated to pH 7.0. High-strength analytical-grade agarose was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Degenerate light chain (a 65-base oligomer, LCCDR3RAN8) and heavy chain primers (a 68-base oligomer, HCCDR3RAN9) were synthesized, 5′-phosphorylated and polyacrylamide gel electrophoresis (PAGE)-purified by Sigma-Genosys (Cambridgeshire, UK). The primer sequences were LCCDR3RAN8: 5′-CTGCAATGTATTTCTGTCTCAG-(NNK)₈TTCGGTGCTGGGACCAAGGTG-3′; and HCCDR3RAN9: 5′-GTC-TATTACTGTGCAAGATGG(NNK)₉-TACTGGGGCCAAGGCACCAC-3′, where N is A, T, C, or G, and K is G or T. The design and construction of the pComb3H-Fab4 plasmid (GenBank® accession number AY254174), the template vector for the library construction, will be described elsewhere. Briefly, it consisted of light and heavy chains (Fab portion) of Mab25.3, a monoclonal antibody that recognizes the human immunodeficiency virus (HIV)-1 capsid protein (22) cloned into the plasmid pComb3H obtained from Dr. Carlos Barbas III (Scripps Research Institute, La Jolla, CA, USA) (23). Several modifications were made to the light and heavy chains to improve the stability and purification behavior of the Fab for optimal expression in *E. coli*. Electrocompetent XL-1 Blue cells prepared in house had a transformation efficiency of 4×10^{10} transformants/μg using pUC19 as control (23).

Library Construction and Testing

Plasmid pComb3H-Fab4 was used as a template for multisite mutagenesis following the manufacturer’s instructions with some modifications. Table 1 shows the parameters for the PCR and the transformation results. The pUC controls included with the kit were performed separately, because pUC optimal PCR conditions were different from the

Table 1. PCR Parameters Used to Optimize the Construction of an rFab Library

Component	Control Reaction (μL)	MultiMix Enzyme Blend (μL)	MultiMix Enzyme Blend Plus <i>PfuTurbo</i> DNA Polymerase (μL)
10× QuikChange Multi reaction buffer	2.5	5.0	5.0
Sterile, Nanopure® water	19.5	35.0	35.0
Double-stranded DNA template pComb3H-Fab4	1.0	2.0	2.0
Mutagenic primers:			
LCCDR3RAN8 (144 ng/μL)	0	2.0	2.0
HCCDR3RAN9 (151 ng/μL)	0	2.0	2.0
dNTP mixture	1.0	2.0	2.0
<i>PfuTurbo</i> DNA polymerase	0	0	1.0
QuikChange Multi enzyme blend	1.0	2.0	1.0
Total Reaction Volume	25.0	50.0	50.0
Transformation Results (cfus/μL)^a			
Plasmid pComb3H-Fab4 template	6.2 × 10 ^{3b,c}	2.8 × 10 ^{5d}	1.5 × 10 ⁶
Triple mutant control using pWS-3 and primers from kit			7.2 × 10 ⁴ blue/ 1.7 × 10 ⁵ total ^{b,c}
rFAB, recombinant antibody fragment.			
^a Three dilutions of cells were prepared with the numbers reported from the plates with <200 colonies. Library size is calculated as mean colony-forming units (cfus)/μL plating volume × 550 μL transformation volume × total reaction volume (25 or 50 μL)/1.5 μL transformed DNA as per Hogrefe et al. (21). For a direct comparison to Reference 21, the library sizes should be divided by 2 for the 50-μL reactions. Transformations were performed in duplicate using the supercompetent cells provided in the kit.			
^b Only one transformation performed.			
^c 25 μL reaction volume.			
^d Average of two transformations.			

library. For the final library, a total of 10 reactions (50 μL reaction volume each) were performed. Optimized cycling parameters using a RoboCycler® (Stratagene) were: 1 cycle at 95°C, 30 cycles of 1 min at 95°C, 1 min 30 s at 63°C, and a final extension cycle of 8 min at 65°C. Five micrograms of the *DpnI*-treated, PCR-purified, single-stranded DNA were transformed by electroporation into 1300 μL *E. coli* XL1-blue (six separate transformations). The panning

procedure for production and selection of phagemid rFab antibody fragments followed standard phage display procedures using Immulon® 2 HB Flat Bottom Microtiter Plates in 100-μL volumes (23).

For ELISA analysis of the selected pComb3H-Fabs, 25 colonies from the panning rounds two and three were selected at random, propagated in 2 mL SB containing 50 μg/mL carbenicillin, grown for 10 h at 37°C, cooled to room

temperature for 30 min, and induced with 0.5 mM IPTG. The supernatants from cultures grown overnight at room temperature were tested by ELISA for the production of pComb3H-Fab specific for the target proteins (results are shown in Table 2) (24). An absorbance ratio of at least 2.5 for Fab incubated with immobilized target to Fab incubated without was scored as a “positive” colony.

RESULTS AND DISCUSSION

The QuikChange Multi Site-Directed Mutagenesis kit uses one to five primers designed to hybridize to the same strand of DNA in noncontiguous regions. The resulting complementary single-stranded DNA fragments are ligated by a thermophilic ligase in situ (21). While the manufacturer’s protocol was largely followed, we found that the yield of DNA could be increased significantly by optimizing the enzyme mixture (Table 1). Since the reaction does not result in binary amplification like a standard PCR, the overall yield of product is low and limits the possible size of the library. As shown in Table 1, matching the volume of QuikChange Multi enzyme blend used with *PfuTurbo* DNA polymerase consistently produced at least a 3-fold higher number of transformants per reaction volume [5.7×10^3 colony-forming units (cfu)/μL without added polymerase versus 2.9×10^4 cfu/μL with added polymerase]. By also allowing the total reaction volume to be doubled (the Multi enzyme blend is limiting in the kit), the final library size could be effectively increased 10-fold. To ensure that the dilution of the enzyme blend with compensating *PfuTurbo* DNA polymerase did not lower the mutation efficiency, the pWS-3 triple-site mutagenesis control reaction provided with the kit was performed. The transformation efficiency (as cfu/μL) for the control triple mutation with the supplemented polymerase was greater than 4-fold better than that reported by Hogrefe et al. (21). The percentage of triple mutants (42%) was lower than the average for the control without polymerase, 55% reported by Hogrefe et al. (21); but

Table 2. Panning Results for Seven Proteins

Protein ^a	Pan Cycle	HEWL	BHMT	CBS	CGL	FigR	MerR	p24
Average cfu/ μ L. Numbers in parentheses are the variances for each set of countable dilutions per pan cycle.								
	1	2.10 (0.47) $\times 10^6$	1.67 (0.83) $\times 10^6$	1.63 (0.41) $\times 10^6$	3.17 (0.43) $\times 10^6$	4.10 (0.74) $\times 10^6$	3.89 (0.05) $\times 10^6$	4.00 (0.08) $\times 10^6$
	2	518 (208) $\times 10^6$	417 (98) $\times 10^6$	843 (224) $\times 10^6$	1005 (368) $\times 10^6$	1970 (89) $\times 10^6$	1110 (57) $\times 10^6$	1450 (83) $\times 10^6$
	3	107 (62) $\times 10^6$	87.4 (32.0) $\times 10^6$	41.9 (18.3) $\times 10^6$	143 (84) $\times 10^6$	0.71 (0.48) $\times 10^6$	53.9 (1.3) $\times 10^6$	8.62 (4.74) $\times 10^6$
ELISA response with a signal-to-noise ratio ≥ 2.5 . Numbers in parentheses are the actual responses.								
	2	(0/25) 0%	(4/25) 16%	(0/25) 0%	(0/25) 0%	(12/25) 48%	(5/25) 20%	(3/25) 12%
	3	(6/25) 24%	(8/25) 32%	(3/25) 12%	(8/25) 32%	(12/25) 48%	(7/25) 28%	(16/25) 64%

^aProteins evaluated were: HEWL, hen egg white lysozyme; BHMT, human betaine homocysteine methyl transferase; CBS, human cystathionine β -synthase; CGL, human cystathionine γ -lyase; bacterial FigR; bacterial transposon 21 MerR; and p24, human immunodeficiency virus (HIV)-1 capsid protein.

cfu, colony-forming unit.

this value would have been 50% if one low colony count were omitted from the average value. Using the optimized conditions, 10 reactions (50 μ L each) were combined and had a nominal diversity of 1.16×10^7 cfus. Varying the ratio of *PfuTurbo* DNA polymerase to the enzyme blend is thus a useful parameter to examine before creating large-scale libraries using this system.

Another significant deviation from the manufacturer's protocol was that much longer mutation primers (longer in length as well as containing more mutations) than recommended were used. Our primers had approximately 20 to 21 bases on the 5' and 3' ends complementary to the DNA flanking the third CDR loops of the heavy and light chains (Figure 1). Twenty-four or twenty-seven bases in the CDR loops were mutated following an NNK scheme, where K equals G and T are in the third codon position. This scheme thus introduced mutations in eight or nine amino acids of the two CDR loops encoding all 20 amino acids with minimal stop codons. As evidenced by the high transformation efficiency and the production of a functional library with ELISA signals to multiple proteins, the primers were clearly functional.

Table 2 shows that we obtained selection of phagmid-rFabs against seven antigen proteins after three panning cycles. Depending

on the target protein (panogen), the number of rFab-phagemids (out of 25 tested) that had positive ELISA signals varied between 12% and 64%. Since the parent rFab was derived from murine Mab25.3 specific for HIV-1 capsid protein p24 (22), p24 was included as a control. We expected to see a substantial selection in the panning of phagemids specific to the p24. Surprisingly, p24 was not significantly better than the other six protein targets in the number of transformants per panning round. The p24 capsid protein did surpass the other protein targets in the percentage of transformants (64%) that carried the phagemid-rFabs specific for the target, but only ranged from 2- to 5-fold greater when compared with the other phagemid-rFabs (Table 2). However, the effective total number of phagemid-rFabs (total number of cfus times the percentage of positive ELISA signals) produced against their target at the end of the third panning cycle has four of the six other protein targets outperforming the p24 capsid protein. As a final check of the panning process, two rFab-plasmids were purified and examined by restriction enzyme analysis (Figure 2). The recovered plasmids contained appropriate restriction sites and fragments corresponding to the light and heavy chain Fab DNA sequences (Figure 1).

Our goal was to create high-affinity rFabs for co-crystallization with novel protein targets in which the basic scaffold of the co-crystallization Fab was unchanged. Thus, random mutagenesis approaches, such as error-prone PCR, were not desirable. While the suggested use of the QuikChange Multi Site-Directed Mutagenesis kit was for multiple sites with two to three nucleotide changes per degenerate primer, 24 and 27 nucleotides were simultaneously mutagenized in our experiment. Antibody libraries made with more laborious approaches have been reported with diversities ranging from 10^6 to 10^{10} (13). Our library of 1.16×10^7 different rFabs was quickly generated and is of a high quality as judged by our ability to obtain ELISA positive clones to a wide variety of proteins. Our results suggest that this mutagenesis kit is quite versatile for preparing large-scale combinatorial libraries.

Figure 1. Restriction enzyme sites and genetic features of the pComb3H-Fab4 plasmid used for building the antibody fragment (Fab) library. Stars represent relative locations of the six complementarity-determining region (CDR) loops in the Fab light and heavy chains. The genes are under the control of a *lac* operator and promoter, and RIBS represents the ribosome binding site. The phage gene III protein (gIII) is in-frame with the Fab heavy chain.

Figure 2. Restriction endonuclease analysis of selected ELISA positive clones from the antibody fragment (Fab) library. Plasmid pComb3H-Fab4 is the starting DNA used for the library construction, pComb3H-FlgR is DNA isolated from a single ELISA positive colony selected from the third pan round against FlgR, and the pComb3H-p24 is similarly selected against the human immunodeficiency virus (HIV) p24. See Figure 1 for the relative location of the specific endonuclease sites. Abbreviations are: SaXb, *SacI/XbaI*; XhSp, *XhoI/SpeI*; SpNh, *SpeI/NheI*; and Eco, *EcoRI* for the respective restriction enzymes used.

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Random priming PCR strategy to amplify and clone trace amounts of DNA

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Here we report a new methodology to study trace amounts of DNA of unknown sequence using a two-step PCR strategy to amplify and clone target DNA. The first PCR is carried out with a partial random primer comprised of a specific 21-nucleotide 5' sequence, a random heptamer, and a 3' TGGC clamp. The second PCR is carried out with a single 19-nucleotide primer that matches the specific 5' sequence of the partial random primer. Using human and Mycoplasma genitalium DNA as examples, we demonstrated the efficiency of this approach by effectively cloning target DNA fragments from 1 pg DNA sample. The cloning sensitivity could reach 100 fg target DNA templates. Compared to the strategy of first adding adapter sequences to facilitate the PCR amplification of unknown sequences, this approach has the advantage of allowing for the amplification of DNA samples in both natural and denatured forms, which provides greater flexibility in sample preparation. This is an efficient strategy to retrieve sequences from trace DNA samples from various sources.

INTRODUCTION

DNA cloning is one of the most commonly used methods in molecular biology. The technique allows one to obtain a large quantity of specific DNA molecules for study or characterization. However, there is a limitation to this powerful technique. In conventional DNA cloning, a significant amount of target molecules is needed to ensure efficient ligation with vectors. With PCR, cloning trace amounts of DNA can be achieved by first amplifying the target DNA, given that partial sequences are known for the DNA segment of interest and that PCR primers can be designed. However, cloning trace amounts of DNA with unknown sequence presents a difficult task in many situations. Conventional or regular PCR-based cloning strategies are typically unable to fulfill this task.

Many amplification strategies using small amounts of DNA or RNA with unknown sequence have been reported (1-5). Random primers tagged by a specific sequence at the 5' end were commonly used to introduce primer sequences to facilitate PCR amplification (4,5). Most of these applications still require samples in nanogram amounts (4,5). To attain higher sensitivity, sequence-independent single-primer amplification (SISPA) was developed to amplify unknown target DNA molecules with PCR and has been

shown to facilitate cloning from 1 pg DNA after amplification (3). SISPA first requires ligation of an adapter onto the target population of blunt-ended DNA molecules. The resulting DNA with common end sequence allows for PCR amplification by a single primer made from the adapter sequence.

Random priming strategies generating single-stranded DNA (ssDNA) molecules are frequently used to randomly multiply DNA or synthesize cDNA from RNA molecules (6). The most common applications include probe labeling, multiplying DNA or RNA from small samples, and generating random in vitro mutagenesis and recombination (1,2,7-10). To clone trace amounts of unknown DNA from various sources of different physical and biological properties, we have developed a two-step PCR method that is partially based on random priming strategy. The first PCR uses a single primer composed of seven random nucleotides flanked by a specific 5' tag and a 3' clamp (Figure 1, Ran5-29). A *Pst*I site (New England Biolabs, Beverly, MA, USA) is included in the 5' tag. The internal seven random nucleotides and the 3' clamp TGGC enable the primer to anneal with any seven nucleotides with GCCA immediately upstream. The TGGC clamp increases the primer's 3'-specific annealing from 7 to 11 nucleotides. The second PCR is performed using a single specific