CSIR NET Life Science Unit 13

On Site-directed Mutagenesis

Site-Directed Mutagenesis

This method is used to create specific, targeted changes in double-stranded plasmid DNA. Many reasons are needed to make specific alterations in DNA like insertions, deletions, and substitutions, the main objective of site-directed mutagenesis is:

- How DNA manipulation would affect protein activity?
- Which mutations (at the DNA, RNA, or protein level) have resulted in the desired property?
- This technique is also used to introduce or remove restriction endonuclease sites or tags. It is an in vitro method for creating a specific mutation in a known sequence. Several PCR-based methods are being used for site-directed mutagenesis such as primers designed with mutations that can introduce small sequence changes, and primer extension or inverse PCR can be used to achieve longer mutant regions. Site-directed mutagenesis techniques allow for observing the impact of sequence changes and it also helps to screen a variety of mutants to determine the optimal sequence for a specific query.

Non-PCR based Techniques

Mentor Guru

- Cassette Mutagenesis

Site-directed mutagenesis includes two classes of methods that use double-stranded DNA cassettes and those that use single-stranded oligonucleotide primers. These techniques can give high yields of the desired mutations. Through the synthesis of chemical DNA, defined oligonucleotides up to ~100 bases can be prepared reproducibly and inexpensively. These synthetic oligonucleotides are used for site-directed mutagenesis, they work as primers for DNA polymerase and as oligonucleotide cassettes. In cassette mutagenesis, a cassette known as a synthetic double-stranded oligonucleotide contains the desired mutations docked between two restriction enzyme sites on a plasmid vector. The ends of the oligonucleotide duplex are complementary to the restriction cleavage sites so the cassette can be easily ligated into the plasmid.

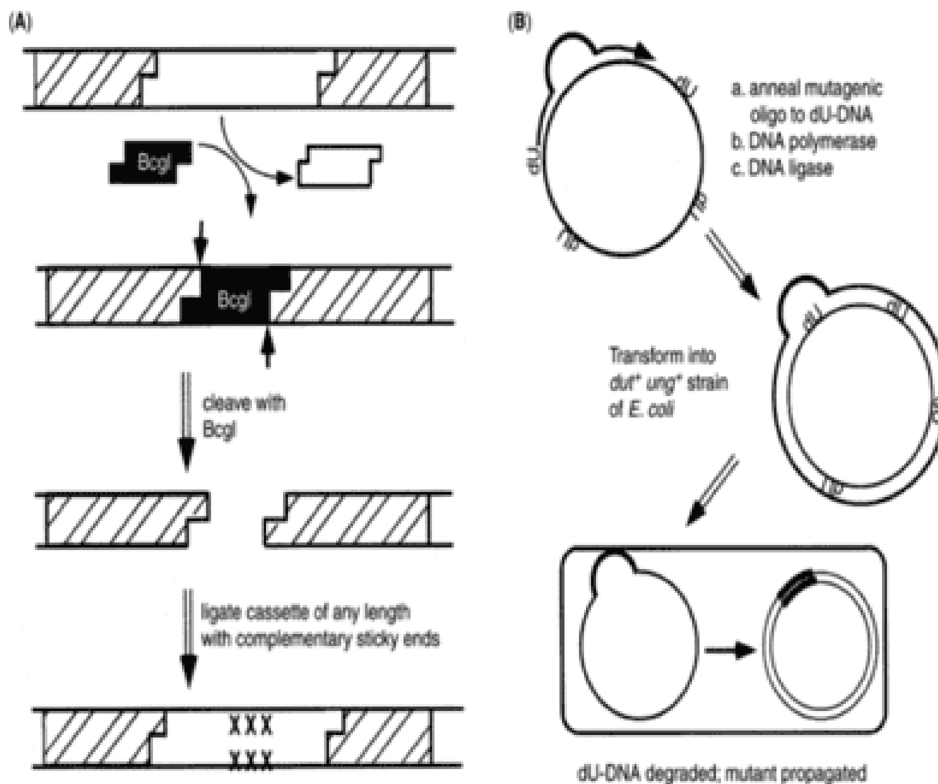

Figure 1. Cassette mutagenesis

- **Primer Extension Mutagenesis**

Mentor Guru

Primer extension methods are one of the early methods of mutagenesis which used uracil-containing circular single-stranded DNA (ss(U)DNA) template. In this method, No phenotypic selection was needed because the nascent synthesized mutant strand contained no uracil so it was thus favoured in bacterial propagation resulting in reported 50% mutagenesis efficiency. This method, also known as Kunkel mutagenesis, was later this method modified to be suitable also for double-stranded (ds) DNA. As a modification of additional nitrocellulose filtering steps for alkali denatured template ds(U)DNA, a second oligo is utilized to destroy a unique restriction enzyme site for template sequence removal and by heteroduplex ds(U)DNA is digested with DpnI, which removes methylated DNA template. Primer extension in site-directed mutagenesis involves incorporating mutagenic primers in independent, nested PCRs before combining them in the final product. The reaction needs flanking primers (A and D) which are complementary to the ends of the target sequence, and two internal primers with complementary ends (B and C) are also required. These internal primers have the desired mutation and will hybridize to the region to be altered. In the

first round of PCR, the AB and CD fragments are created. The products of first-round PCR are mixed for the second round of PCR using primers A and D. in this second PCR The complementary ends of the products hybridize and create the final product, AD, which contains the mutated internal sequence. To create a deletion, the internal primers, B and C, are positioned at either side of the region to be deleted to prevent them from being incorporated within fragments AB and CD from the first round of PCR. The complementary sequences at the ends of these fragments, created by primers B and C, enable the hybridization of AB to CD during the second round of PCR, and the final product with the desired deletion (AD) is created.

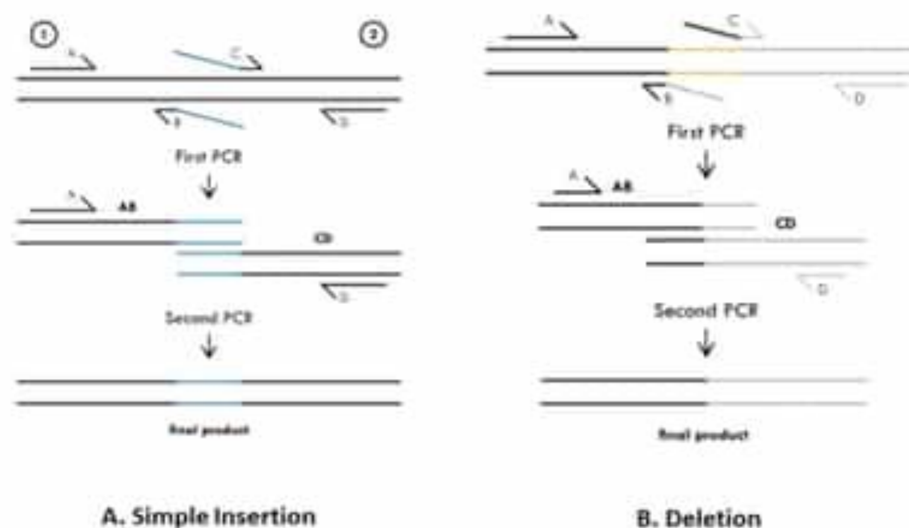

Figure 2. Primer Extension Mutagenesis

PCR based techniques

Overlap extension is a new approach to genetic engineering. In this method, complementary oligodeoxyribonucleotide (oligo) primers and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These overlapping fragments are combined in a subsequent 'fusion' reaction and the overlapping ends are annealed. The fusion product is amplified further by PCR. Specific alterations in the nucleotide (nt) sequence can be introduced by incorporating nucleotide changes into the overlapping oligo primers. this technique of site-directed mutagenesis, is user-generated, to three variants of a mouse major histocompatibility complex class-I gene have been cloned. And other medical practices.

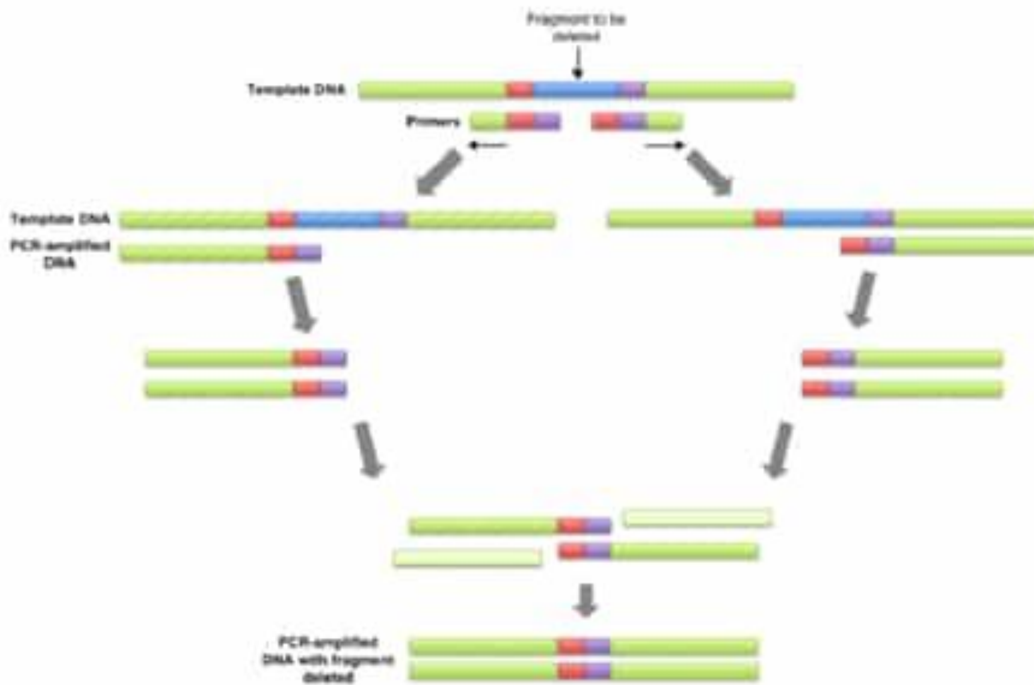

Figure 3. Overlap extension PCR Mutagenesis

Mega primer PCR

Various mutagenesis procedures based on polymerase chain reaction (PCR), the "mega primer" method appears to be the simplest and most versatile. In This method three oligonucleotide primers utilize and two rounds of PCR are performed on a DNA template that contains the cloned gene to be mutated. In this process "flanking" primers that can map either within the cloned gene or outside the gene and internal "mutant" primer have the desired base change. The mutagenic primer may encode any of the following mutations or combinations thereof: single nucleotide or point mutation change, a deletion, or an insertion. The first round of PCR is performed by using a mutant primer and one of the flanking primers. After that double-stranded product (A-M1) is purified and used as one of the primers that's why it is known as a "mega primer". The second round of PCR was performed with the other flanking primer (B). The wild-type cloned gene is used as a template in both PCR reactions. The final PCR product (A-M1-B) contains the mutation that can be used in a variety of standard applications.

Mentor Guru

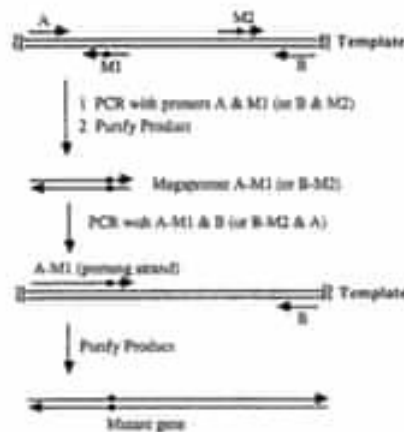

Figure 4. The mega primer PCR method of site-directed mutagenesis. Primers A, B, M1, and M2 (as well as the priming strand of the mega primer, AM1) are indicated by single lines with arrowhead, whereas the double lines represent the template. The dots in M1 and M2 denote the desired mutations (substitution, insertion, or deletion) to be introduced into the product via the mega primer.

- **Inverse PCR, and random mutagenesis**

In inverse PCR mutagenesis, amplification of a region of unknown sequence is done by using primers oriented in the reverse direction. This method can be used to introduce mutations in previously cloned sequences. By using primers for the desired change, an entire circular plasmid is amplified to delete, change, or insert the desired sequence

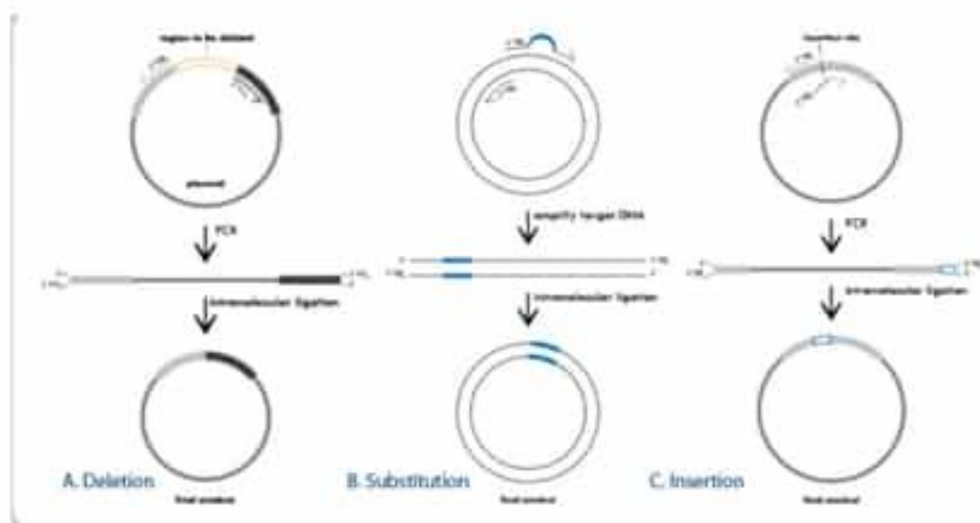

Figure 5. Site-directed mutagenesis by inverse PCR

- **Random Mutagenesis**

Random mutagenesis is a powerful tool in genetic engineering. It is used in generating enzymes, proteins, entire metabolic pathways, or even entire genomes with desired or improved properties. In this technology, genes evolve in vitro through an iterative process consisting of recombinant generation. And the reaction is Coupled with the development of powerful high-throughput screening or selection methods, in this method of treating DNA or whole bacteria with various chemical mutagens, cloned genes pass through mutated strains, by "error-prone" PCR mutagenesis, rolling circle error-prone PCR, and saturation mutagenesis also used.

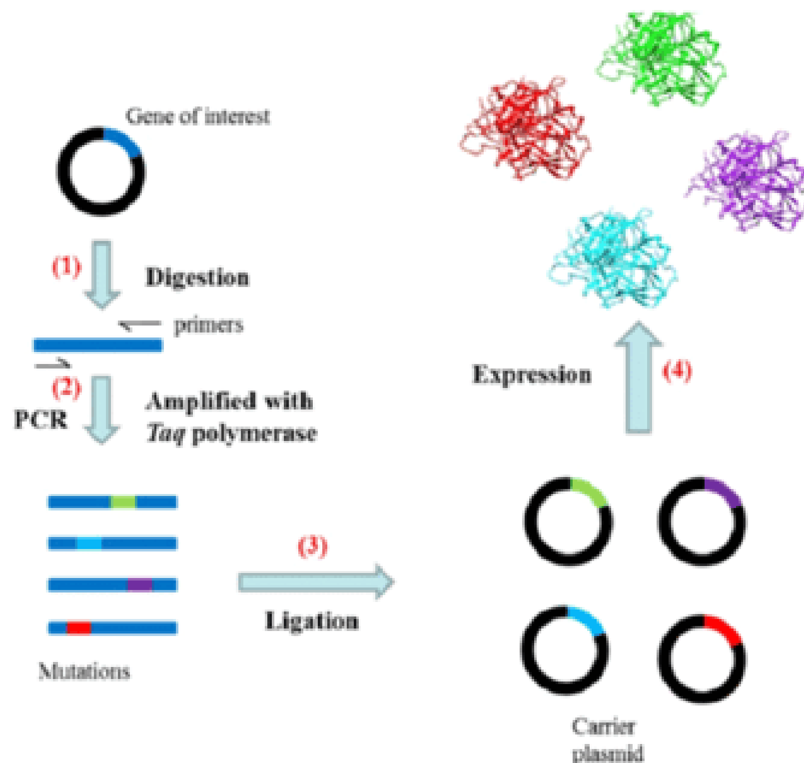

Figure 6. Random Mutagenesis