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Improvements to the Kunkel mutagenesis protocol for constructing primary and secondary phage-display libraries

Renhua Huang, Pete Fang, Brian K. Kay *

Department of Biological Sciences, University of Illinois at Chicago, 845 W. Taylor St., 3240 SES - MC 066, Chicago, IL 60607-7060, USA

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Site-directed mutagenesis is routinely performed in protein engineering experiments. One method, termed Kunkel mutagenesis, is frequently used for constructing libraries of peptide or protein variants in M13 bacteriophage, followed by affinity selection of phage particles. To make this method more efficient, the following two modifications were introduced: culture was incubated at 25 °C for phage replication, which yielded two- to sevenfold more single-stranded DNA template compared to growth at 37 °C, and restriction endonuclease recognition sites were used to remove non-recombinants. With both of the improvements, we could construct primary libraries of high complexity and that were 99–100% recombinant. Finally, with a third modification to the standard protocol of Kunkel mutagenesis, two secondary (mutagenic) libraries of a fibronectin type III (FN3) monobody were constructed with DNA segments that were amplified by error-prone and asymmetric PCR. Two advantages of this modification are that it bypasses the lengthy steps of restriction enzyme digestion and ligation, and that the pool of phage clones, recovered after affinity selection, can be used directly to generate a secondary library. Screening one of the two mutagenic libraries yielded variants that bound two- to fourfold tighter to human Pak1 kinase than the starting clone. The protocols described in this study should accelerate the discovery of phage-displayed recombinant affinity reagents.

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1. Introduction

Several techniques are readily available for site-directed mutagenesis of proteins. Cassette mutagenesis [1], which requires restriction enzyme digestion and ligation to incorporate mutagenic sequences, has been supplanted by the 'QuikChange' method [2,3]. In 'QuikChange', a pair of complementary oligonucleotides, containing the desired mutation(s), are used to amplify the entire plasmid with a high-fidelity polymerase, followed by DpnI digestion to remove the parental strand. A third widely used technique is 'Kunkel mutagenesis' [4–8], where one utilizes uracil-inserted, circular, single-stranded DNA (ssDNA) as a template to synthesize double-stranded DNA (dsDNA) *in vitro* with an oligonucleotide primer that introduces a mutation. After dsDNA is introduced into bacteria, recombinant clones predominate due to cleavage of the uracilated strand *in vivo*. Kunkel mutagenesis is particularly powerful in phage-display experiments that are based on M13 bacteriophage, as the viral particles contain a circular, single-stranded genome [6,9,10].

As the number of the theoretical permutations in a protein engineering experiment can be astronomical, it is desirable to construct phage-displayed libraries that comprise a vast number of mutants, as it has been observed that the size of a phage library is closely correlated with the affinity of the isolated mutants [11]. While the size of the library is a limiting factor in isolating desired clones, the quality of the phage library (i.e., the percentage of the phage particles displaying the recombinant polypeptides out of the total phage pool), also significantly influences the efficiency and the outcome of affinity selections. For example, some studies have found that non-recombinant clones, or target-unrelated clones, can overwhelm the target-binding clones in the library due to the advantages associated with steps of phage propagation or affinity selection [12,13]. Thus, it is widely believed that removing the wild-type clones from the final phage-displayed library should improve the efficiency of affinity selections.

Even with improvements in the size and quality of a phage-displayed library, affinity maturation experiments are usually necessary to fine-tune binders for improved specificity [10,14], affinity [10,15,16], or both [10]. One simple method is to generate secondary (i.e., mutant) libraries through an error-prone polymerase chain reaction (PCR) [17,18], and repeat the affinity selections under more stringent conditions (i.e., less target, longer wash times, more washes). Nevertheless, generating each secondary library can be time-consuming, and unless large, may be inadequate for isolating mutants with dramatically improved properties.

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* Corresponding author.
  E-mail address: bkay@uic.edu (B.K. Kay).

In this study, we describe several modifications to the basic Kunkel mutagenesis protocol for constructing libraries that display the 10th subunit of human fibronectin type III repeat (FN3), also termed 'monobody' [19,20]. With adjustments of the growth conditions of bacterial cultures, the yields of phage particles and single-stranded, circular DNA can be increased two- to sevenfold, which provides an ample source of template DNA for constructing libraries. Furthermore, with insertion of unique restriction endonuclease recognition sites in the FN3 coding region, non-recombinant clones are removed by restriction enzyme digestion, generating naïve (i.e., primary) libraries that are 99–100% recombinant, which should improve the efficiency of affinity selection experiments and the discovery of high-affinity, selective affinity reagents. Finally, to improve the affinity of a previously isolated binder, we construct two secondary libraries using DNA segments generated by error-prone and asymmetric PCR. Affinity selection of one of these libraries yields three variants that exhibit two- to fourfold tighter binding to Pak1 kinase than the original clone.

2. Materials and methods

2.1. Phagemids and Escherichia coli strains

The sequence of the 10th subunit of human fibronectin type III repeat (FN3) was amplified by PCR from a plasmid [21], and sub-cloned into the pAP-III$_6$ vector [22,23]. In this phagemid vector, the Flag (DYKDDDDK) epitope is fused at the N-terminus of the FN3 coding region, thereby allowing convenient detection of the displayed FN3 domain with an anti-Flag antibody (Sigma–Aldrich; St. Louis, MO).

The E. coli strain, CJ236 (New England BioLabs; Ipswich, MA), which lacks functional dUTPase and uracil-N glycosylase, was used for generating uracilated single-stranded DNA template. In E. coli, dUTPase and uracil-N glycosylase serve to play roles in DNA repair and ensure fidelity of DNA replication by removing any uracils incorporated into the bacterial genome [24]. Here in this study, the E. coli strain, TG1 (Lucigen; Madison, WI), which encodes wild-type versions of dUTPase and uracil-N glycosylase, was used to favor propagation of the newly synthesized (i.e., mutated or recombinant) strand. For the sake of clarity, in this report we refer to the circular, single-stranded phagemid genome and the in vitro synthesized circular, double-stranded, heteroduplex product as ssDNA and dsDNA, respectively.

2.2. Extraction of uracilated single-stranded DNA from bacterial cultures grown in different conditions

CJ236 cells, carrying the phagemid, were streaked on a petri plate containing $2 \times$ YT medium (per liter: 16 g tryptone, 10 g yeast extract, 5 g NaCl), 1.5% agar (mass/volume), carbenicillin (50 µg/mL), and chloramphenicol (15 µg/mL). After an overnight incubation at 37 °C, three fresh colonies were inoculated together into 10 mL $2 \times$ YT medium, containing carbenicillin (50 µg/ml) and chloramphenicol (15 µg/mL). The culture was incubated at 37 °C, and shaken at 250 rpm overnight. The next day, 1.8 mL of overnight culture was diluted into 180 mL of fresh $2 \times$ YT medium with carbenicillin (50 µg/mL). After 3–4 h incubation at 37 °C with 250 rpm shaking, when the culture reached an $OD_{600nm} = 0.4$–0.6, M13-K07 helper phage (New England BioLabs) was added at a multiplicity of infection (MOI) of 10. The cells were infected for 1 h at 150 rpm, pelleted, and resuspended in 180 mL fresh $2 \times$ YT medium, containing carbenicillin (50 µg/mL) and kanamycin (50 µg/mL). The infected cells were aliquoted into six 250 mL flasks, 30 mL each. The following six different growth conditions in different combinations were tested for phage replication: incubation at

37 °C or 25 °C, with shaking at 280 rpm or 200 rpm, and in baffled or non-baffled flasks. After 22 h incubation, cells were centrifuged three times to clarify the supernatant, of which 2.5 mL was used for extraction of ssDNA with the QIAprep Spin M13 kit (Qiagen; Valencia, CA). The isolated ssDNA was quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific Co.; Waltham, MA), and evaluated by agarose gel electrophoresis. Phage particles were precipitated from the remaining 25 mL of culture supernatant by adjusting the solution to 5% (mass/volume) PEG 8000 and 300 mM NaCl. The pellet of phage particles was resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$), and processed with the QIAprep Spin M13 kit (Qiagen), which has a binding capacity of 10 µg ssDNA per column.

2.3. Construction of phage-display libraries

2.3.1. Creation of phagemids with stop codons and restriction endonuclease recognition sites inserted into FN3 sequence

Based on a modified protocol of Kunkel mutagenesis [7], two stop codons (TAA and TGA) and a SacII (5′-CCGC$^\downarrow$GG-3′), SmaI (5′-CCC$^\downarrow$GGG-3′), or StuI (5′-AGG$^\downarrow$CCT-3′), site was introduced into each of the BC and FG loop regions of the FN3 coding sequence (Each vector had two copies of the SacII, SmaI, or StuI sites). First, two oligonucleotides (13.2 pmol each; IDT DNA, Coralville, Iowa), each containing the two stop codons and one of the three different restriction endonuclease recognition sites, were phosphorylated by T4 polynucleotide kinase (5 units; New England BioLabs) at 37 °C, for 1 h, in 50 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM ATP and 5 mM dithiothreitol (DTT). In 50 mM Tris–HCl and 10 mM MgCl$_2$, the phosphorylated oligonucleotides were annealed to uracilated ssDNA template, at a molar ratio of 3 (oligonucleotide/ssDNA), by heating the mixture at 90 °C for 2 min, followed by a temperature decrease of 1 °C/min to 25 °C in a thermal cycler. In a solution containing 0.55 mM ATP, 0.8 mM dNTPs, 5 mM DTT, 15 Weiss units of T4 DNA ligase, and 15 units of T7 DNA polymerase (New England BioLabs), the two phosphorylated and annealed oligonucleotides were used to prime in vitro DNA synthesis at 20 °C for 3 h, yielding dsDNA, which was purified with the QIAquick PCR purification kit (Qiagen). In a pre-chilled 0.2 cm cuvette (BioExpress; Kaysville, UT), DNA was electroporated into TG1 cells (Lucigen) at 2400 V, with an electroporator (Eppendorf; Hauppauge, NY). The next day, six single bacterial colonies were inoculated for preparation of phagemid DNA and sequencing analysis.

2.3.2. Comparing different reaction conditions of in vitro dsDNA synthesis for their influences on mutation rate

To examine if different annealing ratios of oligonucleotide to ssDNA and different extension times would change the mutation rate, the following nine different reaction conditions were tested in parallel: three different annealing molar ratios of oligonucleotide to ssDNA (3, 20 and 100), were paired individually with three different extension times (30 min, 3 h and 16 h). Heteroduplex dsDNA, which was generated in these nine parallel reactions, was purified with the QIAquick PCR purification kit (Qiagen) before being electroporated into TG1 cells (Lucigen).

Forty-six bacterial colonies, obtained from transformations with dsDNA product from each of the nine different reaction conditions, were inoculated into a 96 deep-well plate (Thermo Fisher Scientific Co.) and grown overnight in the presence of M13-K07 helper virus particles (New England BioLabs). Anti-M13 bacteriophage antibody (GE Healthcare; Piscataway, NJ) was diluted in PBS to 5 ng/µL for overnight immobilization in the wells of Nunc^{TM} microtiter plates (Thermo-Fisher Scientific Co.). The next day, non-specific binding sites on the plates were blocked for 1 h with casein (Thermo Fisher Scientific Co.), followed by addition of clarified phage supernatant