B’ PHARMACY
SUBJECT-PHARMACEUTICAL BIOTECHNOLOGY
SUBJECT CODE- BP605T

IMMUNOBLOTTING TECHNIQUES

Venkatesh Naik
Assistant Professor
Balaji College Of Pharmacy

OBJECTIVE OF COURSE

Genetic Engineering application in relation to production of Pharmaceutical

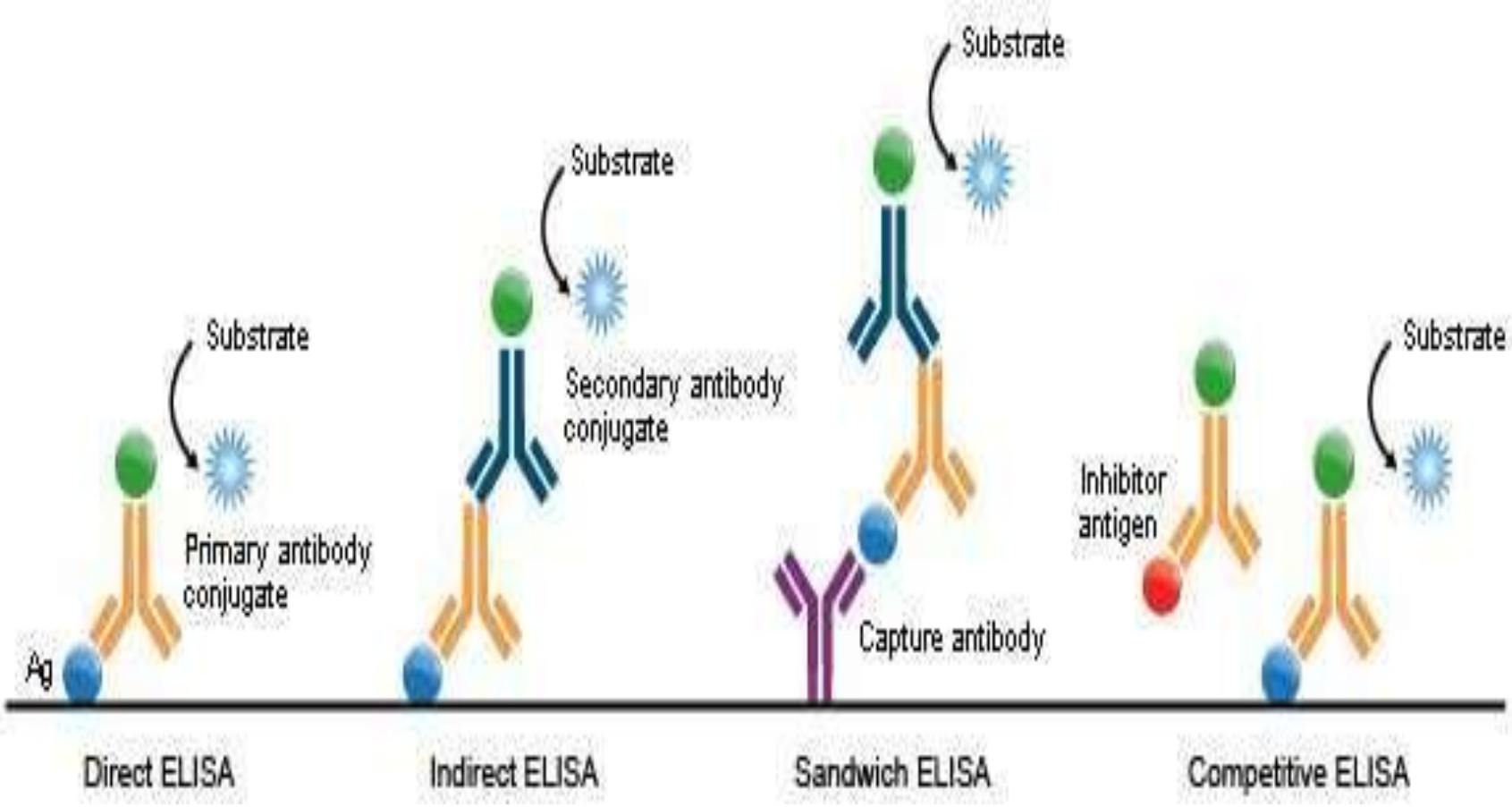
LEARNING OUTCOME:

Students will learn about ELISA and blotting techniques. They will also learn about microbial transformation and mutation.

ELISA

Enzyme-linked immunosorbent assay (ELISA): ELISA stands for "enzyme-linked immunosorbent assay." This is a rapid immunochemical test that involves an enzyme (a protein that catalyzes a biochemical reaction). It also involves an antibody or antigen (immunologic molecules). ELISA tests are utilized to detect substances that have antigenic properties, primarily proteins (as opposed to small molecules and ions such as glucose and potassium). Some of these include hormones, bacterial antigens and antibodies

TYPES OF ELISA



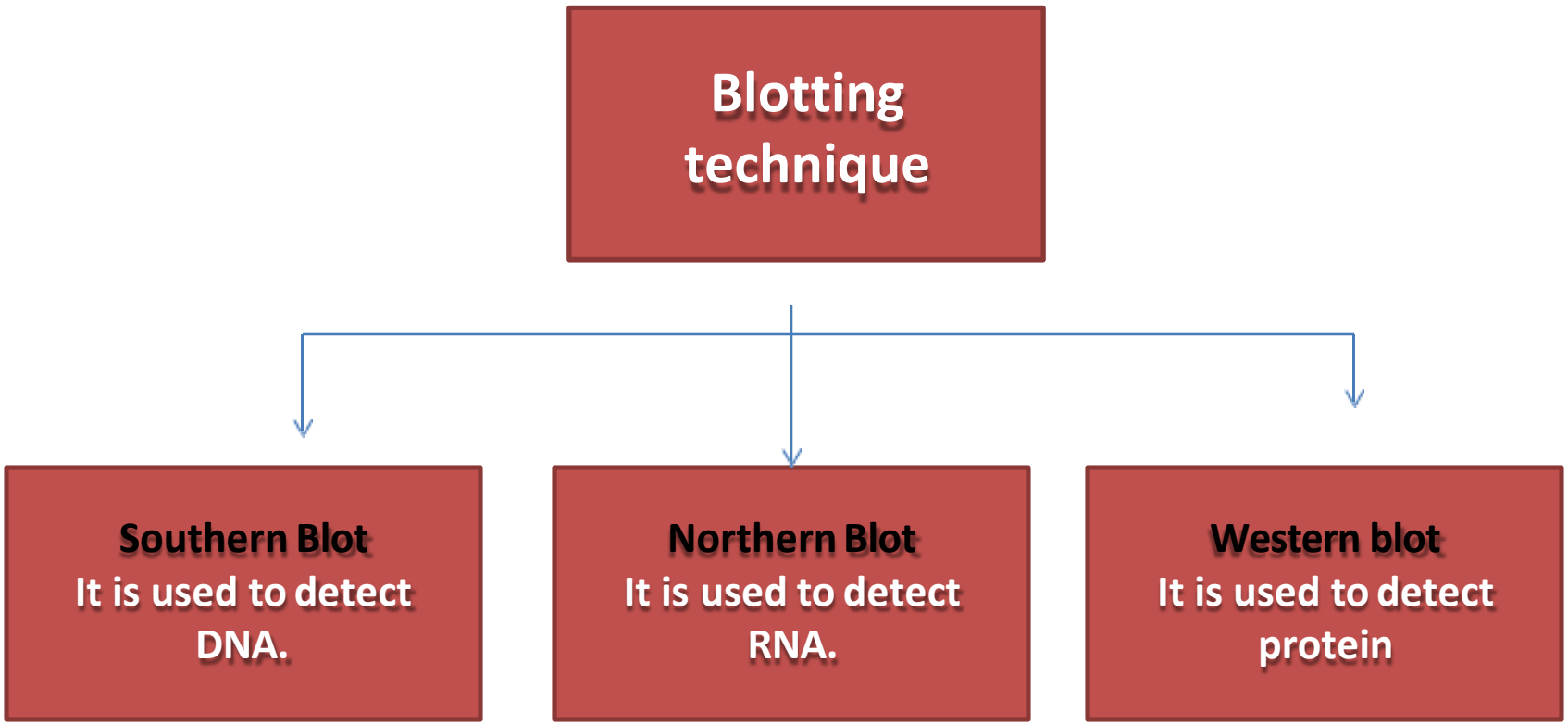
Detection methods	Applications
Indirect ELISA	Gill-rot disease
	Infectious pancreatic necrosis virus
	Reovi virus
	Aeromonas hydrophila
	Turbot hemorrhagic virus
	Lateolabrax vibrio anguillarum
Sandwich ELISA	Grass carp hemorrhage virus
	Rainbow trout IPNV
	Infectious hematopoietic necrosis virus
Dot-ELISA	Aeromonas septicemia
	Bacterial fish disease
mAbs ELISA	VHSV
	WSSV
BAS-ELIS	Vibrio parahaemolyticus

DEFINITION OF BLOTTING

- Visualization of specific DNA , RNA & protein among many thousands of contaminating molecules requires the convergence of number of techniques which are collectively termed **BLOT transfer** .

TYPES OF BLOTTING TECHNIQUES

w



IMMUNOBLOTTING

- Viral antigens are detected with a polyclonal or a MAb onto nitrocellulose paper.
- After incubation, the protein bands (immune complexes) are visualized with peroxidase-conjugated protein and a colour reagent.
- A colour develops in the bands where antibody binds to the antigen.
- Immunoblotting assay mixture of this two technique.

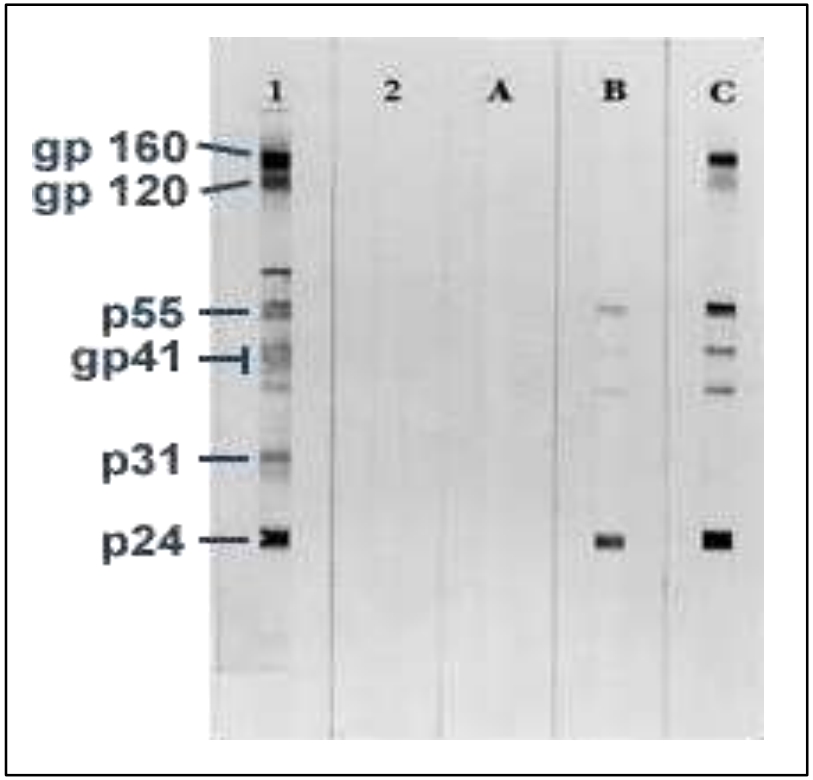
WESTERN BLOTTING

- Western blotting is based on the principles of immunochromatography where proteins were separated into poly acrylamide gel according to the isoelectric point and molecular weight.
- A technique for detecting specific proteins separated by electrophoresis by use of labeled antibodies.
- Immunoblotting is performed chiefly in diagnostic laboratories to identify the desirable protein antigens in complex mixtures.
- An improved immunoblot method **Zestern analysis**, is able to address this issue without the electrophoresis step, thus significantly improving the efficiency of protein analysis.
- Other related techniques include **dot blot analysis, zestern analysis, immunohistochemistry** where antibodies are used to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

WESTERN BLOT

Western Blot

- Lane1: Positive Control
- Lane 2: Negative Control
- Sample A: Negative
- Sample B: Indeterminate
- Sample C: Positive



CONTENTS

- Tissue preparation
- Gel electrophoresis
- Transfer
- Blocking
- Detection
- Analysis
- Applications

TISSUE PREPARATION

- Samples may be taken from whole tissue, from cell culture, bacteria, virus or environmental samples.
- In most cases, samples are solid tissues.
- First broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication.
- Cells may also be broken open by one of the above mechanical methods.
- A combination of biochemical and mechanical techniques, including various types of filtration and centrifugation.
- To encourage lysis of cells and to solubilize proteins, may be employed : **detergents, salts, and buffers**
- To prevent the digestion of the sample by its own enzymes **-Anti Protease and phosphatase**
- To avoid protein denaturing-Tissue preparation is often done at cold temperatures

GEL ELECTROPHORESIS

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point molecular weight, electric charge, or a combination of these factors. Commercially SDS-PAGE gel electrophoresis for protiens.

POLYACRYLAMIDE GEL

- Polymerized gel:

1. **Resolving gels** made in 6%, 10%, 12%, 18%.
2. **Stacking Gel** up to 5% was added to gel and then the wells are created.

- The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample.
- The smaller it is the bigger the percentage.

SDS-PAGE (POLYACRYLAMIDE GEL ELECTROPHORESIS)

- SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology
  - to separate proteins according to their electrophoretic mobility.
  - to separate proteins according to their size, and no other physical feature.
- SDS (the detergent soap) breaks up hydrophobic areas and coats proteins with negative charges thus overwhelming positive charges in the protein.
- Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubilized by the detergent and all the proteins will be covered with many negative charges.

SDS PAGE

- The Pink Strands are the denatured Proteins covered in the negatively charged SDS .
- See varying size they Are traveling to the positive since they have negative charge.
- These strands go throught the tunnel and are seperated by their size.

PAGE

- If the proteins are denatured and put into an electric field (only), they will all move towards the positive pole at the same rate, with no separation by size.
- However, if the proteins are put into an environment that will allow different sized proteins to move at different rates.
- The environment is polyacrylamide.
- the entire process is called polyacrylamide gel electrophoresis (PAGE).
- Small molecules move through the polyacrylamide forest faster than big molecules, big molecules stays near the well.

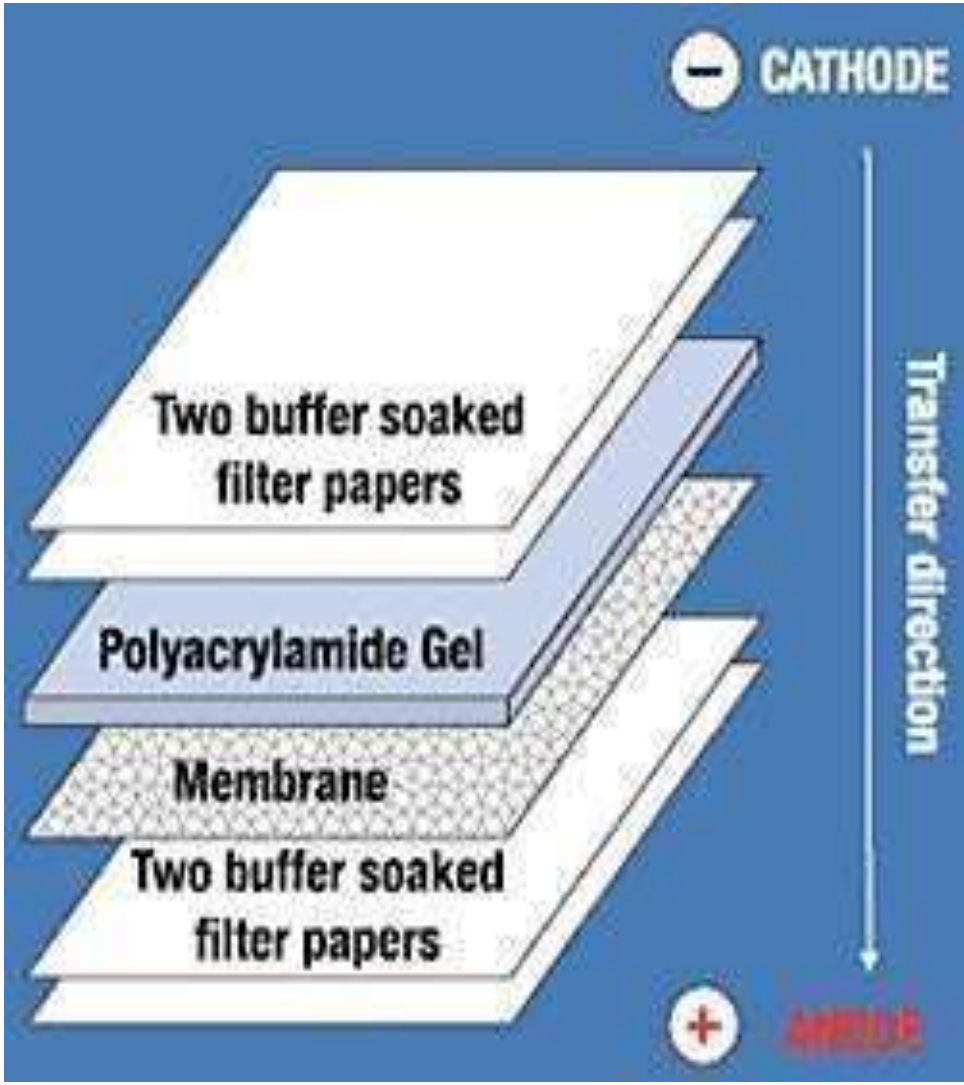
TRANSFER

- In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of **nitrocellulose or polyvinylidene difluoride (PVDF)** . The membrane is placed on top of the gel, and a stack of filter papers placed on top of that.
- The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.
- Another method for transferring the proteins is called **electrobloting** and uses an electric current to pull proteins from the gel into membrane.

- Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.
- The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with coomassie or ponceau S dyes.
- Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.

BLOTTING

- Blotting used to transfer the samples from the gel on to a membrane such as a **nylon membrane** or **nitrocellulose membrane**.
- Analyzed through probing with nucleic acid probes or antibody probes.



BLOCKING

- Steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (since the antibody is a protein itself).
- Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein.
- Typically **Bovin Serum Albumin (BSA)** or **non-fat dry milk** (both are inexpensive), with a minute percentage of detergent such as **Tween20**.

* The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached.

* This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

DETECTION

- The membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour.
- - Two step
- Primary antibody {Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof)}.
- A dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation for anywhere from 30 minutes to overnight at different temperatures.
- The solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA.

- Secondary antibody {After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody }.
- The secondary antibody is usually linked to biotin or to a reporter enzyms such as alkalin phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.
- Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein.
- A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% horseradish peroxidase; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.

❖ ONE STEP

- Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes.
- One-step probing systems that would allow the process to occur faster and with less consumables.
- This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known proteins tags.

ANALYSIS

- In practical terms, not all Westerns reveal protein only at one band in a membrane.
- Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis.
- The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples.
- This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

1. COLORIMETRIC DETECTION :

- This method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody.
- This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane.
- Protein levels are evaluated through densitometry or spectrophotometry.

3. RADIOACTIVE DETECTION

- Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest.
- Very expensive, health and safety risks are high.

4. FLUORESCENT DETECTION

- The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera.
- Allows further data analysis such as molecular weight analysis and a quantitative western blot analysis.
- The most sensitive detection methods for blotting analysis.

APPLICATIONS

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.
- A western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, western blot is sometimes used to confirm FIV+ status in cats.

WHEN SHOULD WB BE USED

- Western blot assay should not be used as a **screening test**.
- Wb should be viewed as a **supplemental test** which can be used to **confirm positive results** obtained from **enzyme immuno assay (EIA).**
- However:
  - Specificity is less than that of EIA.
  - A significant number of indeterminate blots are seen in low risk populations.

DISADVANTAGES

- If a protein is degraded quickly, Western blotting won't detect it well.
- This test takes longer that other existing tests.
- It might also be more costly.

Mutation

Introduction

- Sudden heritable change in genetic material or character of an organism is known as **mutation**
- Individuals showing these changes are known as **mutants**
- An individual showing an altered phenotype due to mutation are known as **variant**
- Factor or agents causing mutation are known as **mutagens**
- Mutation which causes changes in base sequence of a gene are known as **gene mutation** or **point mutation**

History

- English farmer **Seth Wright** recorded case of mutation first time in 1791 in male lamb with unusual short legs
- The term mutation is coined by **Hugo de Vries** in 1900 by his observation in Oenothera
- Systematic study of mutation was started in 1910 when **Morgan** genetically analyzed white eye mutant of Drosophila
- **H. J. Muller** induced mutation in Drosophila by using X- rays in 1927 ; he was awarded with Nobel prize in 1946

Characteristics of Mutation

- Generally mutant alleles are recessive to their wild type or normal alleles
- Most mutations have harmful effect, but some mutations are beneficial
- Spontaneous mutations occurs at very low rate
- Some genes shows high rate of mutation such genes are called as mutable gene
- Highly mutable sites within a gene are known as hot spots.
- Mutation can occur in any tissue/cell (somatic or germinal) of an organism

Classification of mutation

- **Based on the survival of an individual**

1. **Lethal mutation** – when mutation causes death of all individuals undergoing mutation are known as lethal

2. **Sub lethal mutation -** causes death of 90% individuals

3. **Sub vital mutation**– such mutation kills less than 90% individuals

4. **Vital mutation -**when mutation don't affect the survival of an individual are known as vital

5. **Supervital mutation –** This kind of mutation enhances the survival of individual

- **Based on causes of mutation**

1. **Spontaneous mutation-**Spontaneous mutation occurs naturally without any cause. The rate of spontaneous mutation is very slow eg- Methylation followed by deamination of cytosine. Rate of spontaneous mutation is higher in eukaryotes than prokaryotes.
   Eg. UV light of sunlight causing mutation in bacteria

2. **Induced Mutation-**Mutations produced due to treatment with either a chemical or physical agent are called induced mutation .The agents capable of inducing such mutations are known as mutagen.use of induced mutation for crop improvement program is known as **mutation breeding.**
   Eg. X- rays causing mutation in cereals

- **Based on tissue of origin**

1. Somatic mutation-

A mutation occurring in somatic cell is called somatic mutation.

In asexually reproducing species somatic mutations transmits from one progeny to the next progeny

2. Germinal Mutation-

When mutation occur in gametic cells or reproductive cells are known as germinal mutation.

In sexually reproductive species only germinal mutation are transmitted to the next generation

- **Based on direction of mutation**

1.Forward mutation- When mutation occurs from the normal/wild type allele to mutant allele are known as forward mutation

2.Reverse mutation- When mutation occurs in reverse direction that is from mutant allele to the normal/wild type allele are known as reverse mutation

- **Type of trait affected**

1. **Visible mutation-** Those mutation which affects on phenotypic character and can be detected by normal observation are known as visible mutation

2. **Biochemical mutation-** mutation which affect the production of biochemicals and which does not not show any phenotypic character are known as biochemical mutation

Chromosome Mutations

- May Involve:
  - **Changingthe structure**

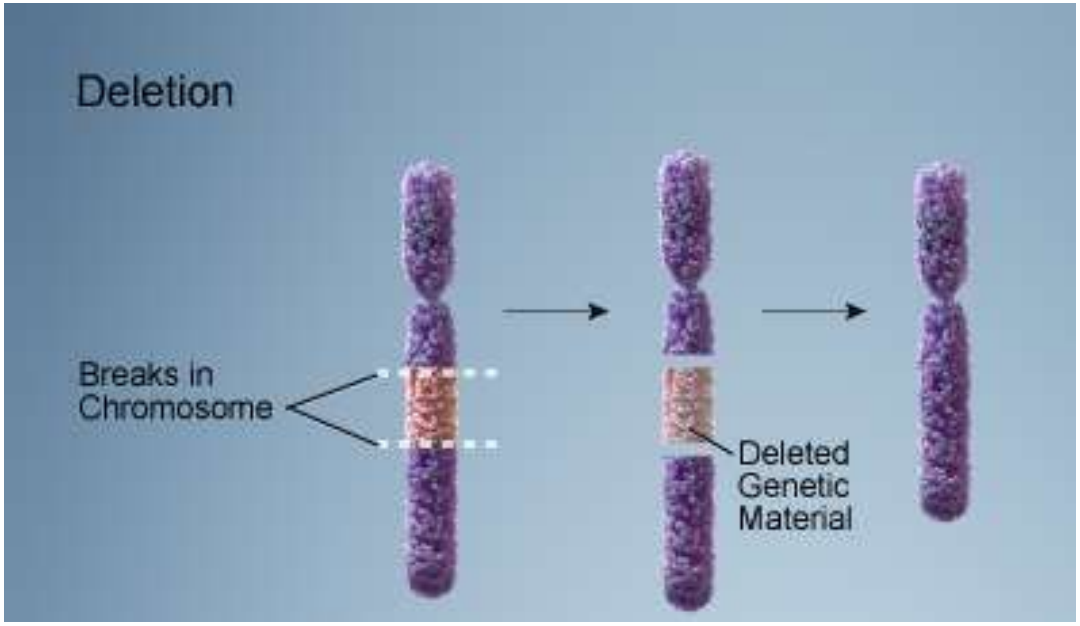
loss or gain

Chromosome Mutations

- Five types exist:
  - **Deletion**
  - **Inversion**
  - **Translocation**
  - **Nondisjunction**
  - **Duplication**

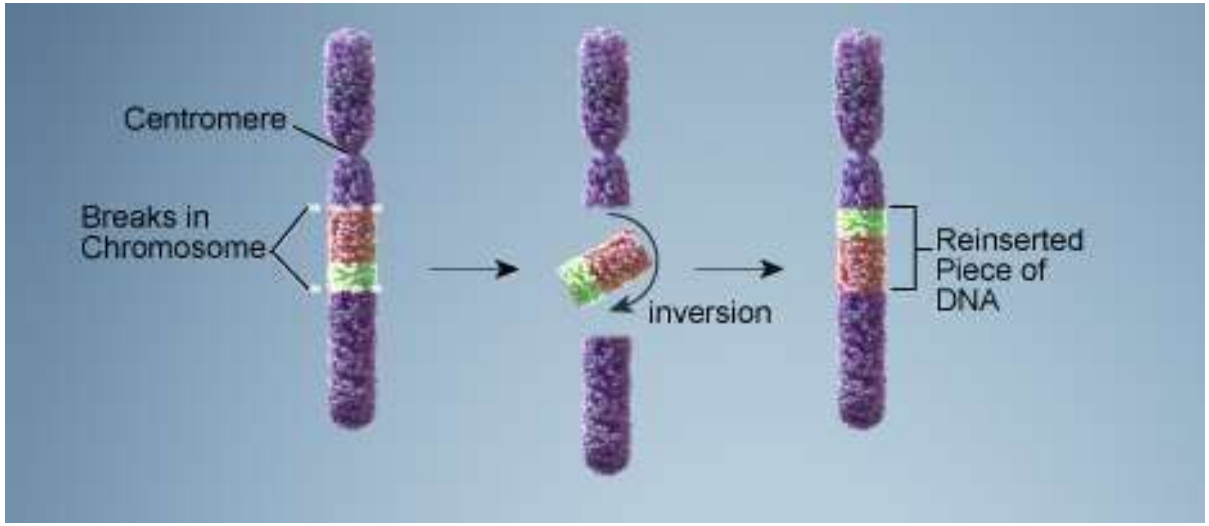
Deletion

- Due to **breakage**
- A **piece** of a chromosome is **lost**



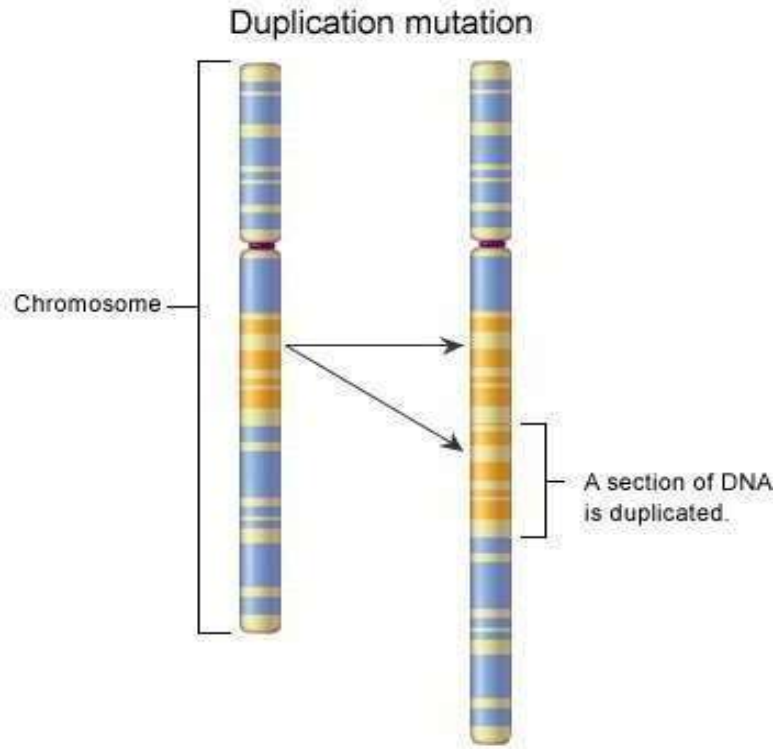
Inversion

- Chromosome segment **breaks off**
- Segment flips around **backwards**
- Segment **reattaches**



Duplication

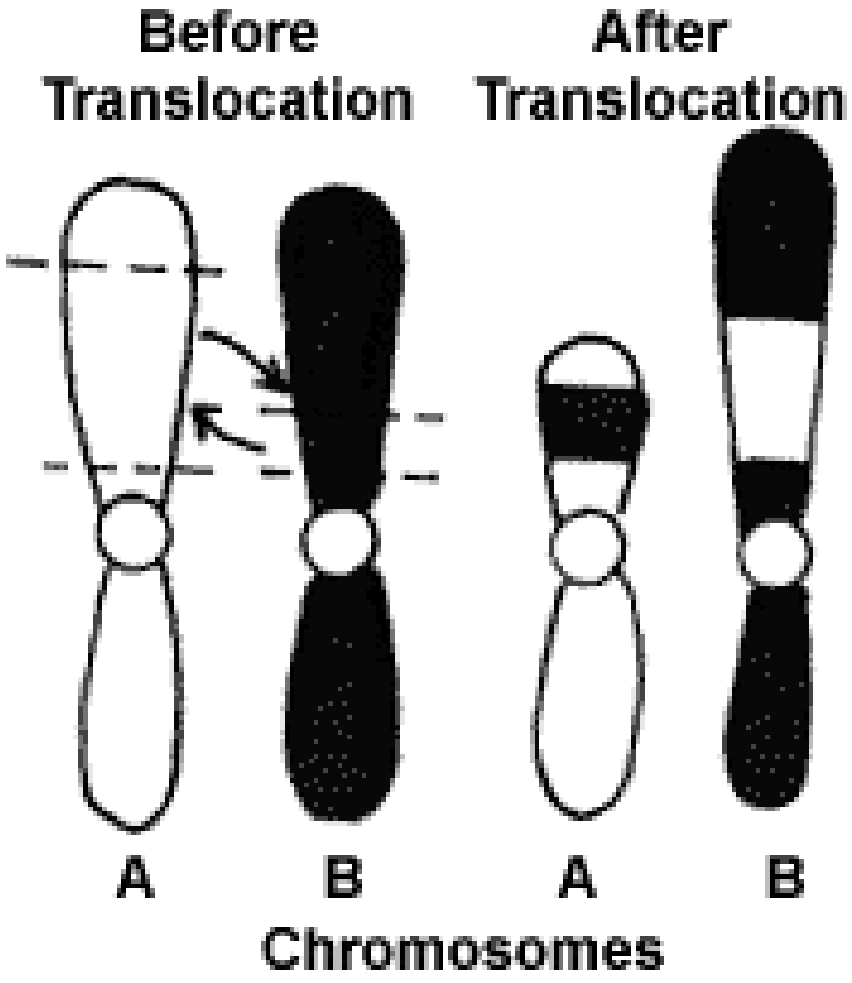
- Occurs when a gene **sequence is repeated**



Translocation

- Involves **two chromosomes** that aren't homologous
- **Part** of one chromosome is **transferred to another** chromosomes

Translocation



Nondisjunction

- **Failure** of chromosomes **to separate** during meiosis
- Causes gamete to have **too many or too few chromosomes**
- **Disorders:**
  - **Down Syndrome –**
  - **Turner Syndrome –**
  - **Klinefelter's Syndrome –**

Types of Gene Mutations

- Include:
  - Point Mutations
  - Substitutions
  - Insertions
  - Deletions
  - Frameshift

Point Mutation

- Change of a **single** nucleotide
- Includes the deletion, insertion, or substitution of **ONE** nucleotide in a gene

Point Mutation

- **Sickle Cell disease** is the result of one nucleotide substitution
- Occurs in the **hemoglobin gene**

Frameshift Mutation

- **Inserting or deleting** one or more nucleotides
- Changes the "**reading frame**" like changing a sentence
- **Proteins** built **incorrectly**

MICROBIAL BIOTRANSFORMATION

BIOTRANSFORMATION

Biotransformations are structural modifications in a chemical compound by organisms /enzyme systems that lead to the formation of molecules with relatively greater polarity.This mechanism has been developed by microbes to acclimatize to environmental changes and it is useful in a wide range of biotechnological processess. The most significant aspect of biotransformation is that it maintains the original carbon skeleton after obtaining the products.

TYPES OF BIOTRANSFORMATION

Biotransformation is of two types:

1. Enzymatic: Microsomal biotransformation is caused by enzymes present within the lipophilic membranes of smooth endoplasmic reticulum .

2.Nonenzymatic: Non-Microsomal Biotransformation involves the enzymes which are present within the mitochondria.

Microbial cells are ideal choice for biotransformation due to certain reasons like:

I. Surface-volume ratio: Microbial biotransformation has high surface-volume ratio.
II. Growth Rate: Higher growth rate of microbial cells reduces the time of biomass transformation.
III. Metabolism Rate: Higher rate of the metabolism in microbes leads to efficient transformation of substrate.
IV. Sterility: It is easier to maintain sterile conditions when microbes are used

APPLICATION OF MICROBIAL BIOTRANSFORMATION

- Transformation of steroids and sterols.
- Transformation of Pollutants.
- Transformation of Non-Steroid Compounds.
- Transformation of Antibiotics.
- Transformation of Pesticides.
- Petroleum Biotransformation.

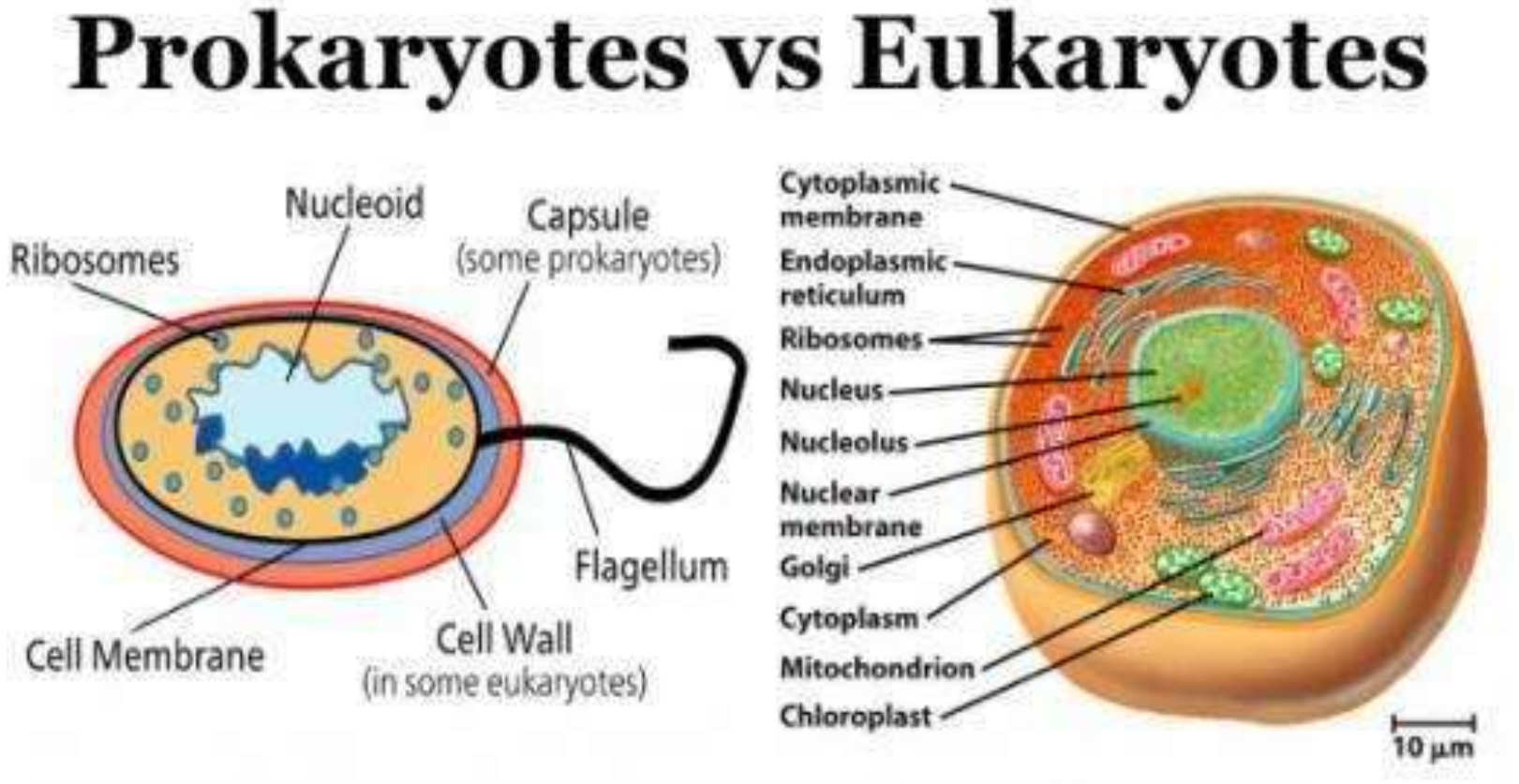
GENETIC ORGANIZATION OF EUKARYOTES AND PROKAROYOTES

EUKARYOTES AND PROKAROYOTES

Prokaryotes are organisms made up of cells that lack a cell nucleus or any membrane-encased organelles.

Eukaryotes are organisms made up of cells that possess a membrane-bound nucleus that holds genetic material as well as membrane-bound organelles.

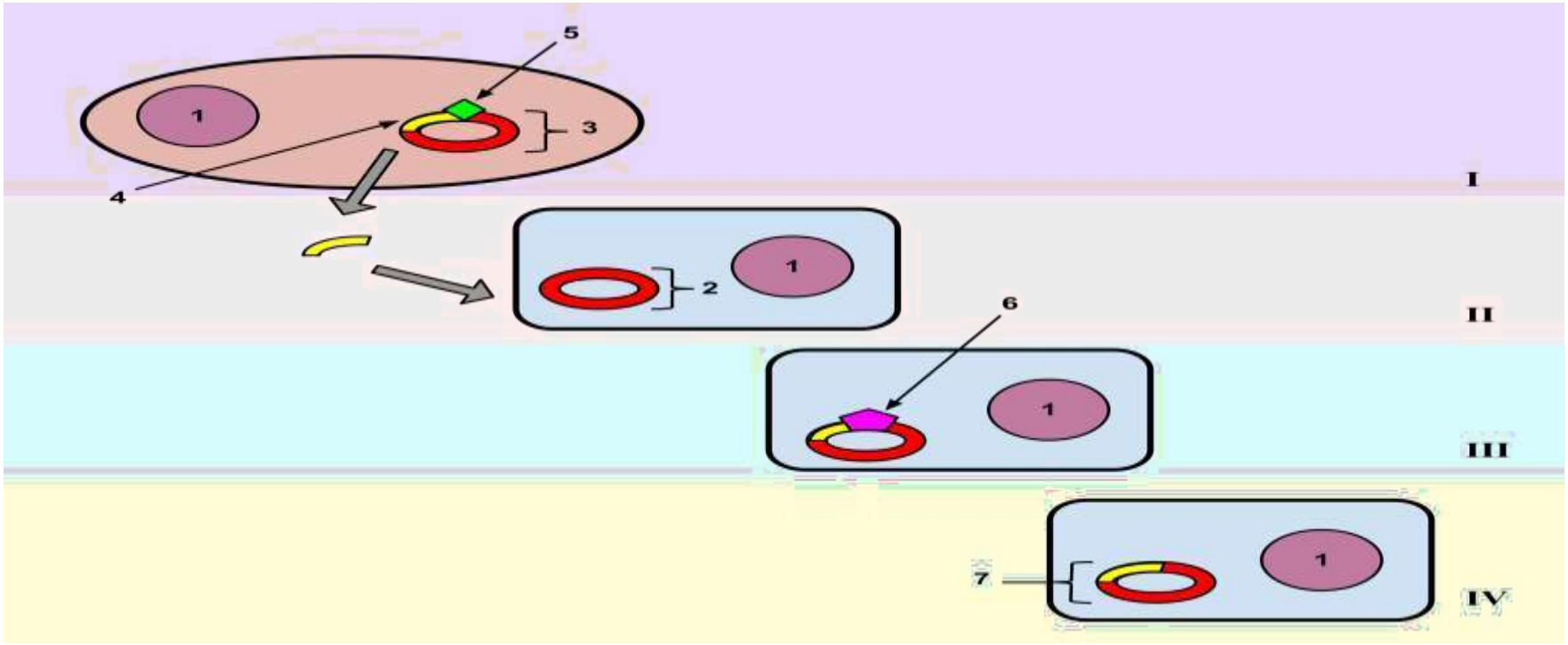
DIFFERENCE BETWEEN EUKARYOTES AND PROKAROYOTES



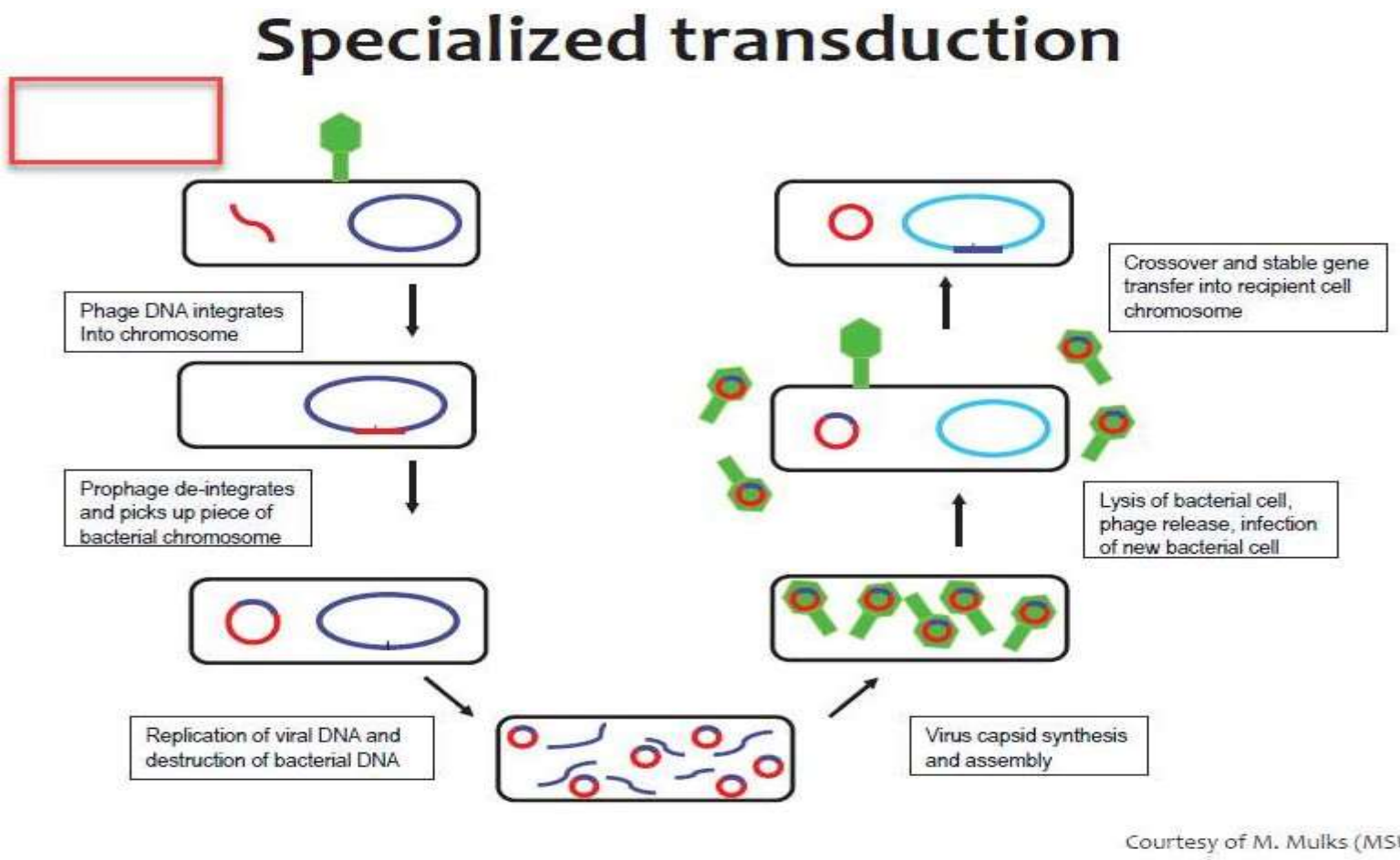
Prokaryotic vs. eukaryotic genomes

Prokaryotic	Eukaryotic
No-nonsense genomes – nearly all coding	Lots of noncoding regions (introns, intergenic regions)
Frequent horizontal gene transfer (HGT)	Less frequent HGT
Circular genome plus plasmids	Distinct linear chromosomes
Operons	More dispersed regulation
Streamlined genomes, few repetitive elements	Abundant repetitive elements
Very rapidly evolving in both sequence and structure	More conservative mode of evolution
Very large population sizes (10^9-10^{10})	Smaller population sizes (~10^3 – 10^4)

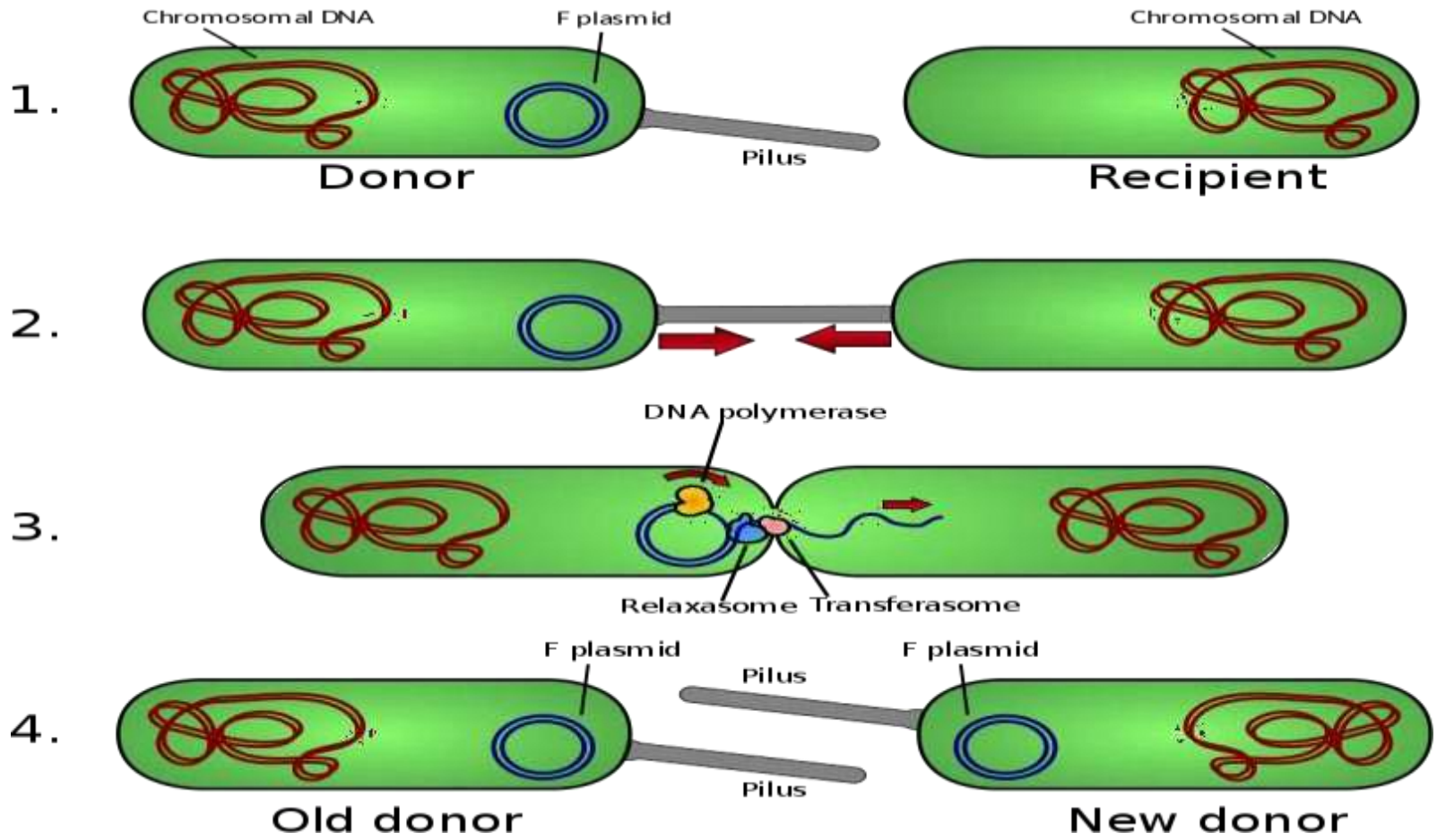
TRANSFORMATION: Transformation occurs naturally in some species of bacteria, and can also be done artificially. ... Introduction of foreign DNA into eukaryote cells is usually called "transfection".



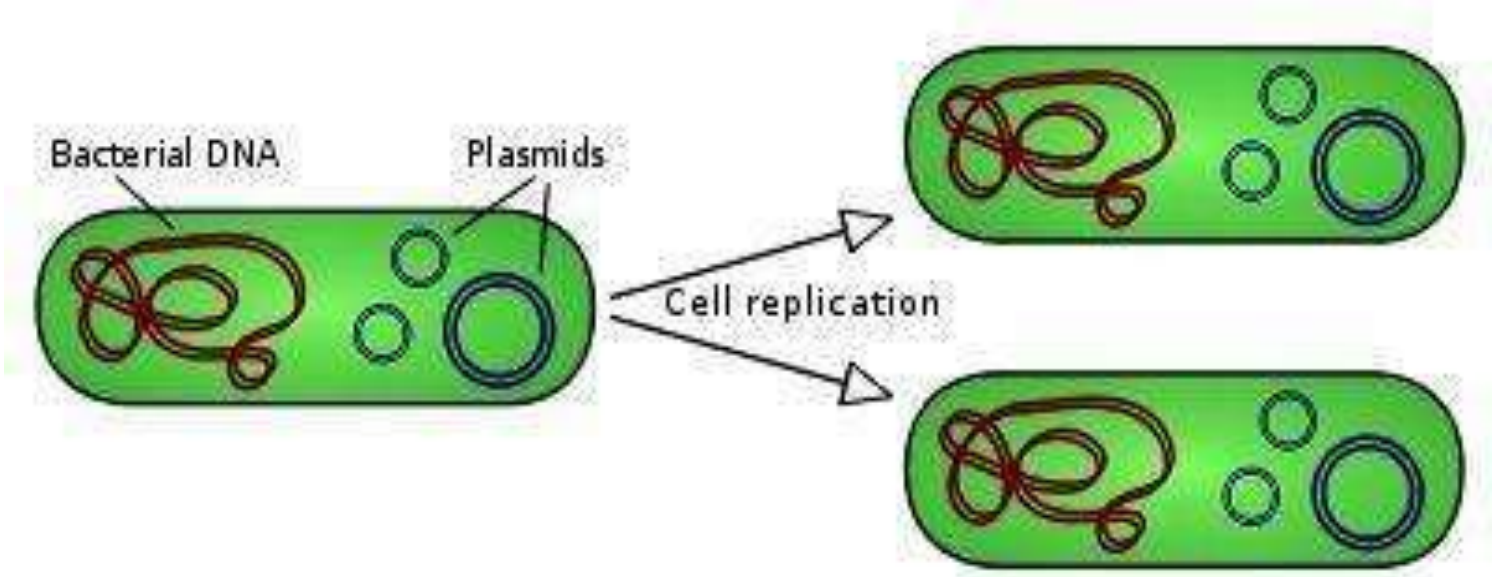
TRANSDUCTION: Transduction is the process by which foreign DNA is introduced into a bacterial cell by a virus or viral vector. An example is the viral transfer of DNA from one bacterium to another and hence an example of horizontal gene transfer.



CONJUGATION: Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells.This takes place through a pilus.



PLASMIDS: A **plasmid** is a small, circular, double-stranded DNA molecule that is distinct from a cell's chromosomal DNA. **Plasmids** naturally exist in bacterial cells, and they also occur in some eukaryotes. Often, the **genes** carried in **plasmids** provide bacteria with **genetic** advantages, such as antibiotic resistance



THANK YOU