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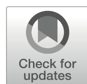

Site-Directed Mutagenesis to Mutate Multiple Residues in a Single Reaction

Saima Usman, Antesar Bushaala, Muy Teck Teh, and Ahmad Waseem

Abstract

Site-directed mutagenesis (SDM) is a technique that allows mutation of specific nucleotide(s) in a codon to study its functional implications in a protein. Commercial kits are available, which require high-performance liquid chromatography purified oligos for this purpose. These kits are expensive, and they are not very efficient, so one has to sequence several clones to get a desired one. We present here a simple method that requires only crude oligos, commercially available high-fidelity enzymes, and the success rate is close to 100%. In addition, up to 6 different mutations can be introduced in one reaction without causing any fortuitous change in the vector backbone. Using this strategy, we have introduced 32 S/T→A substitutions in the N-terminus head and 13 changes in the C-terminus tail domain of vimentin.

Key words DNA polymerase, High-fidelity enzymes, Molecular cloning, Nucleotide substitution, Polymerase chain reaction

1 Introduction

Site-directed mutagenesis (SDM) is a powerful technique that can be used to substitute, delete, or insert specific nucleotide(s) in a DNA sequence cloned in a plasmid. This technique can be used to study relative importance of particular amino acid(s) in structure-function relationship of a protein, to study disease mechanism, disrupt or map protein-protein interactions, mimic or block post-translation modifications, and silence an enzyme activity and mapping regulatory elements (to mutate promoters/enhancers in reporter constructs). Earlier approaches required rescuing single-stranded m13 DNA as a template which was labor intensive and technically challenging requiring several days of work [1–4]. The first protocol requiring double-stranded plasmid DNA for SDM required two primers, a mutagenesis and a selection primer, and both primers after annealing to the template were extended using T7 DNA polymerase [5]. This method was soon replaced by QuikChange SDM protocol from Strategene (now available from Agilent Technologies) requiring the use of overlapping forward and reverse primers containing the mutation and the high-fidelity *Pfu-Turbo* DNA polymerase which was soon replaced by QuikChange

II SDM protocol which employed *PfuUltra* high-fidelity (HF) DNA polymerase with 3′- to 5′-exonuclease activity that enables the polymerase to proofread nucleotide misincorporation errors. Although the QuikChange methods are popular, the use of overlapping primers reduces efficiency due to propensity of primer dimer formation over the template-primer complex formation. To overcome the primer dimer formation, two approaches were developed: in one non-overlapping or partially overlapping primers were employed [6], and in other two separate PCR with forward and reverse primers were carried out, *Dpn*I digested, mixed, denatured, and annealed [7]. Another QuikChange Multi-Site Directed Mutagenesis Kit from Agilent requires one or more primers harboring mutations anneal at the same time and extended by *PfuTurbo* DNA polymerase and the circle closed by Taq DNA ligase. *Dpn*I treatment produces single-stranded closed circular DNA that can be used for bacterial transformation [8]. Although the manufacturers of these kits claim 70–80% success in mutagenesis, the fact remains that efficiency rarely reaches beyond 25%. Therefore, one has to sequence a large number of clones to get a positive clone because one has to take into consideration fortuitous mutations introduced not only during PCR but also due to errors during oligo synthesis.

Here we describe a PCR-based protocol for introducing substitution, deletion, and insertion requiring back-to-back forward and reverse primers using two commercially available DNA polymerases Q5 and Phusion. There is no need to buy expensive kits. At the end of PCR, linear DNA molecules with blunt ends are produced, which can be phosphorylated and ligated in a single step. Using this method, up to 6 substitutions can be made in a single PCR, and in over 100 mutagenesis reactions we carried out, we rarely got 1–2 colonies in control PCR, whereas the PCR with primers had a lot of clones.

2 Materials

All solutions are prepared in ultrapure water (resistivity 18.18 MΩ-cm; referred as water from now on), which was sterilized by autoclaving.

1. Custom-made mutagenesis primers (Sigma Merck Ltd): Desalted with no further purification necessary.
2. Q5 High-Fidelity DNA polymerase 2 x master mix with HF buffer (Cat # M0492L; NEB).
3. Phusion high-fidelity DNA polymerase 2 x master mix with GC buffer (Cat # M0532L; NEB).
4. pBluescript II SK (+) plasmid: Commercially available from Agilent Technologies.

5. Veriti PCR Thermal cycler: Commercially available from Thermo Fisher Scientific.
6. Ligation buffer (10×): 500 mM Tris–HCl, pH 7.5 + 100 mM MgCl2. Make a stock solution of 2 M Tris–HCl buffer, pH 7.5. Dissolve 24.23 g of Tris Base in 60 mL of water, adjust the pH to 7.5 with concentrated HCl, and make up the volume to 100 mL. Make a stock solution of 1 M $MgCl_2$ in water. Dissolve 20.33 g $MgCl_2.6H_2O$ in 60 mL water and make up the volume to 100 mL. All solutions are mixed before use. Take 5 mL 2 M Tris–HCl, pH 7.5 and add 2 mL 1 M $MgCl_2.6H_2O$ and make up the volume to 20 mL. Mix thoroughly, filter sterilize, divide into 2 mL aliquots, and store frozen.
7. Adenosine 5′-tri phosphate (ATP) solution (10×): 20 mM ATP in water. Dissolve 110.23 mg ATP disodium hydrate (A6419-1G; Sigma-Aldrich) in 9 mL water and make up the total volume to 10 mL. Mix thoroughly, filter sterilize, divide into 1 mL aliquots, and store frozen.
8. DL-Dithiothreitol (DTT) solution (10×): Prepare a stock of 1 M in water. Dissolve 1.54 g DTT in 7 mL water and make up the total volume to 10 mL. Mix thoroughly, filter sterilize, divide into 1 mL aliquots, and store frozen. This is 1 M DTT solution. Dilute this solution 1:10 with water to make 0.1 M DTT and aliquot in 1 mL aliquots for ligation reactions.
9. T4 DNA ligase (400 U/μL) (Cat # M0202L; NEB).
10. T4 polynucleotide kinase (10 U/μL) (Cat # M0201L; NEB).
11. Dimethyl sulfoxide (DMSO): Cat # D8418 (Sigma-Aldrich).
12. TE buffer (10 mM Tris–HCl, pH 8.0 + 1 mM EDTA): 100 mM Tris–HCl buffer, pH 8.0 is first made by dissolving 1.21 g Tris Base in 80 mL water, the pH is adjust to 8.0 with concentrated HCl, and the volume is made up to 100 mL. 100 mM EDTA solution is made by dissolving 3.72 g of solid EDTA-disodium salt dihydrate (Cat # E6635; Sigma-Aldrich) in 80 mL water. Once dissolved the volume is made to 100 mL. To make TE buffer 10 mL, 100 mM Tris–HCl is mixed with 1 mL 100 mM EDTA solution and the volume is made to 90 mL. The pH is adjusted to 8.0 with dilute HCl and final volume is made to 100 mL filter sterilized, divided into 10 mL aliquot, and stored frozen.
13. NEB Stable Competent Cells: These are recombination deficient *E. coli* cells that are commercially available from NEB (Cat # C3040H).
14. Preparation of SOB medium: Tryptone (20 g), yeast extract (5 g), NaCl (0.584 g), KCl (0.186 g), $MgCl_2$ anhydrous (0.952 g), and $MgSO_4$ anhydrous (1.204 g) were dissolved in 1 L water and autoclaved.

15. Preparation of SOC medium: To 98 mL SOB, add 2 mL 20% (w/v) glucose, mix, filter sterilize, divide into 5 mL aliquots, and store frozen.
16. Luria-Bertani (LB) medium: Tryptone (50 g), yeast extract (25 g), NaCl (50 g) was dissolved in 5 liters of water, divided into 400 mL aliquots in Duran bottles, and sterilized by autoclaving.
17. Ampicillin solution: 1 g of ampicillin sodium salt powder (Cat # A0166; Sigma-Aldrich) is dissolved in 9 mL water, once dissolved the volume was adjusted to 10 mL and filter-sterilized. The solution is aliquoted and stored-frozen until required.
18. Ampicillin agar plates: 1.6 g of agar was suspended in 100 mL LB medium and melted in a microwave oven. It was allowed to cool to hand warm and ampicillin 1 mL (100 mg/mL) was added and the mixture was poured onto 10 cm plastic plates, allowed to solidify, and stored at 4 °C until required.
19. To make 5 M guanidine hydrochloride (Gu-HCL), weigh 47.8 g of pure guanidinium hydrochloride (Cat # G3272; Sigma-Aldrich) in a beaker and add 20 mL water and 30 mL isopropanol. Make up the volume to 100 mL with water.

3 Methods

1. Mutagenesis Strategy: We never use a cDNA construct in an expression vector directly for mutagenesis, partly because they are larger in size and also there is always possibility of fortuitous mutations in the expression vector backbone during the PCR affecting the expression, which can complicate the interpretation of the data. We always first sub-clone the cDNA into a cloning vector such as pBluescript II SK (+), which is a smaller vector (about 3 kb). This construct is subjected to mutagenesis by PCR, and after confirming the mutation by di-deoxy sequencing, the cDNA is then sub-cloned into a retroviral or lenti viral expression vector for functional analyses. This strategy prevents any fortuitous mutation being transferred into the expression vector backbone.
2. Designing the PCR primers: The forward and reverse primers each should be between 18 and 30 nucleotides long depending on the numbers of amino acids substituted. For 1–2 residues substitution, the primer length can be 18 bases, but for multiple residues, the primer length should be proportionately increased. In amino acid substitution, the codon used should make minimal change, for example, if the wildtype sequence contains “TCC” for a serine and suppose it needs to be

changed to an alanine so the "TCC" should be changed to "GCC" and not to "GCG" or "GCA" (although both code for alanine).

In a primer used for mutagenesis, the mis-match nucleotides should always be at the 5′ end. Suppose one is interested in creating just one substitution, in that case the nucleotide change can be on either forward or reverse primer. However, if two residues are to be substituted, one of mismatch can on the forward primer and the other on the reverse primers. We have successfully changed 6 amino acids in one PCR by using substitution in 3 codons in the forward primers and the same numbers in the reverse primers (*see* Fig. 1). Although we have not tried more changes, they can be introduced if the mutated codons are in close proximity.

GC content for the two primers should be in the range of 50–65% and Tm should be calculated using NEB site using the enzyme (Q5 or Phusion) to be used in the amplification.

3. The mutagenesis primers are dissolved in appropriate volume of TE buffer to 100 μM solution (*see* **Note 1**).
4. We make a primer mix by taking 10 μL forward and 10 μL reverse primer solutions (100 μM each) and diluting to 100 μL with TE (final primer concentration 10 μM each in the mixture).
5. For the PCR, we use 2 x master mix of high-fidelity DNA polymerase enzyme either Q5 or Phusion (both from New England Biolabs). Hot start version can be used, but we have found no difference in the quality of amplified products without the hot start.
6. The PCR mixtures must be set up on ice because both the Q5 and Phusion enzymes have proofreading activities, and if the reactions were set up at room temperature, there was a possibility that the proofreading activity might destroy the single-stranded primers (*see* **Note 2**).
7. *Use of Q5 enzyme with HF buffer:* Take two thin-walled PCR tubes, one control and the other reaction tubes on ice. Add in each 1 μL 30–50 ng cDNA construct in pBluescript II SK (+) and add 1 μL primer mix, 10 μL 2 x Q5 polymerase master mix, and 8 μL water to make it to 20 μL.
8. The control tube with Q5 enzyme and HF buffer will contain everything on ice in **step** 7 except the primers. 1 μL 30–50 ng cDNA construct, 10 μL 2 x Q5 polymerase master mix, and 9 μL water to make it to 20 μL.
9. With some DNA template and primer combinations, Q5 does not work well, and in those cases, we have found that 2 x Phusion enzyme master mixture can be substituted for Q5 (*see* **Note 2**).

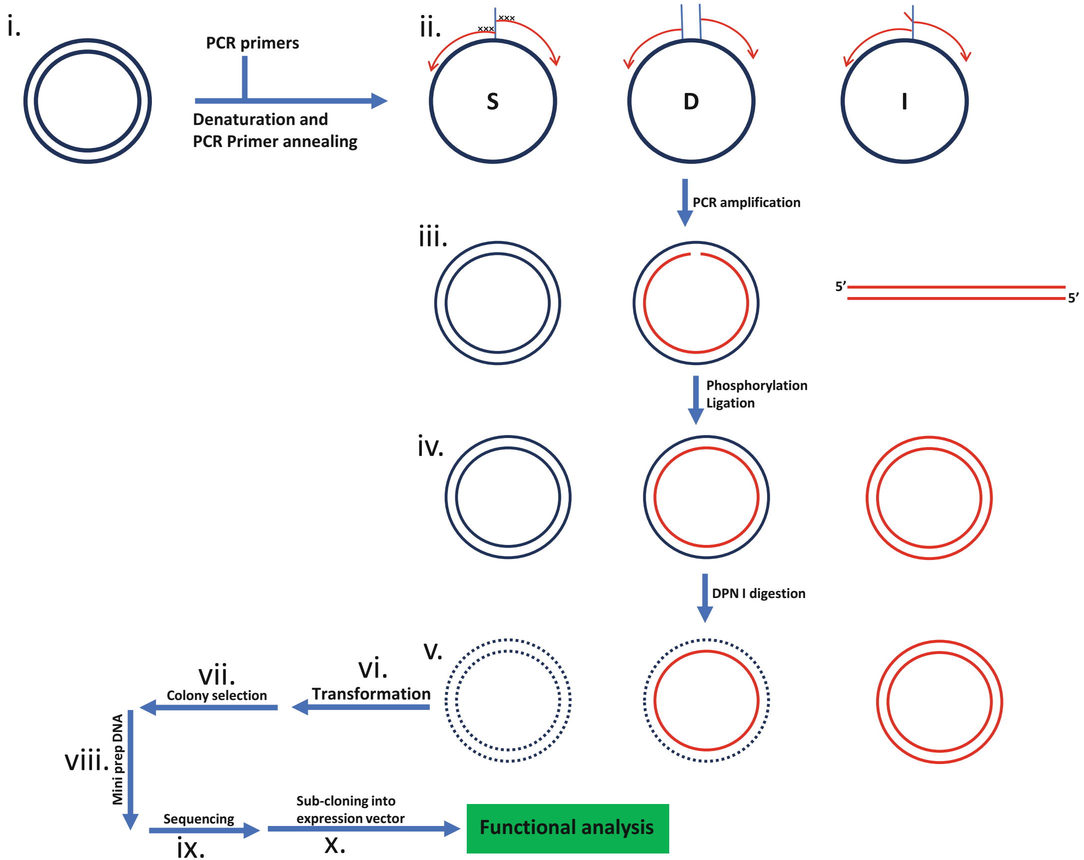

Fig. 1 Different steps in site-directed mutagenesis using back-to-back primers: (i) The circular double-stranded construct containing vimentin cDNA sub-cloned into pBluescript II SK (+). (ii) The construct is heat denatured during PCR and primers anneal to the single stranded circular DNA. The complex of single-stranded circular DNA if the primer is to be used for substitution (S) with three changes at the 5′ end of each primer shown by "x," deletion (D) or insertion (I). (iii) Irrespective of which type of mutation is being created, three kinds of PCR products will be generated. Original double-stranded circular DNA (shown in black lines), hybrid circular DNA containing an original strand (black) and a newly synthesized strand (red) with a nick, and a linear double-stranded DNA with blunt ends. (iv) Phosphorylation and ligation will create close circular hybrid DNA with one original and a newly synthesized DNA strand as well as circular DNA, which will have both newly synthesized strands. This mixture will also contain the original double-stranded circular DNA. All the original DNA strands will be methylated as they are synthesized inside *E.coli*. (v) Treatment with *Dpn*I will remove the methylated DNA leaving the PCR generated DNA intact. (vi) Bacterial transformation using this *Dpn*I digest will generate specific colonies, which will be absent in control PCR without primers. (vii) Colonies will be selected from agar plates containing ampicillin. (viii) The colonies are grown, and mini prep DNA extracted and purified. (ix) The mini prep DNA are subjected to di-deoxy sequencing using pBluescript forward and reverse primers to confirm that mutation has been created. (x) The mutagenized vimentin cDNA is excised and sub-cloned in a suitable expression vector for functional analysis to investigate the effect of mutation on a biological activity

10. *Use of Phusion enzyme with GC buffer:* Take two thin-walled PCR tubes: one control and the other reaction tubes on ice. Add in each 1 μL 30–50 ng cDNA construct in pBluescript II

SK (+) and add 1 μL primer mix, 10 μL 2 x Phusion polymerase master mix, 0.6 μL DMSO, and 7.4 μL water to make it to 20 μL.

11. The control tube with Phusion enzyme and GC buffer will contain everything on ice in **step 9** except the primers. 1 μL 30–50 ng cDNA construct, 10 μL 2 x Phusion polymerase master mix, 0.6 μL DMSO, 8.4 μL water to make it to 20 μL.
12. Run PCR: Denaturation 98 °C for 4 min followed by 20 cycles of denaturation at 98 °C for 10 s, annealing 70 °C for 10 s, extension at 72 °C 1 kb/min (5.5 min for 5.5 kb plasmid), and final extension at 72 °C for 4 min.
13. After the PCR has finished, add into each tube 100 μL a mixture of 5 M guanidine hydrochloride (Gu-HCL) in 30% isopropanol.
14. Apply the reaction and control samples onto Qiagen QIAquick Gel Extraction columns (Cat # 28704). Wash with 500 μL guanidine hydrochloride/isopropanol solution (*see* **step 13**).
15. The columns are washed twice with 750 μL wash solution made with 100 mL 80 mM NaCl +8 mM Tris–HCl diluted to 500 mL with pure ethanol (final concentration 16 mM NaCl, 1.6 mM Tris–HCl + 80% ethanol) (*see* **Note 3**).
16. After washing, the columns are dried by spinning the empty columns at full speed in a microcentrifuge for 1 min.
17. Twenty microliters of TE buffer is added at the center of each column, allowed to incubate for 2 min at room temperature, and spun at full speed in a microcentrifuge to elute the DNA.
18. The concentration of DNA in the reaction and control tubes is measured by a nanodrop spectrophotometer with a setting of DNA-50 (*see* **Note 4**).
19. In the next step, the DNA amplified in the control (minus primer) and reactions (plus primers), and the PCR tubes are phosphorylated and ligated at the same time (*see* **Note 5**).
20. From the control and reaction tubes, 1-2 μL of the PCR amplified DNA is transferred into fresh tubes, 1 μL of 10 x ligase buffer (500 mM Tris–HCl, 100 mM MgCl2), 1 μL 100 mM dithiothreitol (DTT), 1 μL 20 mM adenosine triphosphate (ATP), 1 μL 400 U/μL T4 DNA ligase, 1 μL T4 polynucleotide kinase (10 U/μL), and double-distilled water to 10 μL (*see* **Notes 6 and** 7).
21. The tubes are incubated at room temperature for 2–4 h and then heated at 65 °C for 20 min and cooled to 4 °C (*see* **Notes 8, 9** and **10**).
22. To both the control and reaction tubes is added 8 μL water, 1 μL 10 x rCutsmart buffer (NEB), and 1 μL *Dpn*I (20 U/μL).

23. Incubate at 37 °C for 2 h or for overnight (*see* **Note 11**).
24. The control and reaction mixtures are diluted 2 folds with water and can be stored frozen for later use.
25. Transformation: Fifty μL NEB stable competent *E. coli* cells are taken in 2 mL labelled microcentrifuge tubes, mixed gently with 1 μL ligation mixtures (**step 23**) in separate tubes, and incubated for 30 min. The tubes are heated at 42 °C for 30 s and immediately transferred on ice. SOC medium (500 μL) is also added, and tubes are incubated at 30 °C with shaking for one h. Four hundred μL of the cell suspension is gently plated on agar plates containing 100 μg/mL ampicillin and incubated overnight at 30 °C.
26. Mini prep: Comparison of the number of colonies in control and reaction plates will indicate the success of the mutagenesis reaction. More often than not, the control plates will have no colonies, whereas the reaction plates will have 15–20 colonies. Pick 4 colonies from each reaction plate and inoculates in 10 mL LB medium with 100 μg/mL ampicillin. Grow the clones overnight at 30 °C and next day make mini prep DNA using Qiagen QiAprep Spin Mini prep Kit (Cat # 27104). The DNA is eluted in 30 μL elution buffer.
27. *Sequencing:* All clones are sequenced by di-deoxy method through and through using forward and reverse sequencing primers derived from the Bluescript vector. The sequencing primers we have used successfully are as follows:

    pBluescript_Lab_F TTCCGGCTCGTATGTTGTGTGG
    pBluescript_Lab_R GATTAAGTTGGGTAACGCCAGG

    The sequencing is outsourced to Source Biosciences (UK) which are very efficient with turn around time of less than 24 h. The sequence of mutagenized clone is compared with the wildtype and if correct it is saved otherwise discarded. In our hands, this method of mutagenesis produces correct mutation in every clone in every attempt (*see* **Note 12**).
28. *Sub-cloning into an expression vector:* The mutagenized cDNA from the pBluescript vector is excised out and ligated into an expression vector such as pLPCpuro or pLPChygro in identical restriction sites, usually *Eco*RI and *Bam*HI [9, 10], and used for maxi DNA prep. These constructs can be used for protein expression by western blotting and immunostaining and can be employed for functional analysis.

4 Notes

1. We always use desalted primers from Sigma/Merck and without further purification. The primers should always be dissolved in TE and stored at −20 °C until used. Dissolving oligos in water is not recommended because in the absence of buffer, the pH is lowered and the phosphodiester bond can be hydrolyzed (*see* Subheading 3, **step 3**).
2. Although the reported error rate of both Q5 and Phusion is about 1 in one million bases polymerized [11, 12], in practice the rate is higher. The other source of error is the mis-incorporation of bases during oligonucleotide synthesis. It is because of these two reasons one should sequence at least 2 clones to get a correct one (*see* Subheading 3, **steps 6** and **9**).
3. Instead of using Tris/NaCl/Ethanol mixture, we have found that 20 mL of TE mixed with 80 mL pure ethanol also works well as wash buffer (*see* Subheading 3, **step 15**).
4. If the DNA concentration in column eluate is 3–10 folds higher in the reaction than in the control tubes, the PCR has worked, and it may indicate that mutagenesis reaction was also successful (*see* Subheading 3, **step 18**).
5. The phosphorylation and ligation can be done together because both enzymes T4 PNK and T4 ligase are active in the ligase buffer (*see* Subheading 3, **step 20**).
6. In ligation reaction with T4 ligase, the buffer available commercially tends to go off after few freeze-thaw cycles. We tried aliquoting the commercial 10 x ligase buffer, but it still does not work after few cycles (*see* Subheading 3, **step 20**).
7. We always make a 10 x ligase buffer containing 500 mM Tris–HCl and 100 mM MgCl2, pH 7.5, and 10 x ATP and 10 x DTT separately and mix them just before use. These solutions are stable and are not affected by repeated freezing and thawing producing reproducible results for several years (*see* Subheading 3, **step 20**).
8. Ligation time can also be reduced to less than 15 min at RT by adding 7.5% polyethylene glycol 6,000 in the ligation buffer. In this case, a 2× ligation buffer containing 15% (w/v) Polyethylene glycol 6,000 in 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ is made. Just before ligation, this solution is supplemented with 2 mM ATP and 10 mM DTT as described in **step 20** of method section.
9. Most researchers in the literature carry out ligation at 16 °C overnight and for that they need a water bath in a cold room. We have found that ligation can be achieved by incubating at

RT for 2–4 h. However, if it is 5 pm in the evening and time to go home, one can get perfect ligation by incubating the ligation mix overnight in a cold room at 4 °C.

10. Heating at 65 °C for 20 min inactivates both the T4 ligase and T4 kinase enzymes (*see* Subheading 3, **step 21**).
11. The enzyme *Dpn*I was originally reported to digest only the fully methylated DNA [13]. However, later studies have suggested that the enzyme digests the hemi-methylated DNA 60–100 times slower than the fully methylated DNA [14, 15]. It is for this reason we prefer to incubate the reaction mixture for longer time. However, the difference was very little, for example, 1 h incubation with *Dpn*I produced 1–2 colonies in the control plates vs no colonies when the enzyme mixture was incubated overnight (*see* Subheading 3, **step 26**).
12. We have however noticed fortuitous mutation in other part of the cDNA (perhaps due to mis-incorporation by the DNA polymerase) or in the mutagenesis primer due to errors during oligo synthesis (*see* Subheading 3, **step 27**).

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