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INTRODUCTION TO RECOMBINANT DNA TECHNOLOGY

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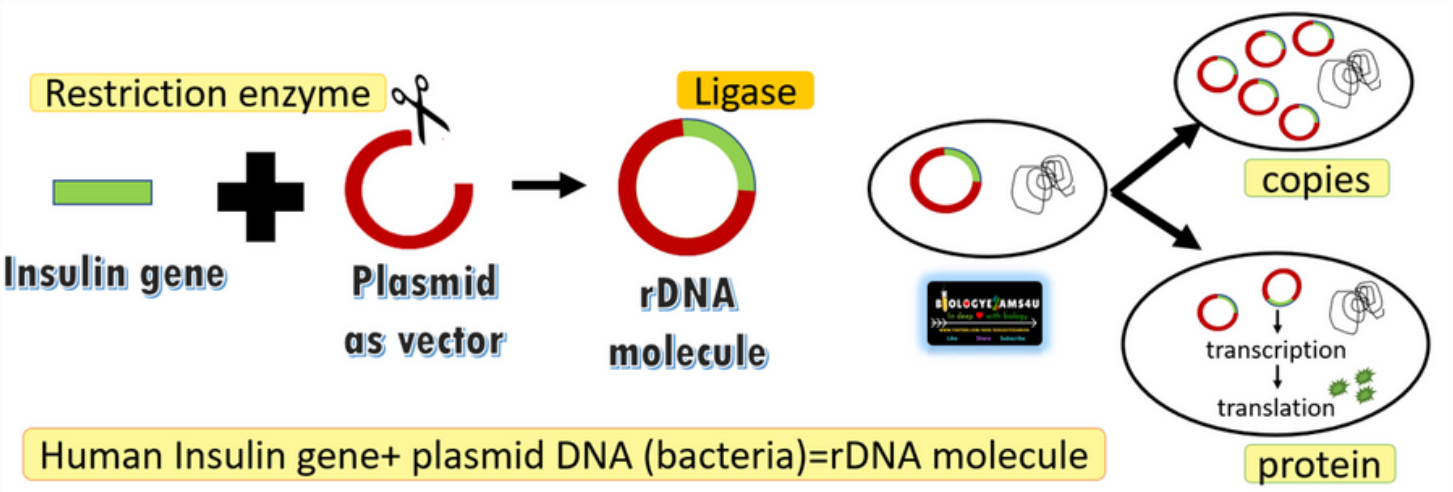
INTRODUCTION

The curd we make in our home, the wine, and flavoring using yeast all are products of biotechnology. What makes recombinant DNA technology different from classical biotechnology? In all the above processes mentioned, we are using the inherent capacity of microorganisms. Lets at take an example of rDNA technology to understand it better. Recombinant insulin is called Humulin, where we have introduced the human insulin gene in microorganisms like bacteria and yeast. These microorganisms serve as biofactories producing insulin protein. 'Flavr Savr' tomato is a genetically modified tomato with increased shelf life. In this case, the ripening gene is partially silenced using antisense technology, thereby, developing a GM tomato with an increased shelf life. In both examples, we have manipulated genes to make genetically modified organisms with desirable qualities. So Gene manipulation is the basis of rDNA technology.

WHAT IS RECOMBINANT DNA TECHNOLOGY?



It is the process of cutting and joining two different DNA molecules and inserting it into a host organism using a vector to produce copies or express the desired gene.

or

It is process that involves the identification, isolation and insertion of gene of interest into a vector such as a plasmid to form a recombinant DNA molecule and production of many copies of that gene fragment or product encoded by that gene inside the host.

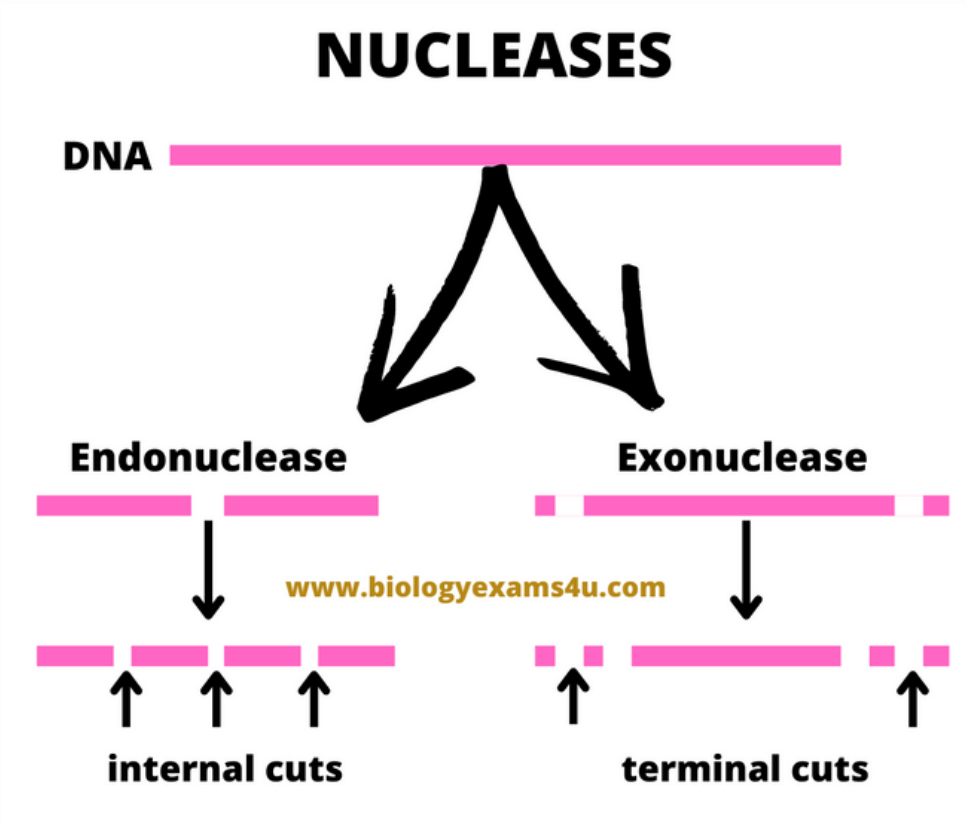
Examples of applications of rDNA technology:
Humulin: Human insulin produced using rDNA technology
Human insulin gene inserted into a vector and transferred to bacterium and bacteria acts as bio factories producing human insulin.

WHAT ARE RESTRICTION ENZYMES?

Restriction enzymes are enzymes that cuts double stranded DNA at unique sequence called recognition
sequence or restriction site

- Also called as molecular scissors, molecular knives or molecular scalpels
- **Hind II** is the first restriction nuclease isolated from *Haemophilus influenzae* bacteria.

Types of restriction Enzymes

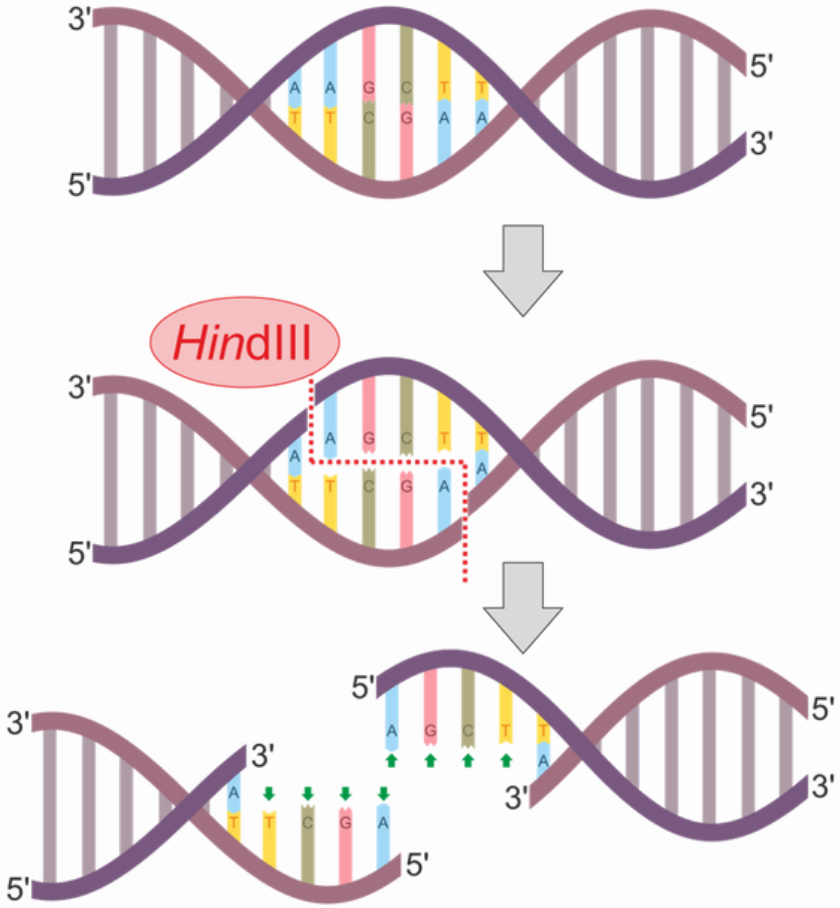


Two types:

1. Restriction endonuclease: makes cuts within the DNA molecule
2. Restriction exonuclease: remove nucleotides from the ends or terminal region of a DNA molecule

WHAT ARE RESTRICTION ENZYMES?

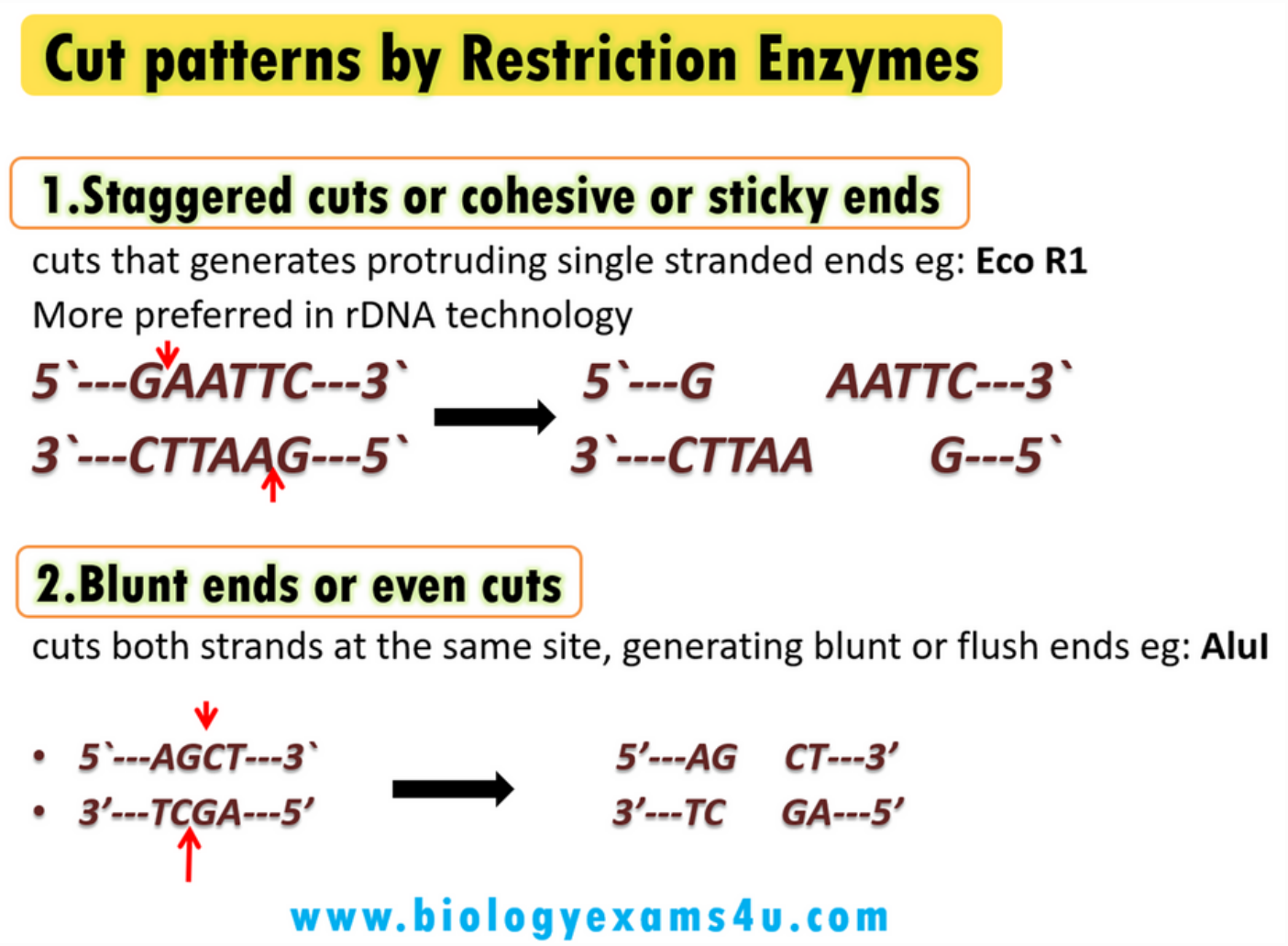
3 types of Restriction endonucleases:



- Type I, II and III
- Type II is used in rDNA technology as its makes cuts within recognition site.
  Eg: Eco R1, Hind III
- Mg2+ is required for cleavage
- Type I and Type III makes random cuts; therefore, rarely used in rDNA technology

WHAT ARE RESTRICTION ENZYMES?

How Restriction Enzymes cut DNA molecule and cut patterns?

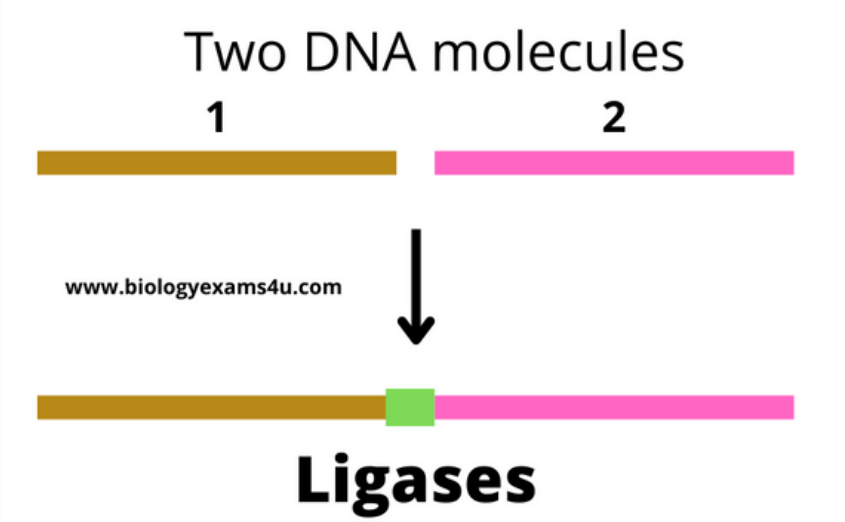


- Lets take an example
- Eco R1is a restriction enzyme isolated from *Escherichia coli* R1 strain
- EcoR1 Restriction site :5`---GAATTC---3`
  3`---CTTAAG---5`

Characteristics of restriction sites or recognition sequences

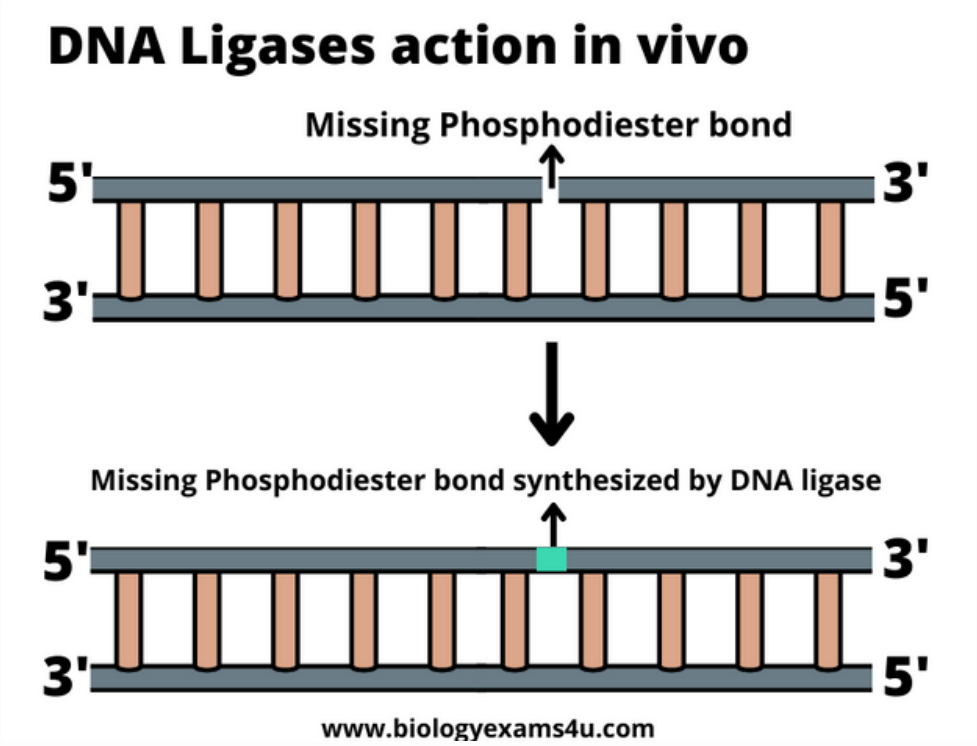
- Recognition sites are often 4-8 base pairs
- They are Palindromic sequences. Palindromic sequence is a DNA sequence of base pairs that reads the same on two strands in 5`-3` & 3`-5`direction.

WHAT ARE DNA LIGASES?



- Ligases are Joining enzyme that joins DNA fragments by forming phosphodiester bond. The process is called ligation.

How DNA ligase joins DNA molecule?



- Phosphodiester bond is between the 5'P of a nucleotide of one DNA fragment and the 3' OH end of the other nucleotide
- Requires ATP or NAD+ for its activity
- Eg: T4 ligase

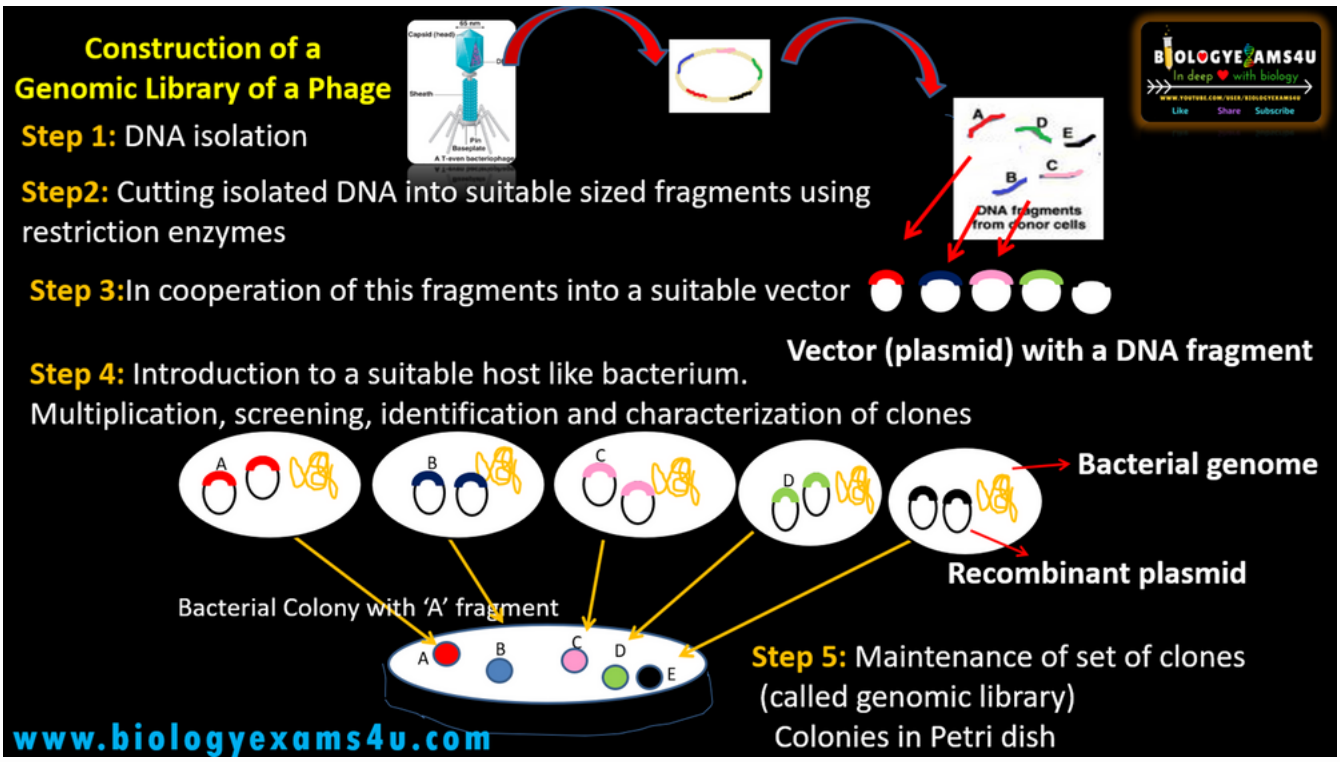
WHAT IS A GENOMIC LIBRARY?

- Genomic library represents an entire genome of an individual animal, bacteria, plant or virus under study.

or

- It is the collection of cloned segments of DNA containing at least one copy of every gene from a particular organism.

Construction of Genomic Library of a Phage



The procedure is same for all other organism.

Step 1: DNA isolation- Isolate complete DNA from the phage (or any cell under study)

Step2: Cutting isolated DNA with restriction enzymes like Eco R1 to suitable sized fragments.

Step 3: Incorporation or cloning of these fragments into suitable vectors like plasmid, cosmid etc forming rDNA molecule.

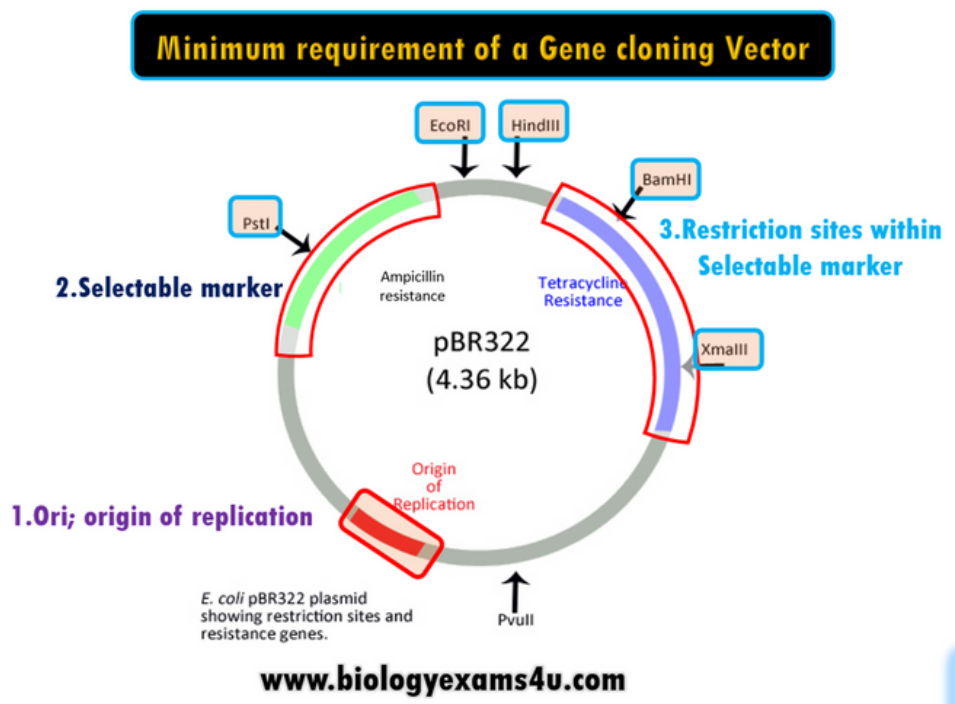
Step 4: Introduction to a suitable host like E.coli bacterium- Multiplication, screening, identification and characterization of clones. Plasmid will multiply inside forming numerous copies. We need to identify the host cells with these fragments from the rest of cells without rDNA molecule.

Step 5: Maintenance of set of clones- The host cell multiplies and forms colonies. Each colony contains cells with a DNA fragment of the phage. Maintenance of such clones or colonies containing all fragments of the phage represents genomic library of the phage.

WHAT ARE GENE CLONING VECTORS?

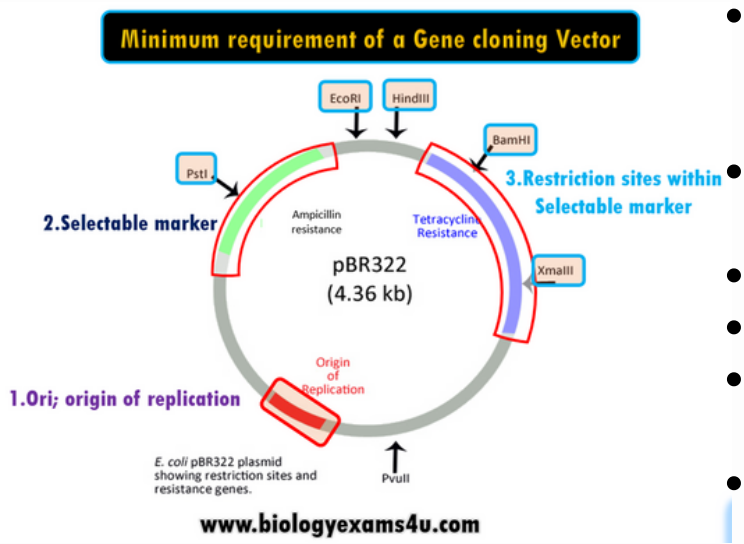
- Any DNA molecule that has the ability to replicate inside the host to which the desired gene is integrated for cloning-
- Cloning Vectors include plasmids, bacteriophages, cosmids, YAC, BAC, yeast vectors, shuttle vectors etc.
- Cloning vectors are used for obtaining millions of copies of the cloned DNA fragments for creating genomic libraries, preparing probes etc.
- Expression vectors are designed for expressing the protein encoded by the inserted gene. this vector contains control elements like promoter sequences

Ideal features of a Gene Cloning Vector



- Should contain an origin of replication for replicating inside host cell.
- Should have unique restriction sites as polylinkers or multiple cloning site (MCS) for inserting gene of interest.
- Should have one or more selectable marker genes for identification and isolation of transformed recombinant bacterial colonies containing recombinant vector.
- Should have a relaxed mode of replication.
- Should have unique restriction sites within selectable markers for selection of transformed recombinant colonies using insertional inactivation

DIFFERENT TYPES OF GENE CLONING VECTORS?



pBR 322:

- First artificial cloning vector (1977) constructed by Boliver & Rodriguez from E.coli plasmid.
- 4362 kb long widely used cloning vector.
- Insert size:~6-15 kb
- Application: protein Expression
- Advantage: Easy to handle, self replicating and stable
- Disadvantages:Small DNA insert size

Cosmid

- Hybrid gene cloning vector works like plasmid inside bacteria
- Cos sites, Plasmid ori, a selectable marker, Unique restriction sites
- Insert size:~45 kb
- Host: bacteria Eg: c2RB, pCP13, pRK290, Supercos 1
- Application: cloning of large genes, cDNA and genomic libraries
- Advantage: Large insert size
- Disadvantages: Phage packaging limitation, unstable inside E.coli

Different Types of Vectors

No	Cloning Vector	Host	Insert Size (kb)
1	Plasmid	E.coli	~10 kb
2	Bacteriophage	E.coli	9- 22 kb
3	P1 phage	E.coli	70-100 kb
4	Cosmids	E.coli	33- 47 kb
5	BAC	E.coli	75-350 kb
6	YAC	Yeast	1000-3000 kb
7	Human Artificial Chromosomes (HACs)	*Cultured Human Cells*	>2000kb

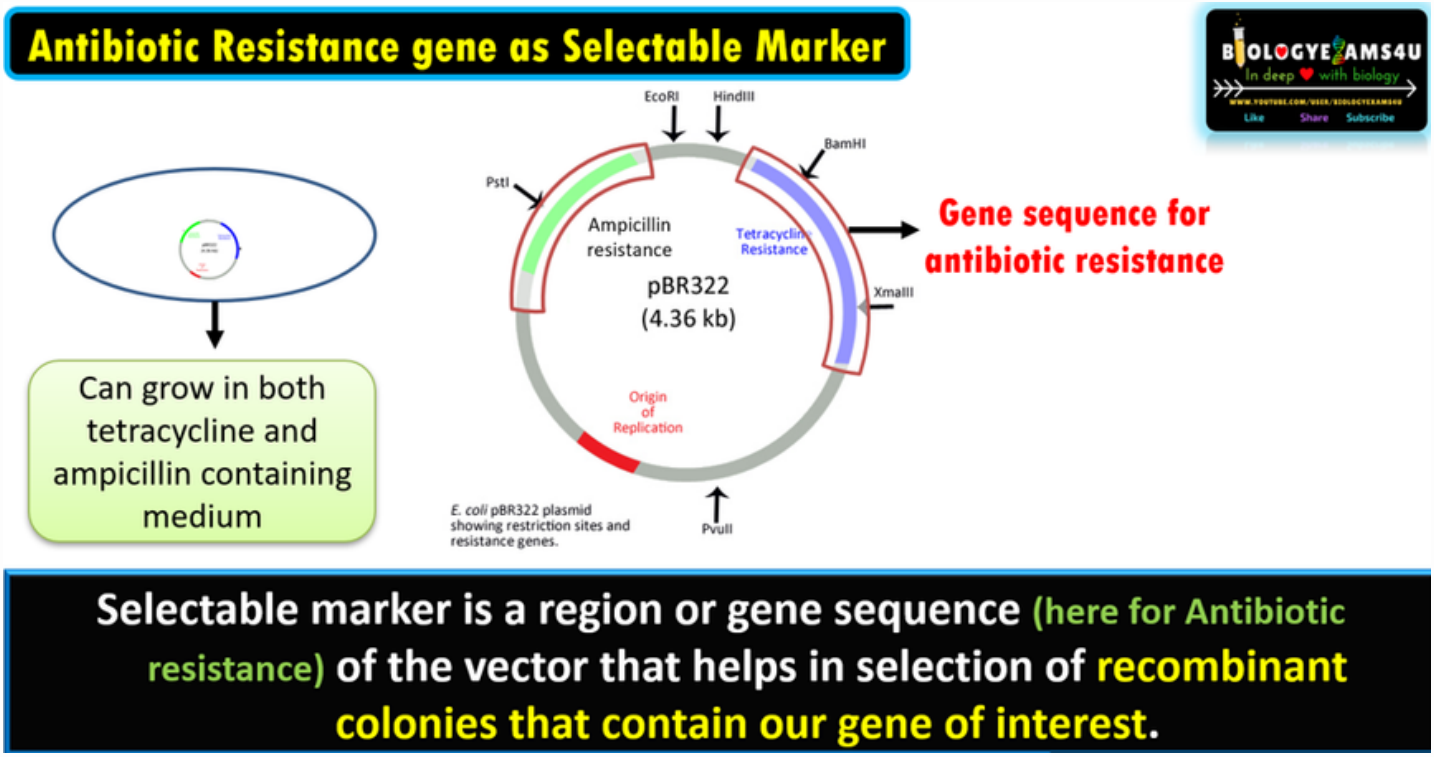
BAC (Bacterial Artificial Chromosome): A vector used to clone DNA fragments of 100-300 kb insert size in E.coli cells. Based on the naturally occurring f factor plasmid found in the bacterium E.coli.

YAC (Yeast Artificial Chromosome): A vector of hundreds to kilobases long used for cloning of large DNA fragment.

WHAT ARE SELECTABLE MARKERS?

Selectable marker is a region or gene sequence (here for Antibiotic resistance) of the vector that helps in
selection of recombinant colonies that contain our gene of interest.

How do Antibiotic Resistance Genes function as Selectable marker?



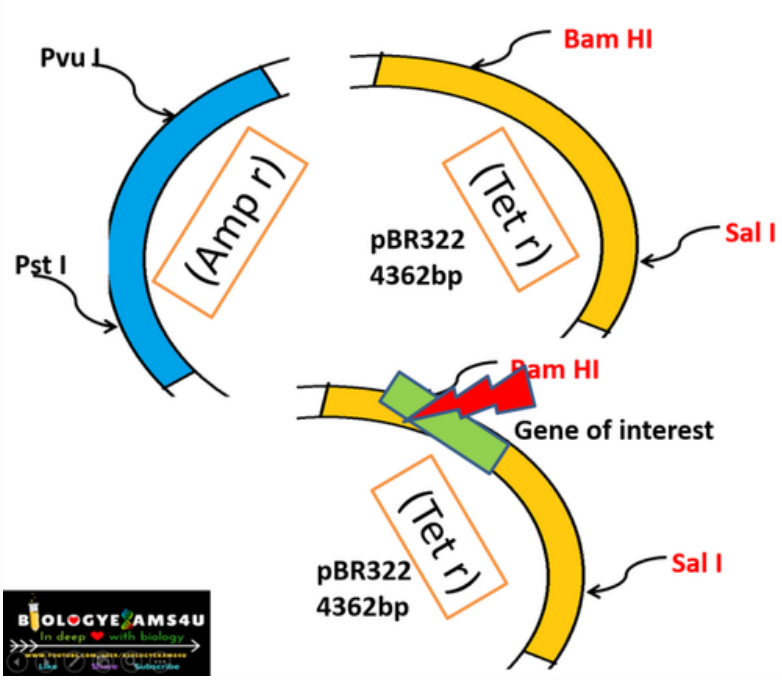
Let's take pBR322 vector as an example to understand this concept. pBR322 vector has genes that encodes polypeptides which confer resistance to ampicillin and tetracycline antibiotics. That means a bacterium with this vector can grow both tetracycline and ampicillin containing medium (Figure above).

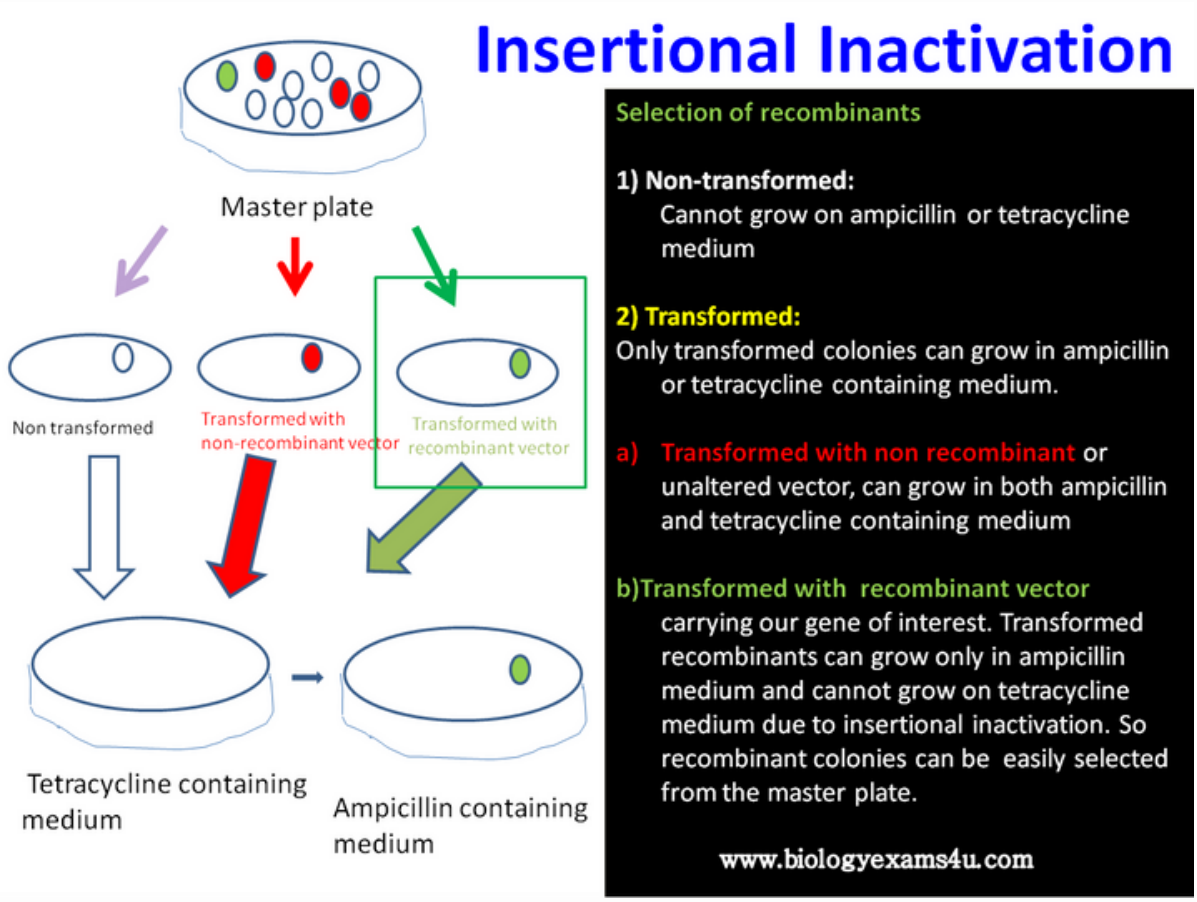
Insertional inactivation helps in the selection of recombinant colonies with our gene of interest.
Insertional inactivation is the inactivation of a gene upon insertion of another gene within its gene sequence.
The inserted gene disrupts continuity of the gene sequence, thereby making it inactive or non-functional.

How do Antibiotic Resistance Genes function as Selectable marker?

In the given example (see this figure), we have inserted our gene of interest in the tetracycline gene coding region. So that, tetracycline resistance gene is no more functional. This process is called insertional inactivation.

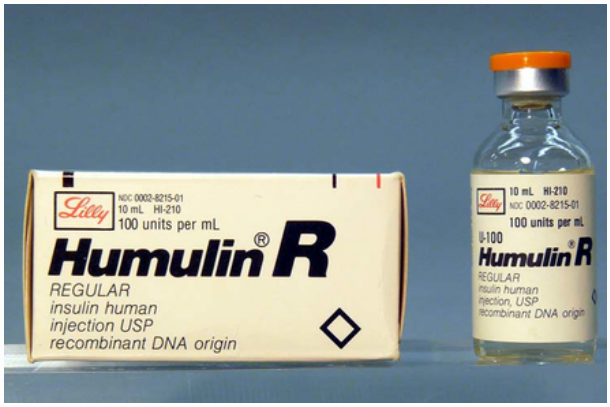
Insertional inactivation is the inactivation of a gene upon insertion of another gene within its gene sequence.





Insertional inactivation helps in the selection of recombinant colonies. Recombinant colonies with desired gene inserted at tetracycline coding region can grow only in ampicillin containing medium, whereas transformed colonies with unchanged vector can grow in both tetracycline and ampicillin medium. We can select the recombinant colonies by comparing the position of the colonies after replica plating with the master plate. (Understand More on replica plating)

Steps in the production of Recombinant Insulin or Humulin

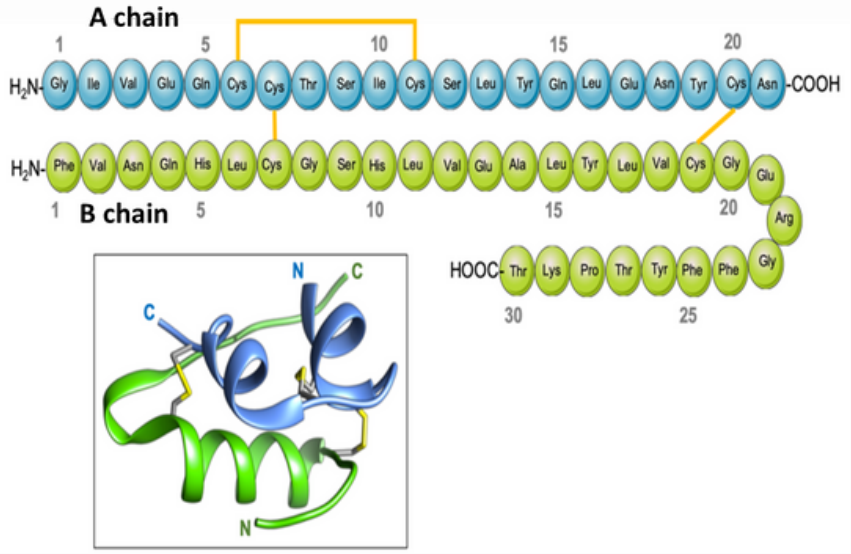


History of recombinant insulin or Humulin

Humulin, is the insulin developed using rDNA technology which is used to treat diabetes. It was developed by David Goeddel and his colleagues of Genetech of USA and later marketed by Lilly under the trade name Humulin. It is the first approved drug created through r DNA technology. Here insulin is synthesized inside bacterium where we introduced human insulin gene. Thus bacterial system just works as bio-factories for the synthesis of insulin.

What are the advantageous of recombinant insulin?

- Large quantities in short time.
- No risk of transferring infections.
- No allergic reactions (compared to cow and pig insulin)
- No ethical issues concerning slaughtering and the use of animals.

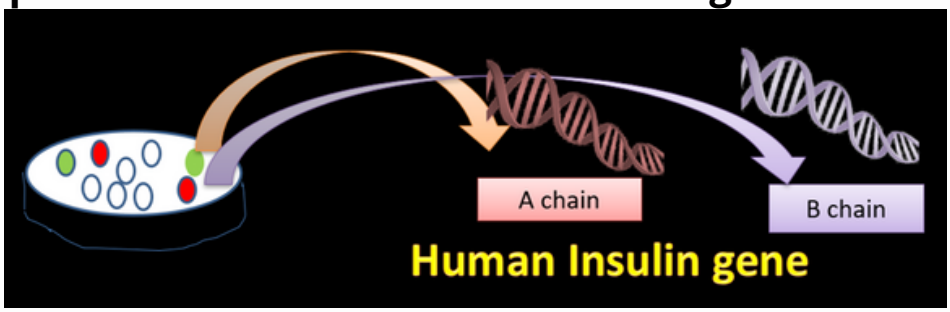


Insulin is a hormone that regulates blood glucose level. Frederick Sanger discovered the **structure of insulin**. Insulin protein is made up of 2 chains; A chain and B chain with 21 and 30 amino acids respectively.

The chains are joined by di-sulphide bond. Insulin is a comparatively simple human protein, enabling its development by r DNA technology.

Steps in the production of Recombinant Insulin or Humulin

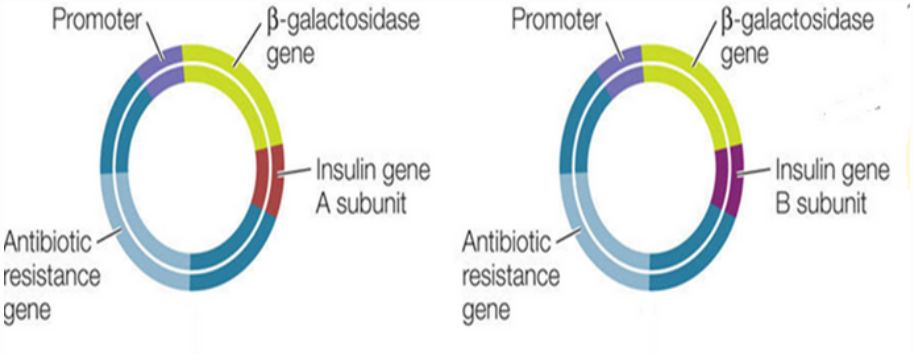
Step1: Identification and isolation of gene of interest



From where we get the desired gene?
In the case of insulin, A chain gene, B chain gene from

- cDNA library (as it has no introns)
- Chemical synthesis of gene. This is the most preferred method.
- Isolate the gene from tissues and gene amplification using PCR

Step II: joining of this gene into a suitable vector (construction of recombinant DNA)

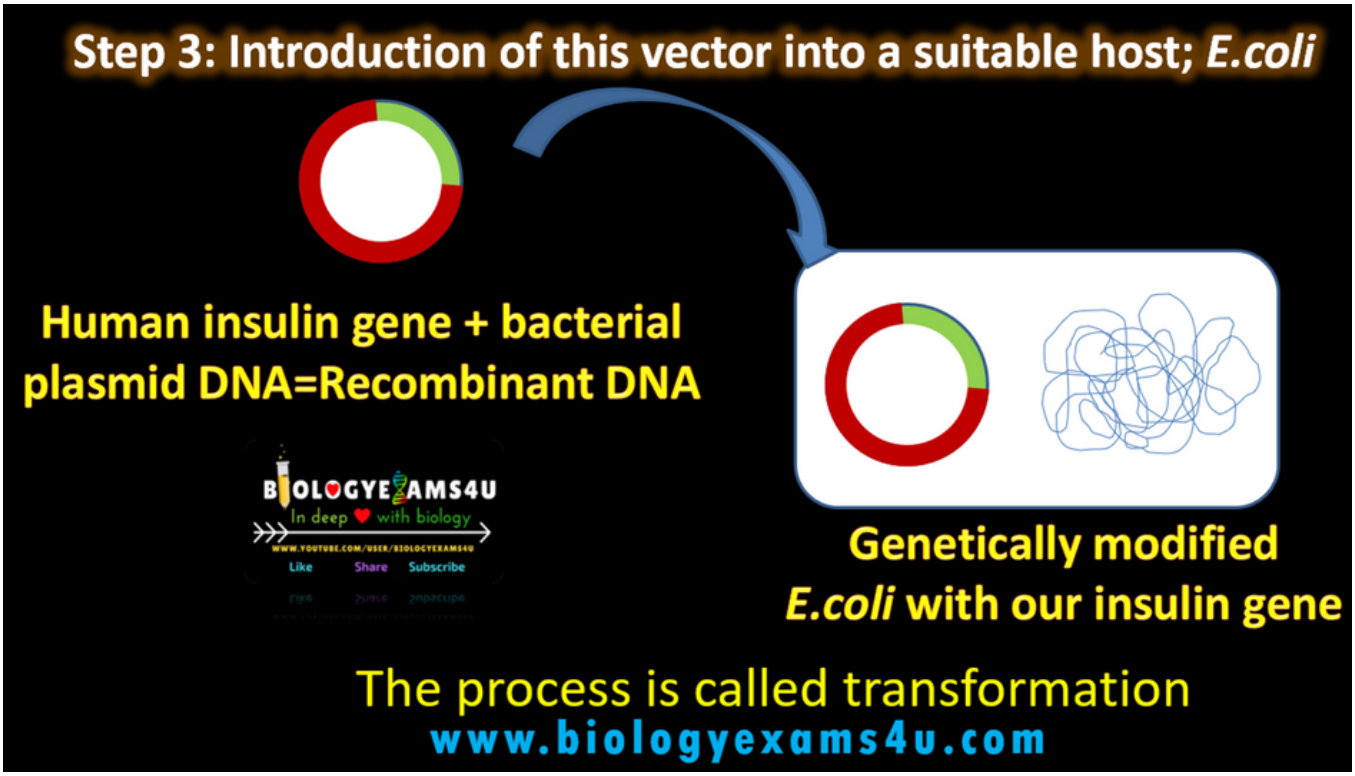


What is a Gene Cloning Vector?

- A vector is any DNA molecule which is capable of multiplying inside the host to which our desired gene is integrated for cloning. The selection of vector depends upon the size of the fragments to be cloned.
- Common vectors include plasmids (Eg: pBR 322)
- In the process, restriction enzymes functions as scissors for cutting DNA molecules. Ligase enzyme is the joining enzyme that joins the vector DNA with gene of interest. The resulting DNA is called the recombinant DNA, chimera or recombinant vector.
- In the case of recombinant Insulin, A chain and B chin are made separately in two cultures
- A gene construct with a promoter, β-galactosidase, insulin A chain gene with other features of vectors like selectable markers (antibiotic resistance gene for selection of transformed colonies), ori etc. Insulin gene is placed next to β-galactosidase to form a fusion protein.
- A second construct with a promoter, β-galactosidase, insulin B chain gene

Steps in the production of Recombinant Insulin or Humulin

Step III: Introduction of this vector into a suitable organism/host



Introduction of recombinant vector into host cell is achieved by different gene transfer methods

Physical gene transfer methods:

- Electroporation
- Microinjection
- Liposome mediated gene transfer
- Silicon Carbide fiber mediated gene transfer
- Ultrasound mediated gene transfer
- DNA transfer via pollen

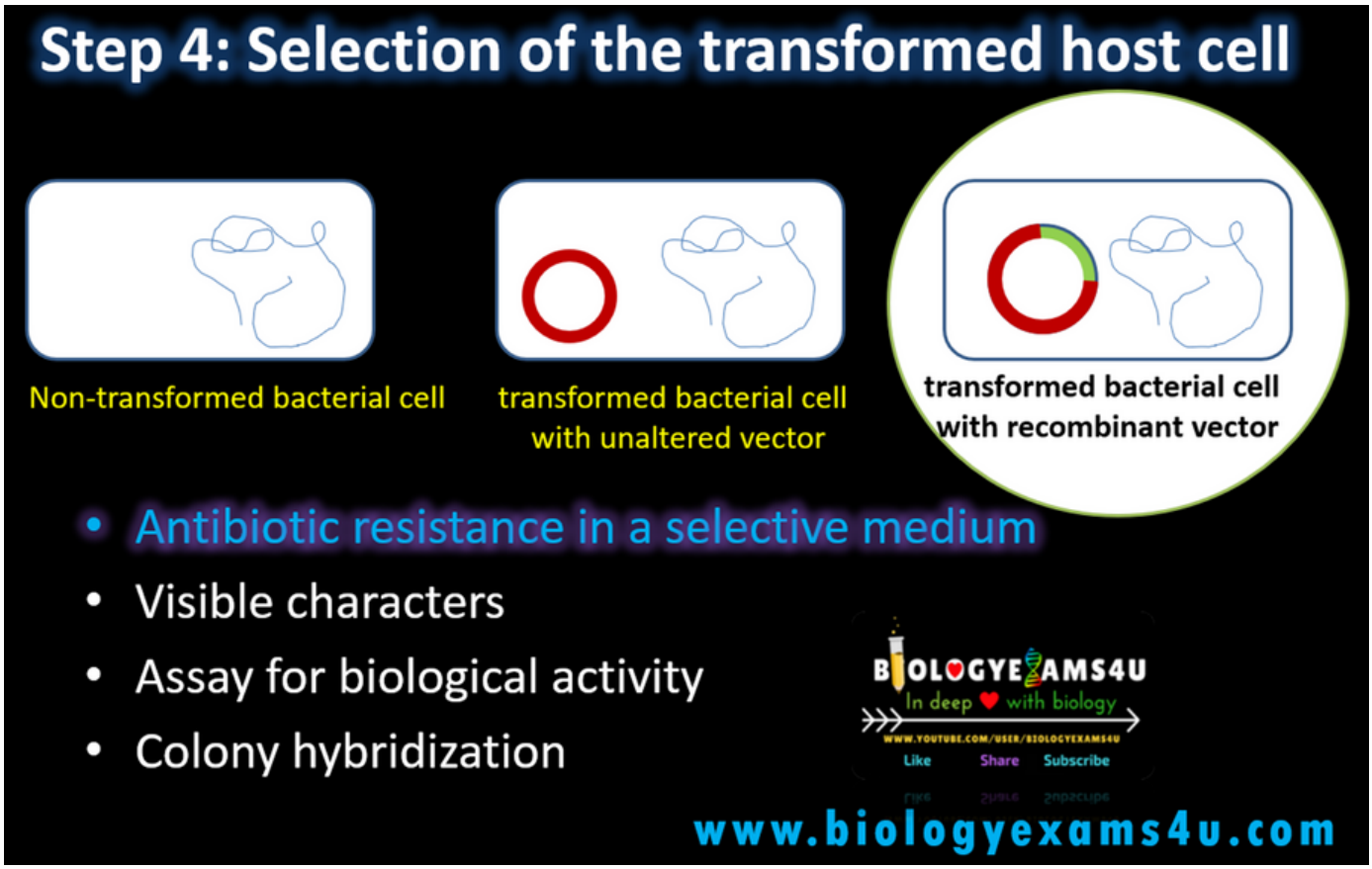
Chemical gene transfer methods:

- Poly Ethylene Glycol mediated (PEG mediated)
- Calcium Chloride mediated
- DEAE dextran mediated gene transfer

DNA imbibition's by cells, tissues or organs: Transformation

Agrobacterium mediated gene transfer in plants

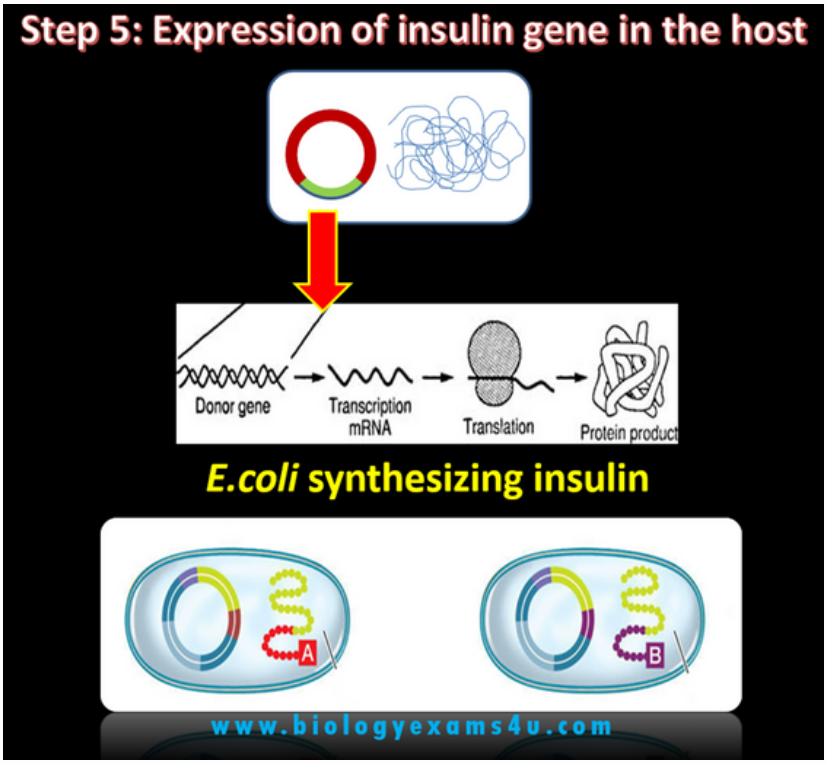
Step VI: Selection of transformed recombinant cells with gene of interest



The number of cells with recombinant vector will be very less. So the next step is to select the transformed recombinant cells with our gene of interest from the sea of non transformed cells.
Several methods are employed for selection of transformed cells:

- Antibiotic resistance,
- Visible characters,
- Assay for biological activity,
- Colony hybridization,
- Blotting test.
- Isolation of DNA from colonies and sequencing to find
- out presence of gene
- DNA isolation followed by PCR amplification using gene specific primers
- The selected cells are cultured in large scale.

Step V: Expression of the gene of interest



For expression of the desired gene, expression vector is used (vector with control elements like promoter).

Lactose, the substrate of β-galactosidase in the medium induces gene expression thus ensuring efficient transcription of our protein of interest also. The product is synthesized in mass cultures in large quantities in fermentation bioreactors. Now Fusion protein is formed in separate cultures.

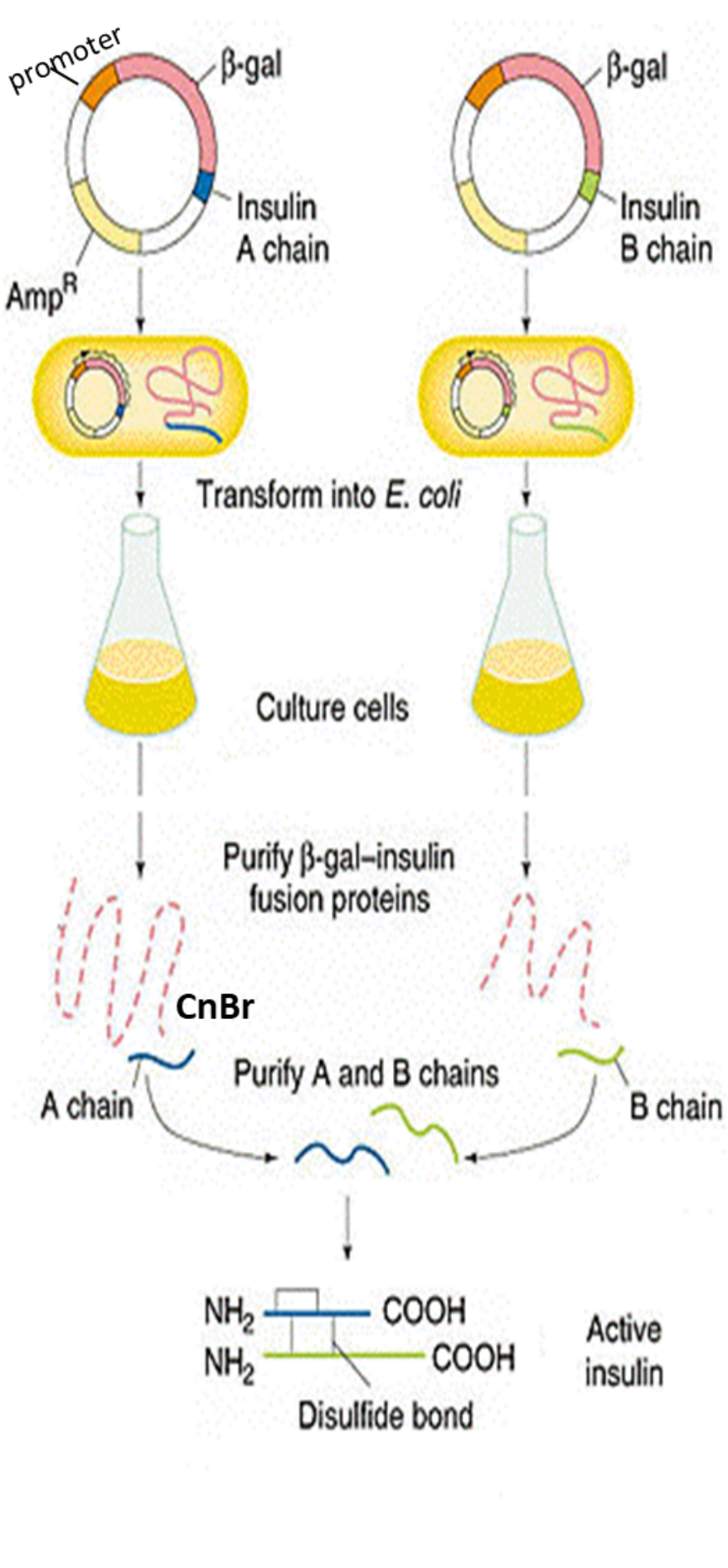
Step VI: Purification of Protein

We get fusion protein upon translation. That is β-galactosidase-Insulin A fusion protein and β-galactosidase-Insulin B fusion protein.

In recombinant insulin production, Fusion protein of Insulin A chain and B chain is formed with fusion partner β-galactosidase. β-galactosidase enables easy purification by affinity chromatography. Finally separate Insulin A chain from β-galactosidase by using cyanogen bromide (CnBr) which cleaves

within methionine residues. Thus we get insulin A chain and B chain in separate cultures. Then the purified A chain and B chain are joined by di-sulphide bond under appropriate condition to form functional insulin.

Steps in the production of Recombinant Insulin or Humulin



PRODUCTS OF RECOMBINANT DNA TECHNOLOGY

rDNA product	Trade name	Application	Company
Insulin	Humulin	Diabetes	Eli Lilly
Human Growth Hormone	Humatrope	Pituitary Dwarfism	Eli Lilly, Genentech
Erythropoietin	Epogen	Anemia	Amgen
Factor VIII	Advate	Hemophilia	Baxter
DNAse I	Dornase alfa	Cystic fibrosis	Genentech
Interferons	Intron A	Hepatitis B, C, multiple sclerosis	Schering-Plough
Tissue Plasminogen Activator (TPA)	Activase	Acute myocardial infarction	Genentech
GM crops			www.biologyexams4u.com
Potato (USA), squash/pumpkin (USA) alfalfa (USA), aubergine (Bangladesh), sugar beet (USA, Canada), papaya (USA and China), oilseed rape (4 countries), maize (corn) (17 countries), soya beans (11 countries) and cotton (15 countries).			

'Flavr Savr' tomato is a genetically modified tomato with increased shelf life.

It was the first commercially grown genetically engineered crop developed using antisense technology.

Recombinant Covid vaccines like mRNA based vaccines

Golden rice: produced through genetic engineering to biosynthesize beta-carotene, a precursor of vitamin A. It is developed with an aim to mitigate dietry Vitamin A deficiency

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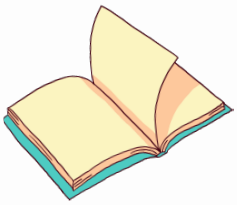

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