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Development of the SapI/AarI Incision Mediated Plasmid Editing Method

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Abstract

Plasmid engineering and molecular cloning is a virtually ubiquitous tool in biology. Although various methods have been developed for ligating DNA molecules or targeted mutagenesis of plasmids, each has its limitations. Many of the commonly used laboratory strategies are inefficient, while commercially available kits are quite costly and often specialized for highly specific circumstances. Here, we describe the SapI/AarI incision mediated plasmid editing (SIMPLE) method, which allows users to perform site-directed mutagenesis, deletions, and even short insertions into any plasmid in a single PCR reaction, using just one restriction enzyme. Additionally, the SIMPLE method can be adapted to insert any sized DNA fragment into a vector using a two-step PCR approach, and can be used to ligate any number of DNA fragments with non-compatible ends in the specific order desired. The SIMPLE method provides researchers an efficient and powerful tool with a broad range of applications for molecular cloning.

Graphical Abstract

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Keywords

Site-directed mutagenesis; Molecular cloning; Restriction endonuclease; Gene insertions and deletions; Plasmid

INTRODUCTION

Despite tremendous advancements in DNA editing technologies in recent years such as the development of zinc finger nucleases¹, TAL effector nucleases², and CRISPR³, PCR-based plasmid editing remains one of the most important tools in molecular biology research. Using plasmids for analysis of gene function is often simpler and more preferable to mutating genomic sequences, as the DNA editing process can be done completely *in vitro*. This allows for the entire plasmid to be sequenced prior to its introduction into cells to ensure accuracy, and avoids the possibility of secondary unwanted mutational events that are a common problem associated with genome editing methods⁴. Furthermore, plasmid-based expression of a gene of interest allows for fine-tuning, as researchers can clone their desired promoter upstream of the gene. Additionally, plasmids are frequently used to produce the protein of interest in a heterologous organism or *in vitro*.

Here we describe a novel approach for performing a variety of different modifications to any plasmid, using a single PCR reaction and one restriction enzyme. Our method, herein referred to as SIMPLE (SapI/AarI incision mediated plasmid eding), can be used for site-specific mutagenesis, deletions of any size, and short insertions (up to 30 base pairs). Furthermore, the SIMPLE method can be adapted to ligate together any number of DNA fragments in the desired order. One of the greatest advantages of the SIMPLE method is that the ligation junction between two fragments can be “patched” perfectly, preventing unwanted nucleotides/amino acids from being introduced, which is a common problem with standard DNA cloning methods. As a proof of concept for the SIMPLE method, we have made two separate point mutations, a partial deletion, and inserted a FLAG epitope into the *Schizosaccharomyces pombe ade6⁺* ORF cloned in pUC19. Our results show that the method is highly efficient and specific, and thus SIMPLE cloning should become a valuable method for plasmid construction and editing for cell and molecular biology researchers.

RESULTS AND DISCUSSION

While multiple different methods have been developed for targeted site-specific mutagenesis, deletions, and small insertions of plasmid DNA, no single currently available approach is able to produce all of these changes in a single PCR reaction^{5,6,7}. In our method, we took advantage of the unique characteristics of a pair of Type II restriction enzymes, SapI and AarI⁸. Both of these enzymes have several properties which make them conducive to the SIMPLE method (summarized in Fig. 1A). First, both have a recognition sequences of 7 base pairs, making their cut sites relatively infrequent in commonly used cloning vectors. Second, they cut on the 3' side of their recognition site rather than the position where they bind. Third and most importantly, there are no constraints for the sequence being cut (Ns), allowing an overhang of any sequence to be generated.

We reasoned that by using these properties of SapI/AarI, we would be able to edit specific nucleotides in any plasmid via PCR. As an example, we show a partial hypothetical DNA sequence on a plasmid, with a single nucleotide that will be targeted for mutagenesis highlighted in red (Fig. 1B). The primers would be designed to contain a 3–4 nucleotide spacer sequence at their 5' end, followed by the SapI (or AarI) recognition site, then 1 (for SapI) or 4 (for AarI) nucleotides of spacer sequence. Directly downstream of this spacer, there are up to 3 (for SapI) or 4 (for AarI) nucleotides that can be engineered to contain any sequence. Lastly, the remaining ~25 nucleotides will be used for targeting the primers to the appropriate sites for PCR amplification. The specific steps for designing primers are outlined in Fig. 1C.

PCR amplification of a plasmid using our primer design scheme would lead to production of a linearized, full length version of the plasmid, flanked by the desired point mutation and the SapI/AarI sites on both the 5' and 3' ends. This PCR product can then be digested with the appropriate enzyme, generating a pair of compatible overhangs which when ligated together, would reconstitute the original plasmid containing the desired mutation (Fig. 2A). Similarly, this approach can be modified to delete any part of a plasmid without the need for specific restriction enzyme sites, generating a perfect sequence without any “scars” left from the cutting and ligation. To perform a deletion, the PCR primers will be designed in the same way as above, but made complementary to the sequences flanking the desired deletion site. The PCR, digestion, and ligation steps will be identical to those used for mutagenesis (Fig. 2B).

As a proof of principle, we decided to use the SIMPLE method to make two separate point mutations (A46→G and C1466→T) and a partial deletion (601-1101) of the *S. pombe ade6⁺* ORF using both SapI and AarI. A short sequence of the relevant region for the C1466→T mutation is shown in Fig. 3A. The A46→G change is not shown. To determine the efficiency of our method, we sequenced 5 possible clones from each of the mutations and deletion, and found that our method had a success rate of 80% (Fig. 3B). We also screened several potential clones for the deletion by restriction enzyme digestion followed by gel electrophoresis (Fig. 3C).

The SIMPLE method can also be adapted to make small insertions in a single PCR step. It is commonly desirable to clone small epitopes such as the HA, MYC, or FLAG to tag gene cloned in a plasmid. However, researchers often run into the issue of having no convenient restriction site at their desired position, or not being able to clone their tag in the proper reading frame. The SIMPLE method has the ability to circumvent all of these issues, as there are no restrictions to where the cut sites can be located. As a proof of concept, we inserted the FLAG epitope into a precisely defined position within the *ade6⁺* ORF. As before, the primers were designed to contain a spacer, followed by the enzyme recognition site, an additional spacer, then half of the FLAG epitope (per primer), and finally the targeting homology sequence (Fig. S1). After the PCR amplification, digestion and ligation, the two compatible ends generate the FLAG tag in the proper reading frame.

The unique properties of SapI and AarI can also be utilized to ligate together multiple large DNA fragments in any desired order. Each fragment can be amplified via primers that, when digested, will contain a specific overhang. By generating pairs of compatible overhangs, multiple fragments can be “stitched” together to construct a plasmid (Fig. S2). In theory, this approach can be used to produce $4^3 = 64$ unique overhangs using SapI, and $4^4 = 256$ unique overhangs using AarI. However, further work needs to be done to assess the practical feasibility of this concept.

Lastly, we propose that additional enzymes with similar properties can also be utilized to perform the SIMPLE method. We have chosen to use SapI and AarI for this study as their recognition sequence of 7 base pairs is relatively rare, and does not occur in our plasmids. However, for researchers who do plan to utilize our approach, we have assembled a list of additional enzymes which have a recognition sequence of at least 6 base pairs, and cut distal to their binding sites on the 3' side (Table S1). Theoretically, any of these enzymes should also be compatible with SIMPLE cloning.

MATERIALS AND METHODS

PCR amplification and cloning

All PCR reactions were performed using the Phusion High-Fidelity DNA Polymerase (NEB), using the manufacturer's recommended specifications. After amplification, PCR products were first digested with DpnI (NEB) to eliminate the parental plasmid, cleaned up over columns (Qiagen), and then digested with either the SapI (NEB) or AarI (Thermo) restriction enzyme, following the manufacturer's specifications. After digestion, DNA products were cleaned up again as before, and 50µg of the DNA was self-ligated using T4 DNA ligase (NEB). Ligations were transformed into chemo-competent *E. coli* cells, and grown at 37°C overnight. Five colonies were screened from each cloning reaction by sequencing (GENEWIZ) or restriction digestion where applicable.

Plasmids

All site-specific mutations and deletions were performed on plasmid RP14 (pUC19-*ade6-M216*), which contains the *S. pombe ade6-M216* mutation(G46A) gene cloned in the SalI site.

Primers

The following primer pairs were used to generate the A46→G mutation in *ade6*: (using the SapI enzyme): CGGGCTCTTCAGGCCGAATGATGGTAGAGGCAGCCCAT and GCGGCTCTTCTGCCCAATTGACCACCTCCAAGGATCCCT; (using the AarI enzyme): CGGGCTCTTCATCGCGTGGAATTACGTTGTTTCATCAACC and GCGGCTCTTCTCGAACCCTCGTAAGCCAATGTTTTACTTT. The following primer pairs were used to generate the C1466→T mutation in *ADE6*: (using the SapI enzyme): CGGGCTCTTCATTTCGAGGTGTCCCTGTGCGCCACTGTTGC and GCGGCTCTTCTGAAGCATCTGAACAATAGAGTGAAGAG; (using the AarI enzyme): CGCCACCTGCGGATTTTCGAGGTGTCCCTGTGCGCCACTGTTGC and GCGCACCTGCCTGACGAAGCATCTGAACAATAGAGTGAAGAG. The following primer pairs were used to delete nucleotides 601-1101 in *ADE6*: (using the SapI enzyme): CGGGCTCTTCAGAATGTGAACGTAGGTATCAGATGCT and GCGGCTCTTCTTTCAACTTTTCCGTCTAAACTGCGTACTA; (using the AarI enzyme): CGCCACCTGCGGATGAATGTGAACGTAGGTATCAGATGCT and GCGCACCTGCCTGAATTTCAACTTTTCCGTCTAAACTGCGTACT. The following primer pairs were used to insert the FLAG epitope tag after nucleotide 510 in *ADE6*: GCCGGCTCTTCTCGATGACGACAAGCTTGGTGATCGTCCGCTTTATGTTG and CGCCGCTCTTCATCGTCTTTGTAGTCTGCTTTGATGGCAGTAGGAATCTCA. The following primers were used for DNA sequencing: TAAAAACCTGTAAATGCTG, GATTCTGATTAAAGCAAG, and GGCCAAGAGAGTTTGGTTA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

Development of SapI/AarI incision mediated plasmid editing (SIMPLE) method.

The SIMPLE method can be used for *in vitro* site directed mutagenesis.

The SIMPLE method can also be used to generate small gene insertion and deletions.

The SIMPLE method is an efficient and inexpensive alternative to commercially available methods.

Figure 1.

Primer design for introducing point mutations using AarI and SapI. **(A)** Recognition sites and cutting patterns for the SapI and AarI restriction enzymes. **(B)** Hypothetical sequence that will be targeted for mutagenesis. **(C)** Primers that can be used to mutate the sequence shown in **(B)** with the SIMPLE method, using the SapI enzyme.

Figure 2.

Schematic diagram showing how the SIMPLE method can be used to perform **(A)** site-directed mutagenesis and **(B)** a deletion within a plasmid. The desired sequence to be targeted for either mutagenesis or deletion is labelled with an Y in both figures, while the desired nucleotide change is labeled with a Z, and sequences flanking the deletion site are labelled with an A and B.

Figure 3. Proof of principle for the SIMPLE method. **(A)** Sanger sequencing chromatograph showing a directed site-specific mutation in the *S. pombe ade6⁺* gene where the C 1466 was changed to a T. The A46→G change is not shown **(B)** Summary of all attempted mutations and deletions using the SIMPLE method. For all methods, we screened 5 plasmids. **(C)** Agarose gel showing the targeted 500 base pair deletion of the *ade6⁺* ORF. M, size markers; 1–10, screened deletions; C, control plasmid. The plasmid was cut with EcoRI which cuts three times: on either side of the *ade6⁺* gene (releasing the *ade6* fragment) and one more time (releasing Fragment 1 and Plasmid backbone). We screened 10 candidates with the deletion. Note the shorter *ade6* fragment.