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A rapid, efficient, and economical inverse polymerase chain reaction-based method for generating a site saturation mutant library

Pankaj C. Jain [a], Raghavan Varadarajan [a,b,*]

[a] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India
[b] Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore 560 004, India

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ABSTRACT

With the development of deep sequencing methodologies, it has become important to construct site saturation mutant (SSM) libraries in which every nucleotide/codon in a gene is individually randomized. We describe methodologies for the rapid, efficient, and economical construction of such libraries using inverse polymerase chain reaction (PCR). We show that if the degenerate codon is in the middle of the mutagenic primer, there is an inherent PCR bias due to the thermodynamic mismatch penalty, which decreases the proportion of unique mutants. Introducing a nucleotide bias in the primer can alleviate the problem. Alternatively, if the degenerate codon is placed at the 5′ end, there is no PCR bias, which results in a higher proportion of unique mutants. This also facilitates detection of deletion mutants resulting from errors during primer synthesis. This method can be used to rapidly generate SSM libraries for any gene or nucleotide sequence, which can subsequently be screened and analyzed by deep sequencing.

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Site-directed mutagenesis (SDM)[1] is widely used for understanding sequence–structure–function relationships and to design novel molecules for biomedical and biotechnological purposes [1–5]. Site saturation mutagenesis is a further advancement of SDM in which each amino acid of a protein or nucleotide in a gene is systematically randomized. A feature of such libraries is that each member is mutated at a single site (nucleotide or codon). A site saturation mutant (SSM) library of a protein can be used to identify residue-specific contributions to protein structure, stability, and function [6–9]. We recently showed that phenotypic screening of such a library coupled with deep sequencing can be used for protein model discrimination [10]. However, in that case the library was constructed by pooling mutants that had been individually made over the course of several years and individually characterized by Sanger sequencing.

Large-scale saturation mutagenesis studies involving single replacements of an amino acid have been carried out previously on a few proteins such as bacteriophage T4 lysozyme [11], lac repressor [12], CfNT2 [13] CcdB [10], HSP90 [14], influenza inhibitor [15], PDZ domain [16], *TEM-1* β-lactamase [17], and rat neurotensin receptor 1 [18]. However, a major bottleneck in all such studies is the process of library generation, which generally is expensive and laborious. The above libraries were generated using diverse techniques including polymerase chain reaction (PCR)-like linear amplification with partially overlapping primer [13,19] or a megaprimer-based approach [10,20] or by overlap extension PCR [5,16,18,21]. Amplification from a uracil-containing single-stranded DNA (ssDNA) template has been employed in the Kunkel method [22] and in its recently published improvement, the PFunkel method [17]. Some non-PCR-based approaches, such as cassette mutagenesis [14] and DNA synthesis [9,15], have also been employed for library generation.

We describe an inverse PCR-based exponential amplification technique in which site saturation mutagenesis is carried out using degenerate primers containing a mutant codon (NNK, where N is A/C/G/T and K is G/T in equimolar ratio) in the forward primer. The goals were to have a library constructed with high mutagenesis efficiency in a short period of time and with the number of individual colonies at least 25- to 30-fold in excess of the number of possible single site mutants.

Materials and methods

Plasmids, host strains, and PCR reagents

The CcdB gene was previously cloned under the control of the arabinose-inducible P_{BAD} promoter in the vector pBAD24 to yield

* Corresponding author at: Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India. Fax: +91 80 2360 0535.
E-mail address: varadar@mbu.iisc.ernet.in (R. Varadarajan).

[1] *Abbreviations used:* SDM, site-directed mutagenesis; SSM, site saturation mutant; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; T4 PNK, T4 Polynucleotide Kinase; WT, wild-type; OE–PCR, overlap extension PCR; AOE–PCR, asymmetric overlap extension PCR; LB, Luria Broth.

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the construct pBAD24CcdB (4.8 kb) [23,24]. pMAL c2x (6.7 kb) and pUC19 (2.8 kb) vectors were used for determining the mutagenesis efficiency by blue–white screening. *Escherichia coli* XLI Blue was used as a host for all of the transformations. KOD DNA polymerase (Novagen), Kapa DNA polymerase (Kapa Biosystems), and Phusion DNA polymerase (Finnzymes) were used for PCR. T4 polynucleotide kinase (T4 PNK), T4 DNA ligase, *Dpn*I, and their respective buffers were obtained from New England Biolabs. X-Gal was obtained from USB Chemicals, and isopropyl-β-D-thiogalactoside (IPTG) was obtained from Calbiochem.

Mutagenesis and sequencing

Primers (21–36 nucleotides long) to generate CcdB and lacZ mutants were obtained from Sigma Genosys, IDT, or the PAN–Oligo facility at Stanford University. The primers for saturation mutagenesis have NNK sequence at the mutant codon, where N is any nucleotide and K is G or T. For mutagenesis in cases where forward and reverse primers were partially overlapping, the overlap region was 15 to 20 bases with the mismatch in the middle of the overlap. Both primers had an additional 10 to 12 bases flanking the mismatch at the 3′ end. For mutagenesis with non-overlapping primers, when the mutation was in the middle of the primer, there were 12 to 15 complementary bases on either side of the mismatch, resulting in a total length of approximately 30 bases. However, when the mutation was at the 5′ end of the primer, there were an additional 21 complementary bases toward the 3′ end, resulting in a total length of 24 bases. The reverse primer in this case was 21 bases long.

Following mutagenesis, plasmids were transformed in high-efficiency chemically competent *E. coli* XL1 Blue cells (efficiency = 10^7 CFU/μg pUC19) prepared by the Inoue method [25]. Following plasmid isolation from individual colonies, the entire coding region of CcdB was subjected to automated Sanger sequencing at Macrogen (South Korea). For the sequencing of PCR products, the gel-purified PCR product was directly sent for sequencing. The sequencing data were aligned using MEGA5 software [26] against the wild-type (WT) CcdB sequence.

Short PCR-based mutagenesis approaches

Overlap extension PCR (OE–PCR) was done by the protocol mentioned previously [21]. However, a low template concentration of 500 pg in a 20 μl reaction mixture was employed. Two sets of primer pairs were used to generate the fragments: (i) vector-specific forward primer and gene-specific reverse primer for a position and (ii) vector-specific reverse primer and gene-specific forward primer for the same position. The primers were approximately 30 bases in length, with gene-specific primers having an overlap of approximately 15 bases and the mutagenic site in the middle of this overlap, as shown in Fig. 1A. The PCR was done in a thermal cycler PTC 200 (Bio-Rad Laboratories) for 20 cycles with an annealing temperature of 60 °C for 45 s and an extension temperature of 72 °C for 30 s. After the PCR, each of the reaction mixtures was subjected to *Dpn*I digestion to remove the starting methylated template (instead of gel band purification) by adding 0.5 μl of *Dpn*I (20 U/μl), 1 μl of NEB buffer 4, and 8.5 μl of PCR product. This was incubated at 37 °C for 6 h to digest the starting template. In contrast to the conventional overlap PCR method [21], we did not do gel purification for each of the fragments and directly proceeded with the overlap extension reaction.

After *Dpn*I digestion, the two overlapping PCR products were mixed and subjected to a second round of PCR. The PCR protocol followed was the same as before, but instead of the template, the overlapping fragments generated in the first round of PCR were added to the reaction mixture at varying concentrations. The primers used were the vector-specific forward and reverse primers. As a control PCR, one PCR product fragment of the first reaction was mixed with vector-specific forward and reverse primers. The same control was repeated for the second PCR product fragment. Ideally, no product is expected in the control PCR program. In this PCR program, the extension time was increased to 45 s.

Another short PCR-based approach employed was asymmetric overlap extension PCR (AOE–PCR) [27]. The basic difference incorporated into AOE–PCR is using the mutant primer in the first PCR at a 10-fold lower concentration than the vector-specific primer. PCR was done for the above two reactions separately with the same program used for the OE–PCR. The PCR products from the two reactions were mixed in equal amounts, denatured at 94 °C for 1 min, and followed by an AOE cycle of 72 °C for 5 min. As a negative control, reactions were carried out using only the appropriate vector-specific primer for each reaction.

Inverse PCR-like linear amplification with partially overlapping complementary primers

To generate a full-length plasmid with partially overlapping complementary primers (12–16 bp overlap in the two primers), the two primers contained mutations approximately 6 to 8 bases from their 5′ ends [19] (Fig. 1B). The final template concentration used was varied from 25 to 50 ng (of pBAD-CcdB, pMAL c2x, or pUC19) in a 20 μl reaction. When using either Phusion DNA polymerase or KOD DNA polymerase, the reaction mixture and PCR procedure were according to the manufacturer's protocol. The PCR was done for 25 cycles with an annealing temperature of 60 °C for 45 s.

The PCR mixture was subjected to *Dpn*I digestion by adding 0.5 μl of *Dpn*I (20 U/μl), 1 μl of NEB buffer 4, and 8.5 μl of PCR product. This was incubated at 37 °C for 6 h.

Inverse PCR with non-overlapping primers

The set of primers used in this PCR were non-overlapping but adjacent to each other [28]. The site of mutation was either in the middle of the forward primer or at the 5′ end of the forward primer. The reverse primer was located immediately adjacent to the forward primers, with no gap between the 5′ position of each primer, as shown in Fig. 1C. The template concentration used was 50 to 500 pg in a 20 μl reaction mixture. PCR was carried out according to the manufacturer's protocol; however, the number of cycles used was 20.

PCR purification/Gel band purification and ligation of PCR product

PCR purification was done using the PCR Cleanup Kit of Amersham Biosciences. Gel band purification was done with the Fermentas Gel Extraction Kit. The DNA was finally eluted in approximately 20 μl of MilliQ water.

Phosphorylation and ligation

Primer (300 μM) was phosphorylated with 2 U of T4 PNK in the presence of 1× T4 PNK buffer (NEB) in a 10 μl reaction mixture and incubated at 37 °C for 1 h. In some cases, instead of the primer, the PCR product after purification was phosphorylated directly in a 10 μl reaction mixture (∼20 ng/μl) by adding 2 U of T4 PNK in T4 ligase buffer. Ligation was carried out by treating the phosphorylated PCR product with 10 U of T4 DNA ligase in the presence of 1× T4 ligase buffer. In cases where the PCR product was phosphorylated in T4 ligase buffer, after phosphorylation T4 ligase was added in the required amount directly to

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