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

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Abstract

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 2- to 7-fold 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 2- to 4-fold 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.

Keywords

Affinity selection; asymmetric PCR; diversity; error-prone PCR; FN3 monobody; Kunkel mutagenesis; library construction; M13 bacteriophage; mutation rate; Mutazyme II; phage display; single-stranded DNA

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

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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.

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 2- to 7-fold, 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 2- to 4-fold tighter binding to Pak1 kinase than the original clone.

2. Materials and Methods

2.1. Phagemids and *E. coli* strains

The sequence of the 10th subunit of human fibronectin type III repeat (FN3) was amplified by PCR from a plasmid [21], and subcloned into the pAP-III₆ 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 *Escherichia 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 2xYT 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 2xYT 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 2xYT 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 2xYT 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 Na₂HPO₄, 1.5 mM KH₂PO₄), 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 a phagemid 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↓GG-3'), SmaI (5'-CCC↓GGG-3'), or StuI (5'-AGG↓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 pmole 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 hour (h), in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM ATP and 5 mM dithiothreitol (DTT). In 50 mM Tris-HCl and 10 mM MgCl₂, 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 minutes (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 volts, 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™ 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 from overnight cultures in the deep-well plates. After 1 h incubation, microtiter plates were washed five times with PBS-0.1% Tween (volume/volume), followed by addition of 100 ng/mL anti-Flag antibody, conjugated to horseradish peroxidase (Sigma-Aldrich). The microtiter plates were washed after 30 min incubation, and the chromogenic substrate, 2,2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS), mixed with hydrogen peroxide, was added and the resulting absorbance was measured at 405 nm with a microtiter plate spectrophotometer (POLARstar OPTIMA; BMG Labtech, Cary, NC). A clone displaying a positive ELISA signal was considered as a recombinant (i.e., both BC and FG loops replaced by mutagenic sequences), whereas a negative ELISA signal was interpreted as the starting template (i.e., carries two stop codons in the coding regions for the two loops). Only phage clones with positive ELISA signals were selected for DNA sequencing. The nine different reaction conditions were tested three times.

2.3.3 Construction of phage libraries that were 99-100% recombinant—

Following the protocol described in section 2.3.1, dsDNA was synthesized *in vitro* with oligonucleotides encoding five NNK (where N is an equimolar mixture of A, G, C, and T, and K is an equimolar mixture of G and T) codons in the BC and FG loop regions of the FN3 coding sequence. The purified heteroduplex dsDNA was then electroporated into TG1 cells (Lucigen) and spread on agar plates containing 2xYT and carbenicillin (50 μg/mL). After overnight incubation at 30°C, colonies were scraped together and DNA was extracted with the Wizard Plus SV Mini-Prep kit (Promega; Madison, WI). The DNA was then digested with SacII, SmaI or StuI for 8 h (New England BioLabs), purified with the QIAquick PCR purification kit (Qiagen), and used to transform TG1 cells (Lucigen). After transformation, cells were recovered at 37°C with 200 rpm shaking for 30 min, before serial dilutions of the recovered cells were plated (to determine transformation efficiency) on 2xYT agar plates with carbenicillin (50 μg/mL), and incubated overnight at 30°C. The resulting colonies were inoculated into 96 deep-well plates (Thermo Fisher Scientific Co.) and grown overnight in 2xYT medium, containing carbenicillin (50 μg/mL), kanamycin (50

μg/mL), and helper virus, M13-K07 (New England BioLabs). Phage ELISA and DNA sequencing of selected clones were carried out as described above (Section 2.3.2).

2.4. Construction of two mutagenic libraries with DNA segments generated by error-prone and asymmetric PCR

Screening a phage display library of FN3 monobodies with the kinase domain of Pak1 kinase [25] led to isolation of a monobody, C12, which bound to the 'open' form of the protein [26]. A secondary mutant library based on the C12 monobody was created and a variant was isolated that bound twice as tight as the original clone. To further increase the affinity of the C12 variant, a mutagenic library of this clone was constructed with a modified protocol of Kunkel mutagenesis. Briefly, 0.3 fmole (1 ng) of phagemid DNA from the C12 variant was mixed with 2.5 units of Mutazyme II (Agilent; Santa Clara, CA), and amplified by PCR over 34 cycles (95°C for 1 min, 62°C for 1 min, and 72°C for 1 min). The resulting DNA segment was resolved by agarose gel electrophoresis and purified with the QIAquick gel extraction kit (Qiagen). Following heat denaturation of the purified DNA segment and annealing to the ssDNA template, the *in vitro* dsDNA synthesis was performed under the reaction conditions described in section 2.3.1, and the resulting dsDNA was electroporated into TG1 cells (Lucigen). Cells were allowed to recover for 30 min at 37°C with 200 rpm shaking, followed by serial dilutions, spreading on petri plates, and overnight incubation at 30°C.

To investigate if the mutation rate and the transformation efficiency of heteroduplex dsDNA could be increased by using a long single-stranded DNA as a primer for *in vitro* DNA synthesis, we employed asymmetric PCR [27] to amplify the preferred strand. One pmole (200 ng) of the purified DNA segment, generated under mutagenic conditions, and 400 pmole of the reverse primer were mixed and incubated for 40 cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min) of asymmetric amplification. The amplified DNA segments were purified with the QIAquick PCR purification kit (Qiagen). Following the same procedure mentioned in section 2.3.1, purified DNA segments were phosphorylated and annealed to ssDNA template. After DNA extension, the resulting heteroduplex dsDNA was electroporated into TG1 cells (Lucigen). The bacterial cells were spread on agar plates, and the next day, 46 colonies from the secondary library were picked and subjected to phage ELISA and DNA sequencing analysis, as described in section 2.3.2.

To increase the mutation rate of error-prone PCR, Mutazyme II was used to amplify the FN3 coding region under conditions that increased the mutation rate of *Taq* DNA polymerase [28]. Briefly, 0.3 fmole (1 ng) of DNA template was incubated in a solution containing 7 mM MgCl₂, 0.1 mM MnCl₂, 1 mM dCTP, dTTP and 200 μM dGTP and dATP, 2.5 units of Mutazyme II and 38 pmole of oligonucleotide primers. Thermal cycling consisted of 36 cycles: 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. Kunkel mutagenesis and electroporation of the resulting dsDNA were performed as described above.

2.5. Affinity selection of tighter binders from a secondary mutagenic library

Phage particles, displaying monobody variants, were amplified (as described in section 2.2), and resuspended in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.5% Tween (volume/volume) + 0.5% bovine serum albumin (BSA; mass/volume). The suspension of phage particles was then mixed with the target, biotinylated 'open' form of human Pak1 kinase (25 nM, final concentration). After 2 h incubation, streptavidin-coated magnetic beads (Promega) were added to the phage solution. Beads were collected with a magnet after 15 min tumbling, followed by six washes with PBS-0.5% Tween, three washes with PBS-0.1% Tween, and three washes with PBS (All the solutions contained the non-biotinylated form of the Pak1 kinase protein at 160 nM). Bound phages

were eluted with 50 mM glycine (pH 2) for 10 min, which was neutralized with Tris-HCl (pH 10) and used to infect mid-log phase TG1 cells (Lucigen) for 30 min at 37°C with 100 rpm shaking. Infected cells were spun down and spread on 2xYT agar plates with carbenicillin (50 µg/mL), and incubated overnight at 30°C. The next day, bacterial colonies were scraped from the plate and inoculated into cultures for amplifying viral particles, which would be used for the next round of affinity selection. To increase the stringency, the second and third rounds of selections were performed with reduced concentration of the target protein (500 pM) and with additional washes (to remove weak and non-binding clones).

2.6. Phage ELISA for identifying FN3 variants that bound tighter to Pak1 kinase

The phage ELISA was performed with a similar protocol as described in section 2.3.2. The target protein, 'open' form of human Pak1 kinase, was immobilized directly on the Nunc™ microtiter plate (Thermo Fisher Scientific Co.). Blocking reagent, casein, was used as the control of background binding. Among the eighty-eight clones tested in the initial phage ELISA, three clones (C2, D3, and E10) together with the original clone were picked for further testing their binding to the Pak1 kinase. First a phage ELISA was performed with anti-Flag antibody (Sigma-Aldrich), so that the amount of phage particles could be normalized in assays that monitored binding of FN3 monobodies to the Pak1 kinase.

3. Results and discussions

3.1. Overview of Kunkel mutagenesis

One method frequently used to construct phage-display libraries is Kunkel mutagenesis (Fig. 1). First, a phagemid genome is introduced into *E. coli* CJ236 cells, which lack functional dUTPase and uracil-N glycosylase. With the aid of a helper virus, M13-K07, the transformed CJ236 cells secrete phage particles, from which single-stranded, circular DNA (ssDNA), containing uracil in place of thymine (i.e., uracilated), is recovered. A pair of mutagenic oligonucleotides are phosphorylated and annealed to the ssDNA template for *in vitro* synthesis of heteroduplex, double-stranded DNA (dsDNA) with T7 DNA polymerase and T4 DNA ligase. The resulting product is purified and evaluated by agarose gel electrophoresis; as seen in Figure 2, the ssDNA can be quantitatively converted to the larger dsDNA species by this technique. The dsDNA is electroporated into *E. coli* TG1 cells, where the parental uracilated strand is cleaved *in vivo* by uracil-N glycosylase, so that only the recombinant strand survives.

3.2. Phage replication at lower temperature improved yields of ssDNA

Infected CJ236 cells often have low yields of ssDNA, and of low purity [7]. To find conditions leading to higher yields and better purity, multiple settings (i.e., growing temperature, shaking speed, different type of flasks) were evaluated. Incubation at 25°C for 22 h consistently yielded 2- to 7-fold more ssDNA than cultures grown at 37°C (Fig. 3). A similar increase in ssDNA yields was also observed for cultures of TG1 cells carrying the M13 bacteriophage genome (data not shown). However, if cells were resuspended in a much higher volume (i.e., 30 fold) of fresh medium after infection, the same growing conditions did not lead to higher yields.

The effects of shaking speed and flask shape on phage yields were also examined. High shaking speed (i.e., 280 rpm), with 37°C incubation, led to lower yields of ssDNA due to excessive degradation of DNA (data not shown). As both greater aeration and higher temperatures lead to rapid cell growth and potential cell lysis [29], these conditions are not recommended for phage replication. When cultures were grown in baffled and non-baffled flasks, there was no significant change in the yields or purity of the ssDNA (data not shown). In summary, keeping the culture volume the same after infection and incubating it

for 22 h at 25°C, with a shaking speed of 200 rpm, contributed to the best results, among all the conditions tested.

3.3. Different reaction conditions for *in vitro* dsDNA synthesis exhibited minimal influences on mutation rate

As mentioned above, it is beneficial to reduce or eliminate phage particles displaying the 'wild-type' form of a scaffold protein, thereby increasing the diversity of a library and enhancing its effectiveness in yielding binders. One way to reduce the number of wild-type clones in a phage library is to identify conditions that improve the efficiency of *in vitro* synthesis of dsDNA during Kunkel mutagenesis. To learn what modifications of the extension step might lead to enhanced yields, nine different conditions, ranging from different annealing ratios of oligonucleotide/ssDNA (3, 20, and 100) to different extension times (30 min, 3 h, and 16 h), were tested. While, none of these conditions appeared to significantly increase the mutation rate, there are two points worth noting. First, the average mutation rate (i.e., replacement of both loops with degenerate sequences) for all nine conditions was $38 \pm 3\%$. Second, for reactions of all three annealing ratios, as the extension time went from 30 min, 3 h to 16 h, there was a 1-8% increase in the mutation rate from 30 min to 3 h, and then a 3-6% decrease from 3 h to 16 h (data not shown). A 16 h extension not only led to a lower mutation rate, but also a significantly lower yield of dsDNA (data not shown). Based on our results, an annealing ratio of 3:1 (oligonucleotide/ssDNA template), with an extension time of 3 h, is recommended for the synthesis of heteroduplex dsDNA *in vitro*.

Curiously, even in the absence of any synthesized oligonucleotide primer, ssDNA is still able to self-prime and generate dsDNA [6] during the extension reaction, which can be how the non-recombinant dsDNA is synthesized. To test the possibility that one could outcompete self-priming with longer oligonucleotide primers to reduce the synthesis of non-recombinant dsDNA, primers of different lengths were tested. No significant difference in the mutation rate was observed, indicating that once a minimum length requirement was met ($T_m=50-55^\circ\text{C}$), longer primers offered no advantage in boosting the mutation rate compared to shorter ones. We also hypothesized that degraded small DNA segments, which might be present in preparations of the ssDNA template, could be responsible for the self-priming. To test this notion, ssDNA was resolved by agarose gel electrophoresis and extracted from any contaminating DNA segments. However, this extra purification step only slightly raised the mutation rate (i.e., from 40% to 50%), implying that the persistence of non-recombinants was somehow inherent to the ssDNA template itself.

3.4 Removal of non-recombinant clones by restriction enzyme digestion

To enhance the efficiency of Kunkel mutagenesis, a set of three phagemid vectors were devised with stop codons and restriction endonuclease recognition sites inserted in the BC and FG loop coding regions. The stop codons are intended to prevent the display of wild-type FN3 domain and its N-terminal fused Flag epitope, whereas inclusion of restriction endonuclease cleavage sites is to permit differential destruction of non-recombinant DNA by digestion with restriction endonucleases.

Three restriction endonucleases were chosen because their recognition sequences were absent from the original phagemid genome and their activities were robust. Vectors were designed with SacII (5'-CCGC↓GG-3'), SmaI (5'-CCC↓GGG-3'), or StuI (5'-AGG↓CCT-3') sites in the BC and FG coding regions. (Note that each vector carried two recognition sites for the same enzyme). For StuI recognition site, even though it contains thymine, which can be replaced by uracil when ssDNA genome is propagated in the CJ236 *E. coli* strain, and thus can make non-recombinant dsDNA resistant to StuI cleavage, we

rationalized that the chance that one or both thymines in the *StuI* site are replaced by uracil is low (i.e., 3-4%), given that only 20-30 uracils are inserted in each ssDNA genome when propagated in CJ236 cells [30].

When the three vectors were used for *in vitro* synthesis of dsDNA, digestion of the dsDNA with the cognate restriction enzyme prior to electroporation led to only a 10% increase in the frequency of recombinants (51% to 61%) obtained. At the moment, we cannot account for this result, even though it is reproducible. However, we noted that the phagemid carrying the two *StuI* sites consistently yielded larger transformation outputs, compared to the phagemids with *SacII* or *SmaI* sites; consequently, we used the phagemid DNA with the two *StuI* sites throughout this study.

Since we could not selectively degrade the *in vitro* synthesized, non-recombinant dsDNA molecules by restriction enzyme cleavage, we decided to first electroporate the dsDNA into TG1 cells, and then digest the purified, replicated DNA. After re-electroporating the digested DNA into bacteria, the resulting transformants were observed to be entirely recombinant (Fig. 4). By this approach, a phage-display library, with 10^{10} members and >99% recombinant, has been recently constructed in the lab by performing 140 electroporations (i.e., 100 electroporations of the *in vitro* synthesized dsDNA, followed by 40 electroporations of the digested DNA, into bacteria).

3.5. Kunkel mutagenesis with DNA segments generated by error-prone and asymmetric PCR

To bypass the time-consuming steps of restriction enzyme digestion, gel purification, and ligation in the conventional approach for generating a secondary mutagenic library, a protocol was devised that utilizes DNA segments amplified from PCR in lieu of oligonucleotide primers. First, an error-prone PCR was carried out with Mutazyme II, a mixture of two mutant DNA polymerases that introduce point mutations at high frequency in the targeted gene [31]. The amplified DNA segments were resolved by agarose gel electrophoresis, gel purified, denatured, and annealed to the ssDNA template. After DNA synthesis *in vitro*, the resulting dsDNA was electroporated into TG1 cells. But as revealed by phage ELISA and DNA sequencing, only 3-8% of the transformants were recombinants. With this method, a secondary library with a diversity of 1.0×10^8 was constructed (Fig. 5). To investigate whether digestion by *StuI* could elevate the incorporation of the mutated strand, the dsDNA was subjected to digestion with *StuI* before transformation. Although digestion did increase the mutation rate to 17-28%, the transformation efficiency decreased 5- to 7-fold.

As Kunkel mutagenesis is usually conducted with one to several 30-60 nucleotide long oligonucleotide primers, we rationalized that a double-stranded 300-bp DNA segment might not anneal efficiently to the ssDNA template for *in vitro* DNA synthesis because of the equimolar presence of its complementary DNA strand. Therefore, we decided to generate DNA segments that were predominately single-stranded through asymmetric PCR [27, 32], and use the resulting mixture to prime DNA synthesis on the template strand (Fig. 5A). DNA segments that were predominantly single-stranded were generated through 40 cycles of DNA synthesis with Mutazyme II. They were subsequently annealed to the uracilated ssDNA template, converted to dsDNA *in vitro*, and electroporated into TG1 cells. A library consisting of 4×10^8 recombinants was created, with a transformation efficiency of 7.4×10^8 cfu/ μ g dsDNA (Fig. 5B), which is comparable to the transformation efficiency, $5-6 \times 10^8$ cfu/ μ g, obtained with dsDNA synthesized with oligonucleotide primers ordered from commercial vendor (data not shown). Upon sequencing 24 recombinants, we observed equal numbers of transition mutations and transversion mutations, 18/35 compared to 17/35, respectively, and an overall point mutation rate of 5.3 mutations per kilobase (i.e., 0.5%).

While conducting our experiments, we witnessed that asymmetric PCR amplification was very sensitive to many factors (i.e., type of DNA polymerase, buffer, thermal cycling conditions). Even with a third primer to perform a nested asymmetric PCR [32], amplification results were not consistent (i.e., the yields of single-stranded product varied). Thus, when asymmetric PCR fails to generate high-quality single-stranded DNA, the double-stranded segment from the error-prone PCR can be used instead for constructing a secondary library.

To elevate the mutation rate, error-prone PCR was performed with Mutazyme II under conditions reported to increase mutation rate of *Taq* DNA polymerase [28]. The resulting DNA segment was used directly as the primer for *in vitro* dsDNA synthesis, as described above (Fig. 5A). Electroporation of the dsDNA yielded 2.8×10^9 transformants, with a transformation efficiency of 5.3×10^8 cfu/ μ g dsDNA. Among the transformants, 3.5% were recombinant, and on the average each recombinant clone had 6.5 mutations, which is equal to a point mutation rate of 2.5% (i.e., 25 mutations/per kb) (Fig. 5B). While the fraction of recombinants among the transformants is low, the diversity of the resulting library is still sufficient (i.e., 1.0×10^8) for generating enhanced binders.

3.6. Identification of stronger binders through affinity selection of a mutagenic library

The mutagenic library containing 4×10^8 variants was screened through three rounds of affinity selection under stringent conditions. After the third round of selection, eighty-eight clones were evaluated by phage ELISA: ~50% of the clones showed tighter binding to Pak1 kinase than the original clone (data not shown). Three clones with the greatest ELISA signals were chosen for further testing, and two variants, C2 and D3, were observed to bind 4-fold stronger to Pak1 kinase than the original clone, while a third variant, E10, bound 2-fold better (Fig. 6). Compared to the primary structure of the original binder, each of the three FN3 variants had three amino acid substitutions (data not shown). Thus, with this example, we demonstrate the utility of our modifications to the Kunkel mutagenesis protocol in generating high quality recombinant affinity reagents.

4. Conclusions

Kunkel mutagenesis is one of the most frequently used methods for constructing phage-displayed libraries. To make this technique more efficient, we optimized the yields of ssDNA by growing infected cells for 22 h at 25°C, with a shaking speed of 200 rpm. By introducing *Stu*I recognition sites in the FN3 coding sequence, we were able to remove non-recombinant clones by restriction endonuclease digestion and subsequently created libraries that were > 99% recombinant. Finally, two mutagenic phage libraries were constructed with DNA segments amplified by error-prone and asymmetric PCR. Screening one of the secondary libraries identified three variants that bound 2- to 4-fold tighter to the Pak1 kinase than the original clone. In the future, we envision that a phage pool, enriched for binders via affinity selection of the primary library, can be used directly as template for mutagenic PCR, and the resulting mutated DNA segment can be used to prime dsDNA synthesis *in vitro*. Thus, in one week's time a secondary library can be constructed and screened to yield enhanced binders.

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Figure 1. Overview of Kunkel mutagenesis

Phagemid DNA is electroporated into the CJ236 strain of *E. coli*. These cells are then infected with M13-K07 helper virus for amplifying phage particles that yield uracilated (U) single-stranded DNA (ssDNA). The ssDNA is annealed to two phosphorylated mutagenic oligonucleotides, which prime the synthesis of heteroduplex, double-stranded DNA (dsDNA) in the presence of T7 DNA polymerase, T4 DNA ligase, and deoxynucleotides. The resulting dsDNA is purified and electroporated into TG1 cells, where the uracilated parental strand is degraded and the mutant strand is preserved and converted into the replicative form of phagemid DNA.

Figure 2. Synthesis of heteroduplex dsDNA *in vitro*

Purified uracilated ssDNA was converted into dsDNA by T7 DNA polymerase and T4 DNA ligase. The extended heteroduplex dsDNA was purified and resolved by agarose gel electrophoresis. The relative mobility of ssDNA and dsDNA are noted with an arrow and a parenthesis, respectively.

Figure 3. Single-stranded DNA extracted from bacterial cultures grown at two different temperatures

Bacterial cultures, which were grown at 37°C and 25°C, were clarified by centrifugation. Single-stranded DNA (ssDNA) were extracted from clarified supernatant and resolved in a 1% agarose gel. The ssDNA depicted here migrates with a size of 5830 nucleotides and its position in the gel is denoted with an arrow. As shown in the figure, the yields of ssDNA from a culture grown at 25°C were 7-fold higher than that grown at 37°C.

Figure 4. Removal of non-recombinant clones by digestion with *StuI*

The phagemid modified with sequences of stop codons and *StuI* restriction enzyme sites was electroporated into CJ236 bacterial cells for generating uracilated ssDNA, which was annealed to mutagenic oligonucleotides (With NNK codons) and converted to dsDNA *in vitro*. Heteroduplex dsDNA was electroporated into TG1 cells, and the next day, transformed cells were scraped off the surface of agar plates. Phagemid DNA was then extracted from the scraped cells, digested with *StuI*, and electroporated back into TG1 cells. Bacterial colonies from electroporation of the synthesized dsDNA and the digested DNA were picked and grown up for phage replication. The following day, a phage ELISA was performed with anti-Flag antibody to identify mutant clones for sequencing analysis. Due to the presence of two amber stop codons encoded by the NNK codons, some recombinant clones appeared negative in the ELISA assay. On the microtiter plate of phage ELISA, the red-circled and green-circled wells correspond to positive and negative controls, respectively.

Figure 5. Construction of two secondary mutagenic libraries with DNA segments amplified by error-prone and asymmetric PCR

A. Error-prone PCR was performed to amplify the coding region of an FN3 variant that had been evolved to bind to Pak1 kinase. The DNA segment, which was gel purified, was either used directly as primer for annealing to the uracilated ssDNA template, or was used as template to carry out asymmetric PCR, yielding DNA segments that were mostly single-stranded segment. This mixture of DNA segments was purified free of primers and annealed to uracilated ssDNA. The annealed ssDNA template, either to the double-stranded or the single-stranded segments was converted into dsDNA *in vitro* by the action of T7 DNA polymerase and T4 DNA ligase. The extended, circular dsDNA was purified and electroporated into TG1 cells, thereby generating a secondary library. **B.** Two mutagenic libraries were constructed with the modified protocol of Kunkel mutagenesis. The number of transformants is the estimated total number of bacterial colonies produced through electroporation. The percentage of recombinants is the ratio of recombinants to the total pool of transformants, as a fraction of 100. The library diversity is the estimated total number of recombinants in the mutagenic library. Mutations/kb is the average number of point mutations per kilobase (kb).

Figure 6. Comparing the original clone and its three variants for binding to Pak1 kinase
 The blue bars represent the binding of phage particles to the Pak1 kinase (target), and the orange bars represent binding to casein (negative control). All binding values were normalized to the display level of the Flag-epitope, which is at the N-terminal of the displayed FN3 monobody. The error bars represent the standard deviation of duplicate measurements.