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 × 10⁷ 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 10⁶ to 10¹⁰ (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). SacI, SpeI, XbaI, and XhoI 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 polyacylamide gel electrophoresis (PAGE)-purified by Sigma-Genosys (Cambridgeshire, UK). The primer sequences were LCCDR3RAN8: 5’-CTGCAATGTTTCTGTCAG-(NNK)₃TTCGGTGCTGGGACCAG-GGTG-3’; and HCCDR3RAN9: 5’-GTC-TATTAAGTGCAAGATGG(NNK)₃-TACTGACGGCAGCACACC-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 AF1254174), 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. Electropotent XL-1 Blue cells prepared in house had a transformation efficiency of 4 × 10¹⁰ 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...
The panning into 1300 DNA were transformed by electroporation.

- **Transformation Results**
  - **Plasmid pComb3H-Fab4 template**: $6.2 \times 10^5$ colonies per reaction volume.
  - **Triple mutant control using pWS-3 and primers from kit**: $7.2 \times 10^4$ blue/white colonies per reaction volume.

**rFAB, recombinant antibody fragment.**

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Control Reaction (µL)</th>
<th>MultiMix Enzyme Blend (µL)</th>
<th>MultiMix Enzyme Blend Plus PfuTurbo DNA Polymerase (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× QuikChange Multi reaction buffer</td>
<td>2.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Sterile, Nanopure® water</td>
<td>19.5</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Double-stranded DNA template pComb3H-Fab4</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mutagenic primers:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCCDR3RAN8 (144 ng/µL)</td>
<td>0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>HCCDR3RAN9 (151 ng/µL)</td>
<td>0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTP mixture</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>PfuTurbo DNA polymerase</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>QuikChange Multi enzyme blend</td>
<td>1.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>25.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

**Transformation Results (cfus/µL)²**

- **Plasmid pComb3H-Fab4 template**: $6.2 \times 10^5$ colonies per reaction volume.
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²Three 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.

²Only one transformation performed.

³25 µL reaction volume.

²Average of two transformations.

### 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 × 10³ colony-forming units (cfu)/µL without added polymerase versus 2.9 × 10⁴ cfu/µL with added polymerase]. By also allowing the total reaction volume to be doubled (the Multi enzyme blend is limited 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

<table>
<thead>
<tr>
<th>Proteina</th>
<th>HEWL</th>
<th>BHMT</th>
<th>CBS</th>
<th>CGL</th>
<th>FlgR</th>
<th>MerR</th>
<th>p24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan Cycle</td>
<td>Average cfu/µL. Numbers in parentheses are the variances for each set of countable dilutions per pan cycle.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.10 (0.47) x 10^6</td>
<td>1.67 (0.83) x 10^6</td>
<td>1.63 (0.41) x 10^6</td>
<td>3.17 (0.43) x 10^6</td>
<td>4.10 (0.74) x 10^6</td>
<td>3.89 (0.05) x 10^6</td>
<td>4.00 (0.08) x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>518 (208) x 10^6</td>
<td>417 (98) x 10^6</td>
<td>843 (224) x 10^6</td>
<td>1005 (368) x 10^6</td>
<td>1970 (89) x 10^6</td>
<td>1110 (57) x 10^6</td>
<td>1450 (83) x 10^6</td>
</tr>
<tr>
<td>3</td>
<td>107 (62) x 10^6</td>
<td>87.4 (32.0) x 10^6</td>
<td>41.9 (18.3) x 10^6</td>
<td>143 (84) x 10^6</td>
<td>0.71 (0.48) x 10^6</td>
<td>53.9 (1.3) x 10^6</td>
<td>8.62 (4.7) x 10^6</td>
</tr>
</tbody>
</table>

ELISA response with a signal-to-noise ratio ≥2.5. Numbers in parentheses are the actual responses.

| Pan Cycle | | | | | | | |
| 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% |

Proteins evaluated were: HEWL, hen egg white lysozyme; BHMT, human betaine homocysteine methyl transferase; CBS, human cystathionine β-synthase; CGL, human cystathionine γ-lyase; bacterial FlgR; bacterial transposon21 MerR; and p24, human immunodeficiency virus (HIV)-1 capsid protein.

cfu, colony-forming unit.

*Proteins evaluated were: HEWL, hen egg white lysozyme; BHMT, human betaine homocysteine methyl transferase; CBS, human cystathionine β-synthase; CGL, human cystathionine γ-lyase; bacterial FlgR; bacterial transposon21 MerR; and p24, human immunodeficiency virus (HIV)-1 capsid protein.
ACKNOWLEDGMENTS

We thank Ladislau Kovari and Michael Rossman for providing us with mouse monoclonal antibody cell line for Mab25.3 (originally obtained from Jan McClure) and Anne Summers and Tim Hoover for the MerR and FlgR proteins. Support for this work was funded by grants from the National Science Foundation (MCB9723244) and the University of Georgia Research Foundation.

REFERENCES

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 specific TGTC 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 PstI site (New England Biolabs, Beverly, MA, USA) is included in the 5’ tag. The internal seven random nucleotides and the 3’ clamp TGTC enable the primer to anneal with any seven nucleotides with GCCA immediately upstream. The TGTC clamp increases the primer’s 3’-specific annealing from 7 to 11 nucleotides. The second PCR is performed using a single specific