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Highly efficient and simple SSPER and rrPCR approaches for the accurate site-directed mutagenesis of large and small plasmids

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Abstract

Advances are needed in the site-directed mutagenesis of large plasmids for protein structure-function studies, as current methods are often inefficient, complicated and time-consuming. Here two new methods are reported that overcome these difficulties, namely the single primer extension reaction (SSPER) strategy that reaches 100% efficiency and the reduce recycle PCR (rrPCR) method that is advantageous in generating single and pairwise combinations of mutations. Both methods are distinguished from current technologies by the addition of a step that easily removes the oligonucleotide primer(s) after the first reaction, thus allowing for the addition of a second reaction in chronological sequence to generate and isolate the appropriate DNA product with the site-directed mutation(s). High efficiency of the methods is demonstrated by generating single and paired combinations of the 11 site-directed mutations targeted on 5 different plasmid DNA templates ranging from 10 to 12 kb and 57–60% GC-content at a rate of 50–100%. Overall, the methods are demonstrated to be (i) highly accurate, allowing for screening of plasmids by DNA sequencing, (ii) streamlined to generate the mutations within a single day, (iii) cost-effective in requiring only two primers and two enzymes (DpnI and a proofreading DNA polymerase), (iv) straightforward in primer design, (v) applicable for both large and small plasmids, and (vi) easily implemented by entry level researchers.

Keywords

Site-directed mutagenesis; Substitution mutation; Amino acid exchange; Method; PCR

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CRediT authorship contribution statement

JM and HJ designed the research experiments, performed research experiments, analyzed the data, and wrote the paper. All authors performed research experiments associated with this paper and have read and approved of the paper.

Declarations of interest

The authors do not have a conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.08.004.

Introduction

Side-directed mutagenesis is a common tool used in molecular biology to study the influence of DNA sequence on downstream biological and biochemical functions [1–3]. The methods used to perform site-directed mutagenesis are varied, but often include PCR to generate point substitutions, deletions, and/or insertions in the DNA. Methods that accurately generate substitution mutations are desirable for protein structure-function studies, as they allow the expression of proteins with targeted alterations in amino acid residues including presumed active sites. The plasmid vectors used for heterologous protein synthesis are varied, but are often large in order to accommodate the elements needed to tightly control target gene expression, select for the plasmid in recombinant *Escherichia coli*, and/or shuttle the plasmid from *E. coli* into an alternative host strain. For example, the pET-based plasmid vectors used to replicate genes for heterologous expression in *E. coli* are commonly over 5 kb [4]. Likewise, the plasmid vectors used for gene expression in alternative hosts are large, such as the > 10 kb *E. coli-Haloferax volcanii* shuttle plasmids used to synthesize extremophilicproteins that are active in high salt and organic solvents [5,6].

PCR-based methods that generate substitution mutations are available, but have several disadvantages when using large plasmids as the DNA templates. Overlap extension PCR can be used, but relies on the generation of two overlapping PCR products that carry the mutation(s), with these products subsequently used as template for a final overlap extension PCR [7–9]. This final PCR product can be difficult to generate and must be inserted into a plasmid vector for expression studies, making overlap extension PCR suboptimal for site-directed mutagenesis studies. An alternative and more traditional approach is to design complementary primer pairs with the substitution mutation(s) positioned at the center of each primer [10,11]. In this standard PCR approach, the expression plasmid serves as the DNA template and is prepared in an *E. coli dam*+ *dcm*+ strain. After PCR, the template is removed from the sample using DpnI, a restriction enzyme that cleaves methylated and hemimethylated DNA, while leaving the unmethylated PCR product intact [12]. Due to the complementary nature of the primer pair, primer-dimers often form during PCR leading to reaction failure. DNA insertions and other artifacts also occur in the PCR products when using this type of primer design. Modifying the primers to be partially complementary can overcome these limitations, but adds complex steps after the PCR (*e.g., in vitro* recombination) to avoid DNA sequence repeats, as the primer ends are homologous [13,14]. Inverse PCR is also an option, with one of the primers designed with the substitution mutation (s) near the 5′ end [15]. The PCR products are phosphorylated and ligated after amplification, with DpnI used to remove the methylated template DNA prior to transformation. While simple in design, inverse PCR is unreliable for site-directed mutagenesis, particularly for large DNA templates, and often results in the generation of DNA artifacts at the site of self-ligation, most likely due to the asymmetric positioning of the substitution mutation in the primer.

PCR-based protocols are available to accommodate the site-directed mutagenesis of large plasmids. One such method, the site-directed mutagenesis for large plasmids (SMLP) [16], relies on a specialized DNA polymerase to generate two large DNA fragments with

overlapping ends by two independent PCRs. The substitution mutation is included in the primers. Once generated, the PCR products are joined through recombinational ligation using an Exnase II kit, which adds complexity and expense to the method as this kit is not commonly used in other molecular biology approaches. Furthermore, the primer design for SMLP is complicated for entry level researchers. A less costly approach (SPRINP, Single-Primer Reactions IN Parallel) has been developed for the site-directed mutagenesis of large plasmids [17]. The method uses the straightforward design of complementary primer pairs with the substitution mutation(s) in the center and avoids the formation of primer dimers by incorporating two parallel single-primer extension reactions. The resulting single stranded (ss)DNA products are mixed, annealed *in vitro*, and digested with DpnI to remove the template DNA. The resulting product is directly transformed into *E. coli*. While this method is reported for plasmids of up to 5 kb [17], it has not been demonstrated for larger plasmids.

Here two PCR-based methods are developed that are found to be highly efficient in generating substitution mutations in large plasmids (> 10 kb) and which overcome the difficulties encountered in current technologies. Included in these two methods are the single primer extension reaction (SSPER) strategy, with the advantage of reaching 100% efficiency, and the reduce recycle PCR (rrPCR) method, that is more flexible in allowing for single and pairwise mutations to be generated in parallel. These methods are distinguished in approach by removing the initial short oligonucleotide primer(s) after the first reaction series and proceeding with a subsequent site-directed mutagenesis step without adding extra template. The methods are straightforward and cost-effective, requiring only two primers and two enzymes, namely a proofreading DNA polymerase, such as Phusion, and DpnI, that cleaves the methylated DNA template prior to transformation. Most importantly, the methods are highly efficient at a 50–100% positive rate and easily performed by entry-level researchers.

Materials and methods

Chemicals

Biochemicals and analytical-grade inorganic chemicals were purchased from Fisher Scientific (Atlanta, GA), Bio-Rad (Hercules, CA) and Sigma-Aldrich (St. Louis, MO). Primers were ordered from Eurofins Genomics (Louisville, KY, USA) and used without further purification. Monarch PCR and DNA Cleanup Kit (5 μg), Phusion DNA polymerase, PCR reagents, and DpnI restriction enzyme were from New England Biolabs, while Hi-Lo DNA standards were from Minnesota Molecular, Inc. (Minneapolis, MN).

Strains, media and growth conditions

Strains, plasmids and primers used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium for routine cultivation. Cells were also grown in SOB medium consisting (per L) of 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl, autoclaved and supplemented with 5 mL of 2 M $MgCl_2$ (10 mM final concentration) after cooling to 55 °C, as indicated. Media was supplemented with 1.5% agar for plates and ampicillin (Ap, 100 mg/L) as required. Liquid cultures were grown with rotary agitation

at 200 rpm. Sanger DNA sequencing of plasmids was performed by Eton Bioscience Inc. (Research Triangle Park, NC, USA) using primers p15, p16 and p10.

Primer design

Primers (Table 1) were designed for site-directed mutagenesis of 1–3 nucleotides within coding sequences. The guidelines used for primer design were: 10–20 nt of unmodified sequence on both sides of the mutation; mutated bases in the center; GC content of 40–70%; Tm of 70–80 °C; at least one G or C at both the 5′ and 3′ ends; and minimal hairpin structures [17]. To assess the robustness of the rrPCR method, primers were also tested that had (i) Tm's outside the 70–80 °C range (p14 and p10), (ii) extensive hairpin structures (p13), and (iii) sequence with an A at the 5′ end (p13). Primer Tm values were calculated according to the NE BioLabs Tm calculator (https://tmcalculator.neb.com) with settings for Phusion high-fidelity DNA polymerase in GC buffer and 500 nM primer concentration. Primer hairpin structures were examined using IDT Technologies OligoAnalyzer (https://www.idtdna.com/calc/analyzer) with settings at: Oligo Conc 0.5 μM, Na+ Conc 50 mM, Mg++ Conc 1.5 mM and dNTPs Conc 0.2 mM.

Plasmid DNA template

Haloferax-Escherichia shuttle expression plasmids pJAM503, pJAM3923, pJAM1208, pJAM3940 and pJAM4016 (Table 1) were used as the methylated DNA templates in the first step of the SSPER or rrPCR methods, as indicated. The plasmids were propagated in *E. coli* TOP10 (*dam*+ *dcm*+) and then isolated from 5 mL overnight cultures using the PureLink Quick Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was eluted into 75 μL of nuclease free water and stored at − 20 °C until use. The concentration of the plasmid DNA was determined using a microvolume spectrophotometer (Take3 microvolume plate with Gen5 software, BioTek Instruments, Winooski, VT, USA) and was at a typical yield of 80–100 ng/μL. The plasmid DNA was diluted in nuclease free water to 20 ng/μL for use as template in either the PCR-1 or single primer extension reactions (the first step of rrPCR and SSPER, respectively).

SSPER and rrPCR methods

SSPER and rrPCR methods are outlined in detail in Supplementary Material. Reaction temperatures were controlled using a MyCycler or iCycler thermal cycler (BioRad, Hercules, CA).

Annealing assay of primers to plasmid template

An annealing assay was developed to compare the efficiencies of annealing plasmid templates to short primers *vs.* amplified megaprimers. The reaction conditions used for the assay (including annealing parameters and primer concentrations) were identical to the SSPER and rrPCR methods. To detect annealing, the primers were 5′-end labeled with 6-carboxyfluorescein (6-FAM, Integrated DNA Technologies, Research Triangle Park, NC, USA) as indicated. The 6-FAM labeled reverse complementary primers p9 and p20 were used for the short primer annealing assay with plasmid pJAM503 as template. For

amplification of the megaprimer, 6-FAM labeled p9 and p11 primers were used with pJAM503 as template. The annealing assay mixture (12.5 μL) included 0.5 μM 6-FAM labeled p9 and p20 for short primer assay, 725 ng (0.39 μM) 6-FAM-labeled megaprimer for the megaprimer annealing assay, 1 ×GC Phusion polymerase buffer, and 2 μg plasmid template (H_2O was used as control by replacing plasmid template). The annealing of reverse complementary short primer strands (6-FAM labeled p9 and p20) was assayed at 69 °C for 7 min 45 s after denaturing at 98 °C for 5 min corresponding to the temperature in the SSPER method. The annealing of the megaprimer was performed at 72 °C for 7 min 45 s corresponding to rrPCR after denaturation at 98 °C for 5 min. The annealed products were separated by 4% polyacrylamide gel electrophoresis (PAGE) at a constant 100 V for 30 min and were visualized using an iBright FL1000 Imaging System (Thermo Fisher Scientific) at 488 nm with an exposure time of 500 msec. ImageJ software [18] was used for quantitative analysis of the density of the gel bands. Experiments were performed in triplicate. Data are expressed as the mean of three independent samples with standard deviation. Probability of the observed differences between sample types was measured by p-value calculated by the two-tailed Student's t-test, based on identifying all variances to be equal across the samples where the means were compared by F-test.

Results and discussion

Standard PCR and its limitation in generating site-directed mutations in large plasmids

To generate substitution mutations in large plasmids (>10 kb), the conventional PCR method was initially used that included two complementary primers with the substitution mutation positioned in the center of each primer. Primer pairs p1/p2 and p9/p15 were designed to be used with templates pJAM1208 and pJAM503, respectively (Table 1, Fig. 1A–B). The plasmid DNA templates were propagated in *E. coli* TOP10 and purified using a conventional miniprep kit. As this *E. coli* strain is *dam*+ *dcm*+ , the plasmids were prepared in a form that is methylated at the common Dam and Dcm sites making the DNA template and any hemimethylated DNA derivates susceptible to DpnI digestion at later stages in the protocol [12]. Standard PCRs were performed by mixing both the mutagenesis primers and the plasmid template in the same reaction. The PCR products were digested with DpnI to remove the template and subsequently transformed into *E. coli* TOP10. By this conventional approach, the number of colonies obtained on the selection plates was reproducibly limited (0–5 CFUs/plate) (Fig. 1B). The few colonies that were detected and analyzed by DNA sequencing carried plasmids corresponding to the original template or had plasmids with unusual rearrangements apparently due to the formation of primer dimers (Supplemental Fig. S1). These findings suggested that the conventional PCR method was not optimal for generating the desired site-directed mutations (SDMs).

Sequential single primer extension reaction (SSPER) method to generate substitution mutations

To overcome this hurdle, the SSPER method was developed. The method was designed to (i) minimize the formation of primer dimers or other artifacts that occur due to the annealing of short complementary ssDNA primers, and (ii) avoid the rearrangements and other problems that may arise when annealing two large strands of DNA *in vitro* [17]. The SSPER used

the conventional primer design, where the substitution mutations were positioned in the center of each primer, and the primers were fully complementary (Fig. 2A). Furthermore, the plasmid DNA template was prepared in a methylated state from an *E. coli dam*- + *dcm*+ strain and later eliminated from the site-directed mutagenesis products by treatment with DpnI (Fig. 2B). However, the feature that distinguished SSPER from other methods was the use of sequential primer extension reactions, with the first primer (pF) removed from the sample after the first primer extension reaction was completed and the second primer (pR) added to this purified sample for the final round of primer extension to generate the desired product (Fig. 2B). An additional distinctive feature of the SSPER protocol was DpnI treatment of the sample prior to the second primer extension reaction. By using DpnI at this intermediate stage of the protocol, the original methylated DNA template was removed and thus the formation of hemimethylated DNA in the second primer extension reaction was avoided. Hemimethylated DNA is more difficult to hydrolyze with DpnI than fully methylated DNA [19]. In contrast to SSPER, conventional site-directed mutagenesis approaches include both ssDNA primers in the same PCR, while SPRINP separates the ssDNA primers into two parallel primer extension reactions but relies upon annealing the two products *in vitro* after the primer extension reactions are separately performed [17].

SSPER method is found highly efficient at generating site-directed mutations in large plasmids

To test and optimize the SSPER method, primer pairs p1/p2 and p9/p15 were used with *E. coli dam*+ *dcm*+ purified plasmids pJAM1208 and pJAM503 of 10–11 kb as templates, respectively (Fig. 1A, B). In all experiments, a long extension time was used to ensure that the > 10 kb products were synthesized. In the fully optimized protocol, the samples were treated with DpnI after the first extension reaction. The newly synthesized ssDNA was then purified by PCR clean up and used as a template in a similar single primer extension reaction with the second primer. To maximize the specificity of annealing the short oligonucleotide ssDNA primers to their target in each of the single primer extension reactions, a gradual reduction in the annealing temperature from 78 °C by 0.5 °C per cycle for 6 cycles followed by 12 cycles of annealing at 75 °C was found to be adequate. Thus, the total thermocycler time for the SSPER method was 5 h (2.5 h for each of the single primer extension steps in the protocol). The number of colonies observed for all SSPER experiments was high and averaged > 250 CFUs/plate for the sitedirected mutagenesis samples when compared to the no primer controls (0–5 CFUs/plate). Further analysis by DNA sequencing, revealed that the substitution mutations were generated at ≥ 50% efficiency, with the fully optimized method reaching the 100% efficiency in generating molecules with the SDMs. While treating the samples with DpnI at the final stage of the SSPER strategy was found to reduce efficiency from 100% to 50–67%, one potential advantage of this latter approach to consider is that the original plasmid DNA would be present to serve as a template in the second primer extension reaction even if the first primer extension reaction failed.

Reduce recycle PCR (rrPCR) method to generate substitution mutations

The substitution mutagenesis strategy was next examined from a different perspective to provide flexibility in generating single and pairwise combinations of mutations. Like

SSPER, this alternative approach also incorporated the removal of short ssDNA primers at an intermediate stage in the protocol, but its distinction from SSPER was that the primers were not complementary and that the second reaction in the series used the PCR products as primers. The lack of complementarity in the initial primer design allowed substitution mutations to be incorporated at one or two distinct sites on the target sequence and the same 'anchor' primer to be used for multiple substitution mutations. This approach was termed the reduce recycle PCR (rrPCR) method, to highlight how the number of synthetic primers is reduced and the template is recycled. The concept behind rrPCR is to design the primers as forward and reverse primers (pF and pR) in a traditional PCR style; however, a substitution mutation(s) is included in the center of one or both primers (Fig. 3A). A traditional PCR is performed using methylated plasmid DNA as template (Fig. 3B). The innovation in the approach is that after the first PCR (PCR-1), the ssDNA primers are removed by PCR clean up and the resulting mixture is used for a second round of PCR (PCR-2) with no external primers or template added. In the PCR-2 stage, the dsDNA product of PCR-1 serves as a megaprimer pair and the recycled methylated plasmid DNA serves again as the template. After PCR-2, the template and any other hemimethylated/methylated DNA fragments are removed by DpnI digestion prior to transformation of *E. coli*. The advantages of the rrPCR approach are that substitution mutations can be incorporated at two distinct sites in parallel, the number of primers needed to generate multiple SDMs at a single site is mimimized, and a thermocycler with touchdown options is not needed.

To demonstrate the efficacy of the rrPCR method, substitution mutations were introduced at 9 different sites in single and pairwise combinations (separated by 69–996 bp) on 4 different plasmid templates (pJAM3923, pJAM503, pJAM3940 and pJAM4016) ranging in size from 10.2 to 11.8 kb and GC content from 57% to 60% (Fig. 4A). Most primers were designed within the guidelines of 10–20 nt of unmodified sequence on both sides of the mutation, mutated bases in the center, GC content of 40–70%, Tm of 70–80 °C, at least one G or C at both the 5′ and 3′ ends, and minimal hairpin structures. However, to test the robustness of the rrPCR method, a subset of primers was constructed with suboptimal features including (i) a Tm outside of the 70–80 °C range (p10 and 14), (ii) extensive hairpin structures (p13), and (iii) sequence with an A (p13) at the 5′ end. Of the 14 different primer pair combinations examined, including the suboptimal primer pairs, all were productive in generating the desired substitution mutation(s) with high efficiency and yield (Fig. 4B). In the case of primer pair combinations p4/5, p4/7, p8/6 and p8/7, transferring 100% of the PCR-1 mixture to the PCR-2 stage was found to generate the desired product. The other primer pair combinations were successful when 50% of the PCR-1 mixture was used for the next round of PCR (PCR-2). Each substitution type could be identified through DNA sequencing of only 2–4 clones (50–100% of the clones had the correct mutation) without the need to pre-screen clones using protocols such as the introduction of 'silent' restriction sites, in which the sequence change creates a restriction site but preserves the coding capacity [20]. When using rrPCR, isolation of plasmids with DNA artifacts or the original DNA sequence was minimal. For comparison, significant rearrangements occurred in the few plasmid DNA products isolated when using the standard PCR with complementary primers p9 and p15 and plasmid pJAM503 as the template, thus, preventing generation of the Pan1

K214 to amber stop codon (TAG) variant by the conventional method (Supplemental Fig. S1).

Comparison of annealing short primers vs. megaprimers to DNA template

The failure of the conventional PCR-based mutagenesis protocol using inverse complementary short primer sets was speculated to be due to the tendency of the short primers to form primer dimers instead of annealing to the plasmid template. The generation and use of a megaprimer pair to incorporate the substitution mutations *via* the rrPCR method likely overcame this hurdle. To investigate this possibility, the tendency of the two types of primer pairs to anneal to a large plasmid template was compared under the conditions of the mutagenesis protocol. The primers were labeled with 6-FAM to detect by fluorescence their annealing after separation of the DNA products by PAGE. Within a single annealing cycle, the megaprimers were reproducibly detected to anneal at a higher (>5-fold) efficiency than the short primer pair after subtracting background in which the template plasmid was replaced by H_2O (Supplemental Fig. S2), suggesting the number of PCR products to be 5^{25} more in a 25-cycle PCR amplification for the megaprimers than the short primers. The observed low efficiency of the short primer pair may be explained by a tendency of these primers to form primer dimers (due to their small molecule size) instead of annealing to the large plasmid template. This tendency to form primer dimers would result in a limited number of effective primers annealing to the template in the PCR amplification and, ultimately, an inability to obtain positive transformants with the desired site-directed mutations. This negative outcome of the conventional PCR-based mutagenesis protocol, with the short ssDNA oligonucleotides tending to form primer dimers, would become more pronounced as the plasmid template size becomes larger. By contrast, the frequency of forming primer dimers between the amplified complementary megaprimers was likely to be lower than that of the short primers; thus, the annealing of the megaprimers to the template could compete to a greater extent with the formation of primer dimers compared to the short primers.

Conclusions

Here two new methods, SSPER and rrPCR, are developed and shown to efficiently introduce substitution mutations in large plasmids. These methods are demonstrated to be highly efficient in generating single and paired SDM combinations by the targeting of 11 SDMs on five different genes carried on dsDNA plasmids of 10–12 kb and 57–60% GC-content. Colonies were routinely detected in high number (>250 CFU/plate) for the experimental samples compared to the no primer controls (≤ 5 CFUs/plate). The substitution mutations were identified at a rate of 50–100% and, thus, allowed for rapid screening by DNA sequencing. The need to redesign primer pairs for a particular mutation was eliminated, as all mutations were generated in a single strategy of primer design. The rrPCR method was demonstrated to be particularly useful in making substitution mutations in a pairwise combination in a single experiment. Overall, the SSPER and rrPCR methods are cost-effective options for generating substitution mutations in large plasmids. They can be performed from the first PCR reaction to the point of transforming the final PCR product into *E. coli* in a single day. The methods are highly accurate, require only two primers per

substitution mutation, use only two enzymes (DpnI and a proofreading DNA polymerase), can be used on GC rich templates, are amenable in design, and are easily performed by entry-level researchers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability

Data will be made available on request.

Abbreviations:

PCR	polymerase chain reaction
SSPER	single primer extension reaction
rrPCR	reduce recycle PCR
Ap^r	ampicillin resistance
ss	single stranded
CFUs	colony forming units
6-FAM	6-carboxyfluorescein
LB	Luria-Bertani
PAGE	polyacrylamide gel electrophoresis
SDM	site-directed mutation
Nv^r	novobiocin resistance

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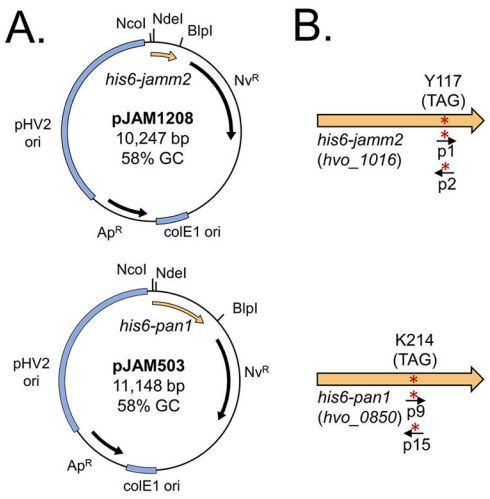


Method	Primer	Plasmid template	CFU/ plate	Positive clones/ sequenced	SDM
Std. PCR[a]	p1 + p2	pJAM1208	0-2[a]	n/a	n/a
Std. PCR[a]	p9 + p15	pJAM503	0-8[a]	0/4 (0%)[a]	n/a
SSPER[b,c]	p1 (step 1) p2 (step 2)	pJAM1208	TNTC[b,c]	1/2 (50%)[b] 2/3 (67%)[c]	JAMM2 Y118(TAG)
SSPER[c]	p9 (step 1) p15 (step 2)	pJAM503	TNTC[c,d]	2/3 (67%)[c] 4/4 (100%)[d]	PAN1 K214(TAG)
Std. PCR[a] & SSPER[b-d]	none	pJAM1208 pJAM503	0-5[a-c] 0[a,c,d]	n/a	n/a

[a]Std. PCR, conventional PCR with primers included in single reaction. [b-d]SSPER, sequential single primer extension reaction with touchdown protocol at 10 and 20 cycles[b] or 6 and 12 cycles[c,d] with DpnI digestion after the second extension reaction (step 2)[b,c] or after the first extension reaction (step 1)[d]. CFU, colony forming units. TNTC, too numerous to count (>250 CFU/plate). n/a, not applicable. SDM, site-directed mutation. (TAG), amber stop codon.

Fig. 1.

Plasmid templates, primers, and results generated by standard PCR compared to the sequential single primer extension reaction (SSPER) methods. A. Restriction map of the 10.2 kb pJAM1208 and 11.1 kb pJAM503 plasmids used as templates. B. Orientation of the primers on the genes targeted for mutagenesis (left) and the tabulated results of the standard PCR and SSPER methods (right). Primers p1 and p2 are designed to anneal to the *his6-hvo_1016* (JAMM2) open reading frame on plasmid pJAM1208, while primers p9 and p15 are designed to anneal to the *his6-hvo_0850* (PAN1) open reading frame on plasmid pJAM503. Red asterisk: site-directed mutations.

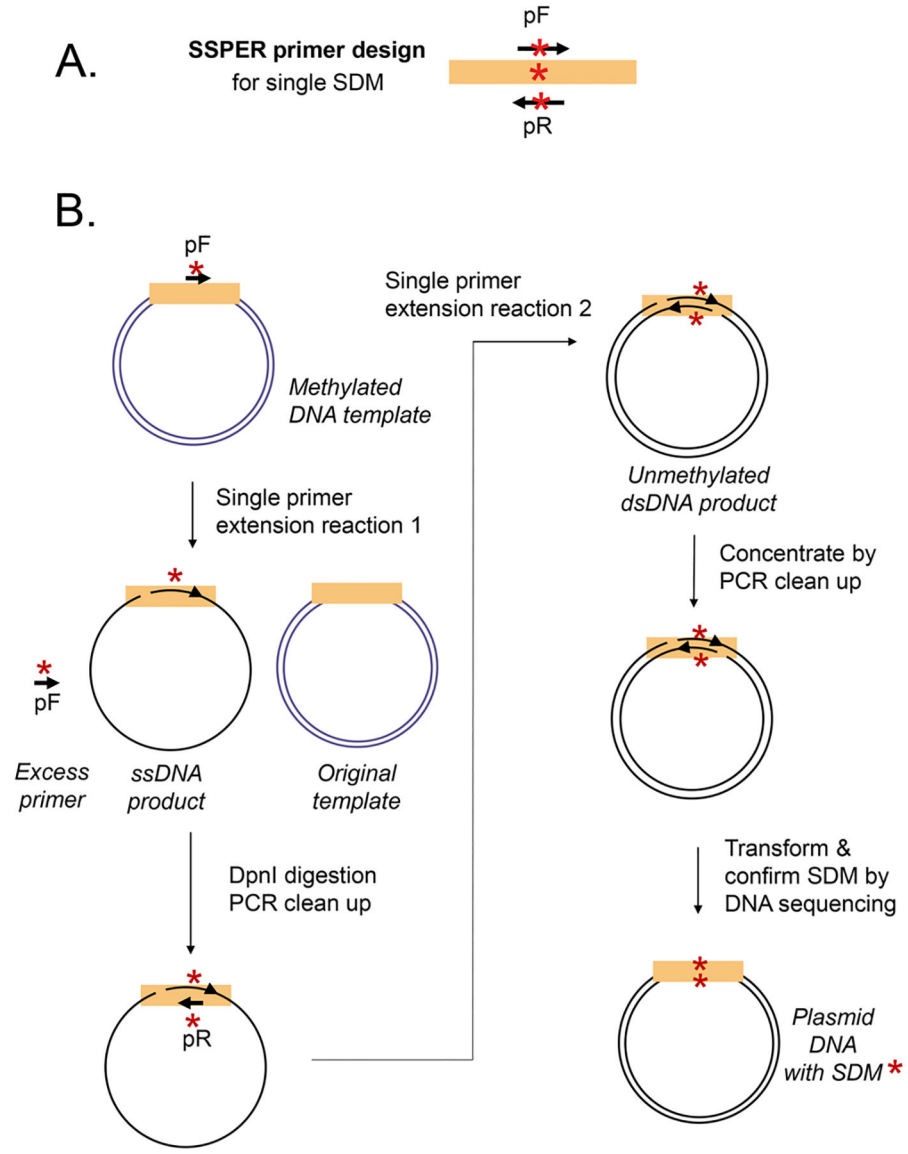


Fig. 2.

Primer design (A) and strategy (B) to incorporate substitution mutations into large plasmids using the sequential single primer extension reaction (SSPER) method. Primers (pF and pR) are designed to be complementary with the substitution mutation in the center of each oligonucleotide. Red asterisk: site-directed mutations. Orange box: gene or region targeted for mutagenesis. Purple circles: methylated DNA strands. Black circles: unmethylated DNA strands. In the SSPER strategy, (i) the original template is fully methylated plasmid DNA, (ii) single primer extension reactions are performed separately, (iii) after the first extension reaction the sample is incubated with DpnI to hydrolyze the methylated DNA template, (iv) the ssDNA product is subsequently purified from the first primer (pF) and the degraded template by PCR clean up, (v) the ssDNA product is then used as template to generate the desired DNA product by a second single primer extension reaction with the complementary primer (pR), (vi) enrichment and purification of the final DNA products by PCR cleanup

prior to transformation of *E. coli* is used to enhance product yield, and (vii) the DNA gaps in the plasmid product with the desired SDMs are sealed by transformation of *E. coli*.

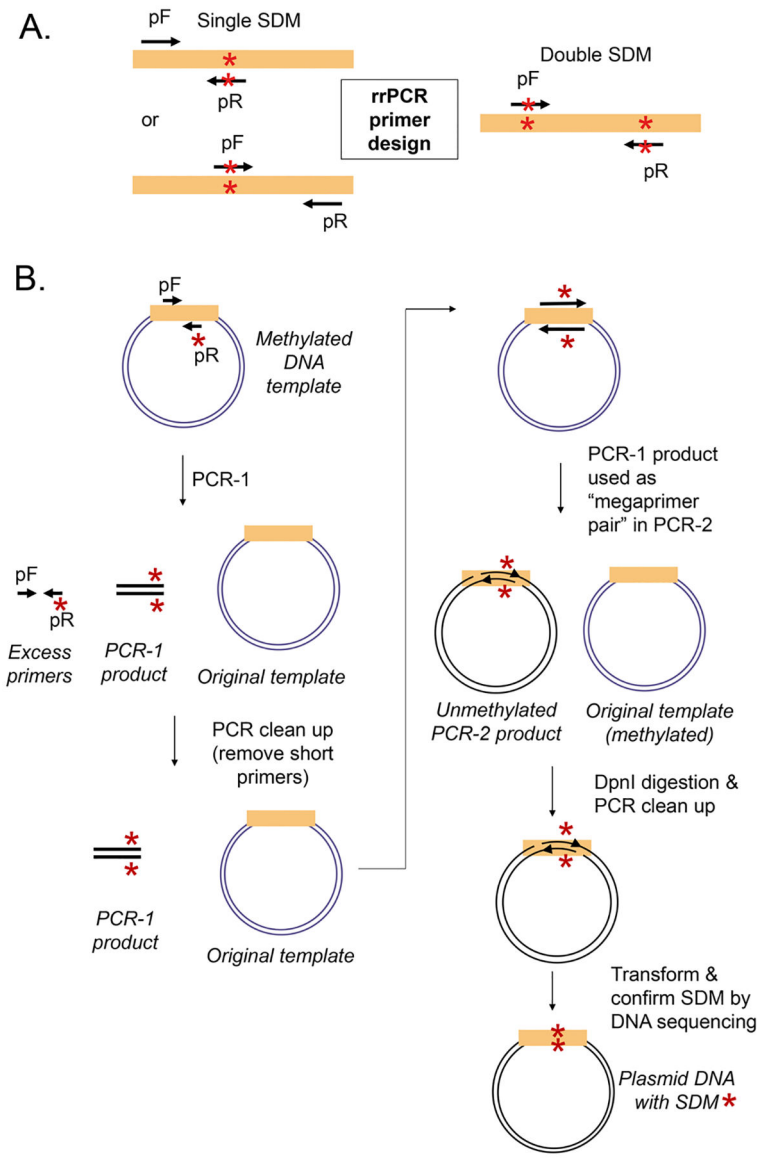
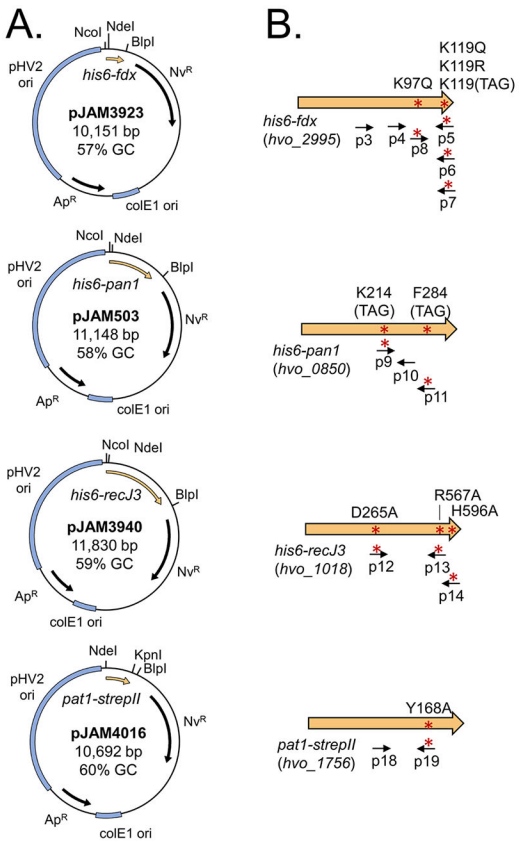


Fig. 3.

Primer design (A) and strategy (B) used to incorporate substitution mutations at single or multiple sites into large plasmids using the reduce recycle PCR (rrPCR) method. pF and pR, single stranded DNA primers used in the first PCR reaction (PCR-1). Red asterisk: site-directed mutations. Orange box: gene or region targeted for mutagenesis. Purple circles: methylated DNA strands. Black circles: unmethylated DNA strands. The short oligonucleotide primers (pF and pR) are removed after PCR-1 by PCR clean up. In the second PCR reaction (PCR-2), the methylated plasmid DNA is reused as template and the PCR-1 product is used as a "megaprimer pair". After PCR-2, the methylated DNA template is removed by DpnI digestion. The unmethylated PCR-2 product is enriched and purified by PCR cleanup prior to transformation into *E. coli*. The DNA gaps in the plasmid are sealed *in vivo* by the cell. A similar method is used to generate two SDMs in parallel with exception that both pF and pR primers used in PCR-1 carry an SDM.



Primer pair	Plasmid template	PCR-1 product	CFU/ plate	Positive clones/ sequenced	SDM
p3/5	pJAM3923	253 bp	TNTC	1/2 (50%)	Fdx K119Q
p3/6	pJAM3923	253 bp	TNTC	2/2 (100%)	Fdx K119R
p3/7	pJAM3923	253 bp	TNTC	2/2 (100%)	Fdx K119(TAG)
p4/5	pJAM3923	148 bp	161 TNTC[a]	0/2 (0%) 2/2 (100%)[a]	Fdx K119Q
p4/6	pJAM3923	148 bp	103	1/2 (50%)	Fdx K119R
p4/7	pJAM3923	148 bp	5 60[a]	0/2 (0%) 2/2 (100%)[a]	Fdx K119TAG
p8/5	pJAM3923	97 bp	14 TNTC[a]	1/2 (50%) n.d.[a]	Fdx K97Q K119Q
p8/6	pJAM3923	97 bp	99 TNTC[a]	0/2 (0%) 2/2 (100%)[a]	Fdx K97Q K119R
p8/7	pJAM3923	97 bp	7 32[a]	0/2 (0%) 2/2 (100%)[a]	Fdx K97Q K119(TAG)
p9/11	pJAM503	222 bp	TNTC	2/4 (50%)	Pan1 K214(TAG) F284(TAG)
p9/10	pJAM503	149 bp	30	3/4 (75%)	Pan1 K214(TAG)
p12/13	pJAM3940	902 bp	TNTC	3/4 (75%)	RecJ3 D265A R567A
p12/14	pJAM3940	989 bp	TNTC	4/4 (100%)	RecJ3 D265A H596A
p18/19	pJAM4016	253 bp	TNTC	3/3 (100%)	Pat1 Y168A
				Avg = 64%	
None	pJAM3923 pJAM503 pJAM3940 pJAM4016	n/a	0, 1[a] 0 12 8	n/a	n/a

[a]100% (vs. 50%) of PCR-1 product and template used in PCR-2. CFU, colony forming units. TNTC, >250 CFU/plate. (TAG), amber stop codon. n.d., not determined. n/a, not applicable. SDM, site-directed mutation.

Fig. 4.

Plasmid templates, primers and positive clones generated using the reduce recycle PCR (rrPCR) method. A. Restriction maps of the 10.2–11.8 kb plasmids used as templates. B. Left, Orientation of the primers on the open reading frames targeted for mutagenesis including *his6-hvo_2995* (Fdx), *his6-hvo_0850* (Pan1), *his6- hvo_1018* (RecJ3), and *hvo_1756-strepII* (Pat1). Red asterisk: site-directed mutations. Right, tabulated results.

Table 1

Lists of strains, plasmids and primers used in this study.[a]

Strain or plasmid (bp)	Description
E. coli TOP10 (Invitrogen)	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 *ΔlacX74 recA1 araD139* Δ(*ara leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG λ-*
pJAM503	Ap^r Nv^r; pBAP5010 carrying P2$_{rrn}$*his6-hvo_0850* (Pan1)
pJAM3923	Ap^r Nv^r; pBAP5010 carrying P2$_{rrn}$*his6-hvo_2995* (Fdx)
pJAM1208	Ap^r Nv^r; pBAP5010 carrying P2$_{rrn}$*his6-hvo_1016* (JAMM2)
pJAM3940	Ap^r Nv^r; pBAP5010 carrying P2$_{rrn}$*his6-hvo_1018* (RecJ3)
pJAM4016	Apr, Nvr; pBAP5010 carrying P2$_{rrn}$*hvo_1756-strepII* (Pat1)

Primer		Sequence (5′−3′)	Length	Primer Tm	Hairpin Tm
p1	JAMM2Y117 >TAGf	CGGCTCGCCGTAgGGCCCCGACGAC	25 nt	78 °C	51.8 °C
p2	JAMM2Y117 >TAGr	GTCGTCGGGGCCcTACGGCGAGCCG	25 nt	78 °C	58.1 °C
p3	FdxF1	GTGAGATGGAAGTGAACCAGGGCGAGTAC	29 nt	70 °C	35.4 °C
p4	FdxF2	CCATCGTGAAGGAGGGCGAAATCGACATG	29 nt	70 °C	49.4 °C
p5	Fdx K119Q	GTAGTCGAGGTGCTgCGCGTTGTAGAC	27 nt	70 °C	36.9 °C
p6	Fdx K119R	GTAGTCGAGGTGCcgCGCGTTGTAGAC	27 nt	72 °C	26.4 °C
p7	Fdx K119TAG	GTAGTCGAGGTGCTaCGCGTTGTAGACGATC	31 nt	70 °C	55.2 °C
p8	Fdx K97Q	GAAGTCAACGAGcAGAACGTCCGCCTC	27 nt	70 °C	40.9 °C
p9	Pan1-K214 >TAGf	CGCCTCGTTCATCtagATGGCCGGCTCCG	29 nt	75 °C	41.7 °C
p10	Pan1-int1	CGATGGCGTCTATCTCGTCGATG	23 nt	64 °C	34.6 °C
p11	Pan1-F284 >TAGr	GCCGCGCTCGTCctaGCCGTCCATCTC	27 nt	75 °C	35.2 °C
p12	RecJ3D265Af	CCGACGGCGcCGGCATGTGCG	21 nt	75 °C	49.0 °C
p13	RecJ3R567Ar	ACGCCGTCGGAGgcGAGGACGGCGAAG	27 nt	78 °C	80.7 °C
p14	RecJ3H596Ar	CCGACGACGAGGgcGCCGCCGCCGGAG	27 nt	82 °C	57.8 °C
p15	Pan1-K214 >TAGr	CGGAGCCGGCCATctaGATGAACGAGGCG	29 nt	75 °C	42.2 °C
p16	HvPrrnF	CGATGCCCTTAAGTACAACAGGGT	24 nt	64 °C	48.5 °C
p17	T7TerR	AACCCCTCAAGACCCGTTTAGAG	23 nt	64 °C	25.5 °C
p18	Pat1_Fwd	AACCTCGTCGACCGCGGCCGCAACTT	26 nt	76 °C	43.0 °C
p19	Pat1_Y168A_Rv	CGAGTTCGCGggcGACGTGGACG	23 nt	73 °C	47.2 °C

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p20	Pan-K214 >TAGr	CGGAGCCGGCCATctaGATGAACGAGGCG	29 nt	75 °C	41.7 °C

[a] Ap^r, ampicillin resistance; Nv^r, novobiocin resistance; Str^r, streptomycin resistance. $P2_{rrnA}$, rRNA promoter used for gene expression. Open reading frames targeted for mutagenesis: *hvo_0850* (Pan1, UniProt D4GUJ7); *hvo_2995* (Fdx, UniProt D4GY89); *hvo_1016* (JAMM2. UniProt D4GVJ3), and *hvo_1018* (RecJ3, UniProt D4GVJ5) were fused to an N-terminal histidine tag (*his6*-), while *hvo_1756* (Pat1, UniProt D4GS81) was fused to a C-terminal StrepII tag (-*strepII*). Primers p1 to p15 were used for SDM with the altered nucleotides written in lowercase characters. p16 and p17 primers were used for DNA sequencing. Primer and hairpin Tm's were calculated as described in methods. For annealing assay, primers p9, p20 and p11 were 6-carboxyfluorescein (6-FAM) labeled at the 5′-end.