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Site-directed *in vitro* mutagenesis: An improved protocol

Understanding functional domains of proteins remains the biggest challenge to proteomic studies[1]. Once cloned and expressed for functional assays, developing mutants for enhanced or superannuated activities is important for scientific as well as commercial interests[2]. This is achieved by site-directed mutagenesis experiments that can alter bases at precise positions on the gene sequence[3]. Existing protocols for introducing site-directed mutations *in vitro*, involve either T4/T7 polymerases[4,5] or thermostable polymerases. Thermophilic polymerases with high fidelity and robust amplification are the enzymes of choice for recent PCR-based mutagenesis protocols[6].

Well-standardized PCR-based protocols generally introduce two mutations, one desired on the gene (primary mutation) and the other somewhere on the vector (secondary mutation) for easy and suitable screening of mutants. Commonly introduced secondary mutations include repair/disruption of antibiotic resistance genes[7–9], introduction/elimination of unique restriction sites[10], deoxyuridine[11,12] or phosphorothioate incorporation[13,14], etc. False clones (clones positive for secondary mutation, but negative for primary mutation) are results of recombination following annealing of wild type, non-mutated upper or lower strands with mutated lower or upper strands respectively.

We suggest a modified megaprimer-based mutagenesis protocol that includes the following prominent features: (i) two mutations, one primary (desirable) mutation and one secondary mutation that introduces a *Not*I site on the mutagenesis vector for convenient screening of mutants, are introduced together; (ii) only the product of the first PCR using primers containing both primary and secondary mutations (megaprimer) is used for the second PCR; (iii) the megaprimer would certainly contain both primary and secondary mutations incorporated through two separate primers; (iv) the megaprimer is gel-purified ensuring that the second PCR is free from contaminating small primers and hybrid, nicked circular molecules having mutations on one strand and not on the other, and (v) for both first and second PCRs, the high fidelity XT–Taq PCR system (Bangalore Genei, India) was used that can successfully amplify DNA fragments up to 5 kb in size. The XT–Taq PCR system is a high-fidelity thermostable enzyme mix that is typically constituted of two DNA polymerases, one contributing to robust $5' \rightarrow 3'$ polymerase activity (Taq DNA polymerase) and the other having a high fidelity mismatch repair system with $3' \rightarrow 5'$ exonuclease activity (Pfu DNA polymerase).

Lower level of polymerase activity associated with Pfu DNA polymerase in combination with fewer cycles of amplification (as followed in Quick Change Protocol, Stratagene, USA) would result into significantly lower level of PCR products being generated. In contrast, higher level of PCR products would ensure: (i) higher proportion of mutants to non-mutants, (ii) PCR products can be visualized on agarose gels to confirm the successful second PCR reactions, and (iii) more colonies following transformation of the second PCR products. Typically, around 2 µg of nicked circular DNA are generated from each 50 µl of the second PCR reactions, and 70% of the transformants are mutants in general.

Ligation-free cloning protocols[15–18] ensure easy and fast generation of clones. The product of the second PCR is a circular DNA containing a single nick on each strand at both ends of the megaprimer. In our studies, we regularly achieved a transformation efficiency of 5×10^4 using nicked, circular second PCR products. The transformants are screened for mutants following an easy and convenient step of restriction digestion (*Not*I). No *Dpn*I-based digestion step is required that degrades wild-type methylated templates as in previously described but otherwise similar protocols. Incomplete *Dpn*I digestion of wild-type template DNA would result into significant percentage of false positive clones (circular template DNA molecules would get transformed more efficiently than nicked circular PCR products). This would necessitate sequencing of multiple transformants to detect mutants. Usage of XT–Taq PCR system, on the other hand, ensures more PCR products and higher number of transformants for simple restriction digestion-based screening of mutants.

As the megaprimer used for the second PCR has both mutations incorporated, the DNA strands generated in the second PCR reactions would typically have both mutations on each strand as well. Thus, the second PCR products would contain majority of double mutants, while only a small proportion of dsDNA molecules would have double mutations incorporated on single strands (heterodimers). Only 25% of the heterodimer population can generate false mutants (positive for secondary mutation and negative for primary mutation) upon host-mediated

mismatch repair system following transformation.

Hypothetically (see Table 1), if 2 µg of nicked circular DNA were generated in the second PCR using 10 ng of wild type circular DNA template, the probability of dsDNA having mutations on both strands (positive clones) would be approximately 99%, while the probability of dsDNA having mutations in either strands (heterodimers) and in no strands (negative mutants) would be around 1 and 0% respectively. Following host-mediated mismatch repair, only 0.25% (25% of 1%) of the secondary mutants can result into false clones and the rest (99.75%) would be double mutants (positive clones). Near 100% double mutants would mean that all transformants that are positive for introduction of secondary mutations (*Not*I site) would be positive for introduction of primary (desirable) mutations as well.

To test the hypothesis, two independent experiments were conducted where *Ssp*I restriction sites were introduced at either 1502nd position or 901st position (primary mutations) and a *Not*I site at 384th position (secondary mutation) on plasmid pUC18. No *Not*I sites and a single *Ssp*I site (at 2501st position) is present on pUC18. Thus, following the introduction of the *Ssp*I site at 1502nd position, two fragments would be generated of sizes 1 kb and 1.686 kb upon *Ssp*I digestion. Similarly, upon introduction of *Ssp*I site at 901st position and *Ssp*I digestion, there would be fragments of size 1.086 kb and 1.6 kb. Introduction of secondary mutation (*Not*I site) would, however, linearize both plasmids.

Primers were designed for introducing primary mutations, i.e. introduction of *Ssp*I sites at positions 1502 (Primer A) and 901 (Primer B), and secondary mutation, i.e. introduction of *Not*I site, at position 384 (Primer C) on pUC18. The primer sequences were as follows:

Primer A: 5′ ctt aac gtg aAt Att cgt tcc ac 3′

Primer B: 5′ tga gcg tcg aAt Att gtg atg ct 3′

Primer C: 5′ acg ttg taa aGc gGc Cgc cag tgc caa gct 3′

(Mutated bases are shown in capital letters.)

Normally, bases are substituted retaining a minimum of 10 perfectly matching bases on both ends (5′- and 3′-) of mutation to ensure annealing of mutated primers to the template. The first PCR amplifications were conducted using Primers A and C as well as Primers B and C following standard PCR cycle conditions. Thirty cycles of PCR using 1.5 units of XT–Taq PCR enzyme system generated enough first PCR products (of size 1142 bp and 541 bp respectively) that were electro-eluted and utilized for the second PCR. Two-fold molar excess of the megaprimer over the template should be provided for the second PCR reaction.

For our experiments, the amount of first PCR product to be used as megaprimer in the second PCR amplification was 850 ng and 400 ng respectively. The second PCR reactions were conducted for 30 cycles following standard cycle conditions, using 10 ng of pUC18 DNA as template and 1.5 units of XT–Taq PCR system. Next, 5 µl of the second PCR products was loaded on 1% agarose gel to confirm successful amplifications, while one µl each was transformed into competent *E. coli* cells (strain DH5α).

Table 1. Total population of single stranded (ss) DNA following second PCR using megaprimer that contains both mutations and wild-type template that contains no mutations would consist of (i) ssDNA with both mutations (*A*) and (ii) ssDNA with both mutations (*B*)

Total dsDNA population can be mathematically represented as follows:

$$\text{Total population (100\%)} = \frac{A^2 + B^2 + 2AB}{(A+B)^2} \times 100.$$

Formula for calculating the frequencies of different clone populations:

$$\text{percentage of double-positive mutants} = \frac{A^2}{(A+B)^2} \times 100;$$

$$\text{percentage of double-negative mutants} = \frac{B^2}{(A+B)^2} \times 100;$$

$$\text{percentage of heterodimers} = \frac{2AB}{(A+B)^2} \times 100.$$

For 10 ng template taken and 2 µg of products generated following the second PCR step:
ssDNA with both mutations (*A*) = 2000 ng and ssDNA with no mutations (*B*) = 10 ng
Thus, there would be ~ 99% double-positive mutants, ~ 0% double-negative mutants and ~ 1% heterodimers.

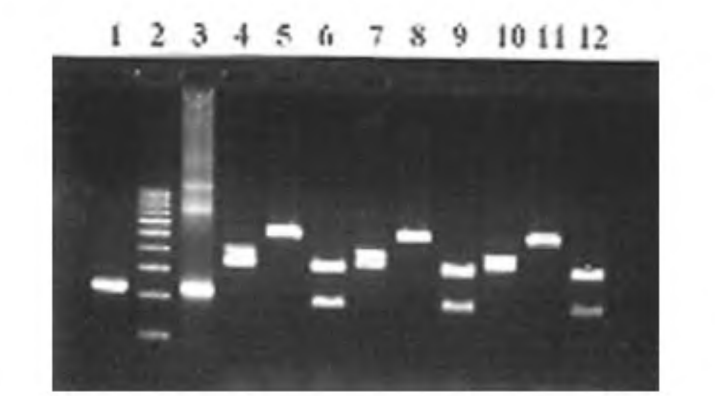

Figure 1. Screening for clones having mutations at positions 384 (*Not*I) and 1502 (*Ssp*I) in pUC18 introduced by modified PCR-based, site-directed mutagenesis protocol. While lanes 1 and 3 showed the products of the first PCR (1142 bp) and second PCR (nicked circular DNA at ~ 3.5 kb) respectively, lanes 4 to 12 contained three different undigested or restriction digested clone DNA samples run on a 1% agarose gel. Uncut plasmid DNA was loaded on lanes 4, 7 and 10, while lanes 5, 8 and 11 contained *Not*I digested samples and lanes 6, 9 and 12 contained *Ssp*I digested samples. Positive clones (mutants) were linearized by *Not*I (2686 bp) and *Ssp*I digestion-released fragments of 1 kb and 1686 bp. 500 bp DNA ladder was loaded on lane 2.

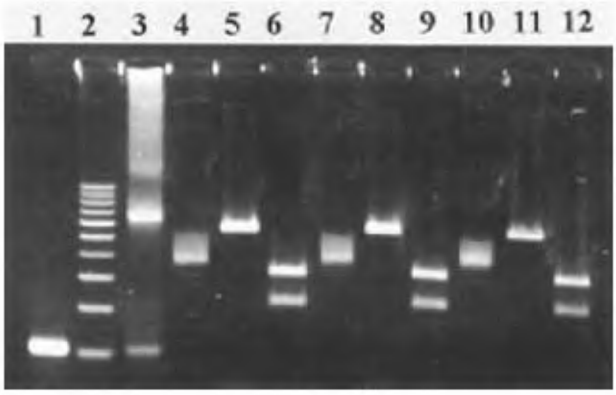

Figure 2. Screening for clones having mutations at positions 384 (*Not*I) and 901 (*Ssp*I) in pUC18 introduced by modified PCR-based, site-directed mutagenesis protocol. While lanes 1 and 3 showed the products of the first PCR (541 bp) and second PCR (nicked circular DNA at ~ 3.5 kb) respectively, lanes 4 to 12 contained three different undigested or restriction digested clone DNA samples run on a 1% agarose gel. Uncut plasmid DNA was loaded on lanes 4, 7 and 10, while lanes 5, 8 and 11 contained *Not*I digested samples and lanes 6, 9 and 12 contained *Ssp*I digested DNA samples. Positive clones (mutants) were linearized by *Not*I (2686 bp) and *Ssp*I digestion-released fragments of 1086 bp and 1600 bp. 500 bp DNA ladder was loaded on lane 2.

For both experiments, plasmid DNA was isolated from individual clones and screened for introduction of secondary mutations (positive for *Not*I digestion). All *Not*I positive clones were further digested with *Ssp*I to screen for introduction of primary mutations. Multiple *Not*I positive clones from individual experiments were screened for introduction of *Ssp*I site at 1502nd and 901st position respectively (Figures 1 and 2 respectively). They all were found to be mutants following restriction digestions (plasmids generated 1.686 kb and 1 kb for *Ssp*I mutants at 1502nd position, and 1.6 kb and 1.086 kb for *Ssp*I mutants at 901st position upon *Ssp*I digestion). We confirmed the statistical data from multiple experiments that were conducted for introduction of mutations at various sites of the plasmids and screened several hundred clones for mutants (data not shown). Multiple mutants were subsequently sequenced to confirm introduction of desirable primary mutations in clones, which were positive for secondary mutations.

We have developed and tested an approach for introducing site-directed mutations on DNA fragments/clones, where mutants can be predicted at a high level of confidence. Clones positive for secondary mutation (*Not*I) would contain the primary mutation with > 99% probability. The whole procedure starting from the first PCR to screening for mutants could be successfully completed in three days.

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