Chapter 8

Directed Mutagenesis and Protein Engineering

Improving Properties of Proteins for Therapeutic and Industrial Applications

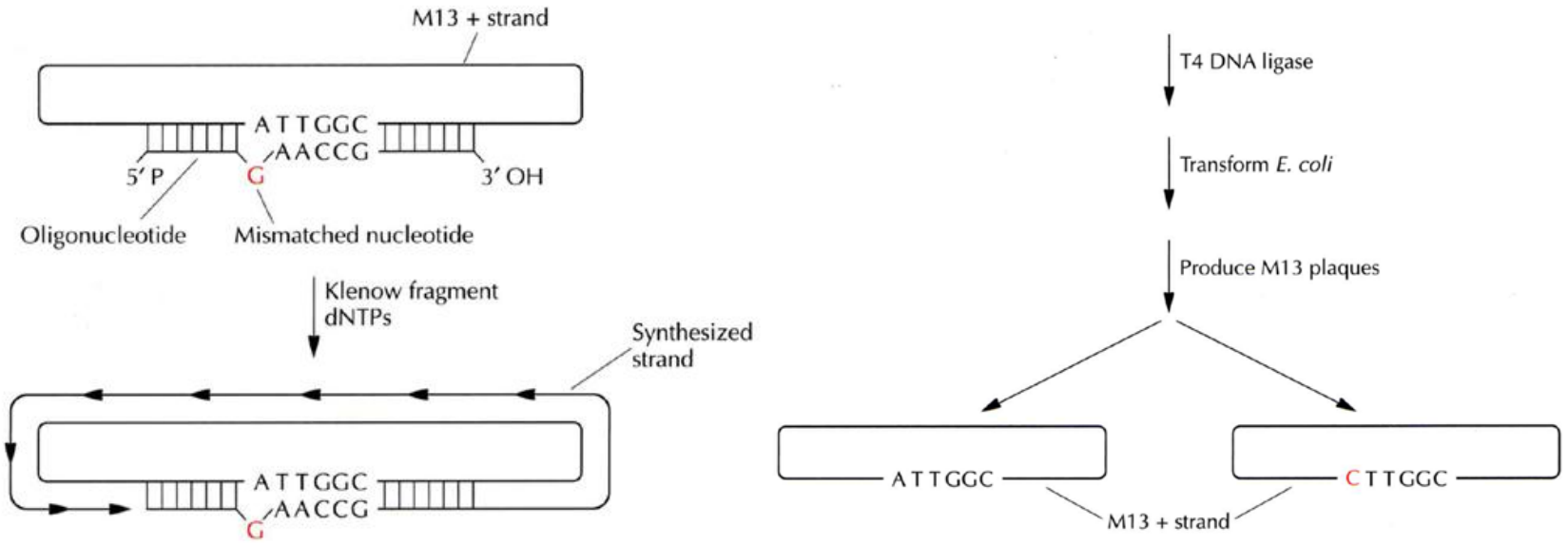
- Increasing catalytic activity
- Increasing stability at high temperature, extreme pH, or in organic solvent
- Elimination of cofactor requirement
- Increasing substrate specificity
- Increasing resistance to cellular protease
- Diminishing feedback inhibition by altering the allosteric regulation

8.1. Directed Mutagenesis Procedures

Mutagenesis

- Site-directed mutagenesis
  - Deciding the sites for mutagenesis
    - Based on structural information
    - Computer-assisted design
  - Site-directed mutagenesis using oligonucleotide
    - With M13 DNA
    - Using PCR
- Random mutagenesis
  - With oligonucleotide analogues
  - Error-prone PCR
  - DNA shuffling

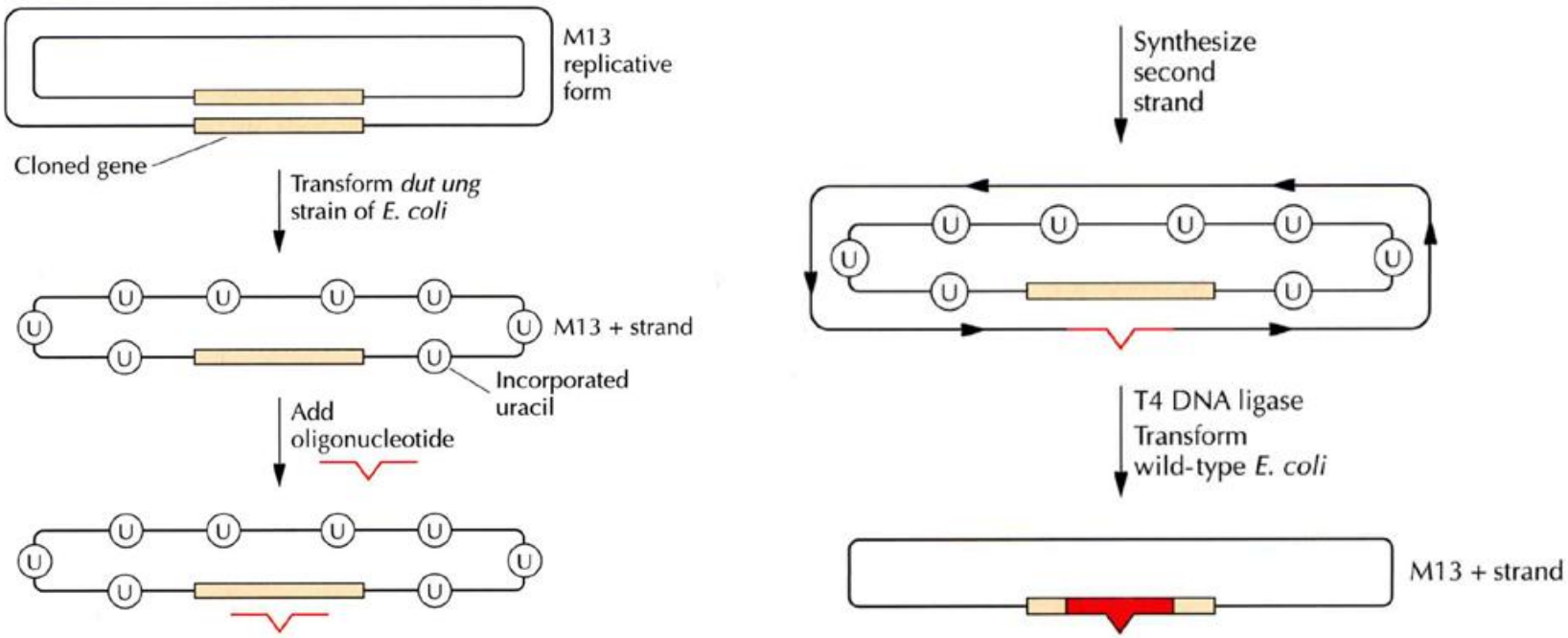
Mutagenesis with M13 DNA



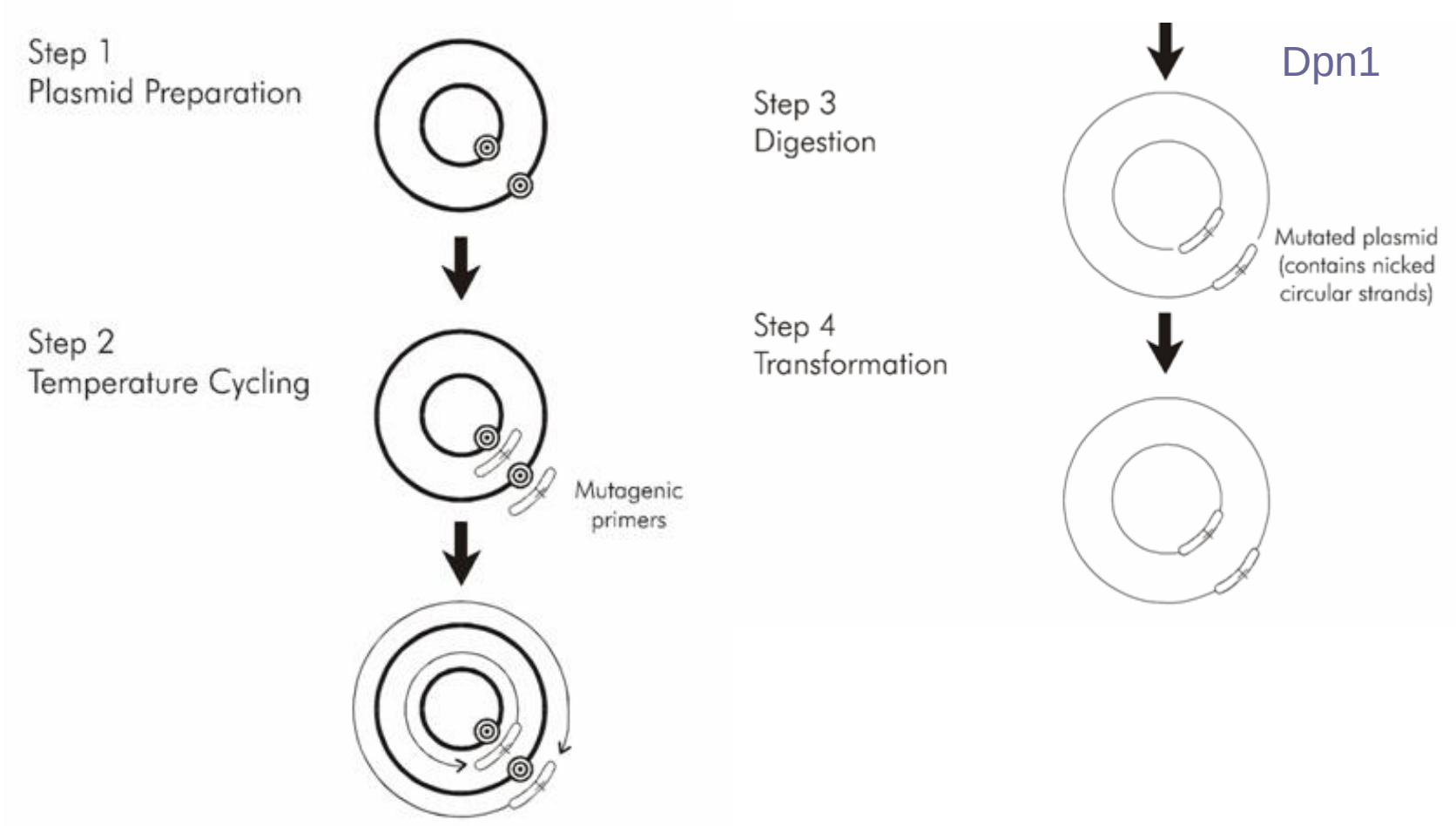
- 50% of chance that M13 have mutation
- Only 1~5% have mutation in actual experiment

Mutagenesis with M13 DNA

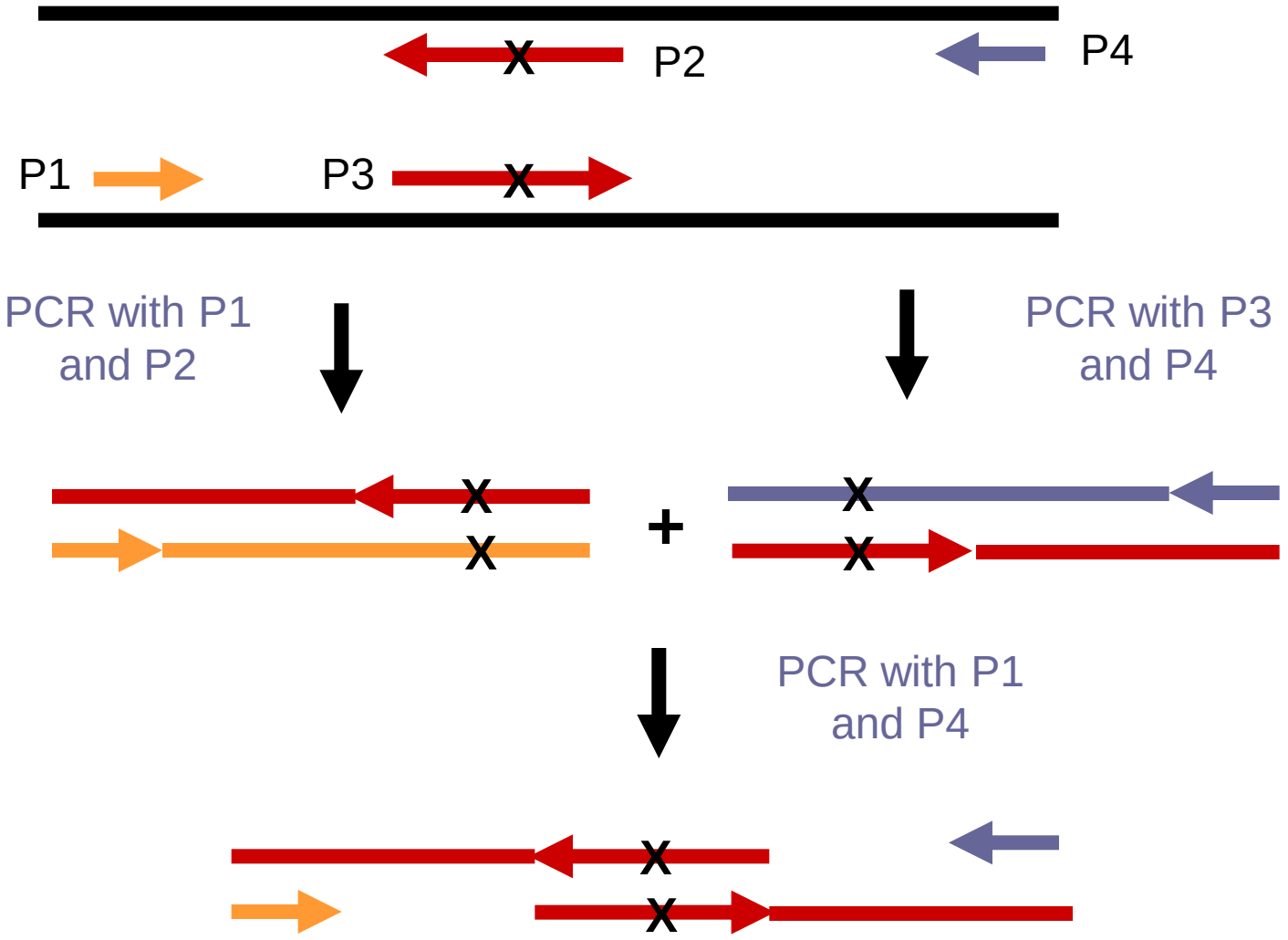
- dut mutant
  - Defective in dUTPase: high cellular concentration of dUTP
- ung mutant
  - Defective in uracyl N-glycosylase: no removal of dUTP from DNA



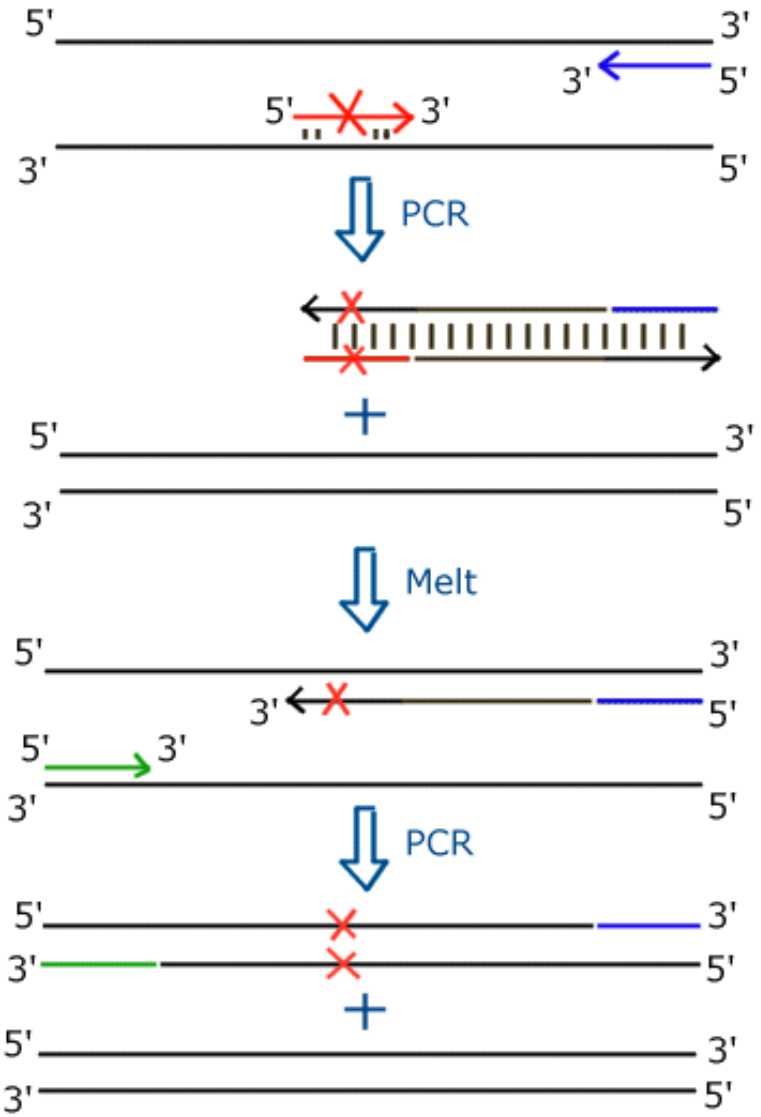
Mutagenesis Using Plasmid



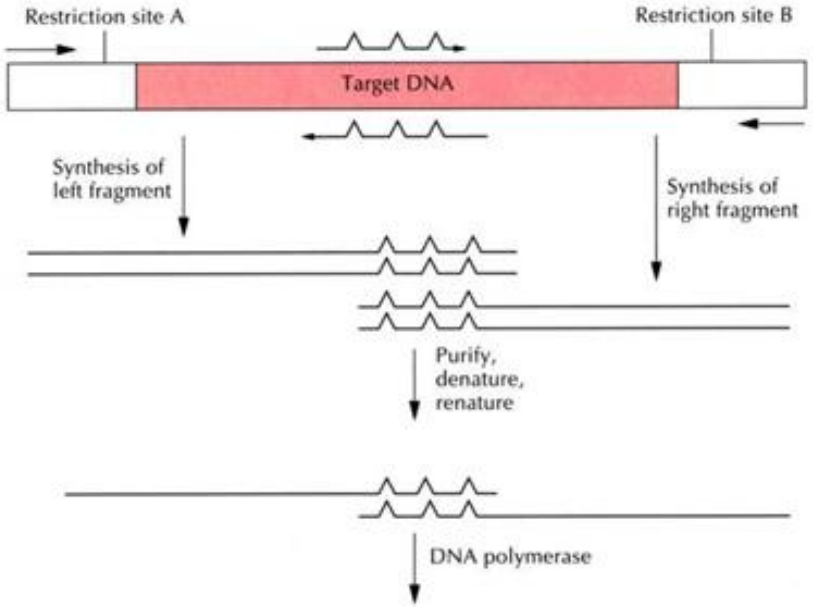
PCR Method of Site-Directed Mutagenesis

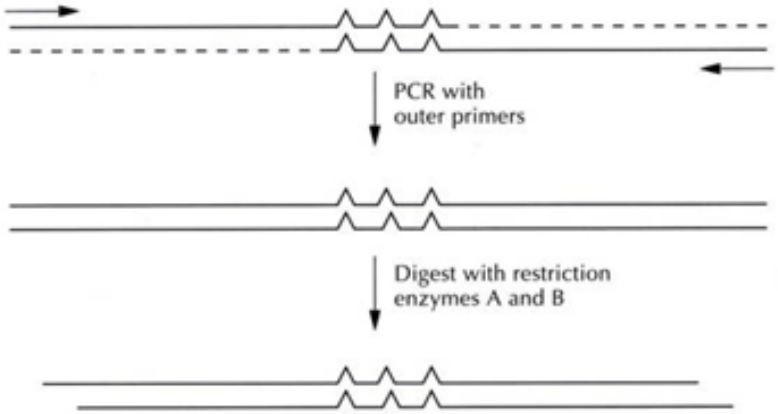


Site-Directed Mutagenesis with Megaprimer



Random Mutagenesis with Degenerate Primers

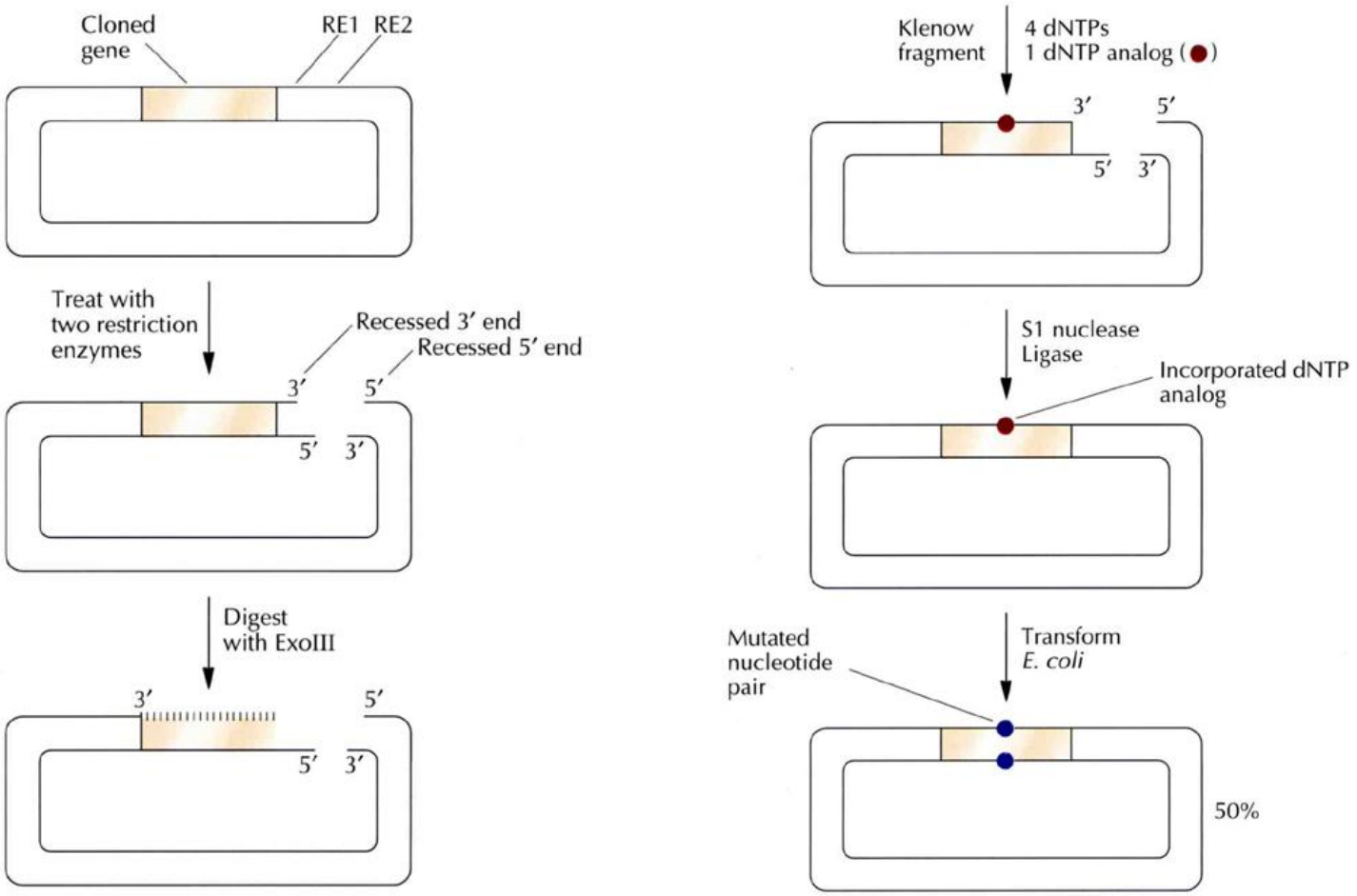




Error-Prone PCR

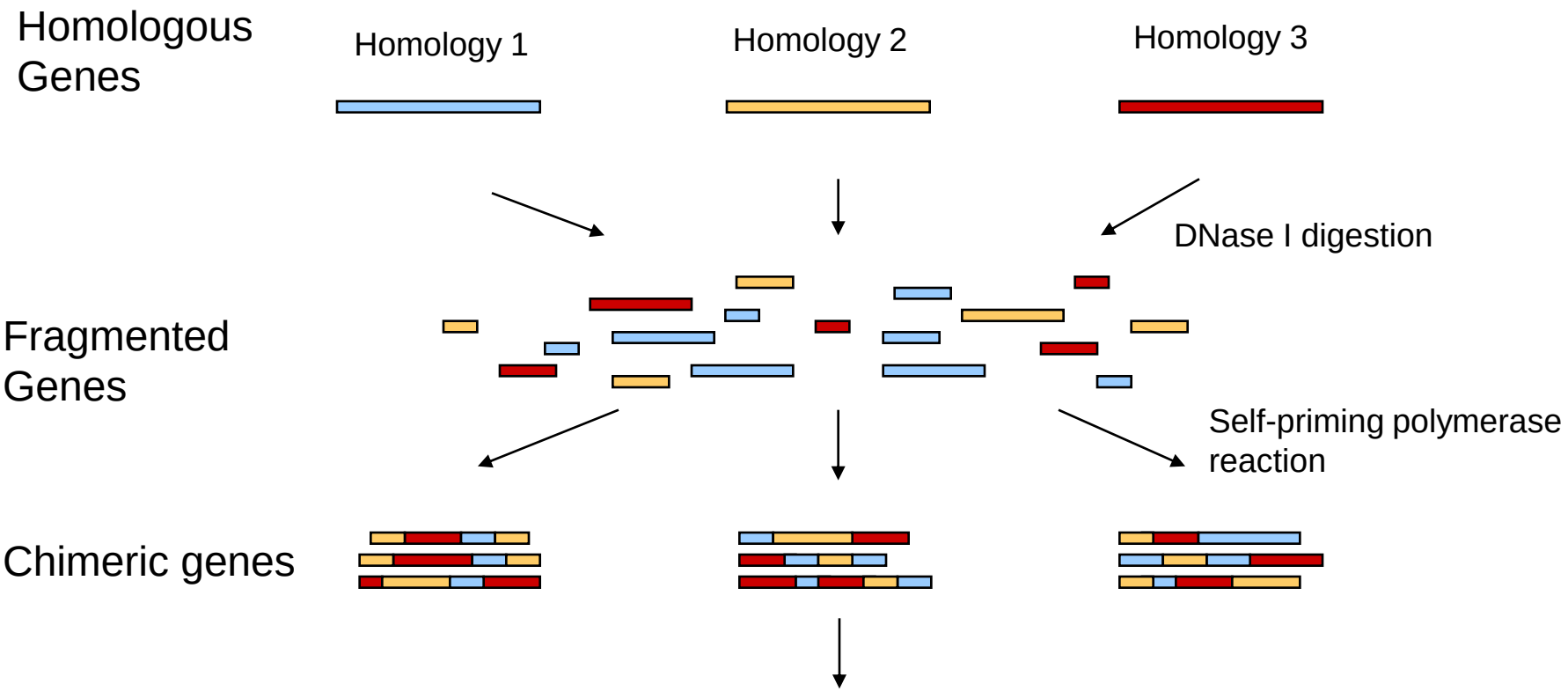
- Reduced fidelity
  - Increasing $MnCl_2$ or Mg concentration
  - Unbalancing dNTPs concentration
- Using nucleotide analogue
- Using ‘mutagenic’ polymerases

Random Mutagenesis with Nucleotide Analogues

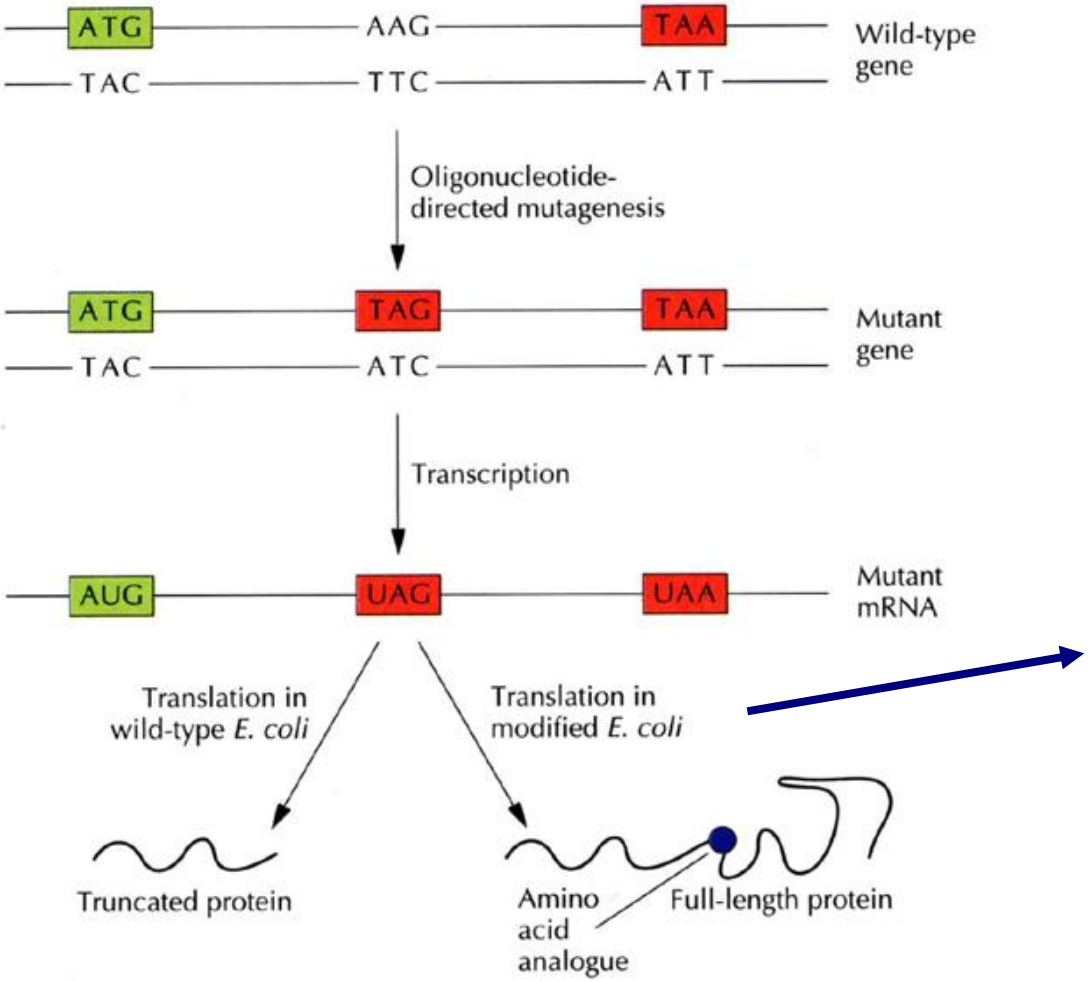


DNA Shuffling

- Recombination of related genes
- Molecular breeding



Mutant Proteins with Unusual Amino Acids



Modified *Methanococcus jannaschii* Tyr-tRNA synthetase

: Charge O-methyl-L-tyrosine-tRNA to amber suppressor tRNA

8.2. Protein Engineering

Protein Engineering

- Protein engineering
  - Generation of proteins suitable for industrial applications
  - Thermostability, resistance to organic solvent etc.
- Industrial enzymes
  - α-amylase — Beer, alcohol production
  - Aminoacylase — Preparation of L-amino acids
  - Cellulase — Alcohol and glucose production
  - Invertase — Sucrose inversion
  - Lipase — Cheese making, preparation of flavoring
  - Protease — Detergent, alcohol production

Protein Engineering

- Adding disulfide bonds
- Changing Asn to other amino acids
- Reducing the number of free sulfhydryl residues
- Increasing enzyme activity
- Modifying metal cofactor requirements
- Decreasing protease sensitivity
- Modifying protein specificity
- Increasing enzyme stability and specificity
- Altering multiple properties

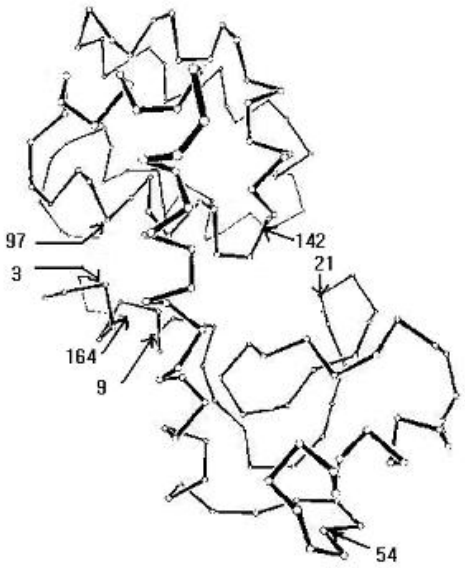
Adding Disulfide Bonds

- Introduction of disulfide bonds
  - Increase in thermostability
  - In general, thermostability correlates with stability to organic solvent and other nonphysiological conditions
- Selection of mutation sites
  - Disulfide bond formation between nearby residues
  - Choose mutation sites outside of the active site

T4 Ligase

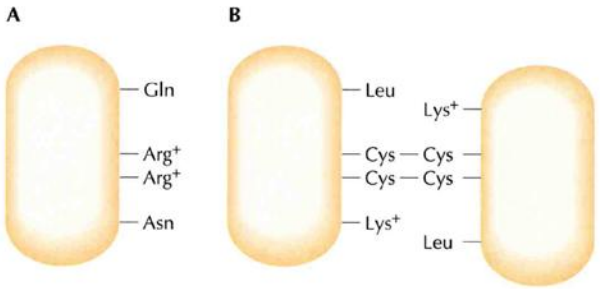
Amino acid at position:

	3	9	21	54	97	142	164	% Activity	Tm (°C)
Wild type	Ile	Ile	Thr	Cys	Cys	Thr	Leu	100	41
Pseudo wild type	Ile	Ile	Thr	Thr	Ala	Thr	Leu	100	41
VariantA	Cys	Ile	Thr	Thr	Cys	Thr	Leu	99	47
Variant B	Ile	Cys	Thr	Thr	Ala	Thr	Cys	101	48
Variant C	Ile	Ile	Cys	Thr	Ala	Cys	Leu	0	49
Variant D	Cys	Cys	Thr	Thr	Cys	Thr	Cys	98	58
Variant E	Ile	Cys	Cys	Thr	Ala	Cys	Cys	0	58
Variant F	Cys	Cys	Cys	Thr	Cys	Cys	Cys	0	66



Introduction of Disulfide Bonds

- Xylanase
  - Degradation of hemicellulose from wood pulp during paper making
  - Replacement of chemical bleaching
  - Treatment after hot alkali treatment : need heat stable enzyme
  - Determination of sites for disulfide bond formation by computer modeling of the structure
- Human pancreatic ribonuclease
  - RNase from bull semen
    - Antitumorigenic activity as a dimer
  - Introduction of disulfide bonds in human RNase

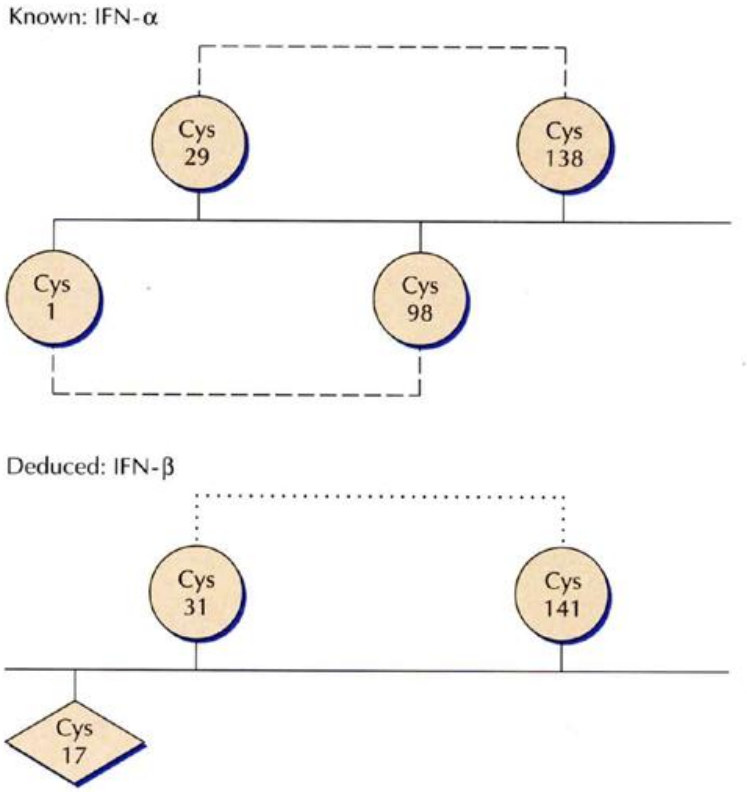


Changing Asn to Other Amino Acids

- Ans and Gln at high temperature
  - Deamination becoming Asp and Glu
  - Localized change in peptide folding
- *S. cerevisiae* triosephosphate isomerase
  - Two Asn residues in subunit interface
  - Asn to Thr or Ile mutation → increase in thermostability and protease resistance
  - Asn to Asp mutation → decrease in stability
- Long lasting human insulin
  - Mutation of an Asp to Gly

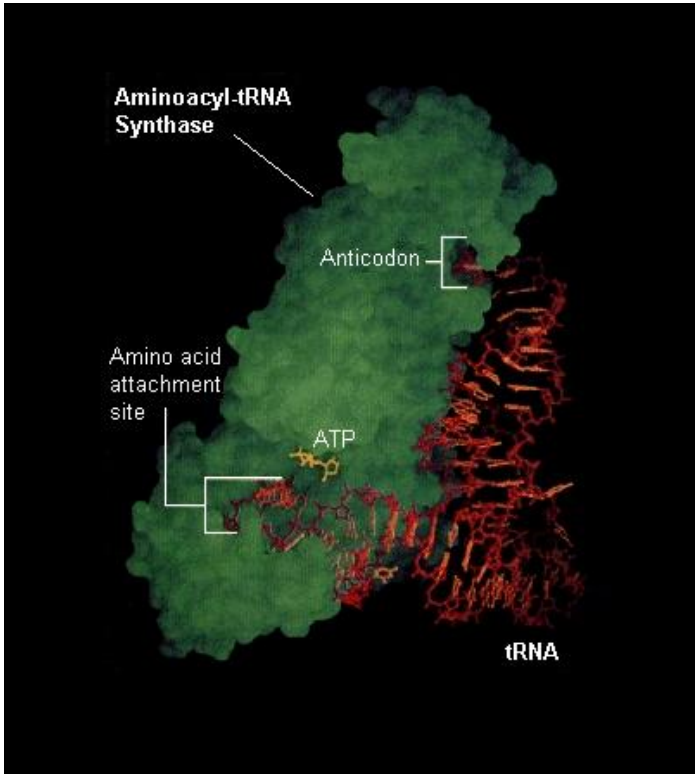
Reducing the Number of Free Sulfhydryl Residues

- Human INF-β produced in *E.coli*
  - Inactivation by intermolecular disulfide bond formation
  - Deduction of Cys residues involved in internal disulfide bond formation by comparison with IFN-α
  - Mutation of Cys 17 to Ser to prevent multimerization



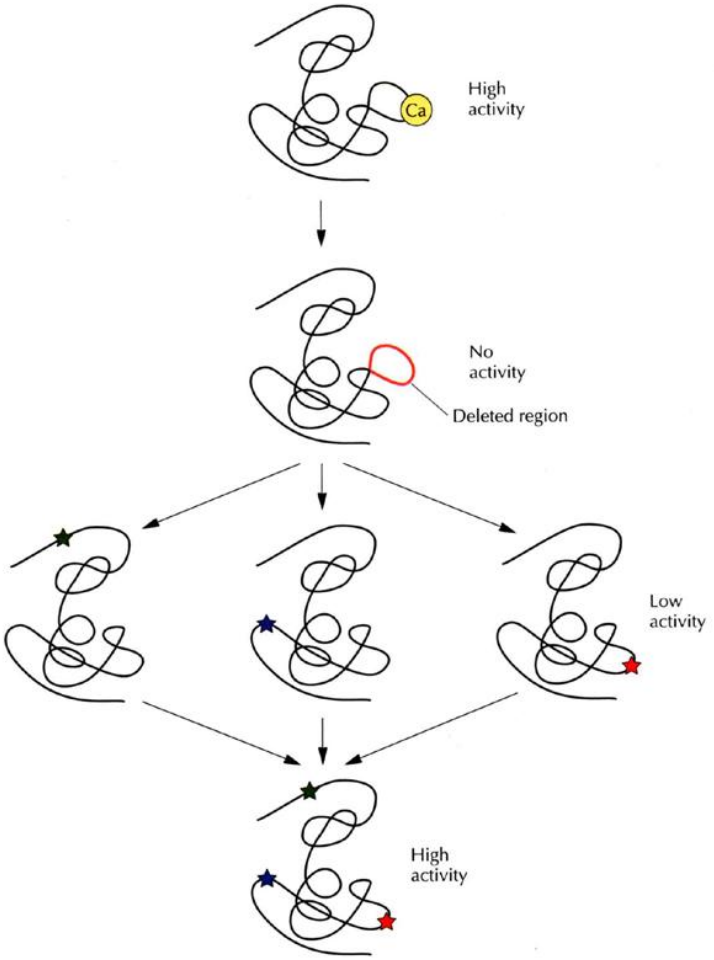
Increasing Enzyme Activity

- *B.stearothermophilus* Tyr-tRNA synthase
  - Tyr + ATP → Tyr-AMP + PPi
  - Tyr-AMP + tRNATyr → Tyr-tRNATyr + AMP
- Thr 51
  - Hydrogen bonding with ribose of Tyr-AMP
  - Mutation to Ala or Pro
    - Improve affinity to ATP and catalytic activity



Modifying Metal Cofactor Requirements

- Subtilisin
  - Produced by Bacillus
  - Used as cleaning agents in laundry detergent
  - Ca^{2+} binding is necessary for enzyme activity
- Engineering of Ca^{2+}-independent enzyme
  - Deletion of metal binding site
  - Selection potential stabilizing sites
  - Random mutagenesis and screening for stable mutants
  - Combination of mutations

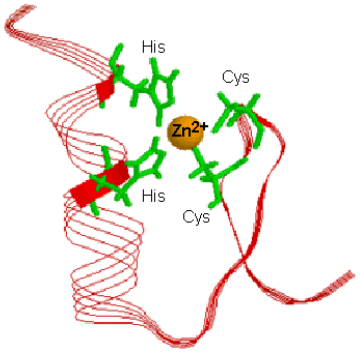


Decreasing Protease Sensitivity

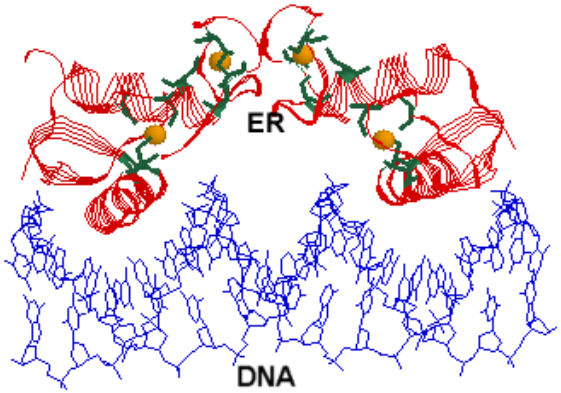
- Streptokinase
  - Produced by pathogenic strain of Streptococcus
  - Binding and conversion plasminogen to active protease plasmin
- Plasmin
  - Degradation fibrin in the blood clot
  - Degradation of streptokinase
- Plasmin resistant streptokinase
  - Mutation of cleavage sites
    - Lys58, 386 to Gln (similar length of side chain to lys)
  - 21-fold increase in protease resistant

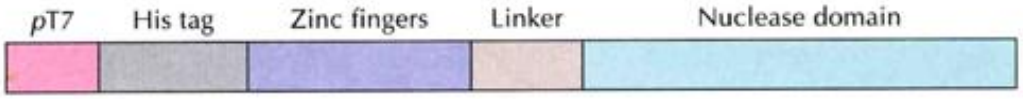
Modifying Protein Specificity

- Restriction endonuclease
  - >2,500 enzymes but ~200 recognition sites
  - Rare cutters are necessary for producing large DNA fragments



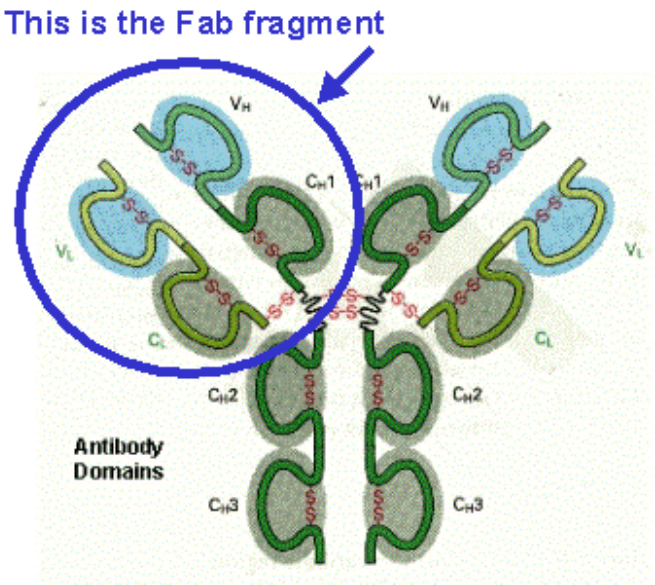
- Protein engineering of FokI endonulcease
  - Expression as a fusion protein with zinc finder domains

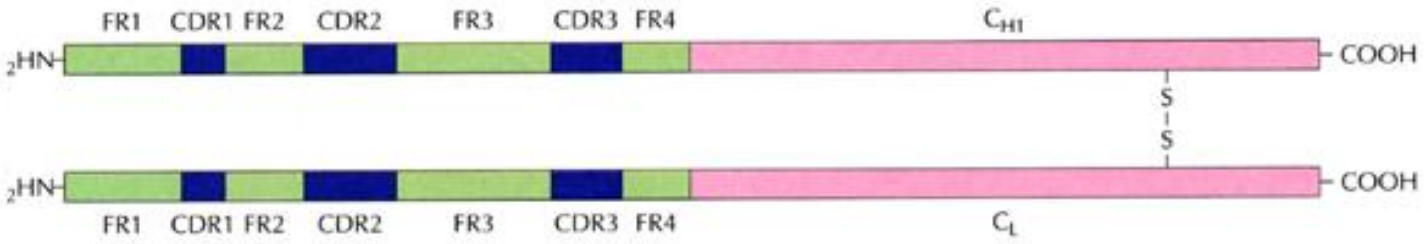




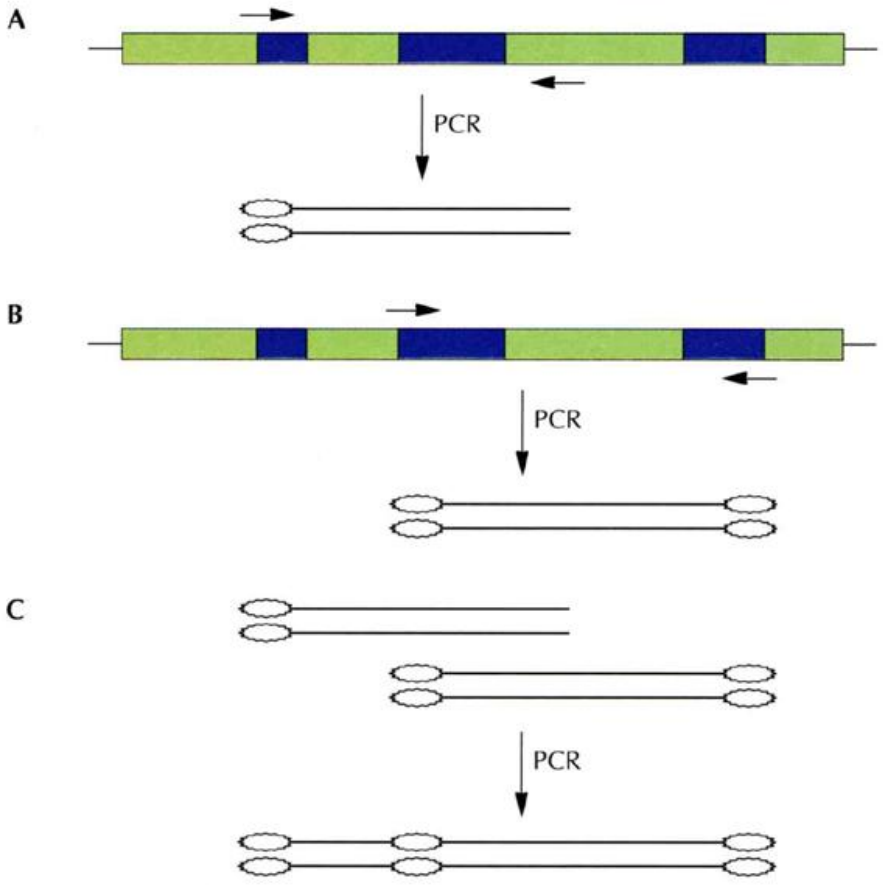
Antibody Engineering

- Fab fragment
  - Binding to antigenic determinant
  - CDR
    - Hypervariable complementarity - determining regions
    - Random mutagenesis and screening for new binding specificity





Random Mutagenesis of Three CDRs

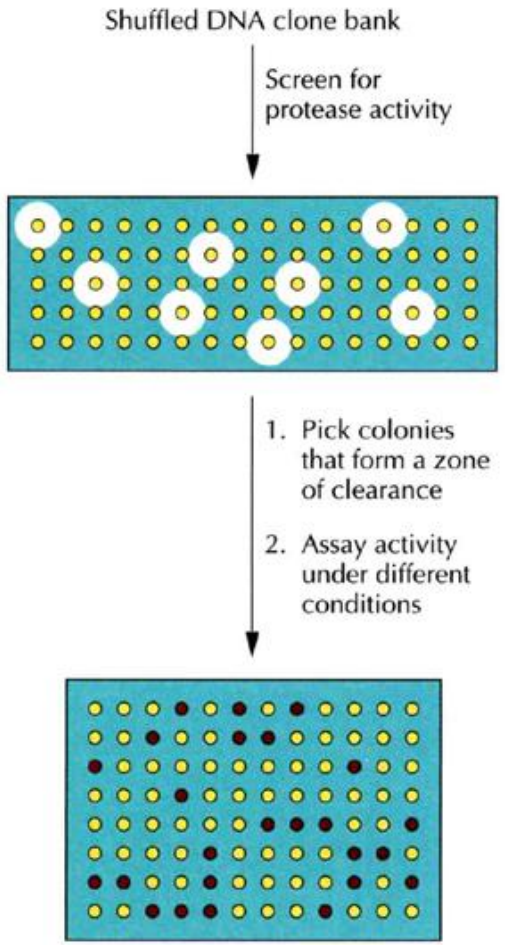


Increasing Enzyme Stability and Specificity

- Tissue plasminogen activator (tPA)
  - Serine protease
  - Dissolution of blood clots
  - Nonspecific internal bleeding at high concentration
- Protein engineering of tPA
  - Increase in stability
    - Thr 103 to Asm
  - Increase in fibrin binding
    - Lys His Arg Arg (296-299) to Ala Ala Ala Ala

Altering Multiple Properties

- Subtilisin
  - DNA shuffling library fro 26 different subtilisin genes
  - Screening of 654 clones for desirable traits
    - Activity at 23 ^{o}C
    - Thermostability
    - Solvent stability
    - pH dependence



Altering Multiple Properties

- Peroxidase
  - From ink cap mushroom Coprinus cinereus
  - Dye transfer inhibitor in laundry detergent
    - Oxidizing (decolorizing) free dyes
  - Bleach-containing detergents
    - High pH, High temperature, high peroxide concentration
- Protein engineering
  - Site-directed mutagenesis and random mutagenesis
  - DNA shuffling of mutants