Presentation from
SOS BIOTECHNOLOGY
for MSc.2 Semester
Subjects: Enzymeology amd
Molecular Biology

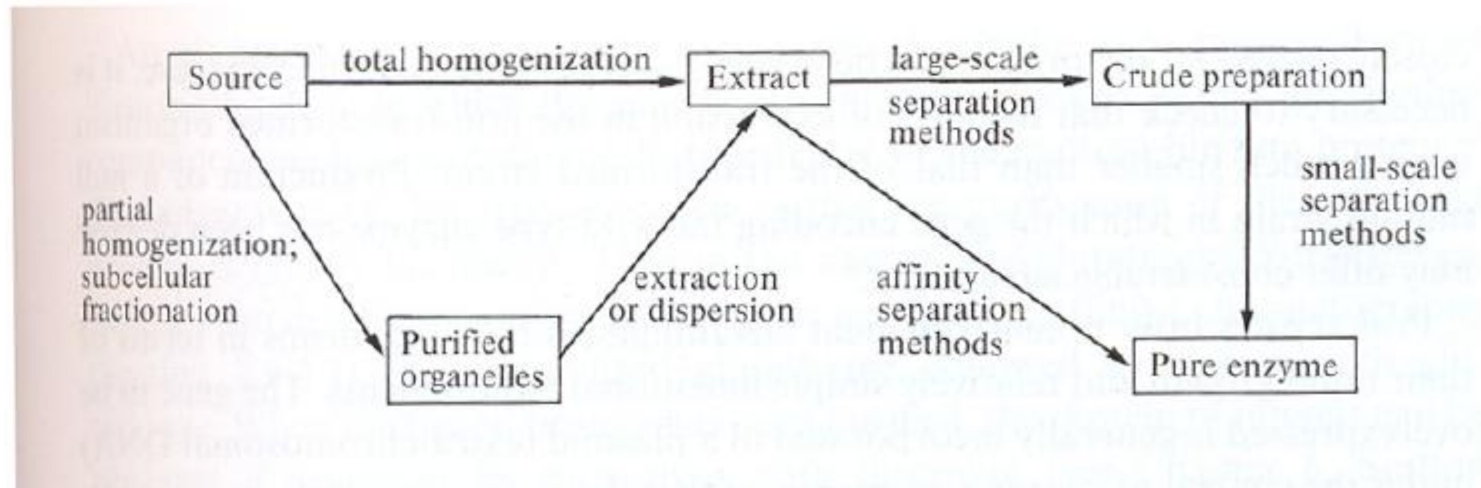
Enzyme Purification

- It is important to study enzymes in a simple system (only with small ions, buffer molecules, cofactors, etc.) for understanding its structure, kinetics, mechanisms, regulations, and role in a complex system
- Also isolating pure enzyme is important to use it for medical and industrial purposes

Objectives of enzyme purification

- Objectives : maximum possible yield + maximu catalytic activity + maximum possible purity
- Assay procedure (Chapter 4)
- History
  - Crystallization
  - Homogenization + large scale separation
  - Attach the affinity tag to enzyme using DNA recombinant technology (ex. $(His)_6$–tag)

Statergy



- Classical approach involves choosing a source containing large quantity of enzymes
  - Acetyl CoA carboxylase (mammary gland)
  - Alkaline phosphatase (kidney)
- Modern approach with DNA recombinant technology
  - 3-phosphoshikimate-1-carboxyvinyl transferase in *E. coli* (1984)
  - 100-fold increase in productivity
- Prokaryotes as host organisms (*E. coli* and *Bacillus*)
  - Rapid growth and simple medium components
  - Disadvantages: lack of post-translational modification (glycosylation) and forming inclusion bodies

Yeasts as enzyme source

- *Saccharomyces cerevisiae* rarely forms inclusion bodies, but grow slowly and make hyperglycosylation
- *Kluyveromyces lactis* and *Pichia pastoris* are also being developed

Insect cell with baculovirus vector

- It can employ many of the protein modification, processing, and transport system in higher eukaryotic cells

'Fusion Protein'

- Glutathione-S-transferase, maltose binding protein, or His-tag are popularly used
- They greatly enhance the power of purification and sometimes solubility of protein

- Mechanical methods
  - High pressure homogenizer* (55 MPa) : cooling is important
  - Wet grinding by mills or glass balls
- Non-mechanical methods
  - Drying
  - Lysis by osmotic shock, detergents, or enzymes
  - Ultrasound*
- Cooling and protease inhibition are important to recover the enzyme

- Animal cells (organs)
  - It is easy to homogenize due to the lack of cell wall
  - Fat and connective tissue must be removed before homogenization
- Bacteria and Fungi
  - Cell wall must be digested by enzymes (Protoplasts can be made by treating lysozyme or chitinase/3-glucanase)
- Plant
  - Disruption of vacuole can damage enzymes
- Membrane proteins
  - Usually detergent (anionic, cationic, or neutral) is added
  - Detergent must be chosen by considering the choice of purification method, especially column chromatography

Methods for separation

Table 2.1 Principal separation methods used in purification of enzymes

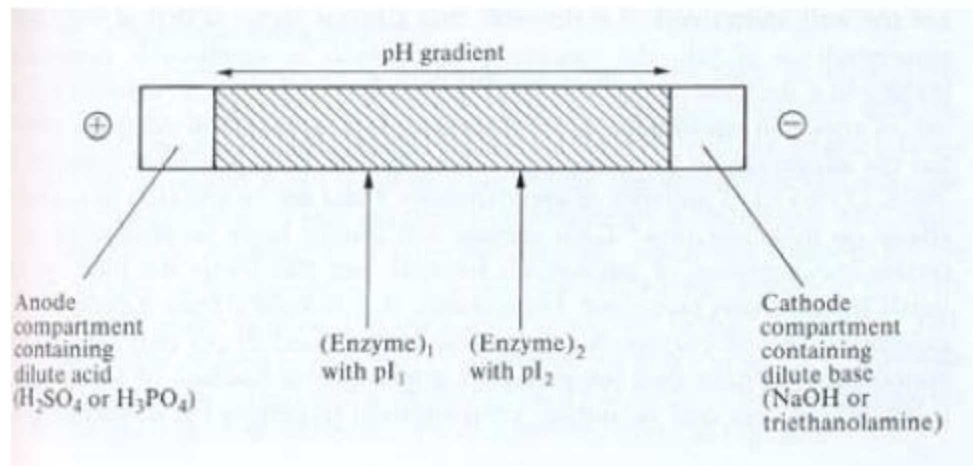
Property	Method	Scale
Size or mass	Centrifugation	Large or small
	Gel filtration	Generally smal
	Dialysis; ultrafiltration	Generally smal
Polarity		
(a) Charge	Ion-exchange chromatography	Large or small
	Chromatofocusing	Generally small
	Electrophoresis	Generally small
	Isoelectric focusing	Generally small
(b) Hydrophobic character	Hydrophobic chromatography	Generally smal
Solubility	Change in pH	Generally large
	Change in ionic strength	Large or small
	Decrease in dielectric constant	Generally large
Specific binding sites or structural features	Affinity chromatography	Generally smal
	Immobilized metal ion chromatography	Generally smal
	Affinity elution	Large or small
	Dye–ligand chromatography	Large or small
	Immunoadsorption	Generally smal
	Covalent chromatography	Generally smal

Methods for Sepration by Polarity

Electrophoresis

- Separation by movement of charged molecules
- Capillary electrophoresis (cross section less than 100μm)

Isoelectric focusing



Hydrophobic interaction chromatography

- Depending on the nonpolar amino acid on the surface of enzyme
- Octyl- or phenyl-Sepharose with high ionic strength
- Desorption by lowering ionic strength or adding organic solvents (or detergents)

Based on solubility

Change in pH

- Enzymes are least soluble at pI because there is no repulsive force between enzymes
- Enzyme must not be inactivated in a range of pH

Change in ionic strength

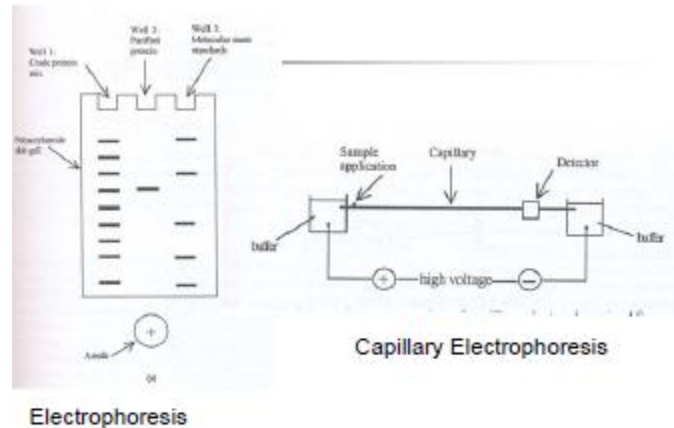
- Large charged molecules are only slightly soluble in pure water: Addition of ion promotes solubility (Salting in)
- Beyond a certain ionic strength, the charged molecules are quickly precipitated (Salting out)
- Ammonium sulfate is popularly used
- 10-fold increase in purity
- Fructose-bisphosphate aldolase from rabbit muscle can be purified in high purity by ammonium sulfate

Decrease in dielectric constant

- Addition of water-miscible organic solvent (ethanol or acetone)
- Decrease dielectric constant
- Sometimes deactivate the enzyme
- Work at low temperature
- PEG (poly ethylene glycol) ~ M_r 4000 to 6000 is commonly used

Table 2.2 Some commonly employed analytical methods to check the purity of enzyme preparations

Method	Comments
Ultracentrifugation (Chapter 3, Section 3.2.1)	Not very satisfactory for detecting impurities at the ≤5% level. Problems can arise from associating–dissociating systems (Chapter 3, Section 3.2.5)
Electrophoresis (Section 2.6.2.2)	A good method for examining enzymes composed of non-identical subunits (Chapter 3, Section 3.5.1.5)
Electrophoresis in the presence of sodium dodecylsulphate (Chapter 3, Section 3.2.3)	A good method for detecting impurities that differ in terms of subunit M_r; excellent for detecting proteolytic damage. Problems arise from enzymes composed of non-identical subunits, which give rise to multiple bands (Chapter 3, Section 3.5.1.5)
Capillary electrophoresis (Section 2.6.2.2)	A powerful analytical technique which can be used in a variety of modes, including isoelectric focusing. Equipment required is specialized and relatively expensive
Isoelectric focusing (Section 2.6.2.3)	A very sensitive method for detecting impurities. Artefacts can arise suggesting apparent heterogeneity[illegible]
N-terminal analysis (Chapter 3, Section 3.3.2.4)	Should indicate the presence of a single polypeptide chain. Some enzymes have a blocked N-terminus (Chapter 3, Section 3.3.2.7); others consist of multiple polypeptide chains held together by disulphide bonds (e.g. chymotrypsin)
Mass spectrometry (Chapter 3, Section 3.2.4)	A very powerful but specialised technique. Subunit M_r values can be obtained very precisely, confirming the authenticity of the primary structure (Chapter 3, Section 3.3.2.10). Post-translational modifications can be identified



Electrophoresis

Capillary Electrophoresis

Test for purification

Tests for catalytic activity

– By enzyme assay

– Check cofactors and inhibitors

Stabilizing factors

– Neutral pH, storage in 50% glycerol may help

– 2-mercaptoethanol or DTT(Dithiothreitol)*

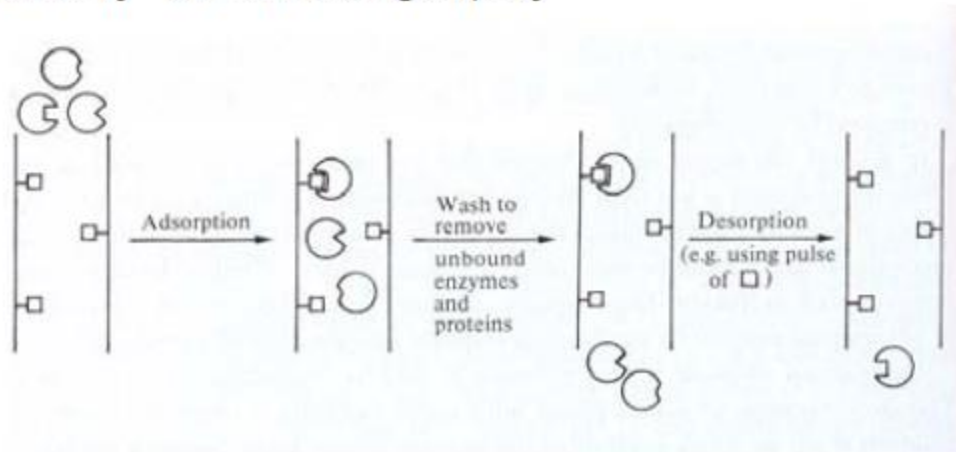
– Protease inhibitor PMSF (Phenylmethylsulfonyl flouride)

Active site titrations

- Checking the proportion of active enzyme in the purified enzyme

Based on affiniy

Affinity chromatography



Substrate or inhibitor is linked to a matrix

Desorbed by a pulse of substrate or changed pH, ionic strength

Problems

- Attaching a suitable substrate or inhibitor to the matrix can be difficult
- Linking b/n substrate and matrix itself may inhibit the binding b/n enzyme and substrate: Spacer arm (diaminehexane) may be needed
- Binding affinity b/n enzyme and substrate must be in a proper range
- Special attention is necessary to separate the enzymes using same substrate or using more than one substrate

Fusing proteins to solve the problems

- Glutathione-S-transferase : glutathione
- Maltose binding protein : maltose
- Hexahisitidine : Ni^{2+} (Elution by imidazole or thrombine cleavage site is added after the tag)

Affinity elution

- Affinity occur at desorption step
- Can solve some problems of affinity chromatography and easy to scale up

Dye-ligand chromatography

- Cibacron Blue F3G-A can bind to a number of dehydrogenases and kinases
- Procion Red HE-3B binds well with $NADP^+$-dependent dehydrogenase

Immunoadsorption chromatography

- Immobilize the antibody to CNBr treated Sepharose
- Achieve much higher purity

Site directed Mutagenesis

•The first site-directed mutagenesis(SDM) experiment was performed into the year 1974, in the laboratory of *Charles Weissmann*. The induced mutation was from GC to AT. However, the mutation was inserted randomly.

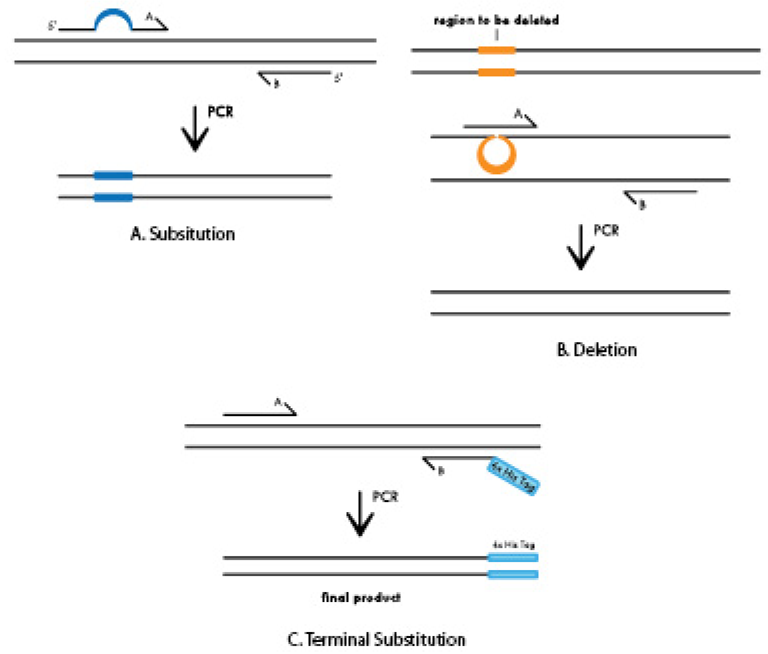
•The basic principle of site-directed mutagenesis is simple, DNA primers along with the desired mutation are artificially synthesised and used to amplify the gene of interest.

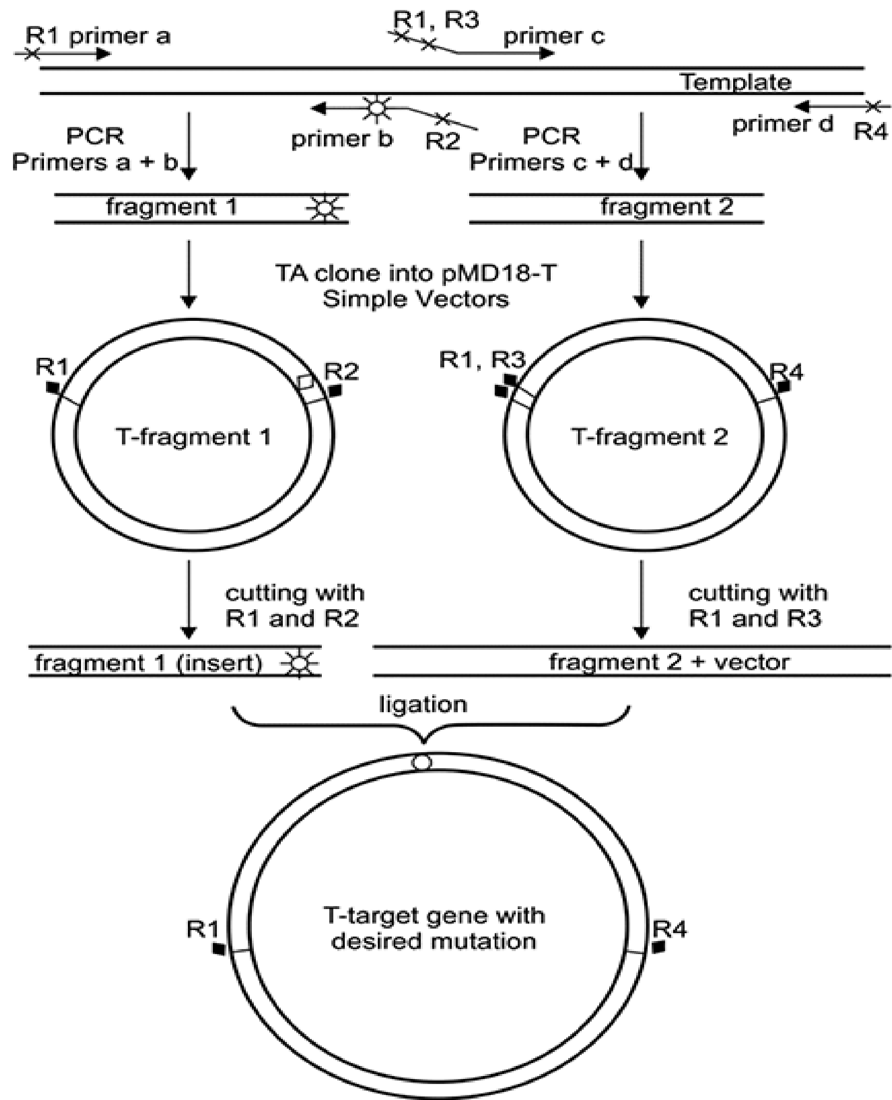
•Site-directed mutagenesis is an *in vitro* method for creating a specific mutation in a known sequence. While often performed using PCR-based methods, the availability of custom-designed, synthetic, double-stranded DNA (dsDNA) fragments can drastically reduce the time and steps required to obtain the same sequence changes.
In this article we describe several PCR-based methods for site-directed mutagenesis. Primers designed with mutations can introduce small sequence changes, and primer extension or inverse PCR can be used to achieve longer mutant regions. Using these site-directed mutagenesis techniques allows researchers to investigate the impact of sequence changes or screen a variety of mutants to determine the optimal sequence for addressing the question at hand.

Traditional PCR

When PCR is used for site-directed mutagenesis, the primers are designed to include the desired change, which could be base substitution, addition, or deletion . During PCR, the mutation is incorporated into the amplicon, replacing the original sequence.

Mutations introduced by PCR can only be incorporated into regions of sequence complementary to the primers and not regions between the primers [1].



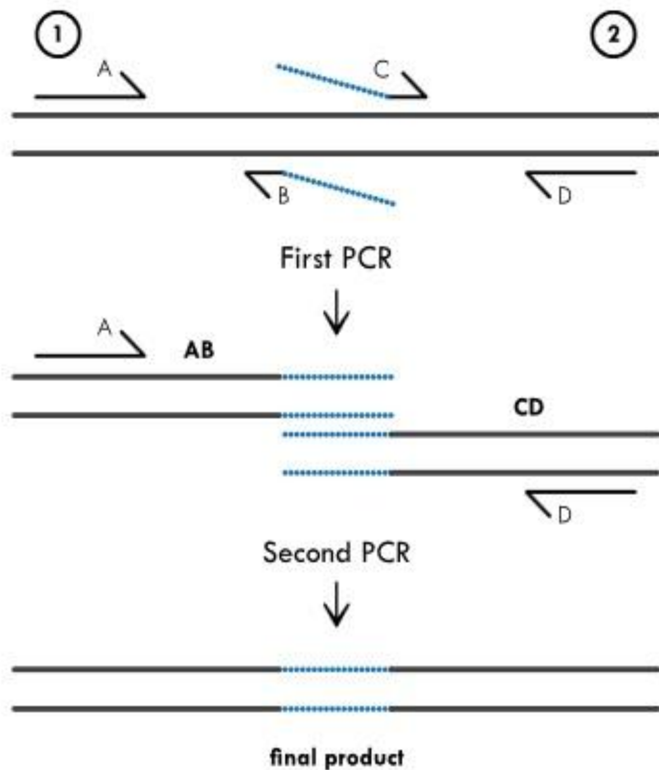


R1 primer a
R1, R3
primer c
Template
primer b
R2
primer d
R4
PCR
Primers a + b
PCR
Primers c + d
fragment 1
fragment 2
TA clone into pMD18-T
Simple Vectors
R1
R2
T-fragment 1
R1, R3
R4
T-fragment 2
cutting with
R1 and R2
cutting with
R1 and R3
fragment 1 (insert)
fragment 2 + vector
ligation
R1
T-target gene with
desired mutation
R4

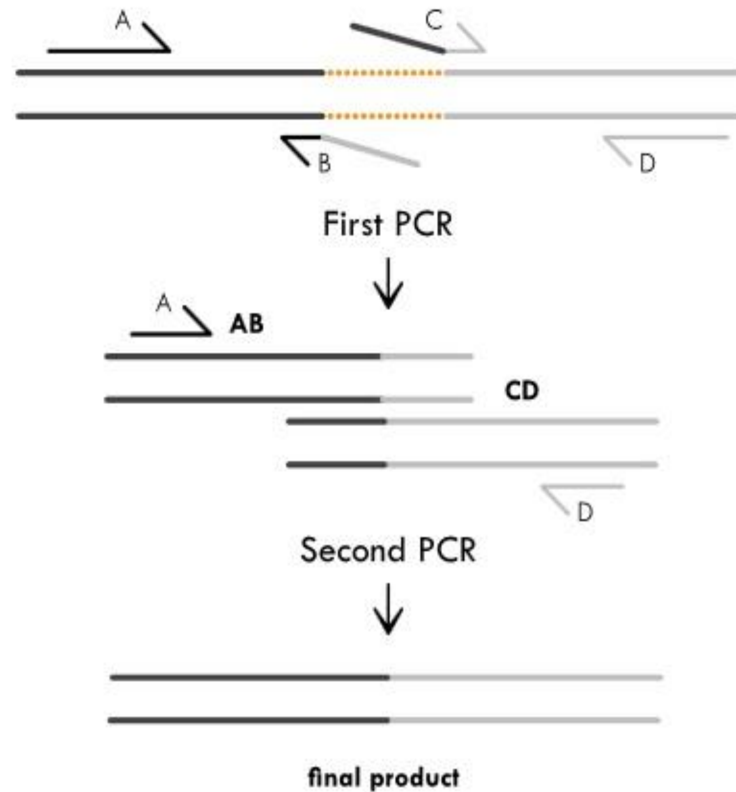
Primer extension

Site-directed mutagenesis by primer extension involves incorporating mutagenic primers in independent, nested PCRs before combining them in the final product [2]. The reaction requires flanking primers (A and D) complementary to the ends of the target sequence, and two internal primers with complementary ends (B and C). These internal primers contain the desired mutation and will hybridize to the region to be altered. During the first round of PCR, the AB and CD fragments are created. These products are mixed for the second round of PCR using primers A and D. The complementary ends of the products hybridize in this second PCR to create the final product, AD, which contains the mutated internal sequence . Longer insertions can be incorporated by using especially long primers,

To create a deletion, the internal primers, B and C, are positioned at either side of the region to be deleted to prevent it from being incorporated within fragments AB and CD from the first round of PCR. The complementary sequences at the ends of the these fragments, created by primers B and C, enable hybridization of AB to CD during the second round of PCR, and the final product with the desired deletion (AD) is created



A. Simple Insertion



B. Deletion

Inverse PCR

Inverse PCR enables amplification of a region of unknown sequence using primers oriented in the reverse direction. An adaptation of this method can be used to introduce mutations in previously cloned sequences. Using primers incorporating the desired change, an entire circular plasmid is amplified to delete , or inser the desired sequence.

Applications of site-directed mutagenesis:

The site-directed mutagenesis helps to improve the quality of the protein by removing harmful elements from it.

Also, the modified, genetically engineered proteins have high commercial value.

The tool is used in the study of a gene characteristics in which by introducing specific mutations, the properties of the DNA, its encoded protein and post-translational modifications of that, can be studied.

The method is the first choice in gene synthesis and gene-editing technology.

It is used in the cloning as well.

By mutating the promoter or the regulatory regions of a gene, one can construct the map of regulatory elements of a gene.

It is also useful in the screening of SNPs

Attenuation is a regulatory mechanism used in bacterial operons(unit of genetic material that functions in a coordinated manner and is transcribed as one unit) to ensure proper transcription and translation.

Prokaryotic transcription attenuation mechanisms are described in which different metabolic signals and sensing events are used to regulate transcription termination at sites preceding structural genes.

- Genes in this class contain transcription termination signals in the region upstream of the coding sequence.
- termination/antitermination of transcription in response to external signals
- The activity of these regulatory termination signals is controlled through a variety of mechanisms.
- These include modification of RNA polymerase to a terminator-resistant, or terminator-prone form,
- Alterations in the structure of the nascent transcript, to determine whether the stem-loop structure of an intrinsic terminator or an alternate antiterminator is formed.
- Structural alterations in the transcript can be controlled by the kinetics of translation of the RNA, by binding of specific regulatory proteins, and by mRNA-tRNA interactions.

- Attenuation is a mechanism utilized by bacteria to regulate unnecessary gene expression.
- Attenuators are characterized by the presence of stop signals within the DNA sequence that can result in either transcriptional-attenuation or translational-attenuation.
- Transcriptional-attenuation is characterized by the presence of an attenuator within the DNA sequence that results in formation of mRNA-stem loops that prevent further transcription from occurring.
- The non-functional RNA produced prevents proper transcription.Translational-attenuation is characterized by the misfolding of the Shine-Dalgarno sequence.
- The Shine-Dalgarno sequence, responsible for ribosomal binding to allow proper translation, is inaccessible because it is folded into a hairpin-loop structure, thus, translation cannot occur.

Transcription-attenuation is characterized by the presence of 5'-cis acting regulatory regions that fold into alternative RNA structures which can terminate transcription. These RNA structures dictate whether transcription will proceed successfully or be terminated early, specifically, by causing transcription-attenuation.

The result is a misfolded RNA structure where the Rho-independent terminator disrupts transcription and produced a non-functional RNA product. This characterizes the mechanisms of transcription-attenuation. The other RNA structure produced will be an anti-terminator that allows transcription to proceed .

Translation-attenuation is characterized by the sequestration of the Shine-Dalgarno sequence.

The Shine-Dalgarno sequence is a bacterial specific sequence that indicates the site for ribosomal binding to allow for proper translation to occur. However, in translation-attenuation, the attenuation mechanism results in the Shine-Dalgarno sequence forming as a hairpin-loop structure. The formation of this hairpin-loop structure results in the inability of the ribosomal complexes to form and proceed with proper translation. Hence, this specific process is referred to as translation-attenuation.

Constitutive and inducible promoters

- Certain genes are transcribed at all times and circumstances

-Examples – tRNAs, rRNAs, ribosomal proteins, RNA polymerase

-Promoters of those genes are called constitutive

- Most genes, however, need to be transcribed only under certain circumstances or periods in cell life cycles

-The promoters of those genes are called inducible and they are subject to up- and down- regulation

Regulation at promoters

- Promoters can be regulated by repression and/or activation
- Many σ^{70} promoters are controlled both by repression and activation, whereas, for example σ^{54} promoters are controled solely by activation

Mechanisms of repression

- Repression by steric hindrance
- Inhibition of transition to open complex
- Inhibition of promoter clearance
- Anti-activation
- Anti-sigma factors

- The *lac* operon of *E. coli* contains genes involved in lactose metabolism. It's expressed only when lactose is present and glucose is absent.
- Two regulators turn the operon "on" and "off" in response to lactose and glucose levels: the *lac* repressor and catabolite activator protein (CAP).
- The ***lac* repressor** acts as a lactose sensor. It normally blocks transcription of the operon, but stops acting as a repressor when lactose is present. The *lac* repressor senses lactose indirectly, through its isomer **allolactose**.
- **Catabolite activator protein** (**CAP**) acts as a glucose sensor. It activates transcription of the operon, but only when glucose levels are low. CAP senses glucose indirectly, through the "hunger signal" molecule **cAMP**.

Lac Operon

3 The *lac* operon

An operon consists of a cluster of genes (called structural genes) that encode proteins (enzymes) to facilitate a common metabolic pathway. The *lac* operon consists of three structural genes needed for the transport and metabolism of lactose in *E. coli*. These structural genes are *lacZ*, *lacY*, and *lacA*, which encode β-galactosidase, lactose permease and thiogalactoside transacetylase, respectively.

β-galactosidase has two functions (Figure 1). One is to convert lactose into allolactose. Allolactose is an isomer of lactose that serves as an inducer by complexing and inactivating the repressor, a product of the nearby regulatory gene *lacI*. Its other function is to break down the disaccharide lactose (as well as free allolactose) into two monosaccharides, glucose and galactose. Glucose serves as the primary source of energy.

Lactose permease is a carrier molecule, which binds to the bacterial membrane to transport lactose from the environment into the cell (Figure 2). Thiogalactoside transacetylase functions in cellular detoxification.

Figure 1 Dual functions of β-galactosidase: conversion of lactose into allolactose and digestion of lactose (as well as free allolactose) into glucose and galactose (see online version for colours)

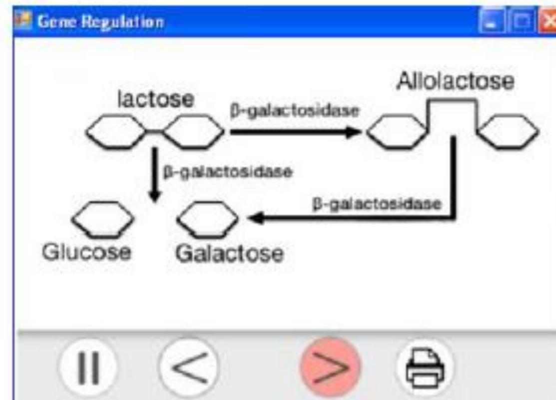
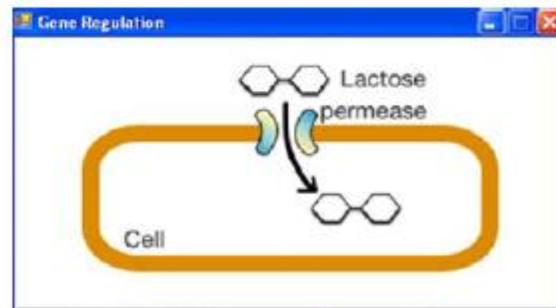


Figure 2 Function of lactose permease: carrying lactose into the cell (see online version for colours)



It should be noted that when the *lac* operon is turned on, the three structural genes, *Z*, *Y*, and *A*, are transcribed, yielding a single mRNA. Since the mRNA is the product of more than one gene, it is polycistronic. The polycistronic RNA, in turn, is translated into the three individual enzymes described above (Figure 3).

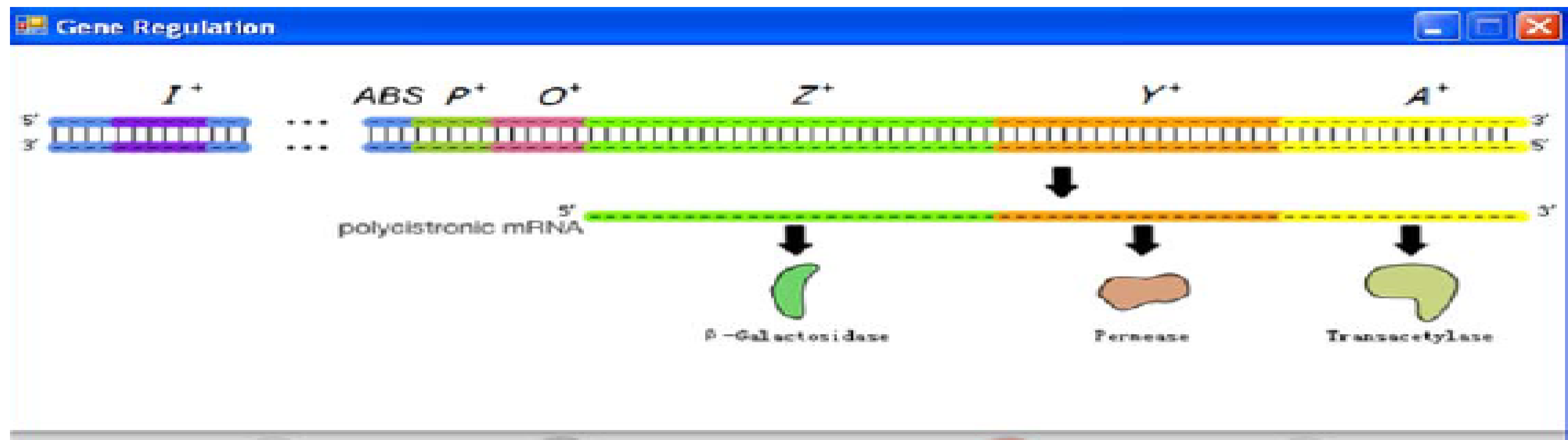
All three structural genes are under the control of a promoter *P*, which contains an upstream activator binding site (*ABS*) along with another control element called the operator *O*. Binding of an activator to the activator binding site enhances the binding of RNA polymerase to the promoter for transcription. On the other hand, binding of a repressor protein (product of the regulatory or repressor gene I^{+}) to the operator blocks the binding of RNA polymerase to the promoter, preventing transcription.

Thus, the *lac* operon is controlled not only by the *lac* promoter and the *lac* operator, but also by the separate regulatory gene *lacI*, which itself requires a promoter for expressing its product, the repressor. The general structure of the *lac* operon is shown in Figure 4. Unless otherwise indicated, all genes and control elements are assumed to be in the wild-type (+) form.

Like all living organisms, bacterial cells use glucose as their primary energy source. However, glucose is not always available in the natural environment. If glucose is not present, or is used up, lactose, when present, can be digested by β-galactosidase to provide an alternative way for generating glucose for the bacteria. Therefore, there is a selective advantage for bacteria, such as *E. coli,* to have two systems for controlling the *lac* operon:

- a positive system to turn on the *lac* operon when lactose is present (and glucose is absent)
- a negative system to turn off the *lac* operon when lactose is absent (or glucose is present).

Figure 3 Elements in the *lac* operon and its structural gene products at the transcription and translation levels (see online version for colours)



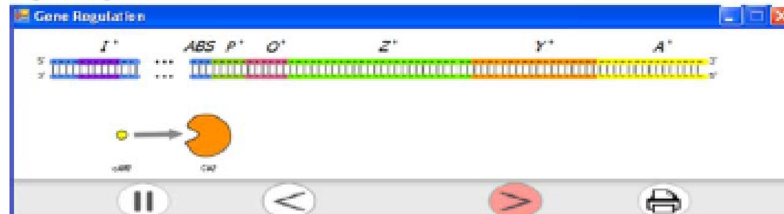
4.1 Positive regulation

During positive regulation, the structural genes are turned on. The following conditions must be met for the *lac* genes to turn on:

(a) Availability of the activator: *E. coli* cells generally contain high levels of cAMP (cyclic adenosine monophosphate) and CAP (catabolic activator protein). These molecules come together to form an activator, the cAMP-CAP complex, which binds to *ABS* to facilitate *lac* gene expression (transcription). Figure 5 shows the stepwise transcription of the *lac* operon when an activator is present.

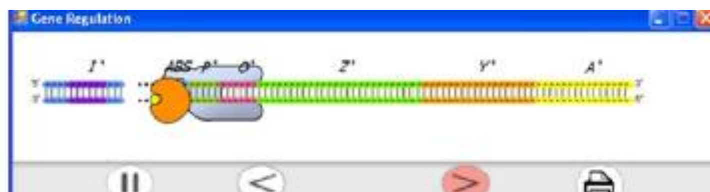
Figure 5 Positive regulation of the *lac* operon by the activator (see online version for colours)

Step 1: Drag cAMP to CAP



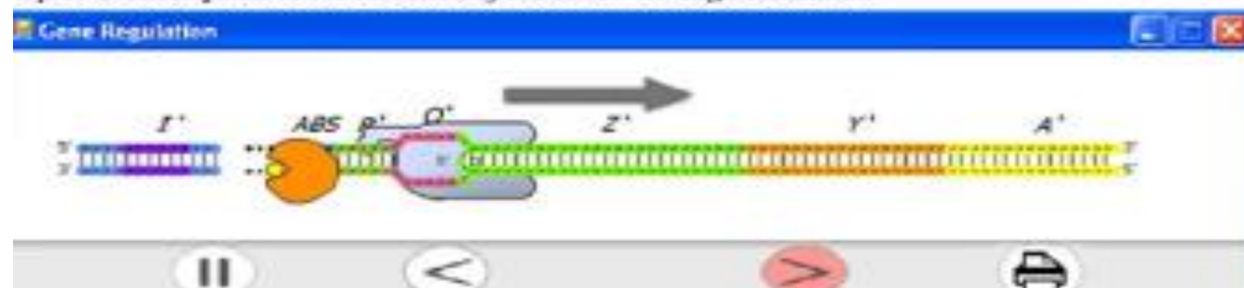
Step 2: Form cAMP-CAP complex (activator) through animation

Step 3: Drag the cAMP-CAP complex to the activator binding site (ABS) of the promoter

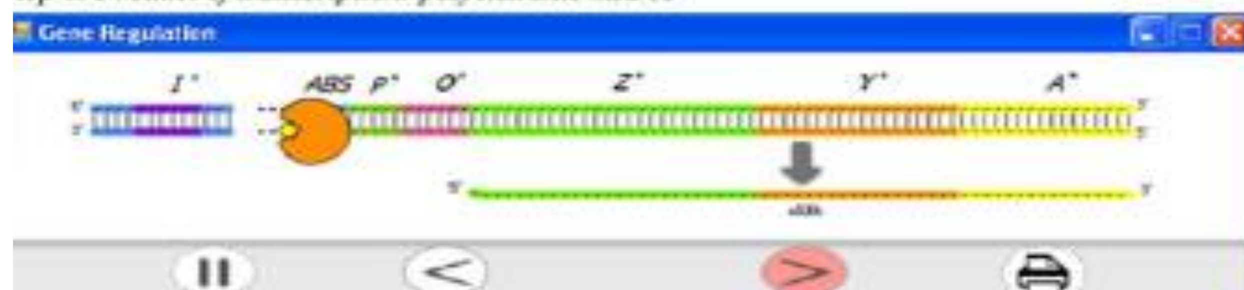


Positive regulation of the *lac* operon by the activator (see online version for colou
(continued)

tep 5: Transcription in the direction of the arrow through animation



tep 6: Product of transcription: polycistronic mRNA



(b) The presence of inducer (allolactose): Allolactose, which is converted from lactose by β-galactosidase, serves as an inducer by complexing to the repressor, resulting in an inducer-repressor complex, which can no longer bind the operator. Thus, the free operator allows the binding of RNA polymerase to the promoter (Figure 5: step 4). Figure 6 shows the stepwise transcription of the *lac* operon when an inducer is present.

Figure 6 Positive regulation of the *lac* operon by the inducer allolactose (see online version for colours)

Step 1: Click RNA polymerase (already attached to the I^+ gene) to initiate transcription (followed by translation) of the I^+ gene to encode the activator

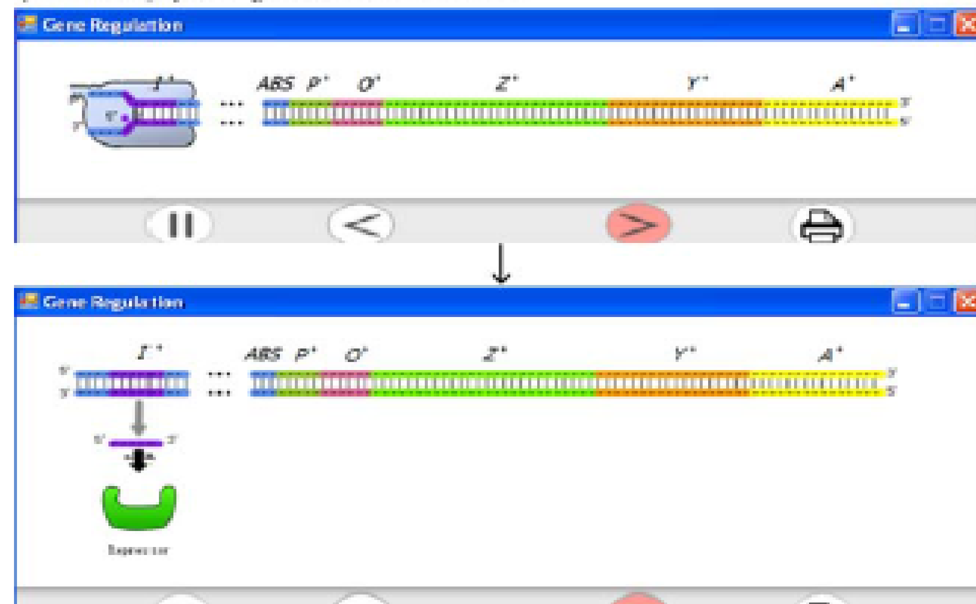
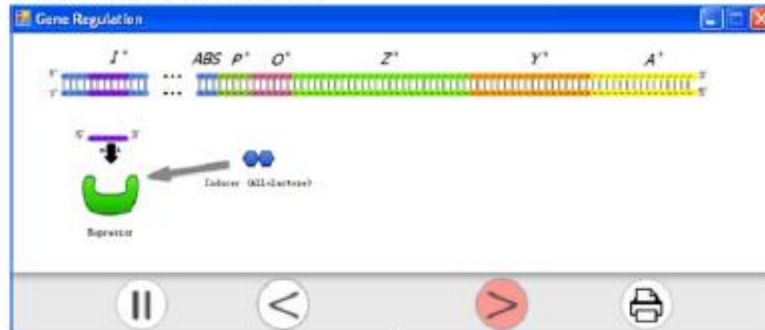
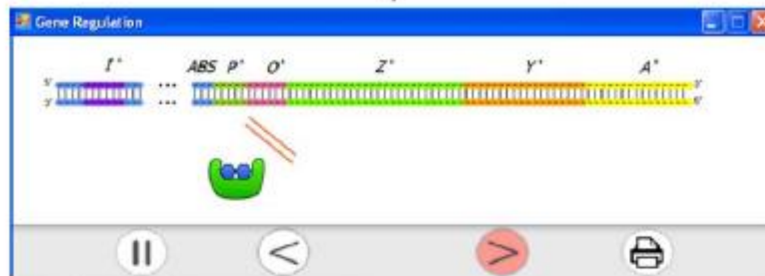


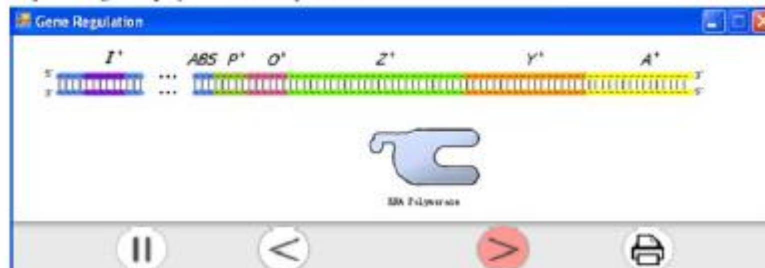
Figure 6 Positive regulation of the *lac* operon by the inducer allolactose (see online version for colours) (continued)

Step 2: Drag the inducer (allolactose) to the repressor to form an inducer-repressor complex, which can no longer bind to the operator





Step 3: Drag RNA polymerase to the promoter



Step 4: Bind RNA polymerase to the promoter (which is not blocked by the repressor) in preparation for transcription through animation (assume that an activator is bound to the ABS)