

PROTEIN ENGINEERING

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Protein engineering is the process of developing useful or valuable proteins.

Protein Engineering is a second generation of recombinant DNA technology.

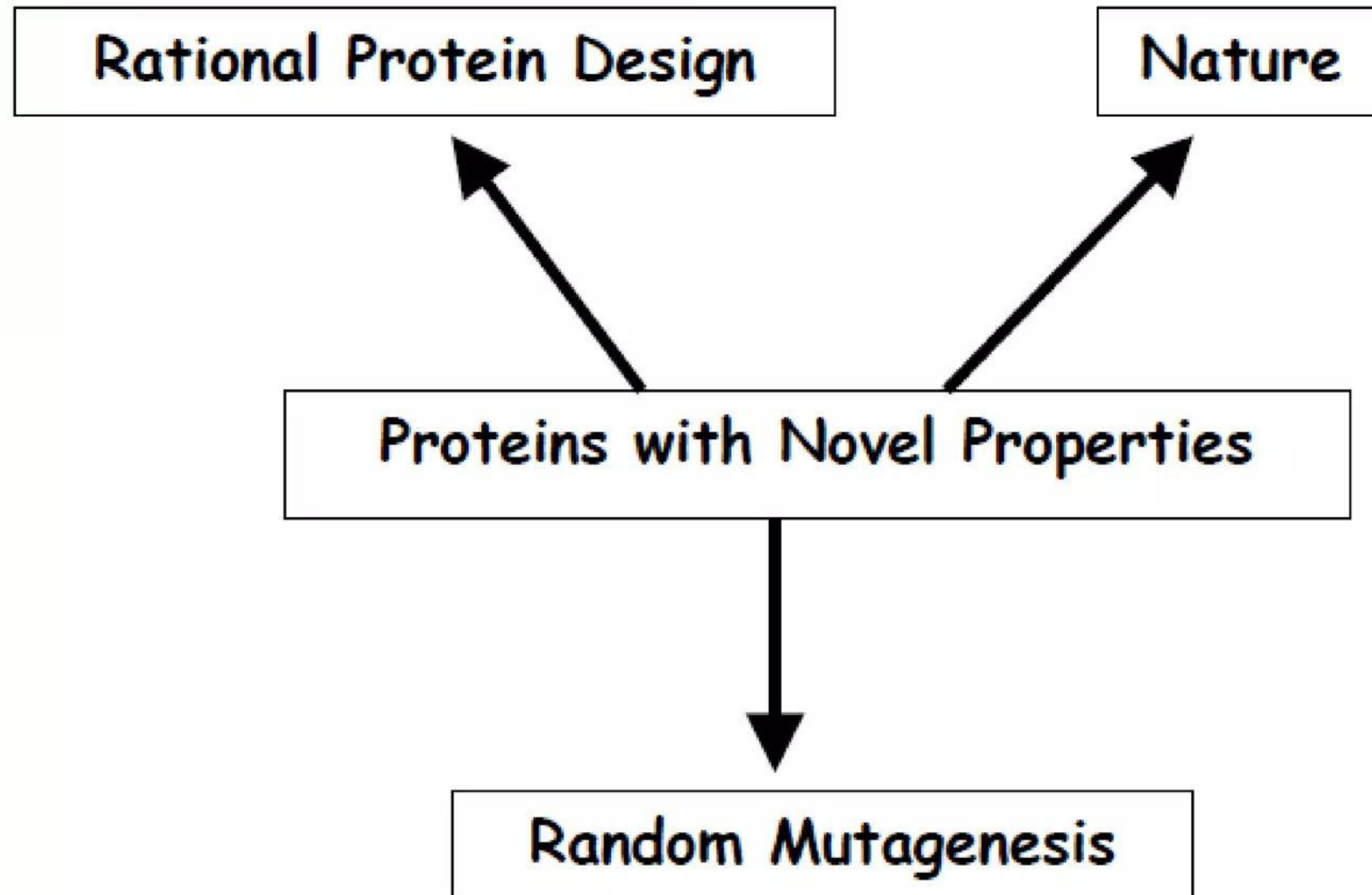
It involves altering cloned DNA in vitro by novel mutational technique so that translated proteins have slightly altered properties.

PROTEIN ENGINEERING

Protein engineering is merging of several disciplines like molecular biology, protein chemistry, enzymology, structural chemistry to alter catalytic or structural stability of protein, enzyme. With the advances in genetic engineering, genes can be isolated from organism and used for the synthesis of naturally occurring proteins. Some of these serve as enzymes.

PROTEIN ENGINEERING

Obtain a protein with improved or new properties



PROTEIN ENGINEERING

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- Increasing substrate affinity to enzyme

2

- Makes the enzyme thermal tolerant (active at high temp) and P^H stable.

3

- Enhances the substrate specificity by modifying the substrate binding site of the enzyme.

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- Designing the enzyme to make it resistant to proteolytic degradation.

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- Synthesizing enzyme that is stable and active in non-aqueous solvents.

PROTEIN ENGINEERING

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- Changing the enzyme in order to make it independent of cofactor for its function.

7

- Improving the stability of the enzyme to heavy metals.

8

- Fusing the enzymes needed in the reactions to give a final product.

9

- Produce hybrid enzymes.

10

- Make isolation and purification of enzymes simpler.

PROTEIN ENGINEERING

TECHNIQUES

Techniques used for protein engineering fall in two basic categories

(1) Genetic modifications:

- (i) Site directed mutagenesis
- (ii) Localized random mutagenesis

(2) Chemical modifications:

- (i) Change in functional group on side chain
- (ii) Modification & replacement of original protein

SITE DIRECTED MUTAGENESIS

MUTATION

- In molecular biology and genetics, mutations are accidental changes in a genomic sequence of DNA.
- Mutations can involve large sections of DNA becoming duplicated, usually through genetic recombination.
- Mutation is defined as a change in the nucleic sequence (bases) of an organism's genetic material (a change in the genetic material of an organism).
- Site directed mutagenesis may be define as a change in the nucleic acid sequence (or genetic material) of an organism at a specific predetermined location.

SITE DIRECTED MUTAGENESIS

- Site-directed mutagenesis is the technique for generating amino acid coding changes in the DNA (gene). By this approach specific (site-directed) change (mutagenesis) can be made in the base (or bases) of the gene to produce a desired enzyme.
- A large amount of experimental procedures have been developed for directed mutagenesis of cloned genes.
- A synthetic oligonucleotide complimentary to the area of the gene of interest but has the desired nucleotide change.
- An oligonucleotide is a short piece of DNA usually 10-30 nucleotide long.
- Directed mutagenesis can be done using:
 - M13 Plasmid DNA, PCR, Random primers, Degenerate primers, Nucleotide analogs

SITE DIRECTED MUTAGENESIS

□ THE SINGLE PRIMER METHOD

- In the technique of oligonucleotide-directed mutagenesis, the primer is a chemically synthesized oligonucleotide (7-20 nucleotides long).
- It is complementary to a position of a gene around the site to be mutated. But it contains mismatch of or the base to be mutated.
- The starting material is a single-stranded DNA (to be mutated) carried in an M_{13} phage vector.
- On mixing this DNA with primer, the oligonucleotide hybridizes with the complementary sequences, except at the point of mismatched nucleotide.
- Hybridization (despite a single base mismatch) is possible by mixing at low temperature with excess of primer, and in the presence of high salt concentration

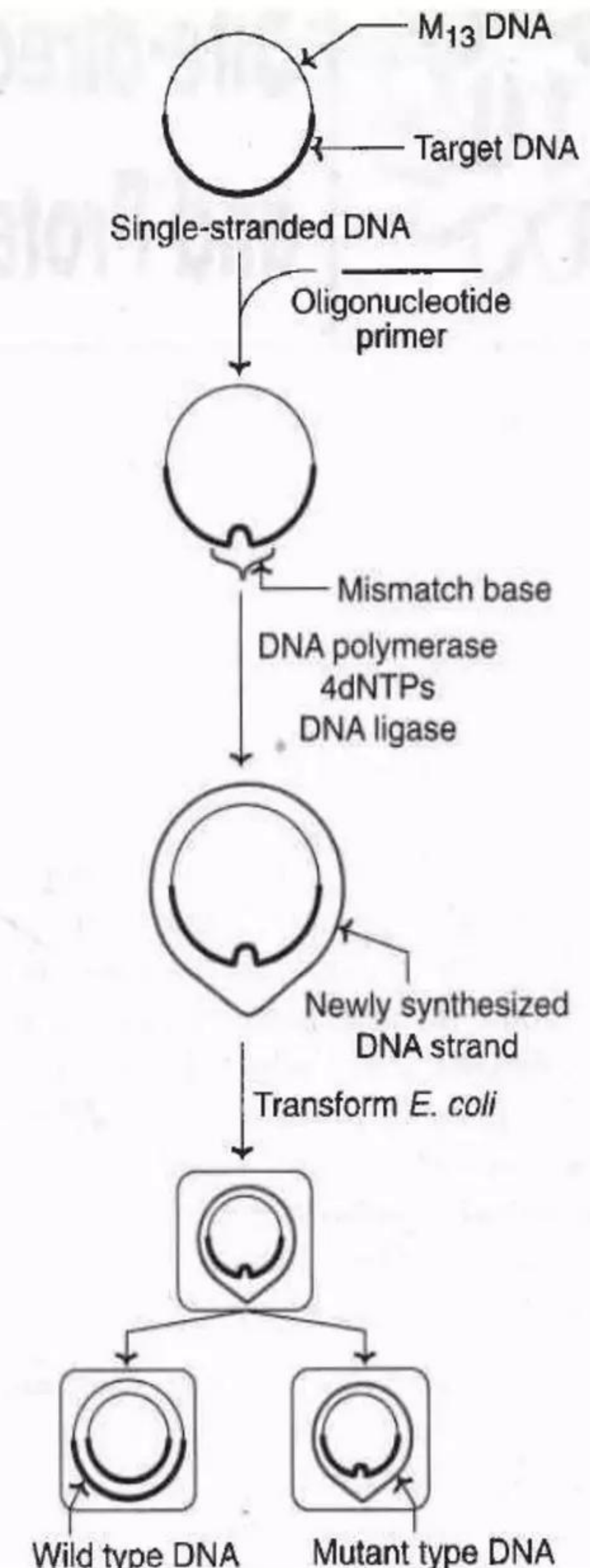


Fig-2: Single Primer Method

SITE DIRECTED MUTAGENESIS

- ❑ The addition of 4-deoxyribonucleoside triphosphates and DNA polymerase(usually klenow fragment of *E.Coli* DNA polymerase) replication occur.
- ❑ The oligonucleotide primer is extended to form a complementary strand of the DNA.
- ❑ The ends of the newly synthesized DNA are sealed by the enzyme DNA ligase.
- ❑ The double-stranded DNA (i.e. M phage molecule) containing the mismatched introduced by nucleotide into *E.coli* transformation .
- ❑ The infected *E. Coli* cells produce M₁₃ virus particles containing either the original wild type sequence or the mutant sequence.
- ❑ It is expected that half of the phage M₁₃ particles should carry wild type sequence while the other half mutant sequence (since the DNA replicate semiconservatively).
- ❑ The double-stranded DNAs of M₁₃ are isolated.
- ❑ Oligonucleotide –directed mutagenesis by using plasmid DNA (instead of M₁₃) is also in use.

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SITE DIRECTED MUTAGENESIS

- There are some variations in use in the oligonucleotide directed mutagenesis.

A) Multiple point mutagenesis

B) Insertion mutagenesis

C) Deletion mutagenesis

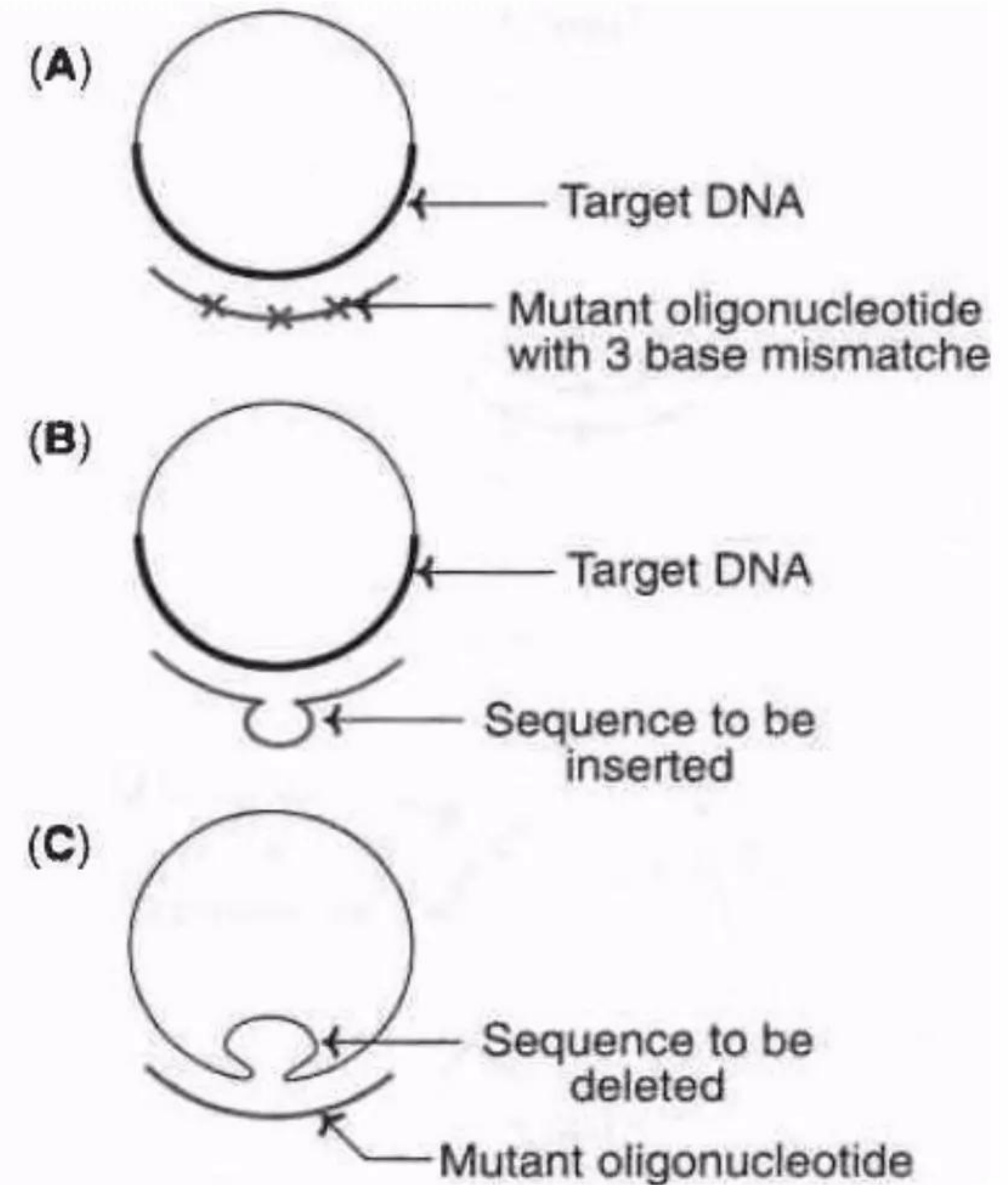


Fig.No.3- Variations in oligonucleotide-directed mutagenesis

SITE DIRECTED MUTAGENESIS

□ CASSETTEE MUTAGENESIS

- In cassette mutagenesis a, synthetic double stranded oligonucleotide (a small DNA fragment i.e., cassette) containing the requisite/desired mutant sequence is used.
- Cassette mutagenesis is possible if the fragment of the gene to be mutated lies between two restriction enzyme cleavage sites.
- This intervening sequence can be cut and replaced by the synthetic Oligonucleotide (with mutation).
- The plasmid DNA is cut with restriction enzymes (such as EcoR1 and Hind111).

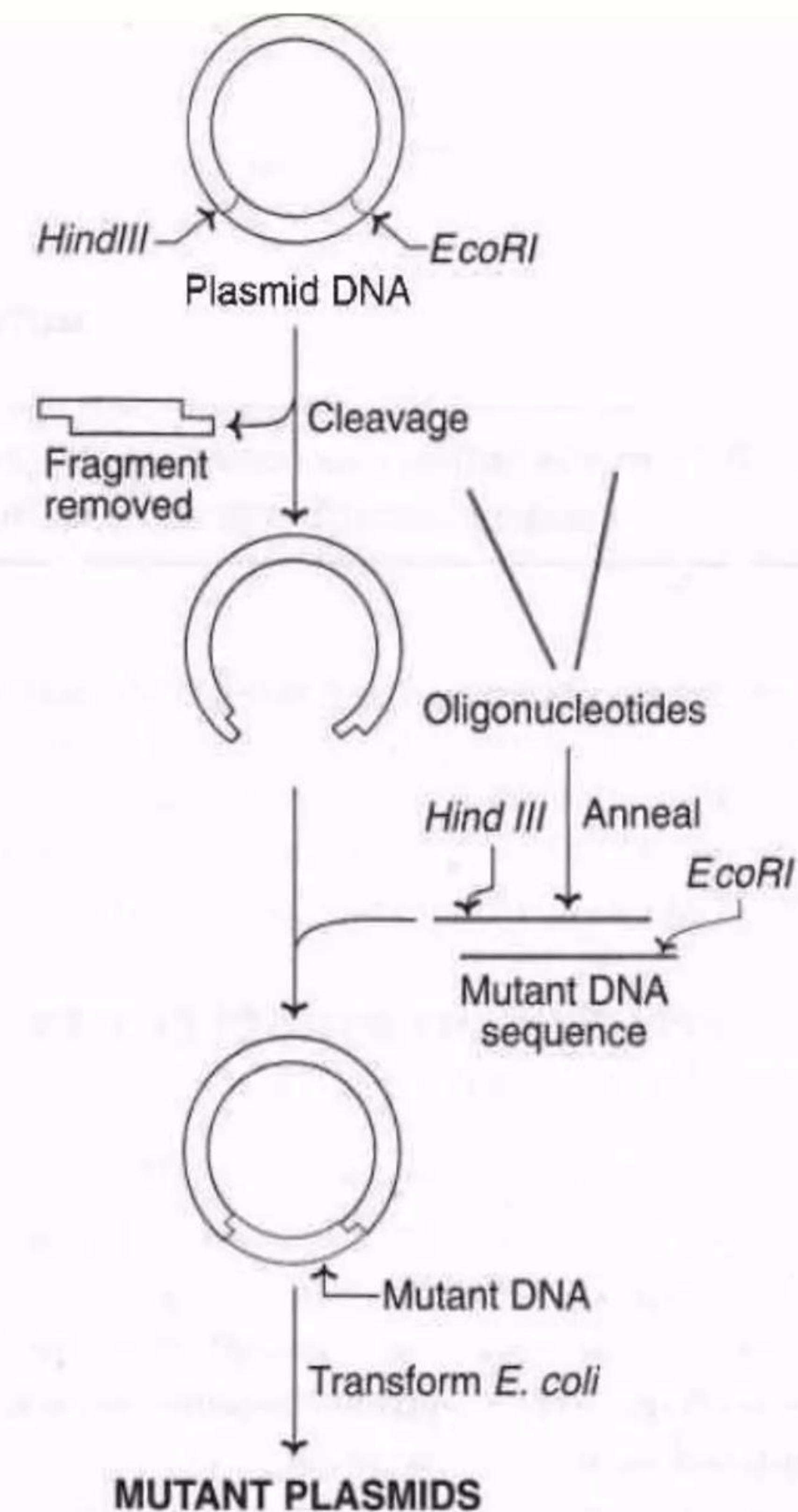


Fig.No.4- Cassette mutagenesis

SITE DIRECTED MUTAGENESIS

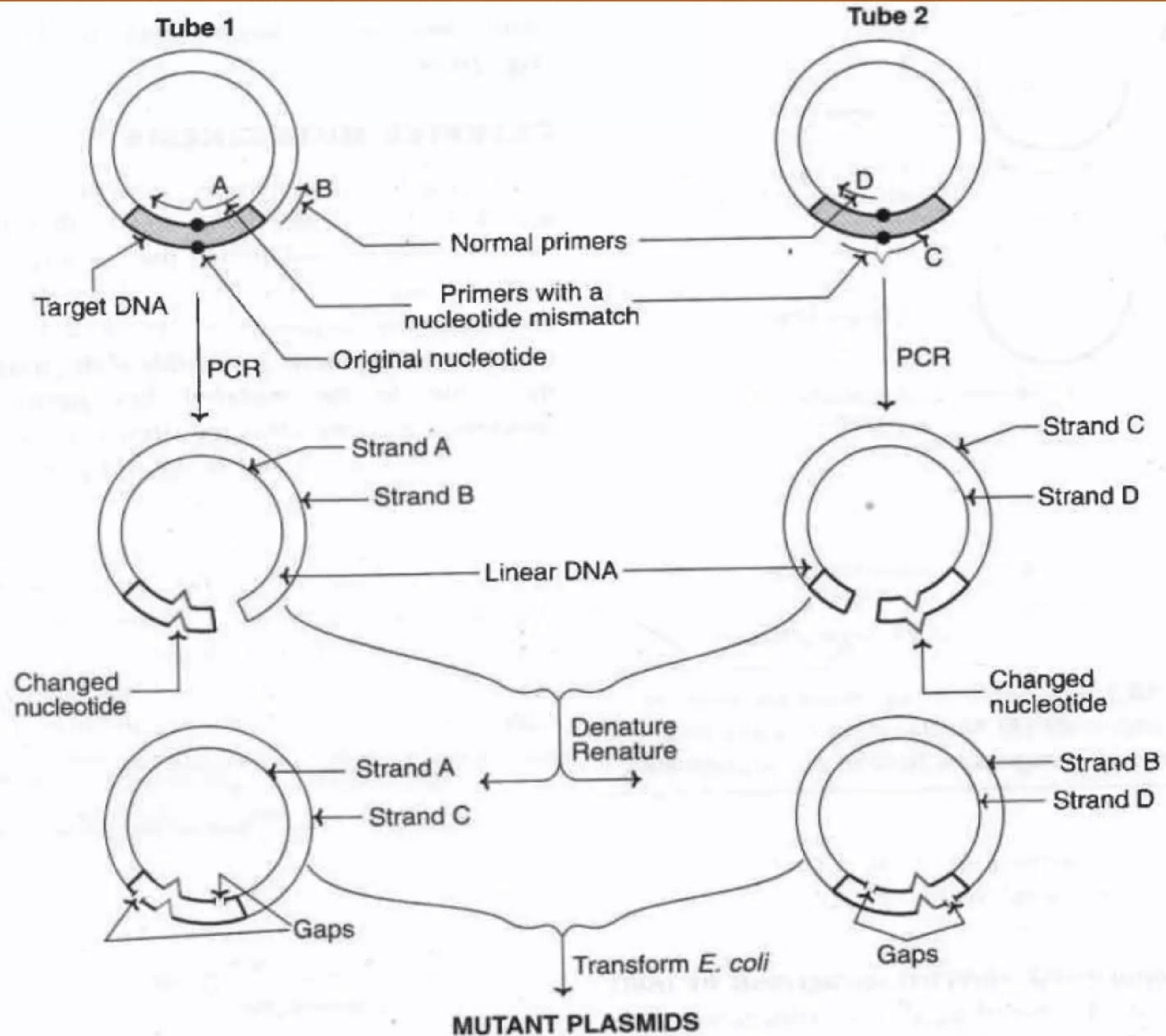


Fig.No.5- PCR-based mutagenesis

SITE DIRECTED MUTAGENESIS

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MUTATIONS

- The PCR-based mutagenesis technique commonly employed is depicted in First the target DNA (gene) is cloned on to a plasmid vector and distributed in to two reaction tubes.
- To each tube are added two primers (oligonucleotides synthesized by using PCR).
- One primer (A in tube1 and C in tube 2) is complementary to a region in one strand of the cloned gene except for one nucleotide mismatch (i.e. the one targeted for a change).
- The other primer (B in tube 1 and D in tube2) is fully complementary of a sequence in the Other strand with in or adjacent to the cloned gene.
- The placement of primers for hybridization (with the DNA strands) in each tube is done in opposite direction.
- The PCR technique is carried out for amplification of the DNA molecule.
- The products of PCR in the two reaction tubes are mixed.
- The DNA molecules undergo denaturation and renaturation.
- A Strand from one reaction tube (strand A) hybridizes with its complementary strand from other reaction tube (strand C).

CHEMICAL MODIFICATIONS

1. Functional group on side chain of natural enzyme may be changed
2. Total chemical synthesis of an enzyme is only a theoretical possibility. Practically it is expensive and knowledge also is still insufficient.

Advantages:

1. Attachment of coenzyme to enzyme is possible.
2. It can be applied in conjunction with genetic methods.

Disadvantages:

1. It is applicable to amino acids that have reactive side chains and only certain alterations can be made
2. Only amino acids at surface of protein are available for modifications.
3. Little specificity. Modification of single amino acid is difficult.

Investigative tools - specific mutations in DNA allow the function and properties of a DNA sequence or a protein to be investigated in a rational approach.

- **Commercial applications** - proteins may be engineered to produce proteins that are tailored for a specific application.
- Example, commonly-used laundry detergents may contain subtilisin whose wild-type form has a methionine that can be oxidized by bleach, inactivating the protein in the process.
- This methionine may be replaced by alanine, thereby making the protein active in the presence of bleach.

Increasing the stability and Biological activity of proteins

Addition of Disulfide bonds: increases thermostability of enzymes

T4 Lysozyme: By changing 2, 4, 6 aminoacids to cysteine residues and forming 1, 2, 3, disulfide bonds makes it very stable with good biological activity.

Xylanase: It is catalytically active at high temperature. Introduction of disulfide bonds at 1, 2, 3 makes it thermostable and substantially improves its functional efficiency.



Changing Asparagine to other Amino acids: At high temperature, amino acids asparagine and glutamine undergoes deamidation and form formic acid and glutamic acid – loss of biological activity.

Reducing the free sulfhydryl groups: Sulfhydryl groups reduces protein activity. The protein stability and its activity can be increased by reducing the number of sulfhydryl groups.

Human β -interferon: It is produced by genetic engineering & exist as dimer and oligomers which are inactive. This is due to formation of cysteine. Replacing cysteine with serine reduces free sulfhydryl groups and makes Human β -interferon more stable.

Single amino acid changes: - improves stability and biological activity

α_1 Antitrypsin: Inhibits the action of neutrophil elastase (elastase damages lung tissues). α_1 Antitrypsin binds to elastase and prevents its action. In this process it gets cleaved as serine & methionine and makes α_1 Antitrypsin a poor inhibitor of elastase.

Insulin: In the neutral solution, therapeutic insulin is present as zinc containing **hexamer**. By introducing single amino acid substitution, insulins were found to be in **monomeric state** with good stability and biological activity.

Tissue plasminogen activator: Used to lyse blood clots. Due to its shorter half-life tPA has to be repeatedly administered. By replacing asparagine residue with glutamine, the half-life can be increased.



Hirudin: A protein secreted by leech salivary gland and is strong thrombin inhibitor. By replacing asparagine with lysine, the potency of hirudin can be increased several fold.

Dihydrofolate reductase: It catalyzes the conversion of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. This results in synthesis of nucleic acids and amino acids. The inhibition of DHFR by folate analogue such as methotrexate will restrict the growth of tumor cells. By employing site-directed mutagenesis, replacement of glycine by alanine was found to produce DHFR inactive.

T4 Lysozyme: Replacement of glycine by any other amino acid in the protein structure, decreases the stability. On the other hand, proline residues increase protein stability.



THANK YOU