

Sequence saturation mutagenesis (SeSaM): a novel method for directed evolution

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ABSTRACT

Sequence saturation mutagenesis (SeSaM) is a conceptually novel and practically simple method that truly randomizes a target sequence at every single nucleotide position. A SeSaM experiment can be accomplished within 2–3 days and comprises four steps: generating a pool of DNA fragments with random length, ‘tailing’ the DNA fragments with universal base using terminal transferase at 3′-termini, elongating DNA fragments in a PCR to the full-length genes using a single-stranded template and replacing the universal bases by standard nucleotides. Random mutations are created at universal sites due to the promiscuous base-pairing property of universal bases. Using enhanced green fluorescence protein as the model system and deoxyinosine as the universal base, we proved by sequencing 100 genes the concept of the SeSaM method and achieved a random distribution of mutations with the mutational bias expected for deoxyinosine.

INTRODUCTION

Inspired by Darwinian evolution in nature, directed evolution methods have been developed for tailoring proteins to our needs and elucidating structure–function relationships (1). An important step in a directed evolution experiment is to efficiently explore sequence space through random mutagenesis. The sequence space of a truly randomized library is, by its nature, not limited by a preselection for function under physiological conditions and allows us to adapt proteins to non-natural environments such as biotechnological processes.

Among random mutagenesis methods, error-prone PCR (epPCR) methods based on inaccurate amplification of genes have been very successful and are generally employed in directed enzyme evolution experiments due to their simplicity and versatility (1). EpPCR methods can be divided into (a) methods that reduce the fidelity of the polymerase by unbalancing nucleotides concentrations and/or adding of chemical compounds such as manganese chloride (2–4), (b) methods that employ nucleotide analogs (5,6), (c) methods

that utilize ‘mutagenic’ polymerases (7) and (d) combined methods (8).

Though versatile and simple, all known epPCR methods are significantly limited in their ability to create diversity on the gene level (9). Instead of changing one amino acid to all other 19 possible amino acids, an average of less than five other amino acids can be obtained with current epPCR methods. The reasons lie mainly in the redundant genetic code and the biased mutational spectra of DNA polymerases (7). Additionally, most directed evolution experiments are performed at low to moderate mutation frequencies in order to avoid a large portion of inactive clones. Thus only 1–3 nt have been mutated per 1000 bp (7). Statistically these mutations rarely occur next to each other. Furthermore, mutational bias of the employed DNA polymerases [methods (a), (c) and (d)] significantly reduces the diversity of the nucleotide substitutions and their random incorporation in the target gene. *Taq* DNA polymerase and Mutazyme (Stratagene), the commonly used polymerases in epPCRs, are biased toward transitions. For instance 40.9% of all observed substitutions employing *Taq* polymerase under epPCR conditions are A→T or T→A substitutions (7). The incorporation of nucleotide analogs occurs in a PCR at preferential sites and results in reduced diversity [methods (c) and (d) (4,5)]. An additional drawback of these methods is the inefficient incorporation of nucleotide analogs by polymerases (10), resulting in limited mutation frequencies (11) and often low product yields.

As a truly random mutagenesis method, sequence saturation mutagenesis (SeSaM) overcomes limitations caused by biased polymerases. Moreover, SeSaM can target a nucleotide species of the selected sequence and each nucleotide species can be exchanged in a controlled manner since SeSaM regulates the mutational spectra through a universal base.

MATERIALS AND METHODS

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany), Applichem (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). pEGFP plasmid was purchased from BD Biosciences (Heidelberg, Germany).

A thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) and thin-wall PCR tubes (Multi-Ultra tubes; 0.2 ml; Carl Roth, Germany) were used in all PCRs. The PCR volume was always 50 µl; larger volumes were prepared in multiple 50 µl PCRs. The amount of DNA was quantified

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using a NanoDrop photometer (NanoDrop Technologies, Delaware, USA).

Preparatory step 1: single-stranded EGFP template preparation

For each PCR (94°C for 3 min, 1 cycle; 94°C for 1 min/59.5°C for 1 min/72°C for 75 s, 31 cycles; 72°C for 10 min, 1 cycle), 2.5 U Taq DNA polymerase (Qiagen, Hilden, Germany), 0.2 mM dNTP mix (New England Biolabs, Frankfurt, Germany), 20 pmol forward primer (5'-fluorescein-CCAATACGAAACCGCCTCTCC-3'), 20 pmol 5'-biotinylated reverse primer (5'-CGTATCACGAGGCCCTTAAGG-3') and 250 ng pEGFP were used. After PCR, biotinylated reverse strands were isolated using Dynabeads MyOne Streptavidin (DynaL Biotech, Oslo, Norway) at room temperature. Fifty microliters of Dynabeads (10 mg/ml) were washed twice using 100 µl B&W buffer 1 (10 mM Tris-HCl pH 7.5, 1.0 mM EDTA, 2.0 M NaCl) each time. Washed Dynabeads were resuspended in 100 µl B&W buffer 1 and 100 µl of PCR product was added. After incubating for 20 min, the biotinylated DNA was immobilized on the Dynabeads, and the Dynabeads were washed with 100 µl B&W buffer 1. Non-biotinylated DNA fragments were released after incubating in 100 µl DNA melting solution (0.1 M NaOH) at 37°C for 10 min and bead washing [three times with 100 µl of DNA melting solution and once with 100 µl B&W buffer 2 (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 M NaCl)]. The single-stranded template was released from Dynabeads according to the manual by boiling in 0.1% SDS. Template DNA was desalted using a Microcon YM-10 centrifugal filter device (Millipore, Eschborn, Germany). The 50 µl sample was applied to a Microcon YM-10 sample reservoir and spun at 14 000 g for 10 min. Then, 100 µl EB buffer (1:10 dilution with water; Qiagen) was added and spun at 14 000 g for an additional 15 min. The sample reservoir was inverted and spun at 14 000 g for 3 min to recover the desalted DNA.

Preparatory step 2: cloning vector preparation

Twenty-five micrograms of plasmid pEGFP (QIAPrep, Qiagen) was digested with 30 U EcoRI (New England Biolabs) in a reaction mixture of 100 µl. The reaction mixture was incubated at 37°C for 3 h. The linearized plasmid was purified with the NucleoSpin Extract kit (Machery-Nagel, Düren, Germany) (elution volume of 50 µl for a 100 µl reaction mixture). Fifteen micrograms of linearized plasmid pEGFP was subjected to a second digestion with 20 U AgeI (New England Biolabs) in a total reaction volume of 50 µl. After incubation at 37°C for 3 h, the double-digested plasmid was purified using the NucleoSpin Extract kit (Machery-Nagel).

Step 1a: PCR with dATPαS

For each PCR with dATPαS (94°C for 3 min, 1 cycle; 94°C for 1 min/59.5°C for 1 min/72°C for 75 s, 31 cycles; 72°C for 10 min, 1 cycle) the reaction mixture included 2.5 U Taq DNA polymerase (Qiagen), 0.2 mM dNTP mix (New England Biolabs), 0.2 mM Sp-dATPαS (Biolog Life Science Institute, Bremen, Germany), 12.6 pmol 5'-biotinylated forward primer (5'-GACCATGATTACGCCAAGCTTGC-3'), 12.6 pmol reverse primer (5'-GACCGGCGCTCAGTTGGAATTCTAG-3') and 250 ng plasmid pEGFP.

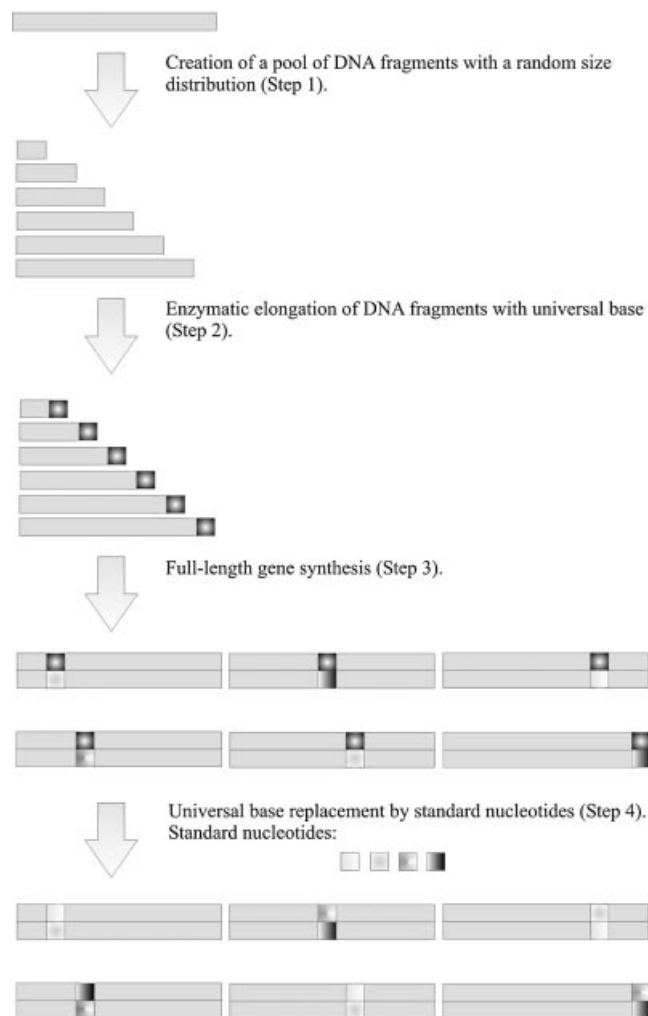


Figure 1. Scheme of the SeSaM method that comprises four steps. Step 1: creating a pool of DNA fragments with a random size distribution. Step 2: enzymic elongation of the DNA fragments with a universal base. Step 3: full-length gene synthesis. Step 4: universal base replacement by standard nucleotides.

Step 1b: iodine cleavage of phosphorothioate backbone

The phosphorothioate bond was cleaved with iodine (dissolved in ethanol; final concentration in the PCR tube, 2 µM). The mixture was incubated at room temperature for 1 h.

Step 1c: preparation of single-stranded DNA fragments with a length distribution

Biotinylated DNA fragments from the forward primer were isolated using Dynabeads MyOne Streptavidin (DYNAL Biotech) by a method identical to preparatory step 1, but without the desalting step.

Step 1d: SDS salt removal from eluted single-stranded DNA fragments

Desalination was done using the NucleoTrap kit (Machery-Nagel). Four hundred microliters of buffer NT2 was added to 100 µl of eluted DNA followed by 15 µl of NucleoTrap suspension. The mixture was incubated at room temperature

for 10 min with gentle shaking every 2–3 min. The sample was then centrifuged at 10 000 *g* for 30 s and, after discarding the supernatant, the pelleted silica matrix was washed twice with 500 μ l of buffer NT3 followed by air-drying for 15 min at 37°C to remove residual ethanol. The silica matrix was resuspended in 55 μ l Tris-HCl buffer (5 mM, pH 8.5), and incubated at 50°C for 5 min to elute the DNA from the silica. The suspension was centrifuged with NucleoSpin Microfilter (Machery-Nagel) at 10 000 *g* for 30 s to separate silica matrix from the DNA-containing solution.

Step 2: enzymic elongation of DNA fragments with the universal base deoxyinosine

Elongation of DNA fragments was performed at 37°C for 30 min in a total reaction volume of 50 μ l. Each reaction mixture consisted of: 5 U terminal transferase (New England Biolabs), 0.25 mM CoCl₂, 0.4 μ M dITP (Amersham Biosciences Europe GmbH, Freiburg, Germany) and 650 ng of desalted DNA fragments from Step 1d. Terminal transferase was subsequently heat inactivated at 70°C for 10 min and the product was purified using QIAquick Nucleotide Removal Kit (Qiagen).

Step 3: full-length gene synthesis

The full-length gene was synthesized by PCR (94°C for 3 min, 1 cycle; 94°C for 1 min/52.7°C for 1 min/72°C for 75 s, 30 cycles; 72°C for 10 min, 1 cycle) using the following reaction mixture: 2.5 U Taq DNA polymerase (Qiagen), 0.2 mM dNTP mix (New England Biolabs), 500 ng elongated

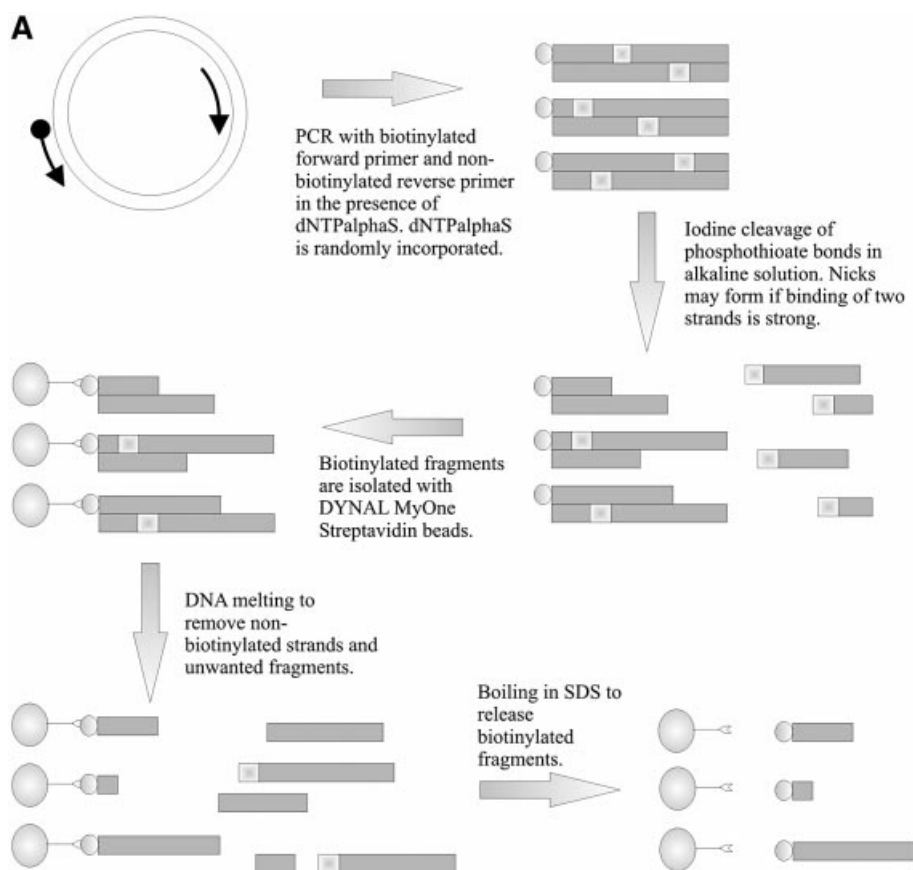
DNA fragments, 20 pmol reverse primer (5'-GACCGG-CGCTCAGTTGGAATTCTAG-3') and 60 ng single-stranded reverse template (preparatory step 1). Full-length genes were purified using the NucleoSpin Extract kit (Machery-Nagel).

Step 4: universal base replacement

Universal base replacement was achieved by PCR (94°C for 3 min, 1 cycle; 94°C for 1 min/52.7°C for 1 min/72°C for 75 s, 30 cycles; 72°C for 10 min, 1 cycle) employing 2.5 U Taq DNA polymerase (Qiagen), 0.2 mM dNTP mix (New England Biolabs), 20 pmol forward primer (5'-GACCATGATTACGC-CAAGCTTGC-3'), 20 pmol reverse primer (5'-GACCGG-CGCTCAGTTGGAATTCTAG-3') and 30 ng full-length gene (Step 3). The PCR products were purified using the NucleoSpin Extract kit (Machery-Nagel).

Cloning of the mutated EGFP library

Two micrograms of purified PCR product (Step 4) was digested with 30 U EcoRI (New England Biolabs) for 3 h at 37°C. Next, 20 U of AgeI (New England Biolabs) was added to the digest and incubated for an additional 3 h at 37°C. The digested product was purified using the NucleoSpin Extract kit (Machery-Nagel). Ligation of the digested PCR product into the linearized pEGFP cloning vector (Preparatory step 2) was performed at room temperature for 1 h using T4 DNA ligase (Roche, Mannheim, Germany). The transformation into *Escherichia coli* XL2 Blue (Stratagene, Amsterdam, Netherlands) cells was performed using the TSS method (12). The cells were plated and grown on LB_{amp} plates.



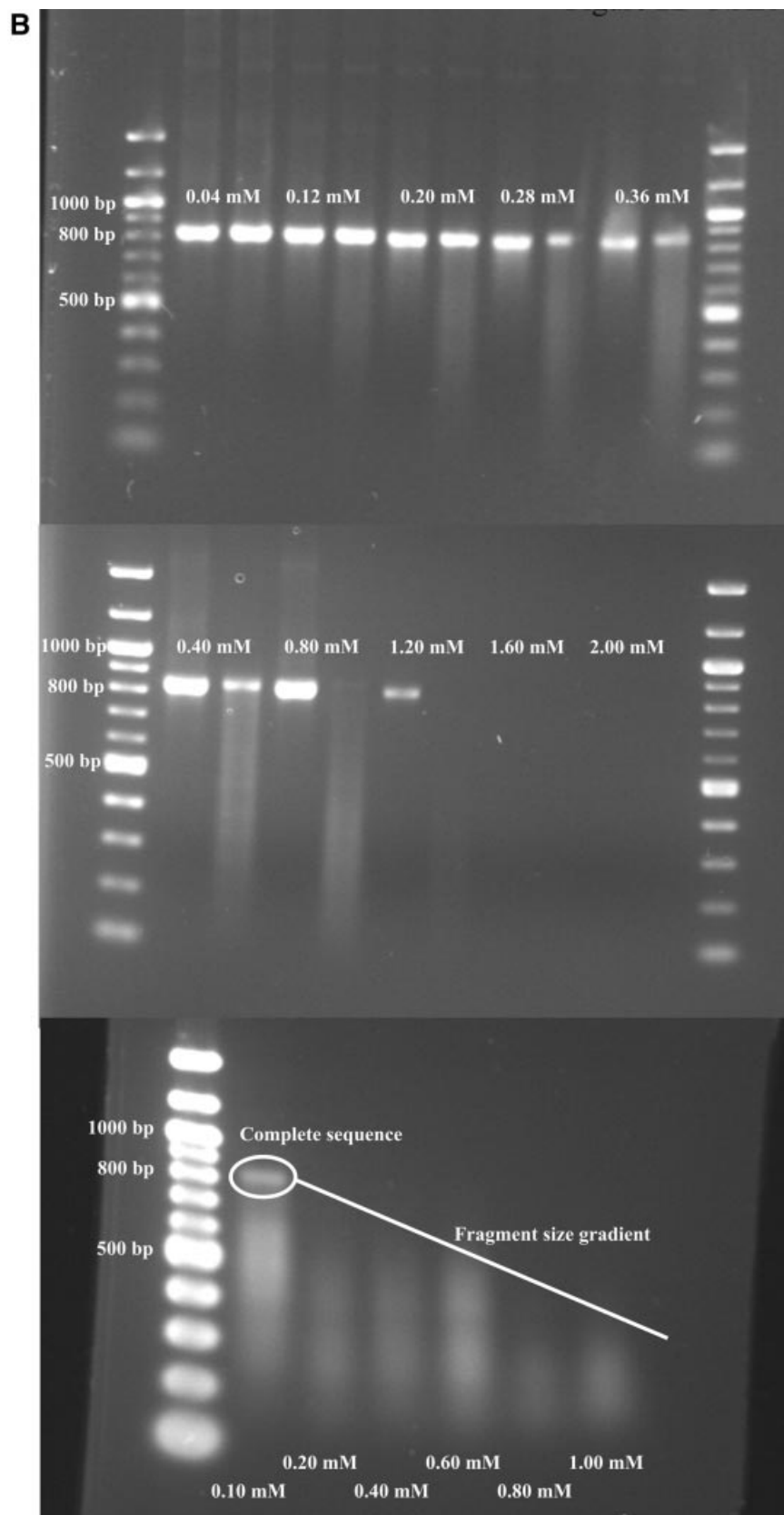


Figure 2. Step 1 of SeSaM: creating a DNA fragment pool with length distribution. (A) scheme of Step 1. (B) Gel pictures: PCR products before (left lane) and after (right lane) iodine cleavage. The PCRs were performed in the presence of the stated dATP α S concentration. Bottom gel picture: DNA fragment size distribution after DNA melting and purification of the cleaved PCR products.

RESULTS

Principle of SeSaM

SeSaM is a four-step method that can saturate every single nucleotide position of the target sequence with all four standard nucleotides (Fig. 1). In the first step a pool of DNA fragments with a random length distribution is generated. In the second step, DNA fragments are 'tailed' at 3'-termini with universal bases using terminal transferase. In the third step the elongated DNA fragments are extended in PCR to the full-length genes by using a single-stranded template and a reverse primer. In the fourth step a concluding PCR is used to replace the universal bases with standard nucleotides.

Step 1: creating a DNA fragment pool with length distribution

In the first step (Fig. 2), a PCR is performed using a biotinylated forward primer and a non-biotinylated reverse primer in the presence of both standard nucleotides and α -phosphothioate nucleotides. α -Phosphothioate nucleotides are similar to standard nucleotides, except that a sulfur atom replaces an oxygen atom at the α -phosphate. The commercially available S-isomer of the α -phosphothioate is randomly incorporated by DNA polymerases while the R-isomer is not accepted as substrate (13,14). The phosphothioate bond is susceptible to iodine cleavage in alkaline solution (15), and cleaving of the PCR product containing α -phosphothioate nucleotides generates a library of fragments that stop at every single nucleotide. Separation of this library from cleaved products is achieved by isolating biotinylated forward primer using magnetic particles with a streptavidin-coated surface in a DNA melting solution. For the success of this method, it is important to fully remove nicked complementary strands in this purification step. Subsequent release of the biotinylated DNA fragments from the solid support is achieved by boiling in 0.1% SDS solution. In order to generate a homogeneous library, the concentration of dATP α S was carefully adjusted to generate a homogeneous smear (DNA fragment pool) that spans through the whole gene length (Fig. 2B).

Step 2: enzymic elongation of DNA fragments with the universal base deoxyinosine

In the second step (Fig. 3), DNA fragments are 'tailed' by terminal transferase with the universal base deoxyinosine (11,16). Deoxyinosine is a well-studied universal base with a strong preference for base pairing with cytosine (11,16). It is used as a replacement for guanine in DNA sequencing (17,18). Terminal transferase is also known to incorporate universal bases with unbiased mutational spectra such as 3-nitropyrrole and 5-nitroindole (19,20). The biased deoxyinosine was selected to ensure that the incorporation of universal bases occurs and that the mutations found can be attributed to bias of deoxyinosine.

Step 3: full-length gene synthesis

The next step is extending the elongated fragment library to full length without amplifying the wild-type gene (Fig. 4) and this has been achieved by using a long single-stranded template. After this elongation step the reverse primer will anneal to the newly synthesized full-length strand and initiate

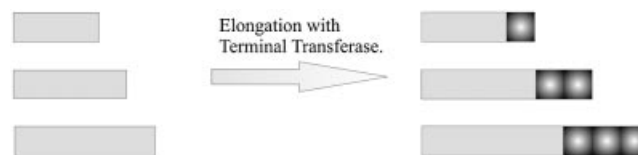


Figure 3. Step 2 of SeSaM: enzymic elongation of DNA fragments with the universal base deoxyinosine.

the synthesis of the complementary strand. The double-stranded DNA synthesized thus contains one strand harboring a nucleotide analog and a complementary strand containing at this position a randomly replaced nucleotide. Using a single-stranded template is crucial for the success of this step. In the case of a double-stranded template, the reverse primer will bind to its complementary template strand, resulting in PCR products that do not contain a nucleotide analog. A large fraction of non-mutated wild-type genes would consequently be generated in Step 4.

Step 4: universal base replacement

To replace nucleotide analogs with standard nucleotides (Fig. 5), a concluding PCR is required where full-length genes containing nucleotide analogs act as a template. In this PCR, nucleotide analogs are replaced by standard nucleotides due to promiscuous base-pairing of the universal bases.

Mutational spectra of SeSaM

Several libraries of 50–60 clones were sequenced for developing the SeSaM and sequencing of 100 clones validated the current protocol. Mutations are randomly distributed along the target gene EGFP (Fig. 6). Sequencing results and mutational spectra of SeSaM are summarized in Figure 7 and Table 1. As expected, most of the mutations (49%) occur at adenine positions because only dATP α S was used to create the DNA fragment pool; 95.8% of the A \rightarrow X substitutions are A \rightarrow G substitutions, reflecting well the base-pair properties of deoxyinosine (11,16).

DISCUSSION

In order to validate the SeSaM method we have to prove that nucleotide substitutions occur at positions of Sp-dNTP α S incorporation, and that universal bases are incorporated and responsible for nucleotide substitutions. For showing the site-specific cleavage we decided to use Sp-dATP α S. Mutations generated by the SeSaM method therefore have to occur preferably at adenine positions in the gene sequence. Equal targeting of all nucleotides in a gene sequence can be achieved by four PCRs (Step 1) using in each a different Sp-dNTP α S and balancing the DNA amount of each fragment library in Step 2.

Figure 7 shows that 49.0% of the mutations occur at adenine positions of the sequence, 16.3% occur at 'adenine' positions in the complementary strand and 24.5% occur at 1 or 2 nt downstream of an adenine position. Substitutions at adenine positions in the complementary strand occur mainly due to nicked DNA that has not been completely removed in the washing step (Fig. 2A). Improvements in the washing procedure reduced the total percentage of these mutations

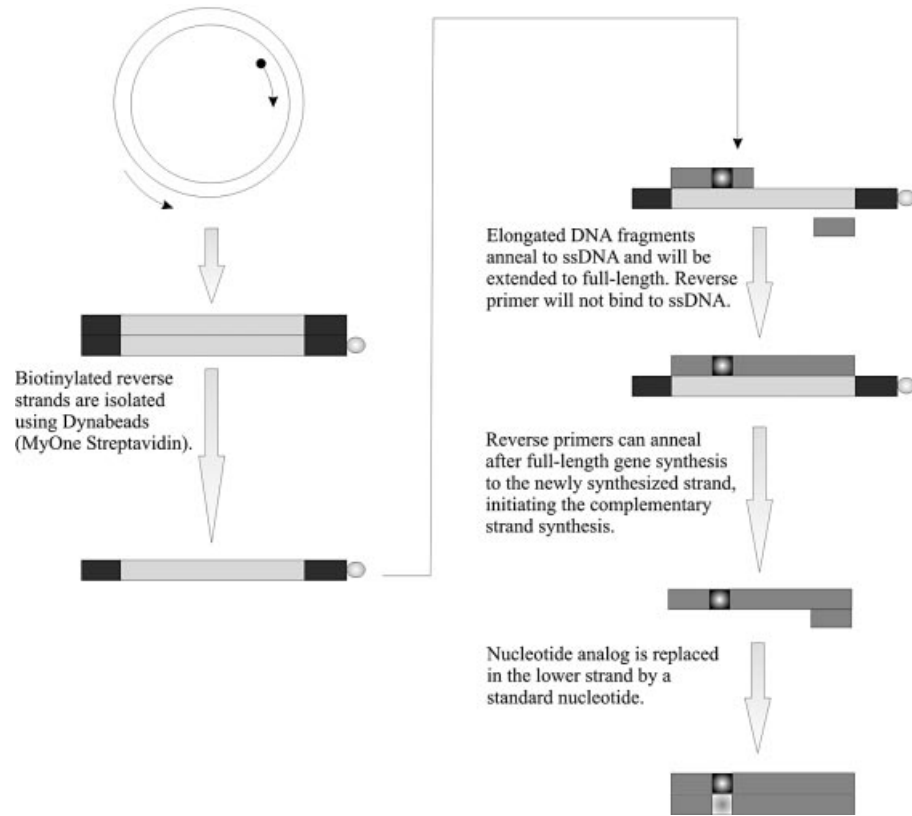


Figure 4. Step 3 of SeSaM: full-length gene synthesis by a PCR employing a single-stranded template and a reverse primer that amplifies the newly synthesized strand.

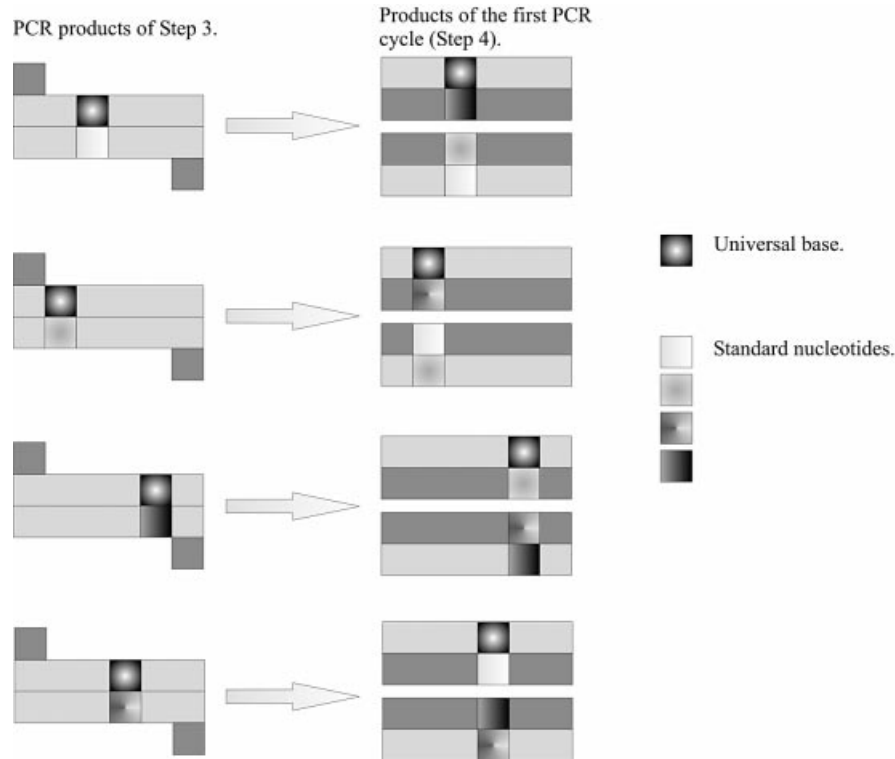


Figure 5. Step 4 of SeSaM: the universal base deoxyinosine is replaced by one of the four standard nucleotides in a PCR.

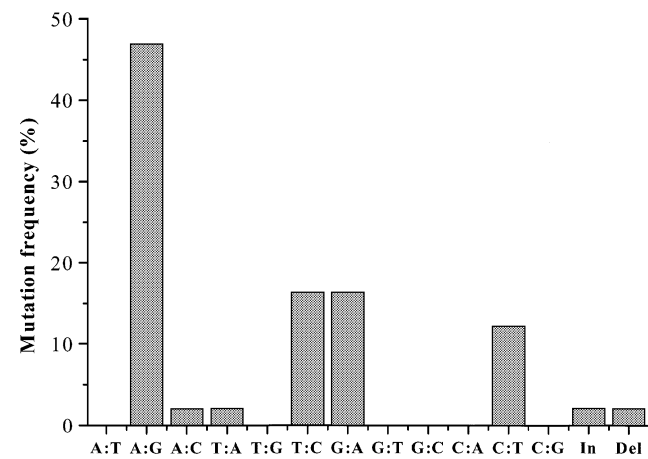


Figure 6. Mutational spectra of the SeSaM method employing dATPαS and deoxyinosine: In, insertion; De, deletion.

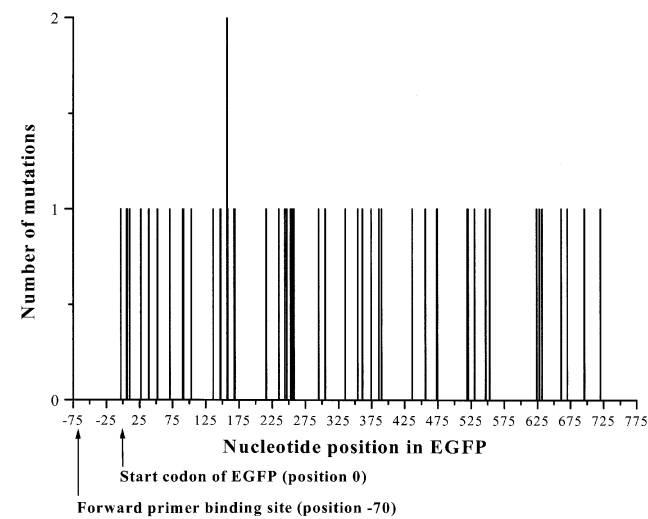


Figure 7. Distribution of the mutations generated by the SeSaM method.

significantly to 16.3%. In order to minimize wild-type formation, conditions were chosen in which statistically more than one degenerated base is incorporated (Fig. 3; Manual of New England Biolabs). We assume that many mutations next to an adenine position are caused by the incorporation of more than one nucleotide analog and that the nucleotide analogs at the adenine positions were replaced by the ‘original’ adenine nucleotide in the subsequent PCR (Step 4; Fig. 5). Elongation of DNA fragments containing more than one universal base incorporated at the 3′-terminus (Step 3; Fig. 4) is, however, a rare event owing to the weak two hydrogen bonds between each deoxyinosine and the template nucleotide. As a result the 3′-end is bending and difficult to be amplified by polymerases. In total up to 88% of the mutations are likely attributed to Sp-dATPαS incorporation.

We selected deoxyinosine as the universal base to prove that incorporation and nucleotide replacement can be attributed to the incorporated universal base. Deoxyinosine has a strong bias to base-pair with cytosine in the complementary strand (11,16). We therefore expect mainly A→G substitutions and the mutational bias found confirms that more than

Table 1. Mutational spectra of SeSaM

| Mutation | Percentage |
|-------------------------------------------------------------|------------|
| A→T | 0 |
| A→G | 46.9 |
| A→C | 2.04 |
| T→A | 2.04 |
| T→G | 0 |
| T→C | 16.3 |
| G→A | 16.3 |
| G→T | 0 |
| G→C | 0 |
| C→A | 0 |
| C→T | 12.4 |
| C→G | 0 |
| Insertion | 2.04 |
| Deletion | 2.04 |
| Total | 100 |
| Mutations at adenine positions | 49.0 |
| Mutation at positions 1–2 nucleotides downstream of adenine | 24.5 |
| T→C (complementary to A→G) | 16.3 |
| Mutations attributed to SeSaM process | 87.8 |

95% of the A→X substitutions are A→G substitutions. Indeed, these findings are in very good agreement with the reported bias (11,16).

DNA fragments smaller than 67 nt were not mutagenized (Fig. 7). The absence of mutations at this starting region is caused by the DNA extraction and purification procedures of the commercial purification kits employed (Machery Nagel NucleoTrap, Qiagen QIAquick) in which small fragments are recovered inefficiently. For library constructions with SeSaM we therefore used a forward primer that binds 70 bp upstream of the EGFP gene.

Compared with currently used epPCR methods, SeSaM has the advantage of being completely independent of the mutational bias of DNA polymerases. In addition, the fragment distribution of a DNA library is controllable by using different concentrations of the individual Sp-dNTPαS or a combination of them. Universal or degenerate bases with different base-pairing preferences can in future be used to tune the mutational spectra. Consequently, we believe that SeSaM is a very beneficial method for the field of directed evolution as it represents a superior tool to truly explore sequence space. Currently, we are extending the scope of SeSaM to randomize every nucleotide position in a target sequence by employing unbiased universal bases and to generate multiple subsequent mutations.

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