Animal Cell culture

(from internet)

Animal tissue culture technology is now becoming a significant model for many scientists in various fields of biology and medicine. Despite the various developments in animal cell and tissue culture since the late 1800s, until the early 1950s progress in animal tissue culture was stalled due to the non-availability of a suitable cell line.

In the early 1950s, for the first time, successful growth of cells derived from the cervical cancer of Mrs Henrietta Lacks was demonstrated. This breakthrough using Mrs Henrietta Lacks's cells in culture successfully transformed medical and biological research, allowing numerous cellular, molecular and therapeutic discoveries, including the breakthrough of the first effective polio vaccine . This culture is now called HeLa, on which there were more than 60 000 publications by 2017, and which has been involved in numerous Nobel prize-winning innovations

Animal cell culture involves isolation of cells from a tissue before establishing a culture in a suitable artificial environment. Initial isolation of the cells from the tissues can be achieved by disaggregation using enzymatic or mechanical methods. The source of the isolated cells is usually an *in vivo* environment, but sometimes cells are also derived from an existing cell line or cell strain. Animal cell culture offers suitable model systems for investigating the following factors:

- **Drug screening and development.**
- **Mutagenesis and carcinogenesis.**
- **Normal physiology and biochemistry of cells.**
- **Potential effects of drugs and toxic compounds on the cells.**

Mammalian cell culture requires an optimal environment for growth. Environmental conditions are divided into nutritional requirements and physicochemical requirements. Nutritional requirements include a substrate or medium that provides support and essential nutrients such as amino acids, carbohydrates, vitamins, minerals, growth factors, hormones and gases (O_2, CO_2). All these factors control physical and chemical factors such as pH, osmotic pressure and temperature.

In animal tissue culture the majority of cells are anchorage-dependent and therefore require a solid or semi-solid support in the form of a substrate (adherent or monolayer culture), whereas others can be cultured in the culture medium, called a suspension culture.

Animal, plant and microbial cells are always cultured in predetermined culture medium under controlled laboratory conditions.

Animal cells are more complex than micro-organisms. Due to their genetic complexity it is difficult to determine the optimum nutrient requirements of animal cells cultured under *in vitro* conditions.

Animal cells require additional nutrients compared to micro-organisms, and they usually grow only when attached to specially coated surfaces. Despite these challenges, different types of animal cells, including both undifferentiated and differentiated ones, can be cultured successfully.

Culturing animal cells, tissue or organs in a controlled artificial environment is called animal tissue culture. The importance of animal tissue culture was initially realized during the development of the polio vaccine using primary monkey kidney cells (the polio vaccine was the first commercial product generated using mammalian cell cultures). These primary monkey kidney cells were associated with many disadvantages such as:

Chances of contamination with adventitious agents (risk of contamination by various monkey viruses is high).

Most of the cells are anchorage-dependent and can be cultured efficiently only when they are attached to a solid or semi-solid substrate (obligatorily adherent cell growth).

The cells are not well characterized for virus production.

A scarcity of donor animals as they are on the verge of extinction.

Broadly, animal tissue culture can be divided into two categories:

- **Cultures that allow cell–cell interactions and encourage communication or signaling between cells.**
- **Cultures in which cell–cell communication or interactions are lost or the signaling between them is missing.**

The first category includes three different types of culture systems: organ cultures, histotypic cultures (3D) and organotypic cultures (complex tisse).

The second category includes cultures in monolayers or as suspensions.

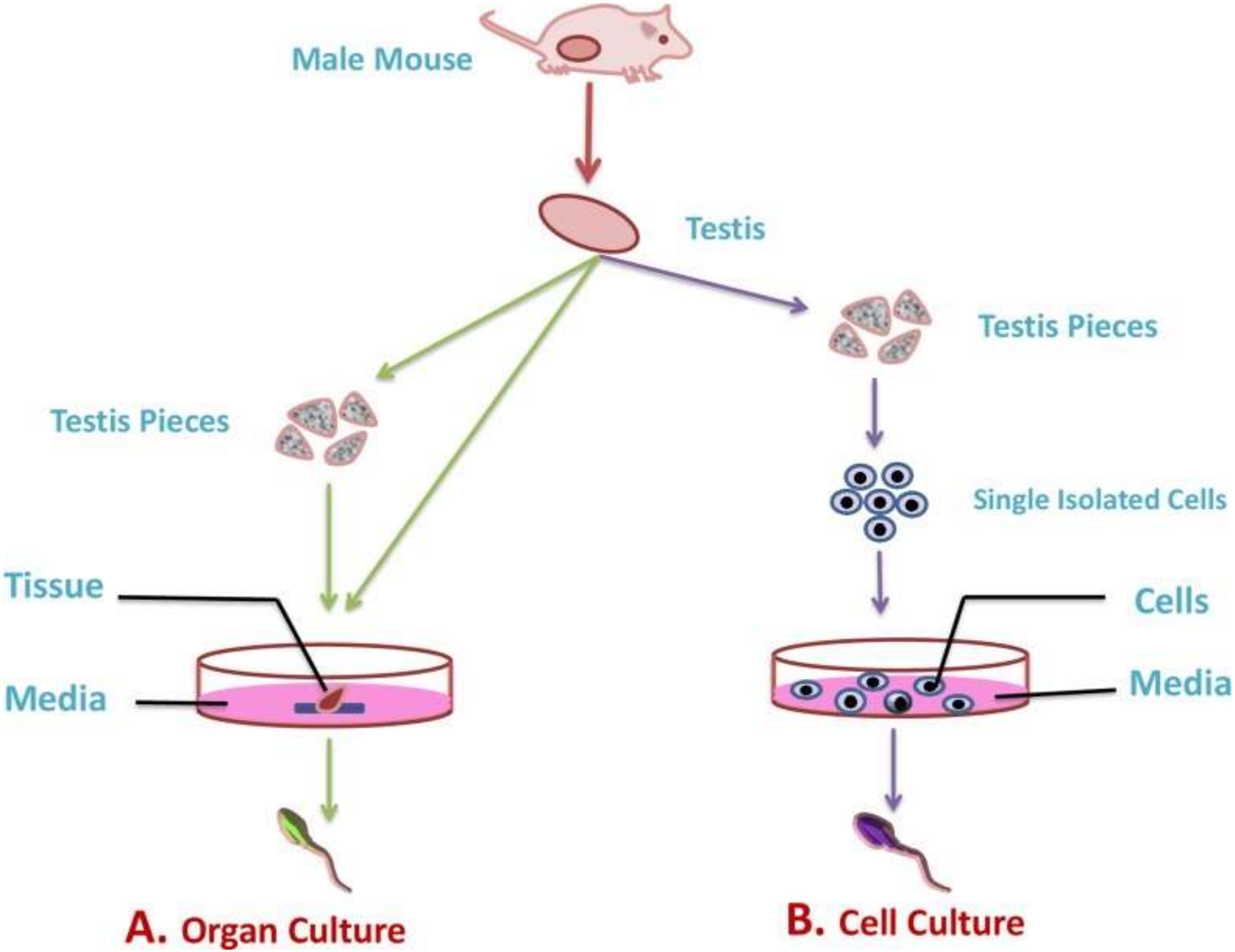
Organ culture is a culture of native tissue that retains most of the *in vivo* histological characteristics, whereas culturing cells for their re-aggregation to yield tissue-like structure is known as histotypic culture. In histotypic cultures, individual cell lineages are initially derived from an organ and then cultured separately to high density in a 3D matrix to study interactions and signaling between homologous cells. In organ cultures, whole embryonic organs or small tissue fragments are cultured *in vitro* in such a manner that they retain their tissue architecture, i.e. the characteristic distribution of various cell types in the given organ.

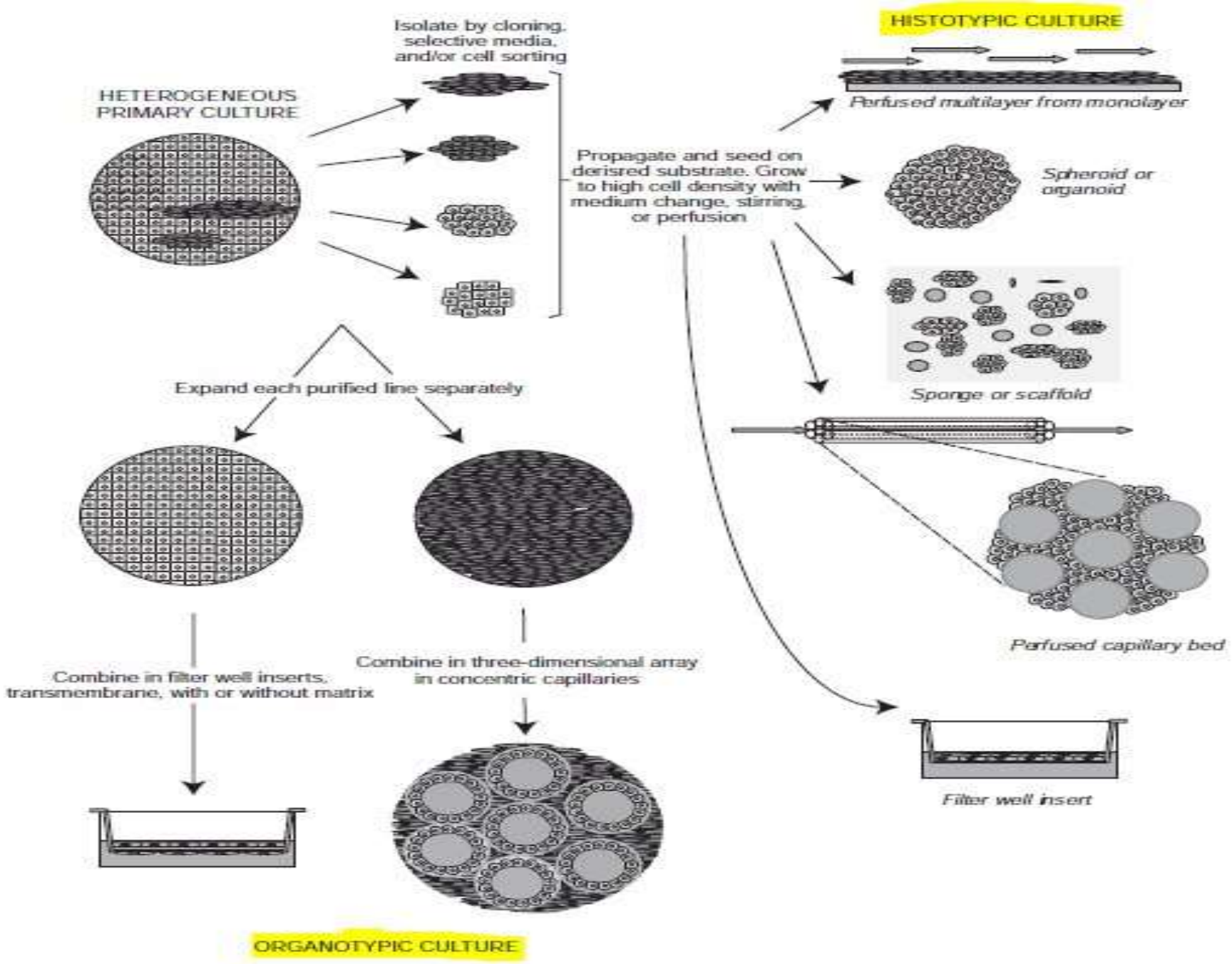
Three main types of techniques

- **Organ culture-** In this type of culture, the whole organs or small fragments of the organs with their spatial and intrinsic properties are used in culture.

- **Histotypic culture- The propagated cell lines grown in three dimensional matrix to high density represent histotypic cultures.**

- **Organotypic cultures-** Cells of different lineages are combined in experimentally determined ratios and spatial relationships to recreate a component of the organ under study.


Male Mouse
Testis
Testis Pieces
Testis Pieces
Single Isolated Cells
Tissue
Media
Cells
Media
A. Organ Culture
B. Cell Culture


HISTOTYPIC CULTURE
Isolate by cloning, selective media, and/or cell sorting
HETEROGENEOUS PRIMARY CULTURE
Perfused multilayer from monolayer
Propagate and seed on derisred substrate. Grow to high cell density with medium change, stirring, or perfusion
Spheroid or organoid
Sponge or scaffold
Expand each purified line separately
Perfused capillary bed
Combine in filter well inserts, transmembrane, with or without matrix
Combine in three-dimensional array in concentric capillaries
Filter well insert
ORGANOTYPIC CULTURE

***Cell culture*. Cell culture is the process of removing cells from an animal or plant and their subsequent growth in an artificially controlled environment.**

Primary cell culture. **This is the first culture (a freshly isolated cell culture) or a culture which is directly obtained from animal or human tissue by enzymatic or mechanical methods. These cells are typically slow growing, heterogeneous and carry all the features of the tissue of their origin. The primary objective of this culture is to maintain the growth of cells on an appropriate substrate, available in the form of glass or plastic containers, under controlled environmental conditions. Since they are directly obtained from original tissue they have the same karyotype (number and appearance of chromosomes in the nucleus of a eukaryotic cell) as the original tissue.**

Once subcultured, primary cell cultures can gives rise to cell lines, which may either die after several subcultures (such cell lines are known as finite cell lines) or may continue to grow indefinitely (these are called continuous cell lines). Usually, normal tissues give rise to finite cell lines, whereas cancerous cells/tissue (typically aneuploid) give rise to continuous cell lines. Nevertheless, there are some exceptional examples of continuous cell lines which are derived from normal tissues and are themselves non-tumorigenic, e.g. MDCK dog kidney, fibroblast 3T3, etc

The evolution of continuous cell lines from primary cultures is assumed to involve mutation, which alters their properties compared to those of finite lines. Serial subculturing of cell lines over time can increase the chances of genotypic and phenotypic variation. Bioinformatic studies based on proteomic phenotypes discovered that the Hepa1–6 cell lines lacked mitochondria, reflecting a rearrangement of metabolic pathways in contrast to primary hepatocytes. With the emergence of newer technologies such as 3D culture, the use of primary cells is becoming increasingly prevalent and achieving improved results. Primary cells which are directly obtained from human or animal tissue using enzymatic or mechanical procedures can be classified into two types:

Anchorage-dependent or adherent cells. **Adherent cells are those cells which require attachment for growth and are also called anchorage-dependent cells. In other words, these cells are capable of attaching on the surface of the culture vessel. These types of cells are often derived from the tissues of organs, for example from the kidney, where the cells are immobile and embedded in connective tissue.**

Anchorage-independent or suspension cells**. Suspension cells do not require attachment or any support for their growth and are also called anchorage-independent cells. All suspension cells are isolated from the blood system, for example white blood cell lymphocytes, and are suspended in plasma.**

For several reasons cells obtained from primary cultures have a limited life span, i.e. the cells cannot be maintained indefinitely. An increase in cell numbers in a primary culture results in exhaustion of the substrate and nutrients, which can influence cellular activity and lead to the accumulation of high levels of toxic metabolites in the culture. This may ultimately result in the inhibition of cell growth. This stage is called the confluence stage (contact inhibition), when a secondary culture or a subculture needs to be established to ensure continuous cell growth.

Secondary cell culture. This simply refers to the first passaging of cells, a switch to a different kind of culture system, or the first culture obtained from a primary culture. This is usually carried out when cells in adherent cultures occupy all the available substrate or when cells in suspension cultures surpass the capacity of the medium to support further growth, and cell proliferation begins to decrease or ceases completely. So as to maintain optimal cell density for continued growth and to encourage further proliferation, the primary culture has to be subcultured. This process is known as secondary cell culture. Major differences between primary and secondary cell cultures are highlighted in table

Differences between primary and secondary cell cultures.

Primary cell culture	Secondary cell culture
Directly obtained from animal or plant tissue.	Originates from a primary cell culture.
Closely resembles the parental tissue.	Does not closely resemble the parental tissue.
The biological response of the cell may be closer to that in an *in vivo* environment.	The biological response of the cell differs from that an *in vivo* environment.
The first culture derived from original cells/tissue (from an *in vivo* environment).	Derived from an existing culture.
Cannot be transformed.	Can be transformed.
Less chance of mutation.	Can increase the chance of mutation or genetic alteration of primary cells.
Acquired through steps of rinsing, dissection, and mechanical or enzymatic disaggregation.	If the primary culture is an adherent culture, the first step is to detach cells from the attachment (the surface of the culture vessel) by mechanical or enzymatic means. Then, the cells have to be detached from each other to form a single-cell suspension.
Finite life span.	Prolongs the life span of cells. Periodic subculturing may produce immortal cells through transformation or genetic alteration of primary cells.
The risk of contamination is high. More difficult to maintain.	The risk of contamination is lower. Comparatively easy to maintain.

Cell line. Once a primary culture is subcultured or passaged it represents a cell line. A cell line that experiences indefinite growth of cells during subsequent subculturing is called a continuous cell line, whereas finite cell lines experience the death of cells after several subcultures.

Cell strain. A cell line is a permanently established cell culture which will proliferate forever if a suitable fresh medium is provided continuously, whereas cell strains have been adapted to culture but, unlike cell lines, have a finite division potential. A cell strain is obtained either from a primary culture or a cell line. This is done by selection or cloning of those particular cells having specific properties or characteristics (e.g. specific function or karyotype) which must be defined.

Primary cell culture

As discussed above, the primary cell culture is the first culture of cells, tissues or organs derived directly from an organism; in other words it is the culture before the first subculture, whereas the cell line is for maintenance or propagation of a culture after subculture. There are certain techniques available for the development of primary cell cultures, such as:

Mechanical disaggregation.

Enzymatic disaggregation.

Primary explant techniques.

Mechanical disaggregation

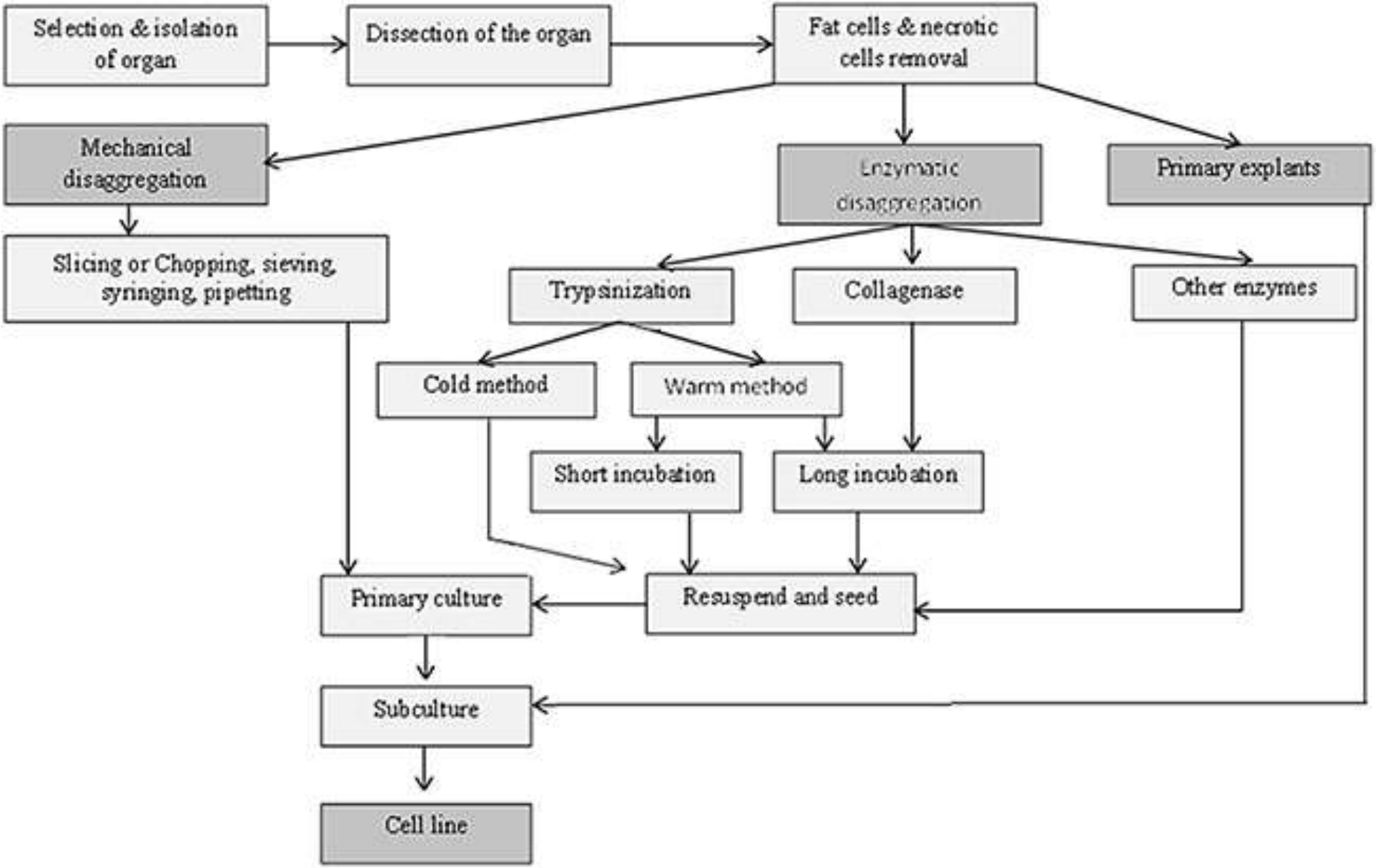
It is necessary to disaggregate soft tissues such as soft tumors. The mechanical approach involves slicing or harvesting tissue and subsequent harvesting of spill out cells. This can be achieved by sieving, syringing and pipetting. This procedure is inexpensive, rapid and simple, however, all these approaches involve the risk of cell damage, thus mechanical disaggregation is only used when the viability of the cells in the final yield is not very important.

Enzymatic disaggregation

This approach involves efficient disaggregation of cells with high yield by using enzymes such as trypsin, collagenase and others. Enzyme based disaggregation allows hydrolysis of fibrous connective tissue and the extracellular matrix. Currently, the enzymatic method is extensively used as it offers high recovery of cells without affecting the viability of cells

Trypsin based disaggregation or trypsinization

This allows disaggregation of tissue using trypsin, usually crude trypsin because this trypsin contains other proteases. In addition, cells can tolerate crude trypsin well and the ultimate effect of crude trypsin can easily be neutralized by **serum or trypsin inhibitor** (supplementation of trypsin inhibitor is required in the case of serum-free media). Pure trypsin can also be utilized for disaggregation of cells, provided that it is less toxic and very specific in its action. An overview of primary cell culture development is shown in figure . Two common approaches, namely warm and cold trypsinization, are described in the following.


Selection & isolation of organ
Dissection of the organ
Fat cells & necrotic cells removal
Mechanical disaggregation
Enzymatic disaggregation
Primary explants
Slicing or Chopping, sieving, syringing, pipetting
Trypsinization
Collagenase
Other enzymes
Cold method
Warm method
Short incubation
Long incubation
Primary culture
Resuspend and seed
Subculture
Cell line

Terms frequently used in animal tissue culture, in particular in the context of cell lines, are defined below:

Adherent cells. Cells with the potential to adhere to the surface of the culture vessel using the extracellular matrix.

Immortalization. Achieving a state of cell culture when cells proliferate continuously.

Attachment efficiency. The proportion of cells that actually adhere to the surface of the culture vessel within a given time after inoculation.

Passaging. The transfer of cells from one culture vessel to another. A more specific term is subculturing where the cells are first subdivided before being transferred into multiple cell culture vessels. A passage number will refer specifically to how many times a cell line has been subcultured. A number of adherent cell cultures will stop dividing when they become confluent (i.e. the stage when they entirely cover the surface of the cell culture vessel), and a number will die if they are retained in a confluent state for longer periods. Thus adherent cell cultures require repeated passaging, which means that when the cells are at the confluent stage, subculturing is required. Regular passaging is required in the case of suspension cultures, where suspended cells use their culture medium rapidly, particularly when the cell density becomes very high. While repeated passaging is essential to maintain cultures, the process is comparatively traumatic for adherent cells since they need to be trypsinized. Thus passaging of adherent cell cultures more than once every 48 h is not recommended.

Split ratio. Divisor of the dilution ratio of a cell culture.

Generation number. The number of doublings that a cell population has undergone. It should be observed that passage and generation number are not the same.

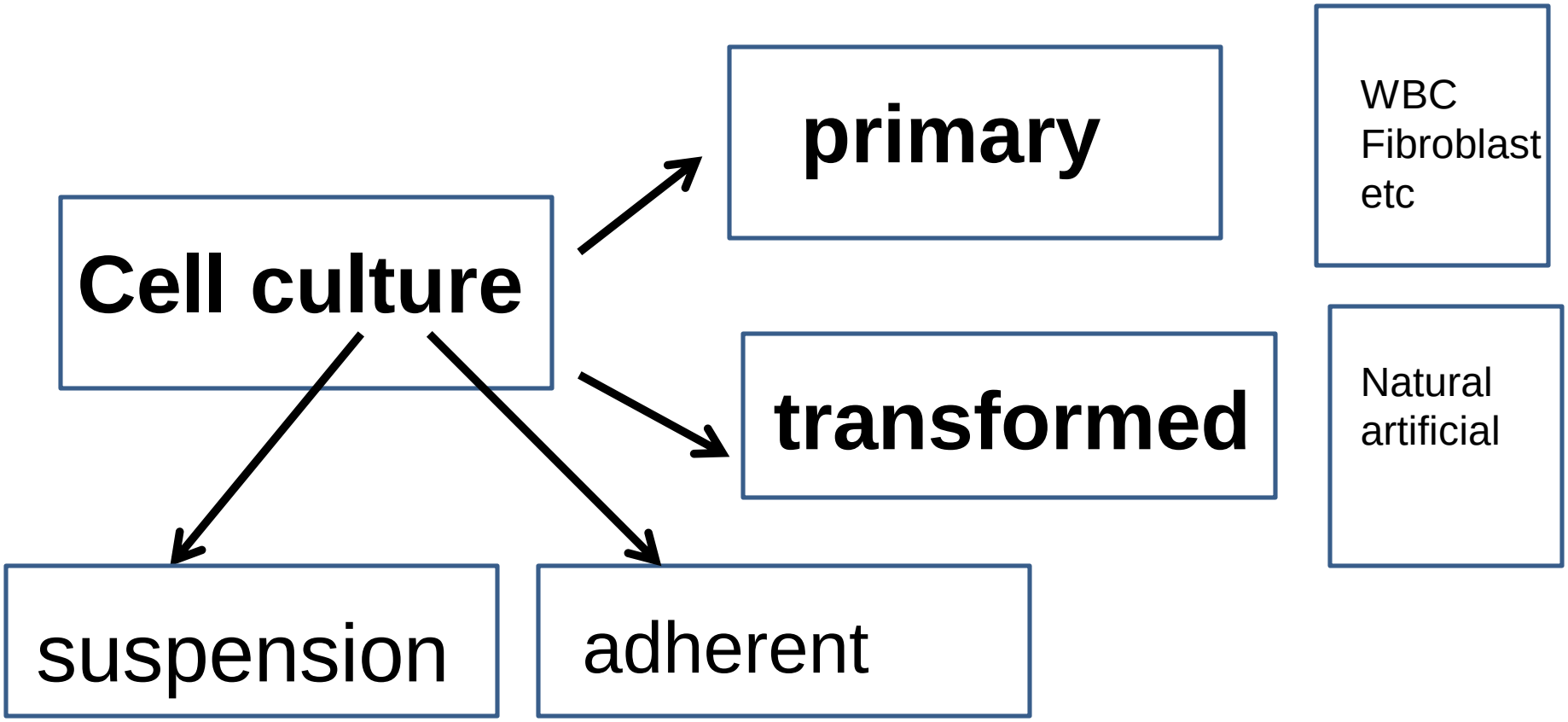
Population doubling time. The population doubling (PD or pd) number is the estimated number of doublings that the cell population has undergone since isolation.

Passage number. The number of times the culture has been subcultured.

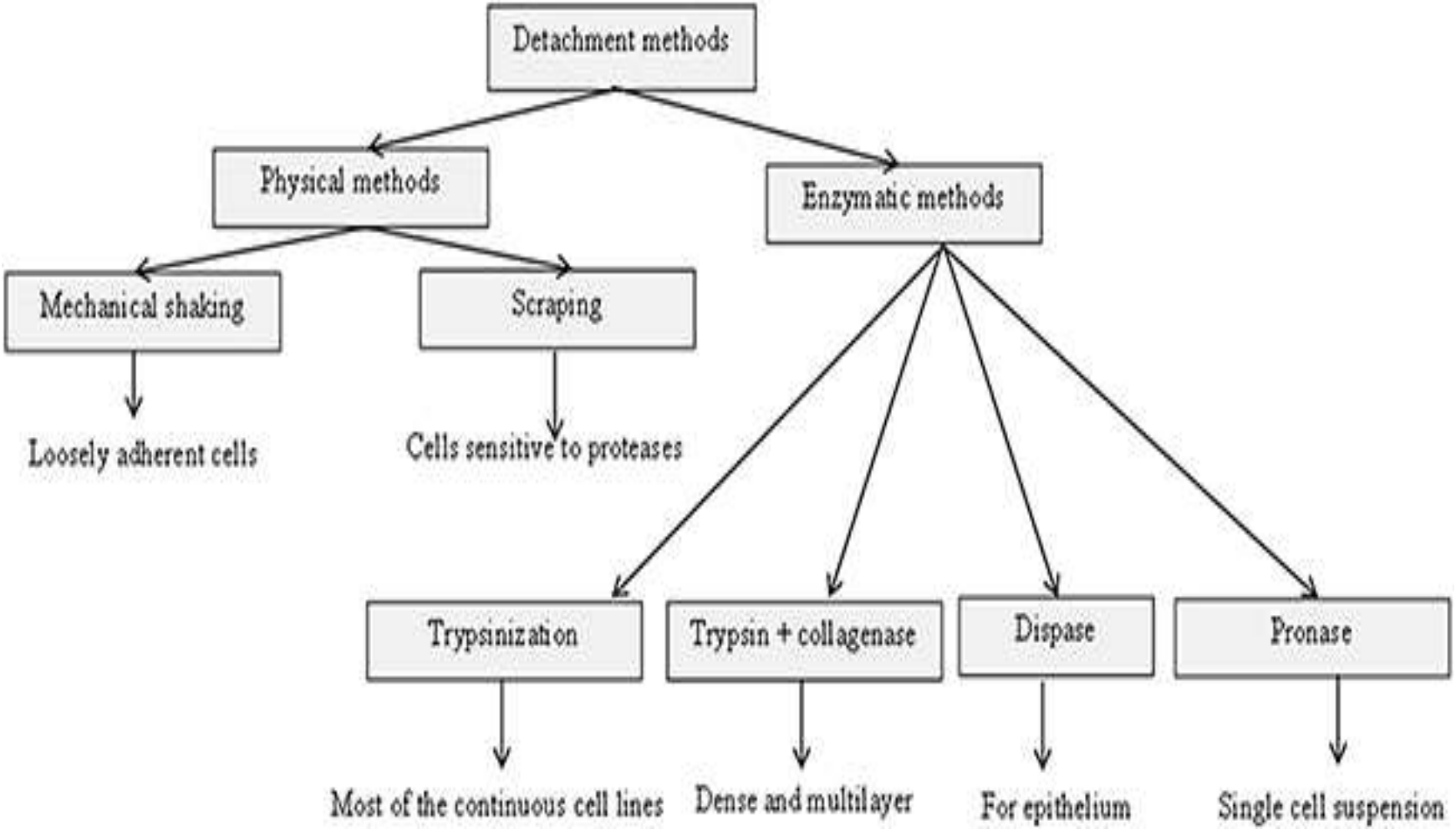
Standard nomenclature of cell lines

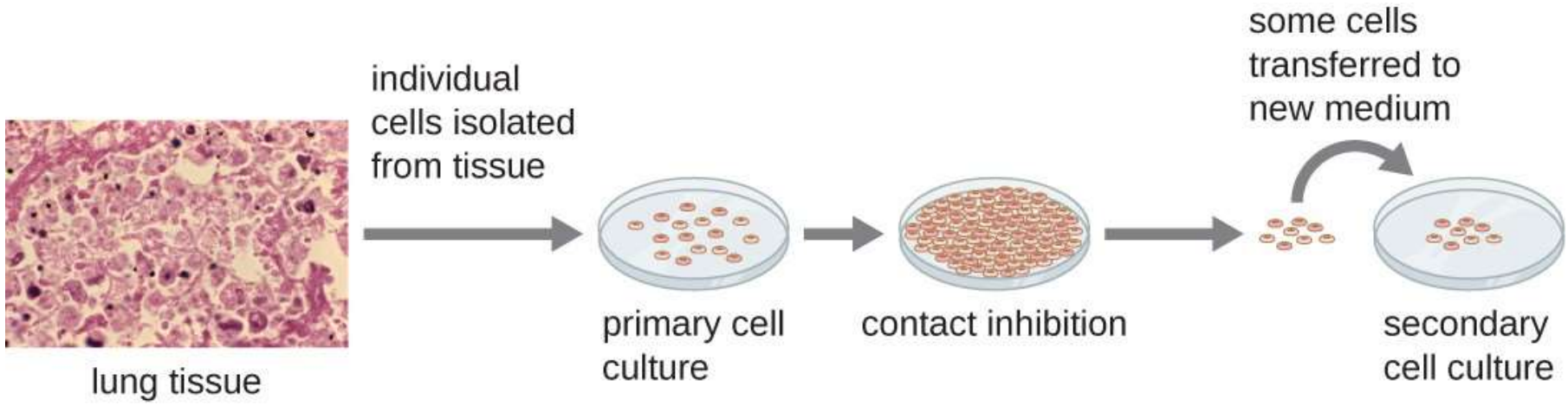
The source and clone number (which represents the number of cell lines derived from the same donor) help in understanding the nomenclature more easily. The basic nomenclature is usually followed by assigning codes or designations to cell lines for their further identification, e.g. HeLa-S3 represents a human cervical tumor cell line, and similarly NHB 2-1 is a cell line derived from normal human brain (NB), followed by cell strain 2 and clone number 1. Another example is the MG-63 cell line. It is the 63rd sample of a tumor that produces a high amount of interferon beta. Therefore, its nomenclature is 'human tumor-63', or in Dutch, 'menselijk gezwell-63' or MG-63

Recently cell lines have transformed scientific study and are used for several purposes, such as:
Vaccine production.
Examining drug metabolism.
Cytotoxicity.
Antibody production.
Investigating gene function.
Development of artificial tissues (e.g. artificial skin).
Production of biological compounds (e.g. therapeutic proteins).
Cell line requirements can be assessed through recent publications using specific cell lines. The American Type Culture Collection (ATCC) cell biology collection contains information on almost 3600 cell lines derived from 150 species. Although they are a useful tool, researchers must be careful when using cell lines instead of primary cells. The simultaneous use of cell lines and primary cells has been supported recently.

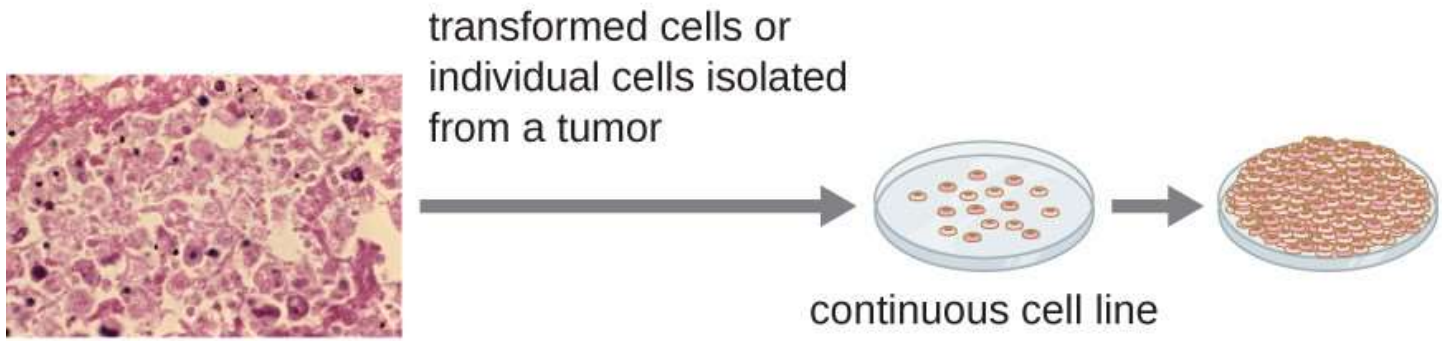

Cell culture
primary
transformed
suspension
adherent
WBC
Fibroblast
etc
Natural
artificial

Methods for dissociation of cells



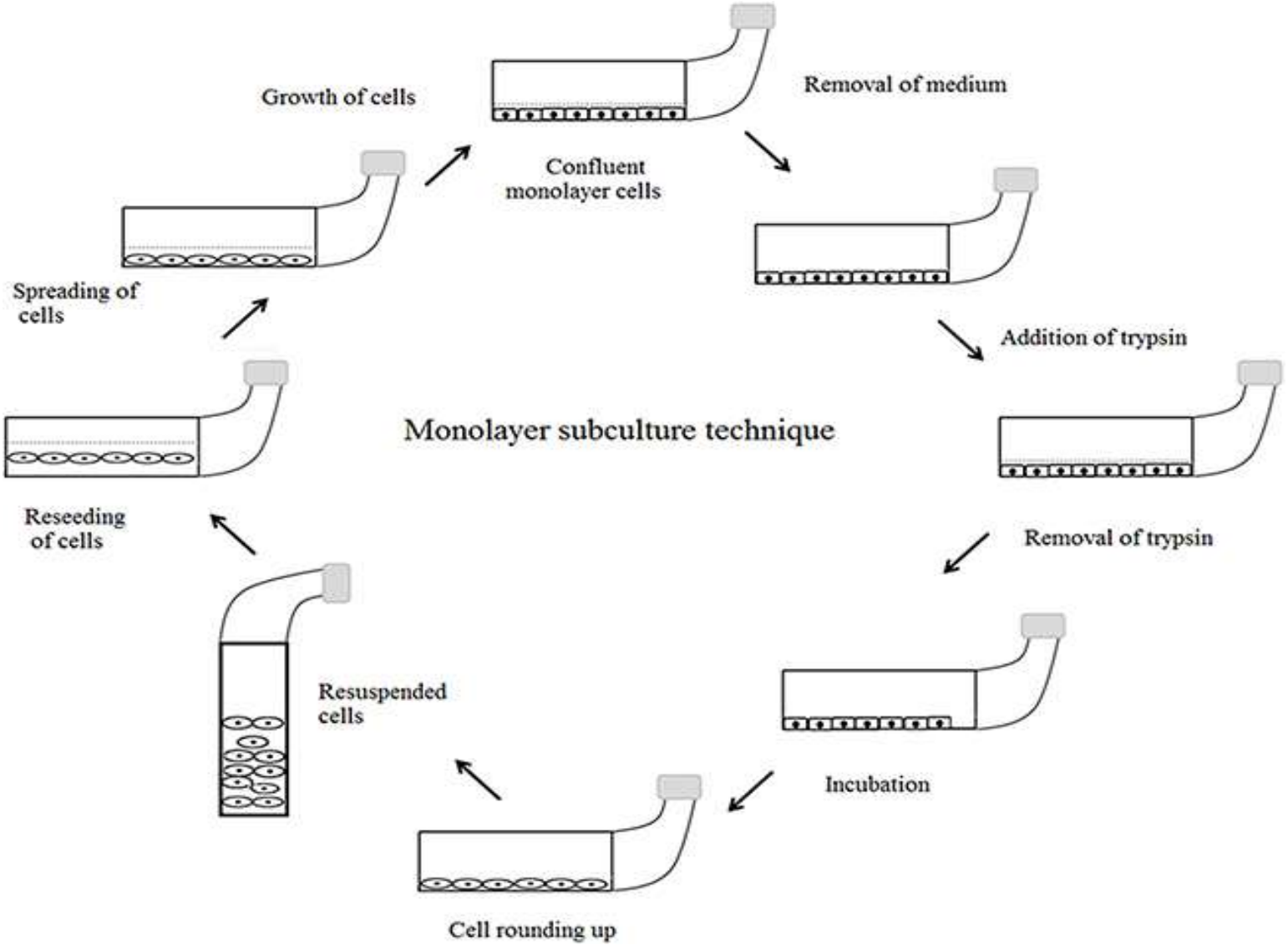

some cells
transferred to
new medium
individual
cells isolated
from tissue
lung tissue
primary cell
culture
contact inhibition
secondary
cell culture

(a)


transformed cells or
individual cells isolated
from a tumor
lung tissue
continuous cell line

(b)

Process of monolayer subculture



Suspension cultures

Most cell lines are grown as monolayer adherent cells, which grow only on the surfaces of culture vessels, however, certain cells are not adhesive, such as cells derived from leukemic tissue. Moreover, certain cells do not require support for their growth. These cells can be mechanically kept in suspension, and such cultures are referred to as suspension cultures. Transformed cells are usually subcultured using this method. Suspension culture of animal tissues is similar to the method used to subculture bacteria or yeast. There are a number of advantages to suspension cultures over monolayer cultures:

Bulk production or production in mass can be achieved.

The cultured cell has access to nutrition from all directions.

Easy to maintain.

Frequent replacement of medium is not required.

The lag period is short.

The process of propagation is fast.

Scale-up is convenient.

Trypsin treatment, or any other enzyme treatment, is not required.

animal cell culture for isolation of virus

Cultured **cells**, eggs, and laboratory **animals** may be used for **virus isolation**. Although embroyonated eggs and laboratory **animals** are very useful for the **isolation** of certain **viruses**, **cell cultures** are the sole system for **virus isolation** in most laboratories

Cell cultures are developed from **tissue** samples and then disaggregated by mechanical, chemical, and enzymatic methods to extract **cells** suitable for **isolation** of **viruses**. With the recent advances in technology, **cell culture** is considered a gold standard for **virus isolation**

Role of

Growth factors:

Sterilization:

Medium : Phenol red

Humidified:

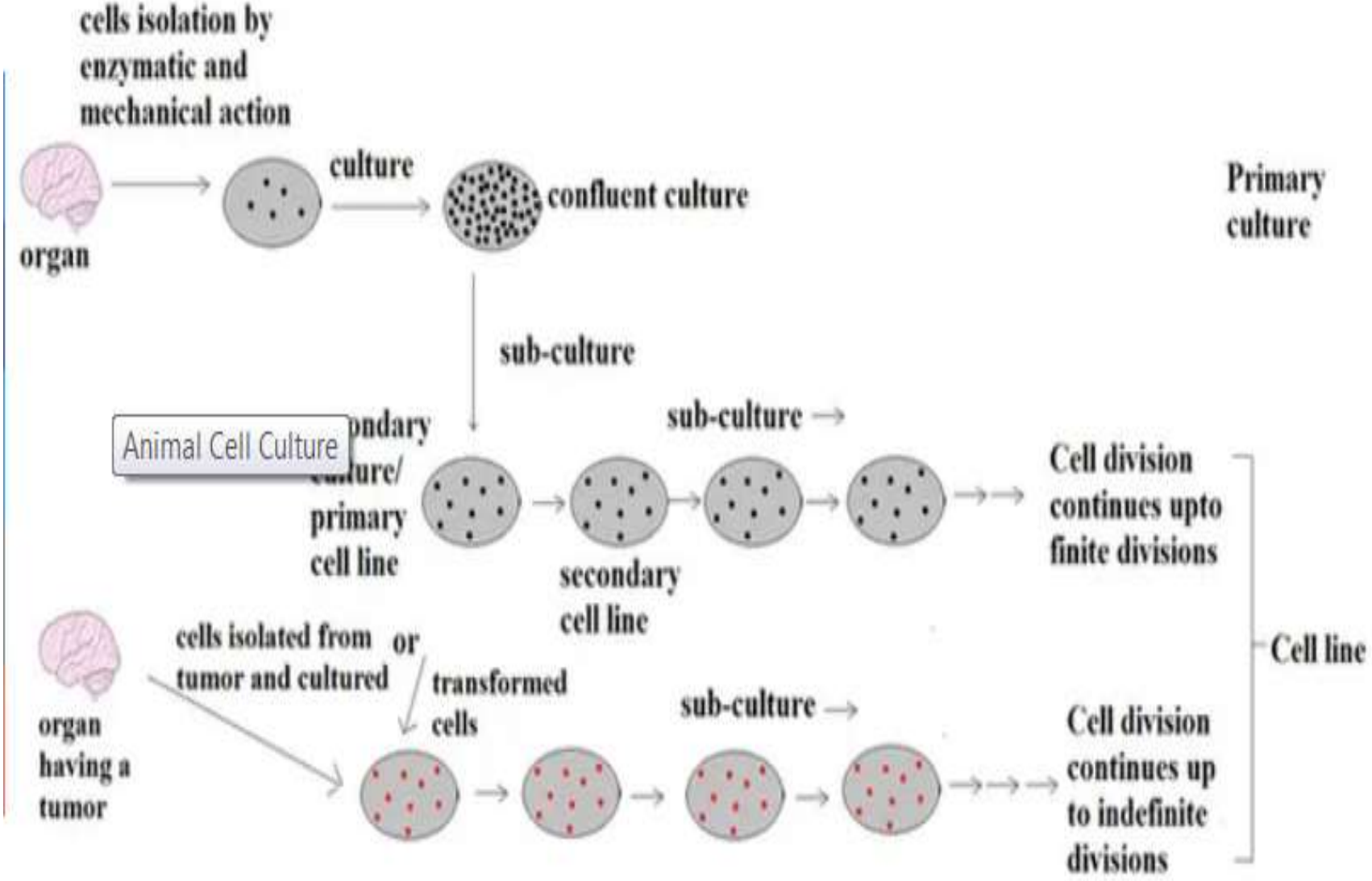


fig: animal cell culture

Primary cells : Lymphocyte culture :

Culture medium: constituents and storage

(1) Basal medium: RPMI 1640 (low thymidine content)

(2) Supplements

(3) Fetal calf serum (5-30% of complete medium)

(4) Antibotics

Gram-positive bacteria: penicillin (100U/ml)

Gram-negative bacteria: streptpmycine (100µg/ml)

(5) L-Glutamine: essential and unstable, amino acid

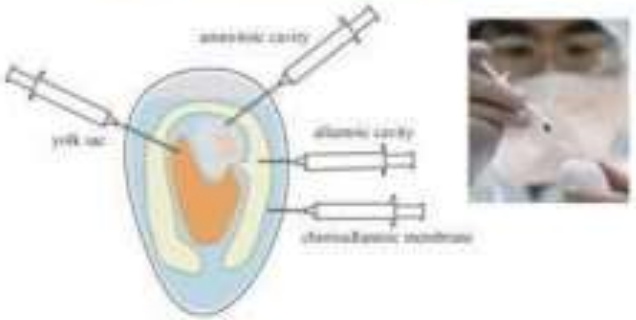
(6) Buffering systems: HEPES and sodium bicarbonate

(7) Phytohaemagglutinin (PHA)

Monoclonal antibody Production

Methods for Cultivation of Virus

- Generally three methods are employed for the virus cultivation
  1. Inoculation of virus into animals
  2. Inoculation of virus into embryonated eggs
  3. Tissue culture



Inoculation of Virus in Animals

- Laboratory animals play an essential role in studies of viral pathogenesis
- Live animals such as monkeys, mice, rabbits, guinea pigs, ferrets are widely used for cultivating virus
- Mice are the most widely employed animals in virology

Advantages of Animal Inoculation

- Diagnosis, Pathogenesis and clinical symptoms are determined.
- Production of antibodies can be identified.
- Primary isolation of certain viruses.
- Mice provide a reliable model for studying viral replication.
- Used for the study of immune responses, epidemiology and oncogenesis .

Disadvantages of Animal Inoculation

- Expensive and difficulties in maintenance of animals.
- Difficulty in choosing of animals for particular virus
- Some human viruses cannot be grown in animals , or can be grown but do not cause disease.
- Mice do not provide models for vaccine development.
- Issues related to animal welfare systems.

- **Cell culture** is mostly used for identification and cultivation of viruses.

  - Cell culture is the process by which cells are grown under controlled conditions.
  - Cells are grown in vitro on glass or a treated plastic surface in a suitable growth medium.
  - At first growth medium, usually balanced salt solution containing 13 amino acids, sugar, proteins, salts, calf serum, buffer, antibiotics and phenol red are taken and the host tissue or cell is inoculated.
  - On incubation the cell divide and spread out on the glass surface to form a confluent monolayer.

Types of cell culture

Based on the origin and the chromosome property the tissue culture are classified into 3 types.

1. Primary cell culture:

- These are normal cells freshly taken from animal or human body.
- They are able to grow only for limited time and cannot be maintained in serial culture.
- They are used for the primary isolation of viruses and production of vaccine.
- Examples: Monkey kidney, cell culture, Human embryonic kidney, chick embryo cell culture.

2. Diploid cell culture (Semi-continuous cell lines)

- They are diploid and contain the same number of chromosomes as the parent cells.
- They can be sub-cultured up to 50 times by serial transfer following senescence and the cell strain is lost.
- They are used for the isolation of some fastidious viruses and production of viral vaccines.
- Examples: Human embryonic lung strain, Rhesus embryo cell strain.

3. Continuous cell lines

- They are derived from cancer cells.
- They can be serially cultured so named as continuous cell lines
- They can be maintained either by serial subculture or by storing in deep freeze at -70°c.
- Due to derivation from cancer cells they are not useful for vaccine production.
- Examples: HeLa (Human Carcinoma of cervix cell line), HEP-2 (Humman Epithelioma of larynx cell line), BHK-21 (Baby Hamster Kidney cell line).

Advantages of cell culture

- Relative ease, broad spectrum, cheaper and sensitivity.

Disadvantage of cell culture

- The process requires trained technicians with experience in working on a full time basis.
- State health laboratories and hospital laboratories do not isolate and identify viruses in clinical work.
- Tissue or serum for analysis is sent to central laboratories to identify virus.

VACCINES AND CELL CULTURES

- Traditionally, few available viral vaccines were produced in animal systems, such as
  - calf skin for smallpox,
  - rabbit spinal cord for rabies and
  - mouse brain for Japanese encephalitis, or
  - in embryonated eggs in the case of influenza and yellow fever viruses.
- A major concern, is the fact that when viruses are cultivated through extended passages in hens eggs,
  - there is an evolutionary process in the allantoic cavity of the egg
  - resulting in the selection of a virus subpopulation, antigenically and biochemically distinct from the original inoculum.

Unlike bacteria, many of which can be grown on an artificial nutrient medium, viruses require a living host cell for replication. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and then the growth medium can be harvested as a source of virus. Virions in the liquid medium can be separated from the host cells by either centrifugation or filtration. Filters can physically remove anything present in the solution that is larger than the virions; the viruses can then be collected in the filtrate

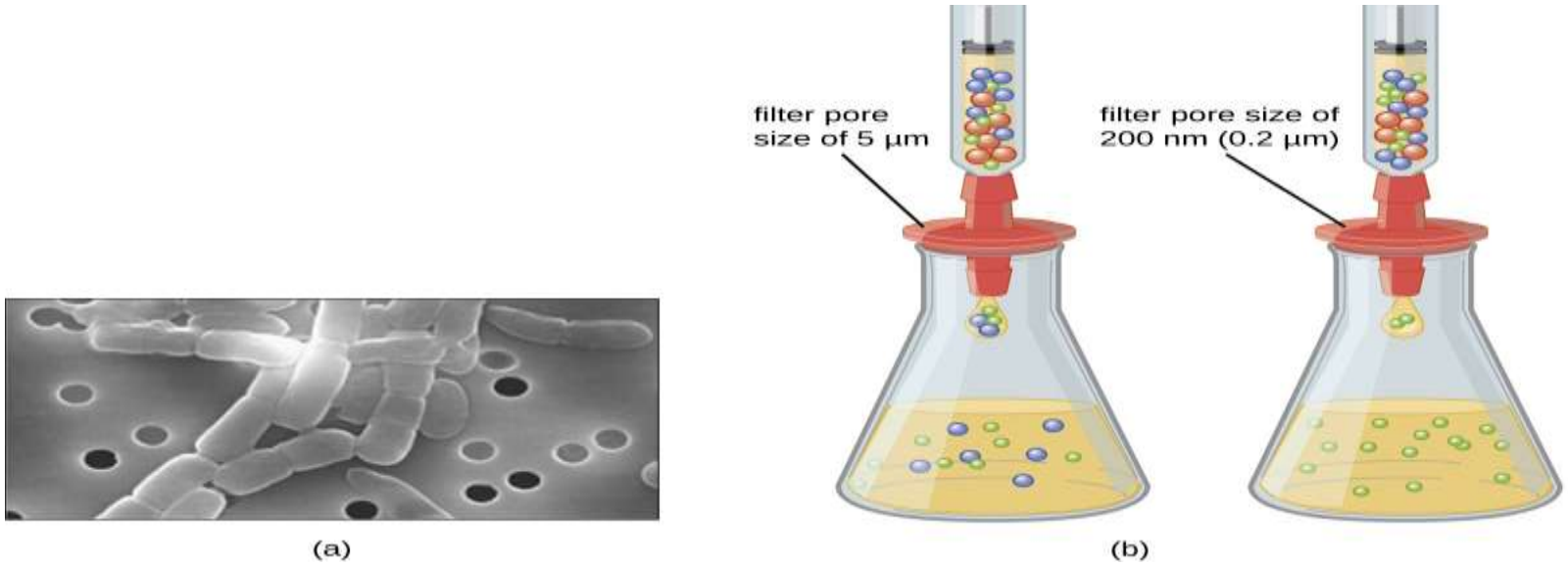
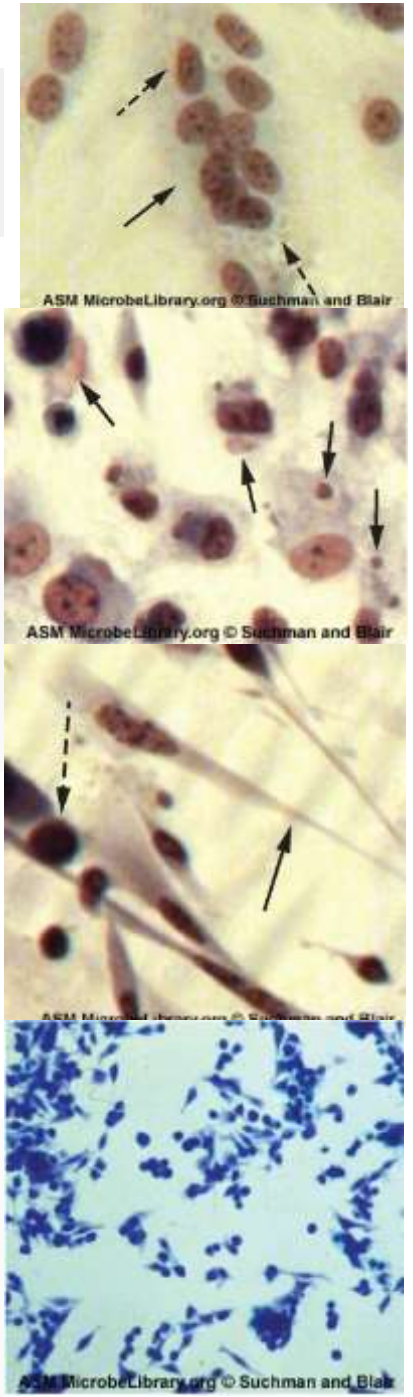


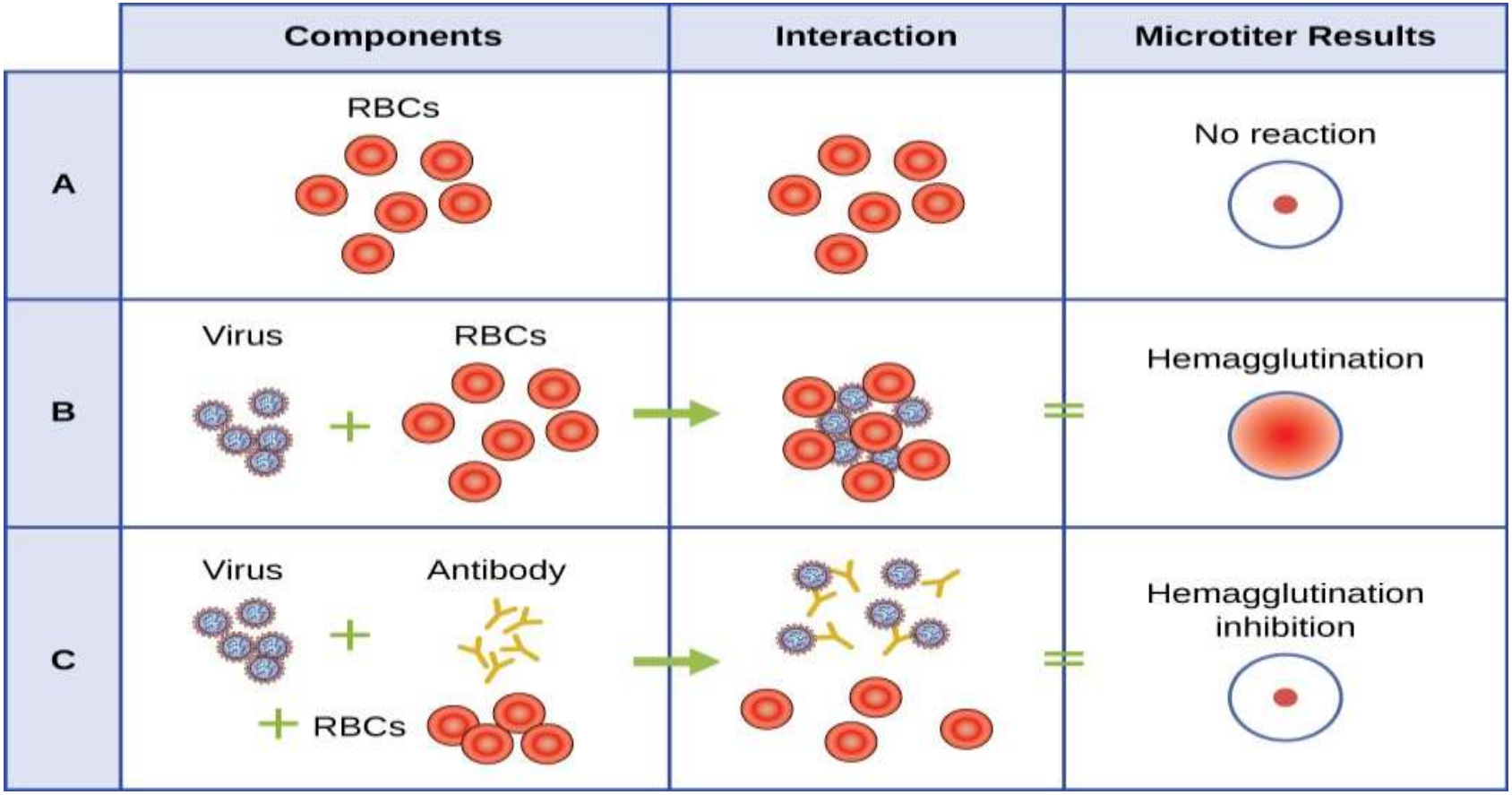
Figure 1. Membrane filters can be used to remove cells or viruses from a solution. (a) This scanning electron micrograph shows rod-shaped bacterial cells captured on the surface of a membrane filter. Note differences in the comparative size of the membrane pores and bacteria. Viruses will pass through this filter. (b) The size of the pores in the filter determines what is captured on the surface of the filter (animal [red] and bacteria [blue]) and removed from liquid passing through. Note the viruses (green) pass through the finer filter

Regardless of the method of cultivation, once a virus has been introduced into a whole host organism, embryo, or tissue-culture cell, a sample can be prepared from the infected host, embryo, or cell line for further analysis under a brightfield, electron, or fluorescent microscope. **Cytopathic effects (CPEs)** are distinct observable cell abnormalities due to viral infection. CPEs can include loss of adherence to the surface of the container, changes in cell shape from flat to round, shrinkage of the nucleus, vacuoles in the cytoplasm, fusion of cytoplasmic membranes and the formation of multinucleated syncytia, inclusion bodies in the nucleus or cytoplasm, and complete cell lysis.

Cytopathic Effects of Specific Viruses		
Virus	Cytopathic Effect	Example
Paramyxovirus	Syncytium and faint basophilic cytoplasmic inclusion bodies	
Poxyvirus	Pink eosinophilic cytoplasmic inclusion bodies (arrows) and cell swelling	
Herpesvirus	Cytoplasmic stranding (arrows) and nuclear inclusion bodies (dashed arrow)	
Adenovirus	Cell enlargement, rounding, and distinctive grape-like clusters	



A serological assay is used to detect the presence of certain types of viruses in patient serum. Serum is the straw-colored liquid fraction of blood plasma from which clotting factors have been removed. Serum can be used in a direct assay called a **hemagglutination assay** to detect specific types of viruses in the patient's sample. Hemagglutination is the agglutination (clumping) together of erythrocytes (red blood cells). Many viruses produce surface proteins or spikes called hemagglutinins that can bind to receptors on the membranes of erythrocytes and cause the cells to agglutinate. Hemagglutination is observable without using the microscope, but this method does not always differentiate between infectious and noninfectious viral particles, since both can agglutinate erythrocytes.



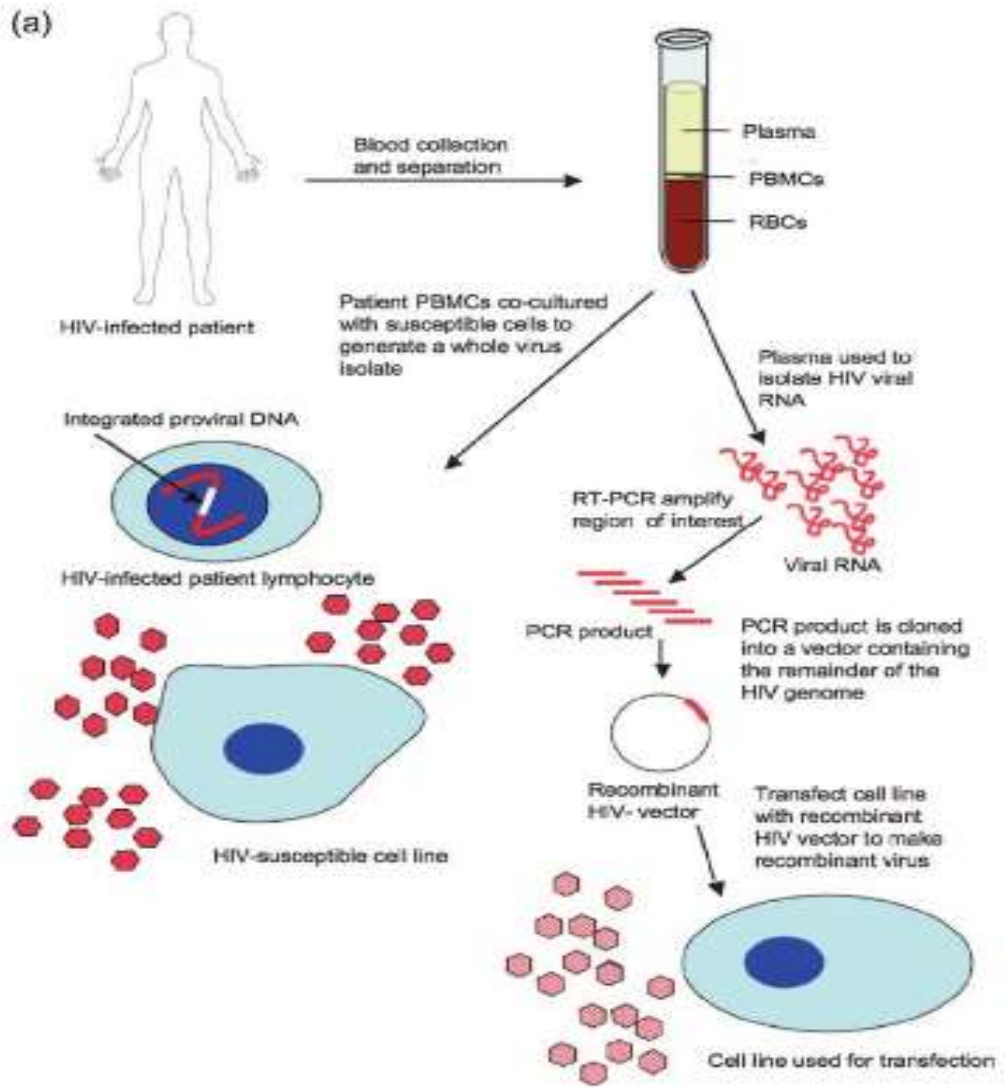
Nucleic Acid Amplification Test

Nucleic acid amplification tests (NAAT) are used in molecular biology to detect unique nucleic acid sequences of viruses in patient samples. **Polymerase chain reaction (PCR)** is an NAAT used to detect the presence of viral DNA in a patient's tissue or body fluid sample. PCR is a technique that amplifies (i.e., synthesizes many copies) of a viral DNA segment of interest. Using PCR, short nucleotide sequences called primers bind to specific sequences of viral DNA, enabling identification of the virus.

Reverse transcriptase-PCR (RT-PCR) is an NAAT used to detect the presence of RNA viruses. RT-PCR differs from PCR in that the enzyme reverse transcriptase (RT) is used to make a cDNA from the small amount of viral RNA in the specimen. The cDNA can then be amplified by PCR. Both PCR and RT-PCR are used to detect and confirm the presence of the viral nucleic acid in patient specimens.

Enzyme immunoassays (EIAs) rely on the ability of antibodies to detect and attach to specific biomolecules called antigens. The detecting antibody attaches to the target antigen with a high degree of specificity in what might be a complex mixture of biomolecules. Also included in this type of assay is a colorless enzyme attached to the detecting antibody. The enzyme acts as a tag on the detecting antibody and can interact with a colorless substrate, leading to the production of a colored end product. EIAs often rely on layers of antibodies to capture and react with antigens, all of which are attached to a membrane filter. EIAs for viral antigens are often used as preliminary screening tests. If the results are positive, further confirmation will require tests with even greater sensitivity, such as a **western blot** or an **NAAT**. EIAs are discussed in more detail in **EIAs and ELISAs**.

Animal **viruses** require **cells** within a host animal or **tissue-culture cells** derived from an animal.



Figure

Caption

FIG. 1. Approaches to measuring HIV-1 replication fitness in cell culture. (a) Production of a virus stock from the peripheral blood of an HIV-infected patient. In this example, peripheral blood is obtained from the patient by venipuncture and separated into its component parts by density gradient centrifugation. A whole-virus isolate is obtained by coculturing the patient's PBMCs with a susceptible cell, either PBMCs from an HIV-negative human donor or an appropriate cell line (left-hand side). The clinical isolate is illustrated as red hexagons and can be harvested by separating the culture supernatant from the cells in culture. A recombinant virus derived from the patient's HIV-1 strain can also be obtained by purifying plasma and amplifying a specific region of the viral genome using reverse transcription followed by PCR (RT-PCR) (right-hand side). The PCR product can then be cloned into a viral vector containing the remainder of the HIV genome. Recombinant virus (illustrated by red striped hexagons) can then be produced by transfecting an appropriate cell line with the recombinant HIV vector. Either of these methods can be used

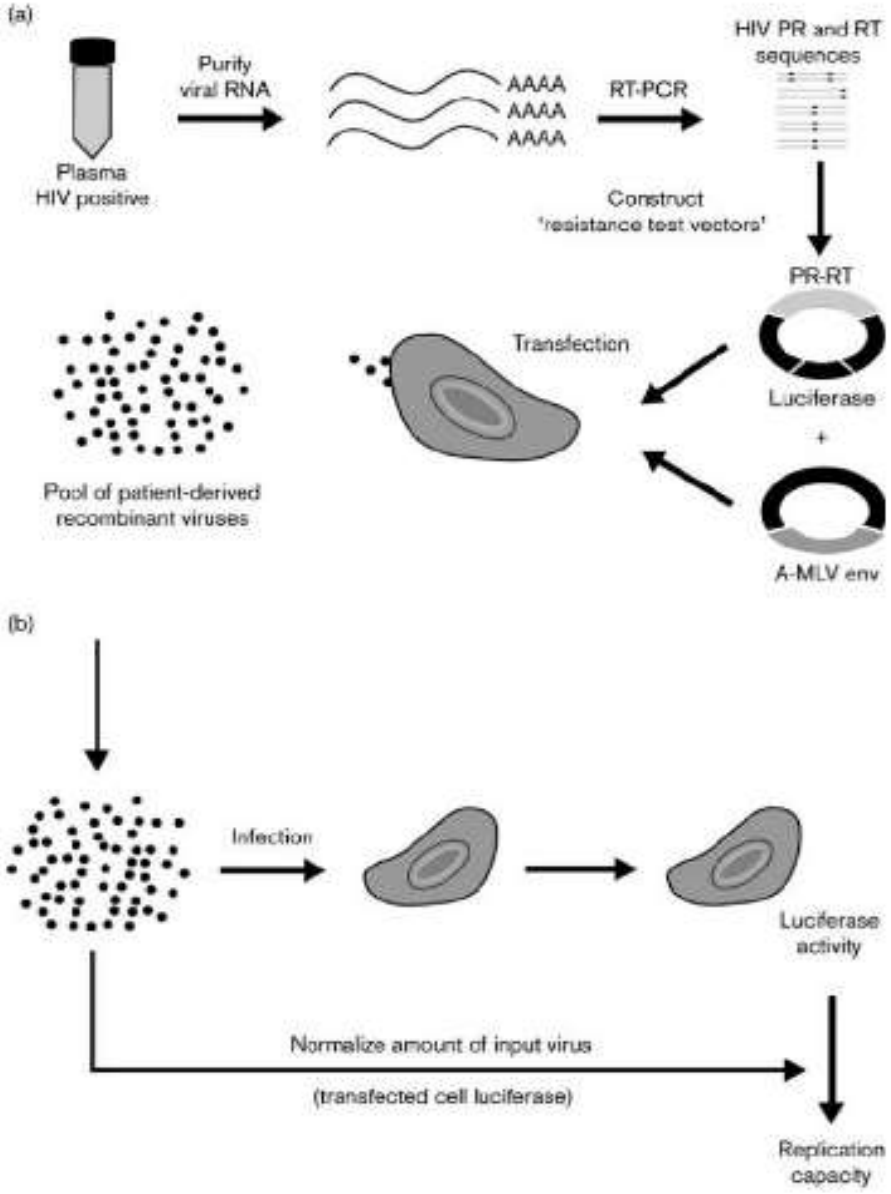
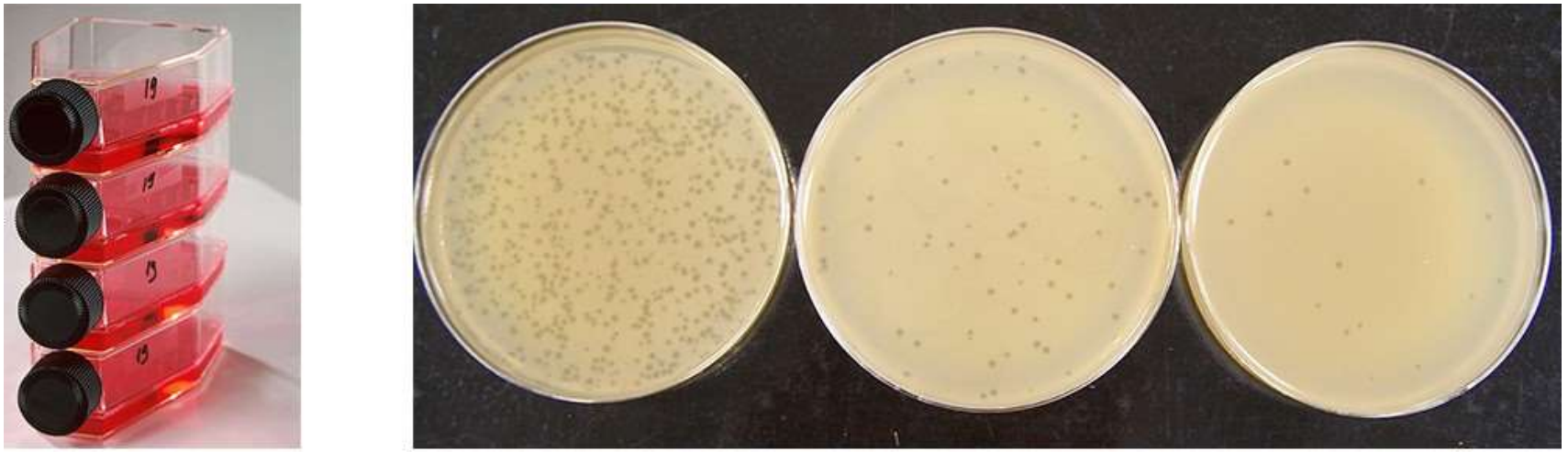


FIG. 2. Design of the Monogram Biosciences RC assay. (a) Production of patient-derived recombinant viruses. HIV-1 genomic RNA is purified from patient plasma. Reverse transcriptase PCR (RT-PCR) is used to amplify a region of the viral genome spanning the 3 J end of gag , protease (PR), and the first 313 codons of reverse transcriptase (RT). The pooled PCR amplicons are cloned into an HIV-1 vector containing a luciferase reporter gene. The resulting recombinant HIV-1 clones are cotransfected together into a mammalian cell line with a plasmid that allows the expression of an amphotropic MLV (A-MLV) envelope. The resultant pool of recombinant pseudotyped viruses will utilize the A-MLV envelope to infect susceptible cells and will express patient-derived protease and reverse transcriptase as well as luciferase. The A-MLV envelope allows the infection of CD4-negative cells. (b) Determination of the replication capacity of patient-derived recombinant viruses. The pool of recombinant viruses is used to infect a cell line; virus replication is quantified by measuring luciferase activity at a single time point. Because the patient-derived recombinant viruses do not encode an envelope protein, progeny viruses will not be infectious, i.e., only a single round of virus replication will occur. (Reproduced from reference 15 with permission of the publisher.)

Cultivation of Viruses

Viruses can be grown **in vivo** (within a whole living organism, plant, or animal) or **in vitro** (outside a living organism in cells in an artificial environment, such as a test tube, cell culture flask, or agar plate). **Bacteriophages** can be grown in the presence of a dense layer of bacteria (also called a **bacterial lawn**) grown in a 0.7 % soft agar in a Petri dish or flat (horizontal) flask . The agar concentration is decreased from the 1.5% usually used in culturing bacteria. The soft 0.7% agar allows the bacteriophages to easily diffuse through the medium. For lytic bacteriophages, lysing of the bacterial hosts can then be readily observed when a clear zone called a **plaque** is detected (see Figure 2). As the phage kills the bacteria, many plaques are observed among the cloudy bacterial lawn



(a) (b)

(a) Flasks like this may be used to culture human or animal cells for viral culturing. (b) These plates contain bacteriophage T4 grown on an *Escherichia coli* lawn. Clear plaques are visible where host bacterial cells have been lysed. Viral titers increase on the plates to the left.

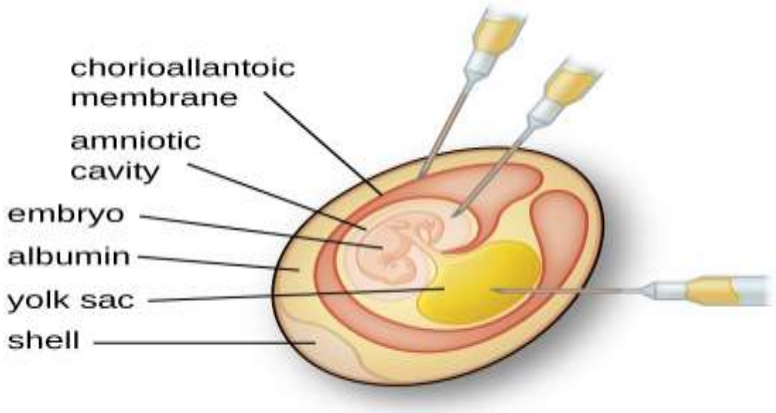
Examples of well-known **cell** types that are standard for most virology laboratories are primary rhesus monkey kidney (RhMK) **cells**, primary rabbit kidney **cells**, human lung fibroblasts (MRC-5), human foreskin fibroblasts, human epidermoid carcinoma **cells** (HEp-2), human lung carcinoma **cells** (A549), and others

- Viral cultivation requires the presence of some form of host cell (whole organism, embryo, or cell culture).
- Viruses can be isolated from samples by filtration.
- Viral filtrate is a rich source of released virions.
- Bacteriophages are detected by presence of clear **plaques** on bacterial lawn.
- Animal and plant viruses are detected by **cytopathic effects**, molecular techniques (PCR, RT-PCR), enzyme immunoassays, and serological assays (hemagglutination assay, hemagglutination inhibition assay).

Animal viruses require cells within a host animal or tissue-culture cells derived from an animal. Animal virus cultivation is important for 1) identification and diagnosis of pathogenic viruses in clinical specimens, 2) production of vaccines, and 3) basic research studies. In vivo host sources can be a developing embryo in an **embryonated bird's egg** (e.g., chicken, turkey) or a whole animal. For example, most of the **influenza vaccine** manufactured for annual flu vaccination programs is cultured in **hens' eggs**.

The embryo or host animal serves as an incubator for viral replication . Location within the embryo or host animal is important. Many viruses have a tissue tropism, and must therefore be introduced into a specific site for growth. Within an embryo, target sites include the **amniotic cavity, the chorioallantoic membrane, or the yolk sac**. Viral infection may damage tissue membranes, producing lesions called pox; disrupt embryonic development; or cause the death of the embryo

(a)



(b)

(a) The cells within chicken eggs are used to culture different types of viruses. (b) Viruses can be replicated in various locations within the egg, including the chorioallantoic membrane, the amniotic cavity, and the yolk sac.

A broad spectrum of cytotoxicity assays is currently used in the fields of toxicology and pharmacology. There are different classifications for these assays: (i) dye exclusion assays; (ii) colorimetric assays; (iii) fluorometric assays; and (iv) luminometric assays.

Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.
Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.
Fluorometric assays: alamarBlue assay and CFDA-AM assay.
Luminometric assays: ATP assay and real-time viability assay.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most commonly used colorimeteric assay to assess cytotoxicity or cell viability . This assay determines principally cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial enzymes such as succinate dehydrogenase . In this assay, MTT is reduced to a purple formazan by NADH. This product can be quantified by light absorbance at a specific wavelength.

Advantages: This method is far superior to the previously mentioned dye exclusion methods because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests .

Disadvantages: MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, prior to measuring the absorbance, an organic solvent such as dimethyl sulfoxide (DMSO) or isopropanol is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error .

Additional control experiments should be conducted to reduce false-positive or false-negative results that caused by background interference due to inclusion of particles. This interference could lead to an overestimation of the cell viability. This can often be controlled by subtraction of the background absorbance of the cells in the presence of the particles, but without the assay reagents .

MTS assay

The MTS assay (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt assay) is a colorimetric assay. This assay is based on the conversion of a tetrazolium salt into a colored formazan by mitochondrial activity of living cells. The amount of produced formazan is depend on the viable cell number in culture and can be measured with spectrophotometer at 492 nm.

Advantages: Previous studies suggest that the MTS in vitro cytotoxicity assay combines all features of a good measurement system in terms of ease of use, precision, and rapid indication of toxicity . MTS assay is a rapid, sensitive, economic, and specific in vitro cytotoxicity assay. Performance of this assay is very competitive to other toxicological tests. This assay provides ideal properties for cytotoxicity measurement because it is easy to use, rapid, reliable, and inexpensive. Therefore, it can be used for onsite toxicological assessments .

Disadvantages: The level of absorbance measured at 492 nm is influenced by the incubation time, cell type, and cell number. The proportion of MTS detection reagents to cells in culture also influences the measured absorbance level. Previous studies suggested a linear relationship between incubation time and absorbance for short incubation times up to 5 hours . Therefore, proper incubation times for this assay are 1–3 hours.

XTT assay

A colorimetric method based on the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) was first described by Scudiero et al. **While MTT produced a water-insoluble formazan compound which required dissolving the dye in order to measure XTT produces a water-soluble dye**. The procedure of XTT is simply for measuring proliferation and is therefore an excellent its absorbance, the solution for quantitating cells and determining their viability. XTT is used to assay cell proliferation as response to different growth factors. It is also used for assaying cytotoxicity.

This assay is based on the ability reduction of the **tetrazolium salt XTT to orange-colored formazan compounds by metabolic active cells**. Orange-colored formazan is water soluble and its intensity can be measured with a spectrophotometer. There is a linear relationship between the intensity of the formazan and the number of viable cells. The use of multiwell plates and a spectrophotometer (or ELISA reader) allows for study with a large number of samples and obtaining results easily and rapidly. The procedure of this assay includes cell cultivation in a 96-well plate, adding the XTT reagent and incubation for 2–24 hours. During the incubation time, an orange color is formed and the intensity of color can be measured with a spectrophotometer .

Advantages: XTT assay is speed, sensitive, easy to use, and safe method. It has high sensitivity and accuracy .

Disadvantages: XTT assay performance depends on reductive capacity of viable cells with the mitochondrial dehydrogenase activity. Therefore, changes of reductive capacity of viable cells resulting from enzymatic regulation, pH, cellular ion concentration (e.g., sodium, calcium, potassium), cell cycle variation, or other environmental factors may affect the final absorbance reading .

WST-1 assay

WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt) cell proliferation assay is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture. **The principle of this assay is based on the conversion of the tetrazolium salt WST-1 into a highly water-soluble formazan by mitochondrial dehydrogenase enzymes in the presence of intermediate electron acceptor, such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate)** . The water-soluble salt is released into the cell culture medium. Within incubation period, the reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase in cell culture and thus, the assay measures the metabolic activity of cells.

To perform the assay, the WST-1 reagent that is ready-to-use is added directly into the media of cells cultured in multiwell plates. The cultures are then given 30 minutes–4 hours to reduce the reagent into the dye form. The plate is then immediately read at 450 nm with a reference reading at 630 nm .

Advantages: It is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests. Furthermore, phenol red indicators in cell culture medium do not interfere with the dye reaction. Because the colored dye which produced at the end of experiment is water-soluble, it is not required a solvent and additional incubation time .

Disadvantages: The standard incubation time of WST-1 time is 2 h. Whether one-time addition of WST-1 can reflect the effect of the testing agents at different time points on the trend of relative cell viability is still unclear

LDH (lactate dehydrogenase) assay

LDH (lactate dehydrogenase) cytotoxicity assay is a colorimetric method of assaying cellular cytotoxicity. LDH Cytotoxicity Assay Kit can be used with different cell types not only for assaying cell-mediated cytotoxicity but also for assessment of cytotoxicity mediated by toxic chemicals and other test compounds. The assay measures the stable, cytosolic, lactate dehydrogenase (LDH) enzyme quantitatively. **This enzyme releases from damaged cells. LDH is an enzyme that is normally found within the cell cytoplasm. When cell viability reduced leakiness of the plasma membrane increase and therefore LDH enzyme is released into the cell culture medium**. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (iodonitrotetrazolium (INT)) into a red color formazan by diaphorase. **In the first step, LDH catalyze conversion of lactate to pyruvate and thus NAD is reduced to NADH/H^+. In a second step, catalyst (diaphorase) transfers H/H^+ from NADH/H^+ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to red formazan .**
The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting red formazan absorbs maximally at 492 nm and can be measured quantitatively at 490 nm.
The detergent Triton X-100 is commonly used as positive control in the LDH assay to determine the maximum LDH release from the cells. In addition, well-known membranolytic particles such as crystalline silica can be used as a positive control in LDH assay .
Advantages: Reliability, speed, and simple evaluation are some of characteristics of this assay. Because, the loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage .
Disadvantages: The major limitation of this assay is that serum and some other compounds have inherent LDH activity. For example, the fetal calf serum has extremely high background readings. Therefore, this assay is limited to serum-free or low-serum conditions, limiting the assay culture period (depending on your cells' tolerance to low serum) and reducing the scope of the assay as it can no longer allow determination of cell death caused under normal growth conditions (i.e. in 10% fetal calf serum). At a minimum, you should always first test the assay with an unused aliquot of the media you intend to use and compare the reading to that from media lacking supplements (e.g. straight DMEM) .

alamarBlue (AB) assay

alamarBlue assay is also known as resazurin reduction assay. **The alamarBlue assay is based on the conversion of the blue nonfluorescent dye resazurin, which is converted to the pink fluorescent resorufin by mitochondrial and other enzymes such as diaphorases.**

Resazurin is a phenoxazin-3-one dye and cell permeable redox indicator that can be used to monitor viable cell number with protocols similar to those utilizing the tetrazolium compounds . It is known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor . It is a nontoxic and cell permeable compound. Color of this compound is blue and it is nonfluorescent. After entering cells, resazurin is reduced to resorufin. Resorufin is red in color and highly fluorescent compound. Viable cells convert continuously resazurin to resofurin, increasing overall fluorescence and color of the cell culture medium. The quantity of produced resofurin is related to the number of viable cells. Ratio of viable cells can be quantified using a microplate reader fluorometer equipped with a 560 nm excitation/590 nm emission filter set. Resofurin can also be measured by absorbance changes, but absorbance detection is not often used because absorbance detection is less sensitive than fluorescence measurement.

The incubation period required to generate a sufficient fluorescent signal above background is usually about 1–4 hours, depending on metabolic activity of the cells, the cell density per well and other conditions such as the culture medium type .

Advantages: alamarBlue (resazurin reduction) assay is relatively inexpensive and more sensitive than tetrazolium assays. Also, it can be multiplexed with other methods such as measuring caspase activity to gather more information about the cytotoxicity mechanism.

Disadvantages: Fluorescent interference from test compounds and the often overlooked direct toxic effects on the cells are possible .

CFDA-AM assay

CFDA-AM (5-carboxyfluorescein diacetate, acetoxymethyl ester) is another fluorogenic dye that is used for cytotoxicity determination. It is indicator for plasma membrane integrity. **The dye CFDA-AM is nontoxic esterase substrate that can be converted by nonspecific esterases of viable cells from a membrane permeable, nonpolar, nonfluorescent substance to polar, fluorescent dye, carboxyfluorescein (CF).** The conversion of CFDA-AM to CF by the cells indicates the integrity of plasma membrane, since only an intact membrane can maintain the cytoplasmic milieu which is needed to support esterase activity .

Advantages: CFDA-AM and alamarBlue assays were shown to be applicable in parallel on the same set of the cells, since both are nontoxic to cells, require similar incubation times, and can be detected at different wavelengths without interferences .

Disadvantages: Fluorescent interference from test compounds is possible.

Protease viability marker assay (GF-AFC assay)
Measurement of a conserved and constitutive protease enzyme activity of viable cells is used as a good indicator of cell viability. **A cell permeable fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) has been recently developed to selectively detect protease activity that is restricted to viable cells . The GF-AFC substrate can penetrate viable cells. In these cells, cytoplasmic aminopeptidase activity removes the gly and phe amino acids to release aminofluorocoumarin (AFC) and produce a fluorescent signal proportional to the number of viable cells** .
When cells die, this protease activity is rapidly loss. Therefore, this protease activity is a selective marker of the viable cell population. The signal generated from this assay approach has been shown to correlate well with other established methods of determining cell viability such as an ATP assay .
Advantages: It is relatively nontoxic to cells in culture. Also, in opposite to exposure of cells to tetrazolium, long-term exposure of the GF-AFC substrate cells results in little change in viability of cells. This assay is suitable for multiplexing with other assays, because at the end of the assay, cell population remains viable and can be used for subsequent assays. Furthermore, the incubation time is much shorter (30 min-1 hour) compared to 1–4 hours required for the tetrazolium assays .
Disadvantages: Fluorescent interference from test compounds is possible.

Luminometric assays

Luminometric assays provide fast and simple determination of cell proliferation and cytotoxicity in mammalian cells. These assays can be performed in a convenient 96-well and 384-well microtiter plate format and detection by luminometric microplate reader . A remarkable feature of the luminometric assays is the persistent and stable glow-type signal produced after reagent addition. This attribute can be harnessed to produce both viability and cytotoxicity values from the same well . Commercial kits of luminometric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

ATP assay

ATP (adenosine tri-phosphate) represents the most important chemical energy reservoir in cells and is used for biological synthesis, signaling, transport, and movement processes. Therefore, cellular ATP is one of the most sensitive end points in measuring cell viability . When cells damaged lethally and lose membrane integrity, they lose the ability to synthetize ATP and the ATP level of cells decreases dramatically . **The ATP assay is based on the reaction of luciferin to oxyluciferin. Enzyme luciferase catalyzes this reaction in the presence of Mg^{2+} ions and ATP yielding a luminescent signal. There is a linear relationship between the intensity of luminescent signal and ATP concentration or cell number** .

The ATP assay chemistry can typically detect fewer than 10 cells per well, and therefore, it has been widely used 1536-well plate format.

Advantages: ATP assay is the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assays. The luminescent signal reaches steady state and stabilizes within 10 min after addition of reagent. It does not have an incubation step for conversion of substrate into colored compound. This also eliminates a plate handling step .

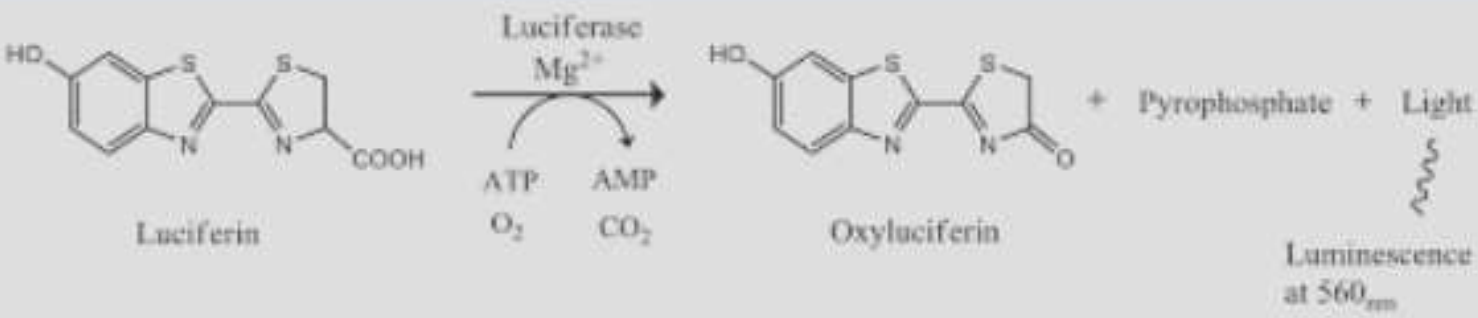
Disadvantages: The ATP assay sensitivity is usually limited by reproducibility of pipetting replicate samples rather than a result of the assay chemistry .

Real-time viability assay

Recently, a new approach is developed to measure viable cell number in real time . **In this assay, an engineered luciferase derived from a marine shrimp and a small molecule pro-substrate is used. The pro-substrate and luciferase are added directly to the cell culture medium as a reagent. The pro-substrate is not a substrate of luciferase. Viable cells with an active metabolism reduce the pro-substrate into a substrate, which used by luciferase, to generate a luminescent signal**. The assay can be performed in two formats: continuous read and endpoint measurement. **In the continuous read format, the luminescent signal can be repeatedly recorded from the sample wells over an extended period to measure the number of cells in "real time" .**

Advantages: This assay is the only assay which allows to real-time measurement of cell viability/cytotoxicity. The rapid decrease in luminescent signal following cell death enables multiplexing this assay with other luminescent assays that contain a lysis step that will kill cells. The decrease in luminescence following cell death is important to eliminate interference with subsequent luminescent assays .

Disadvantages: A limitation of the real time assay results from the eventual depletion of pro-substrate by metabolically active cells. Generally, the luminescent signal generated correlates with the number of metabolically active cells. However, the length of the time the luminescent signal will be linear with cell number will depend on the number of cells per well and their metabolic activity. Therefore, it is recommended that the maximum incubation time to maintain linearity should be empirically determined for each cell type and seeding density .


HO
S
S
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COOH
Luciferin
Luciferase
Mg^{2+}
ATP
AMP
O_2
CO_2
HO
S
S
N
N
O
Oxyluciferin
+ Pyrophosphate + Light
Luminescence
at 560_{nm}

For chromosome ananlysis :

Harvesting lymphocyte culture

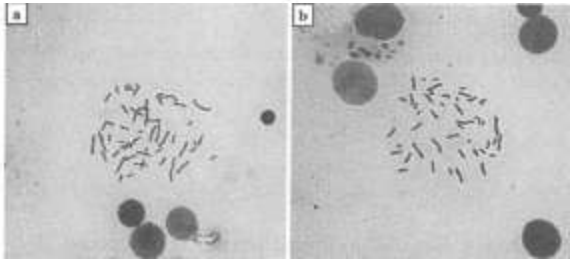
The point of harvest is engineered so that a maximum number of cells are in metaphase.

Increase the mitotic index:

2. Estimation of when most cells are dividing, 24-h interval peak mitotic activity at approximately 72 h
4. Addition of a spindle inhibitor, colchicine, vinblastine, colcemid.

p Cell is subjected to a hypotonic solution (0.075M KCl) -- causing cells to swell and ensuring that chromosome are adequately dispersed within the lymphocytes

p First fixation (methanol: acetic acid = 3 : 1), second fixation and third fixation



Karyotyping
FISH
Chromosome abberation

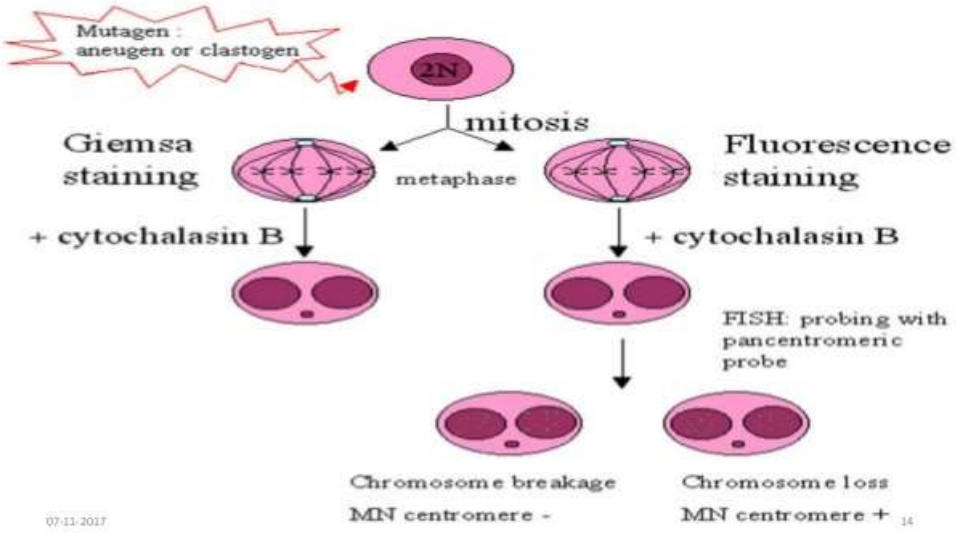
MICRONUCLEUS TEST

- Micronuclei is either the whole chromosome or a chromosomal fragment as a small body outside nucleus

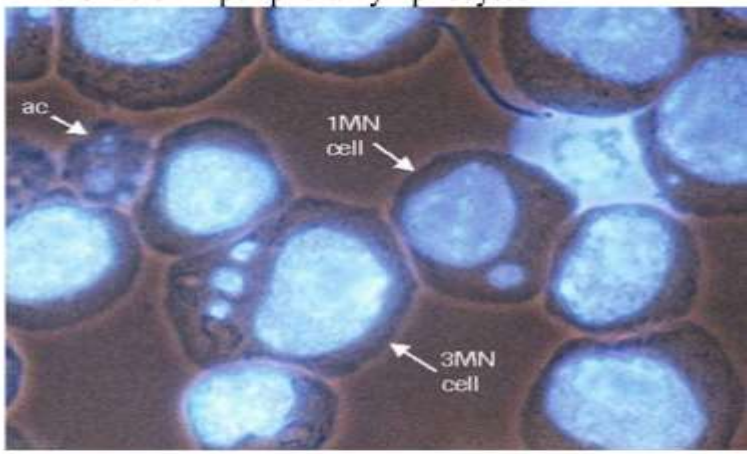
- **Principle**: detection of the frequency of micronuclei:

- Cell cultures of human or other mammalian origin are exposed to the test chemical, → formation of micronuclei in interphase cells.
- Harvested and stained interphase cells are analysed for the presence of micronuclei.
- treated with a cytokinesis blocker, this is easily achieved by scoring only binucleate cells.

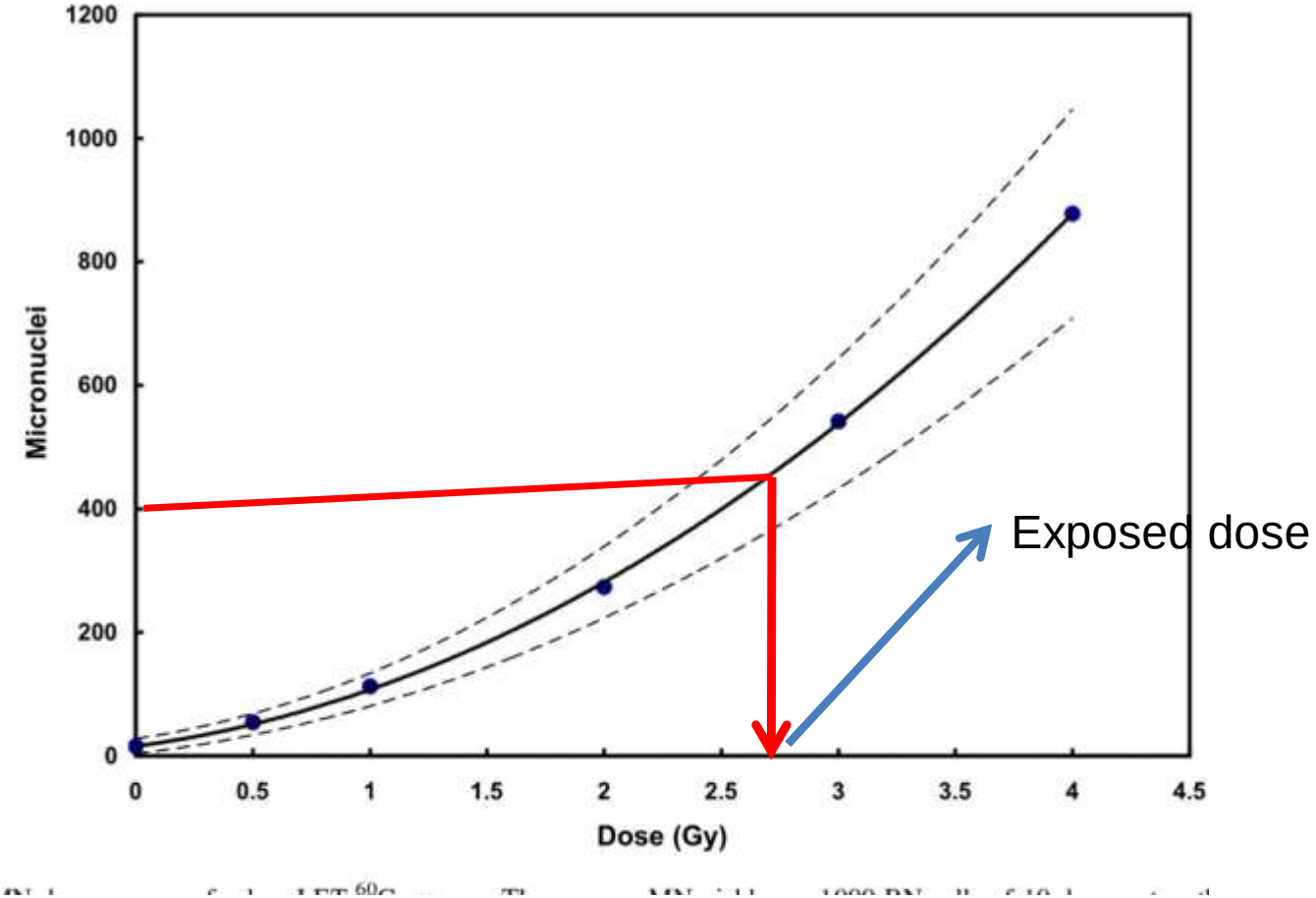
- assay detects the activity of clastogenic and aneugenic chemicals

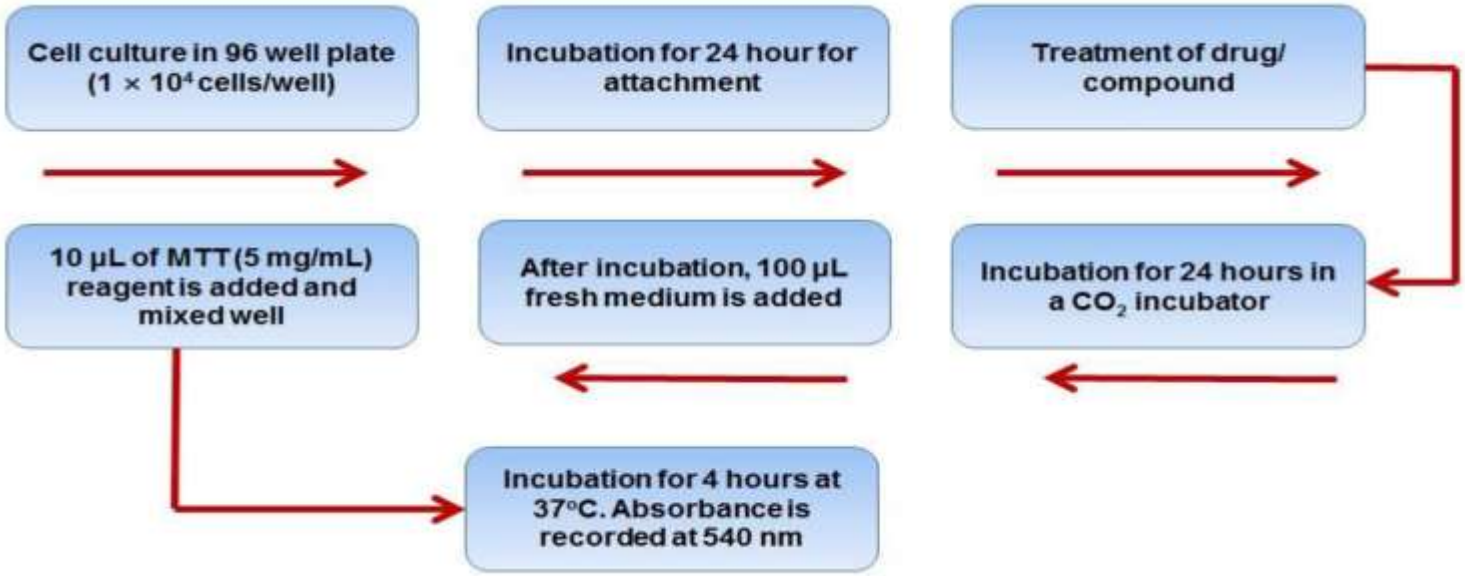


Micronuclei in peripheral lymphocytes

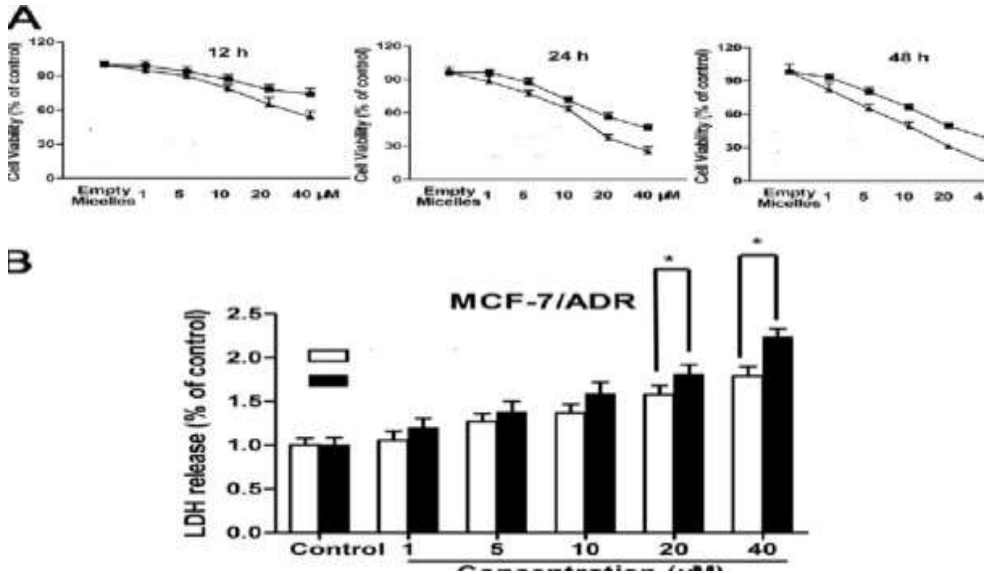


Biological dosimetry, based on the analysis of **micronuclei** (MN) in the cytokinesis-block **micronucleus** (CBMN) assay can be used as an alternative method for scoring dicentric chromosomes in the field of radiation protection. ... in case of a large-scale radiation accident.





Schematic representation of MTT assay protocol



Clonogenic survival assay

(A) Plating before treatment

1) Collect cells and re-plate the appropriate number of cells per dish or per well. The number of cells depend on the severity of the treatment, if you do not know the appropriate effect, use different dilutions of different cell numbers.
2) Incubate the cells for a few hours in a CO_2 incubator at 37°C and allow them to attach to the dish or plate.
3) Treat the cells as needed with chemicals, radiation or a combination of both.
4) Incubate the cells in a CO_2 incubator at 37°C for 1-3 weeks until the control dishes form sufficiently large colonies (50 cells per colony is the minimum for counting).

(B) Plating after treatment

1) Collect cells after treatment. 100 or up to 10^4 cells can be pipetted into the test wells. It is always better to keep the cells on ice before re-plating. If you do not know the severity of the treatments, prepare serial dilutions with different numbers of cells.
2) Incubate the cells in a CO_2 incubator at 37°C for 1-3 weeks until the control dishes form sufficiently large colonies (50 cells per colony is the minimum for counting).

Fixation and Staining

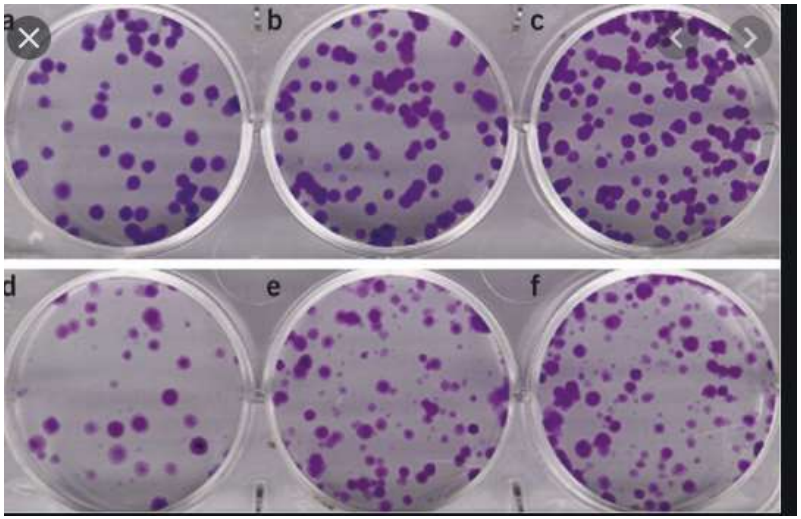
1) Remove the medium and rinse carefully with 10 mL PBS.
2) Remove the PBS and add 2-3 mL of fixation solution.
3) Incubate at room temperature for 5 min.
4) Remove the fixation solution and add 0.5% crystal violet.
5) Incubate at room temperature for 2 hours.
6) Remove the crystal violet carefully and rinse with tap water. Instead of placing the dishes or plates under the running tap, fill the sink with water and immerse the dishes or plates carefully.
7) Air-dry the dishes or plates at room temperature.

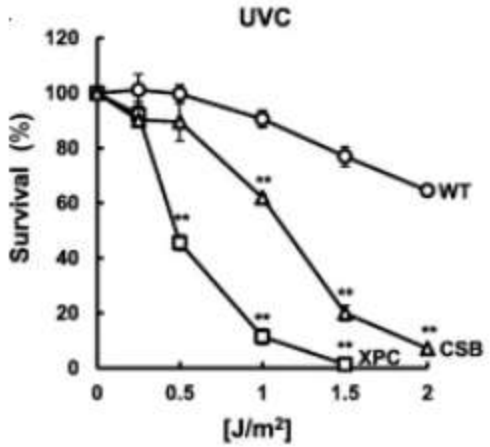
Counting the Colonies

1) Count the number of colonies using a stereomicroscope or an automatic counting "colony counter".
2) Calculate the plating efficiency (PE) and surviving fraction (SF).

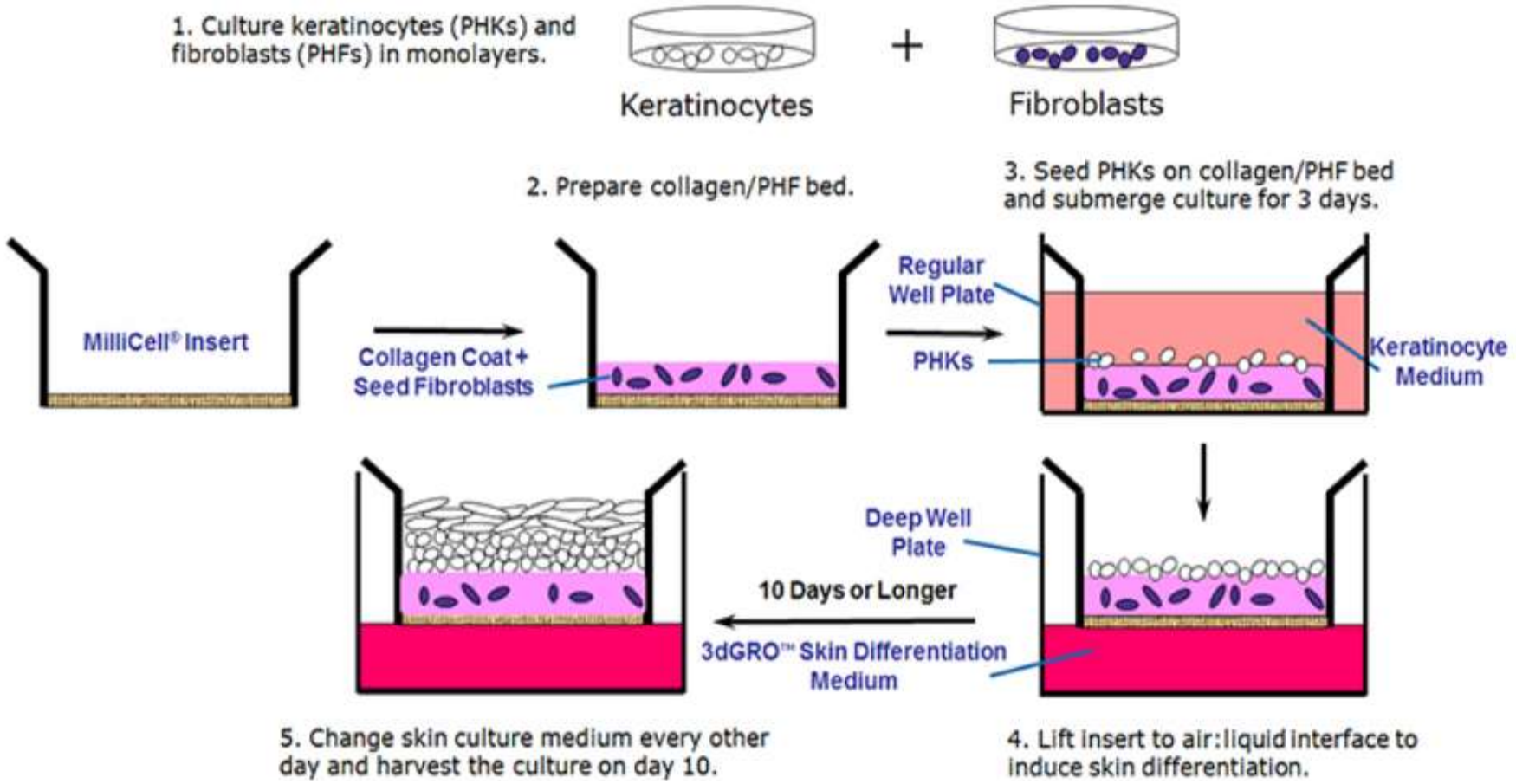
$$\text{Plating efficiency (PE)} = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\%$$

$$\text{Survival fraction (SF)} = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded x PE}} \times 100\%$$





Organotypic human skin culture



Embryonic stem cells, or ES cells, have a number of unique properties that distinguish them from regular adult cells. Therefore, scientists have devised special culturing techniques to maintain or take advantage of these properties. When cultured properly, ES cells can divide indefinitely to make more of themselves. At the same time, by altering the culturing conditions, ES cells can be directed to differentiate into almost any cell type found in our body; this ability of ES cells is called "pluripotency."

Cell Culture Techniques for ESC

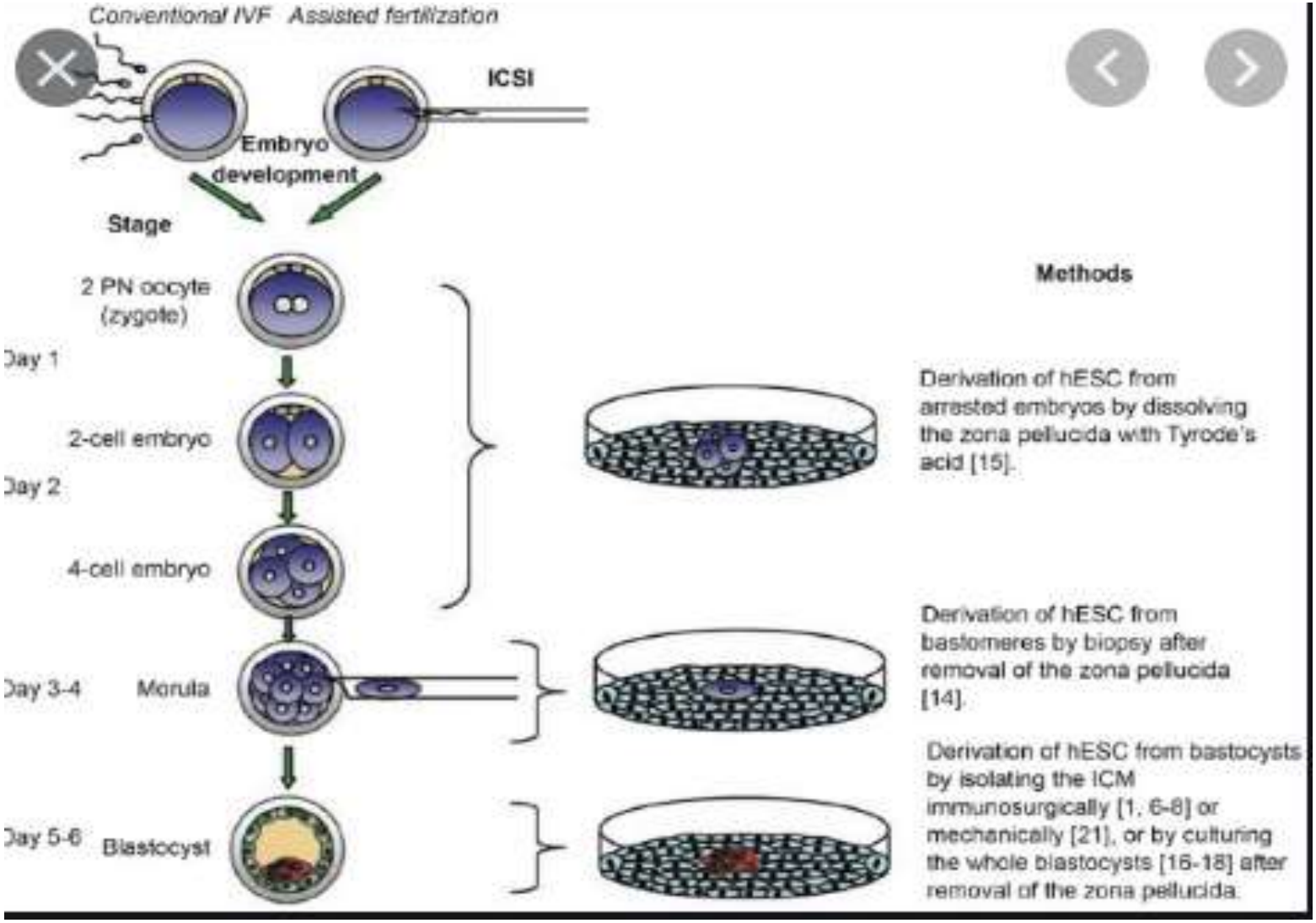
- Isolate & transfer of inner cell mass into plastic culture dish that contains culture medium

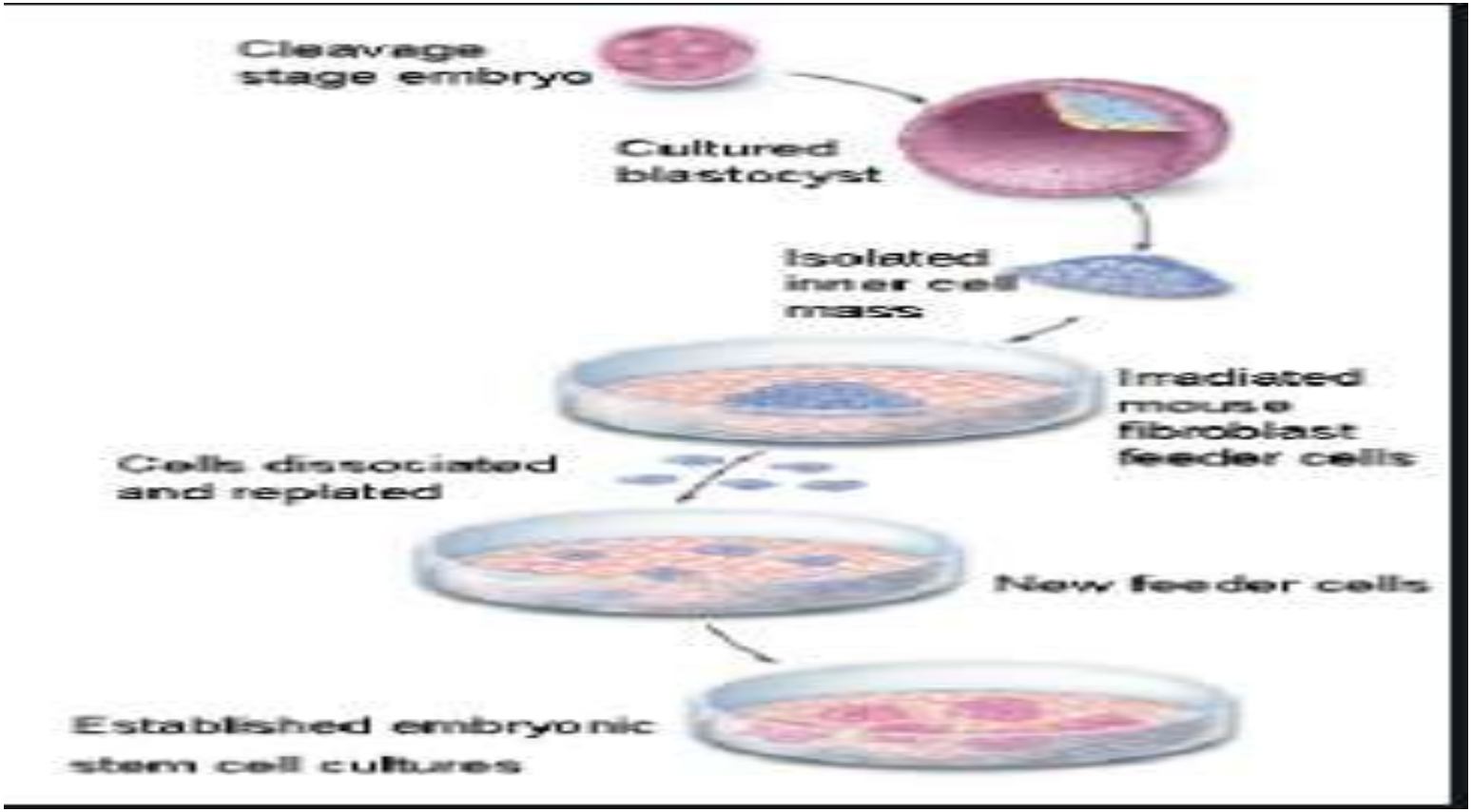
- Cells divide and spread over the dish
Inner surface of culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide

- This coating is called a FEEDER LAYER

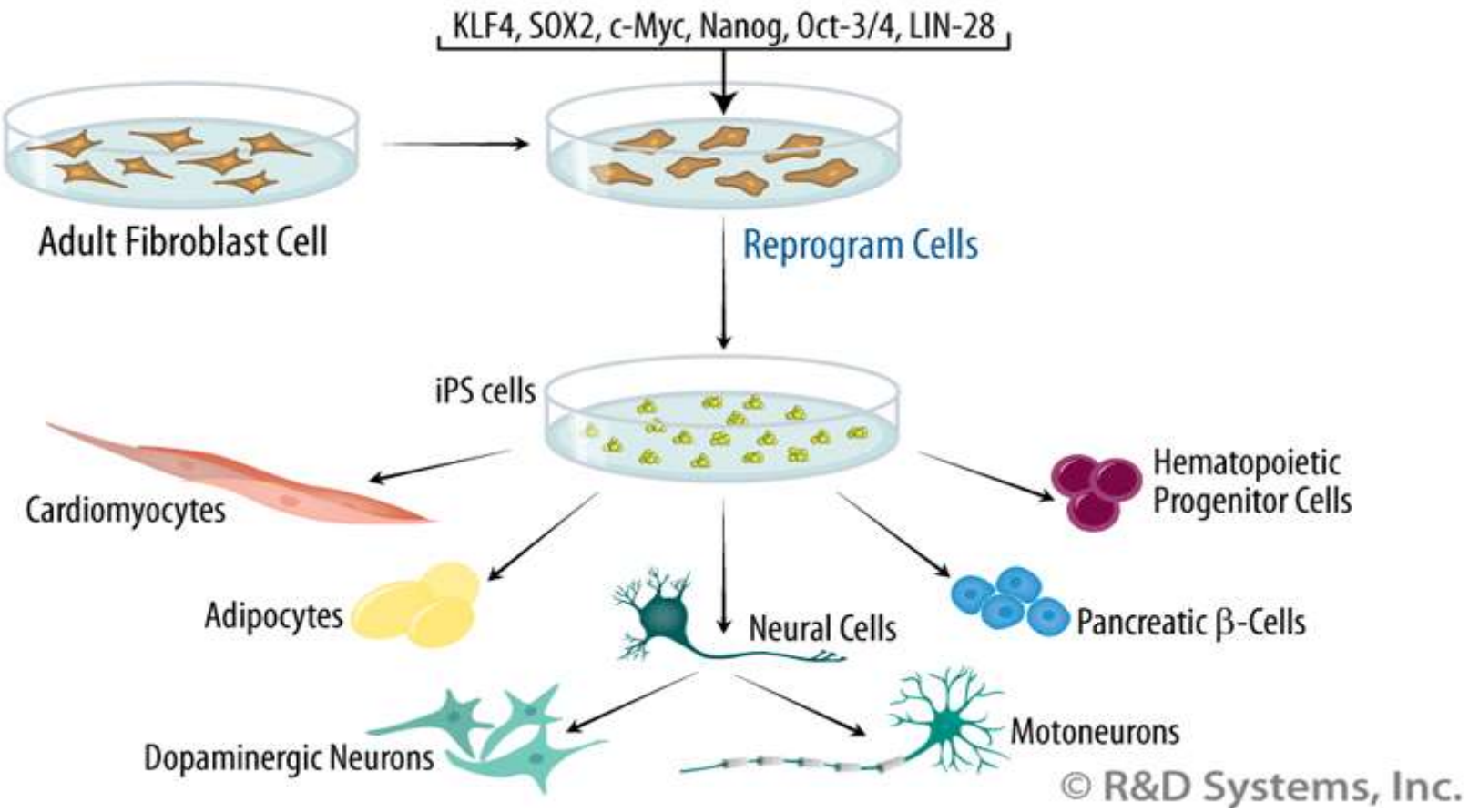
    - Feeder cells provide ES cells with a sticky surface for attachment
    - Feeder cells release nutrients

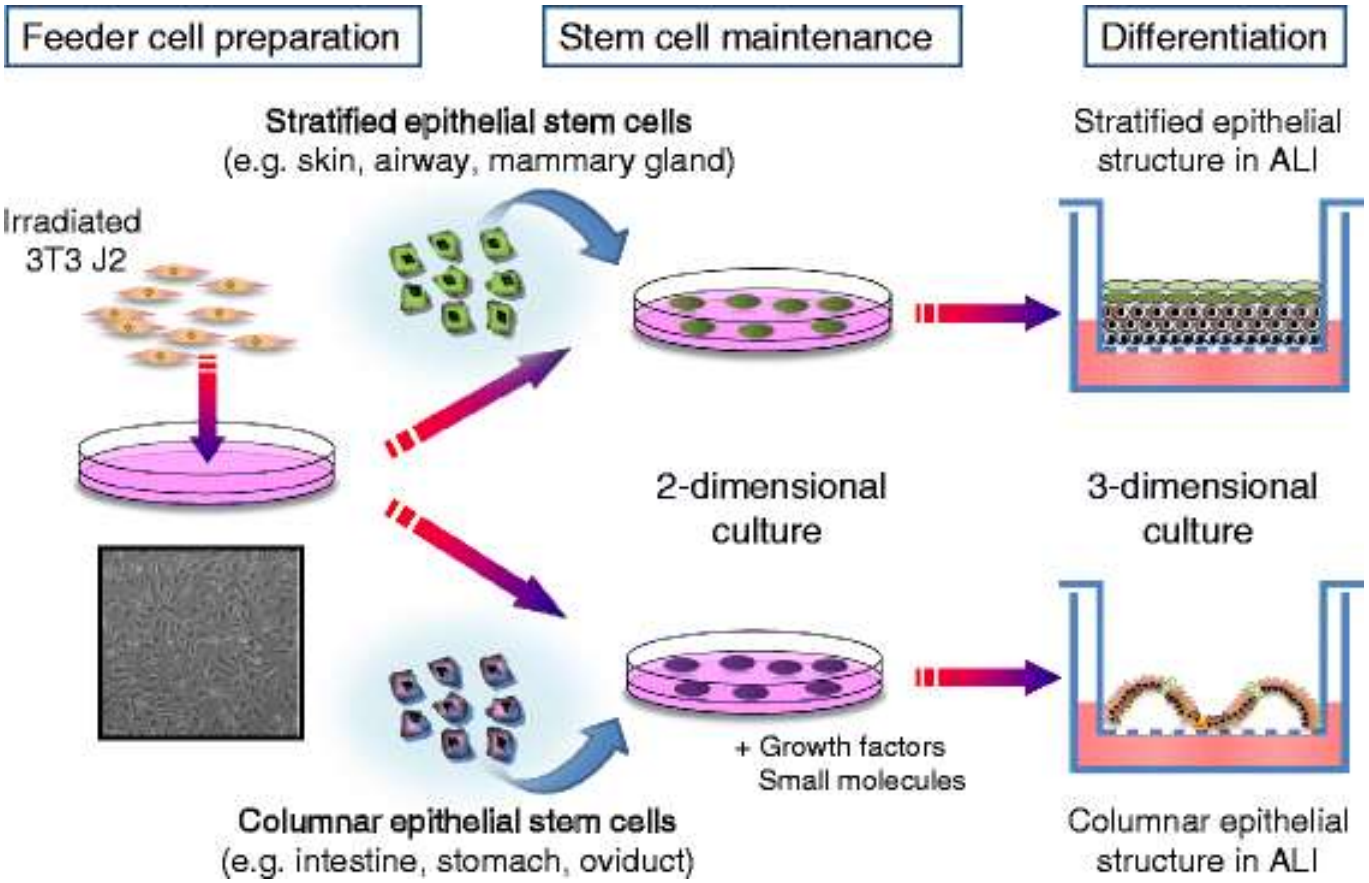
embyonic stem cell culture




Cleavage stage embryo
Cultured blastocyst
Isolated inner cell mass
Irradiated mouse fibroblast feeder cells
Cells dissociated and replated
New feeder cells
Established embryonic stem cell cultures

The four classical transcription factors that have been demonstrated to **induce pluripotency** are Oct4, Sox2, cMyc, and Klf4. These factors are also known as **Yamanaka factors**, after the researcher who discovered their reprogramming effects. Multiple methods can be used to **induce** expression of these transcription factors.





Schematic of the cell culture process for human stratified and columnar epithelial stem cells on a 3T3 mouse feeder layer. For stratified epithelial stem cells, they are isolated from biopsy or surgical specimens are plated on a 3T3 layer for a long-term culture. For columnar epithelial stem cells, they are plated on a 3T3 layer with defined factors which are essential for stem cell growth and maintenance. Morphologically immature colonies (packed colonies with small cells) of epithelial stem cells are mechanically picked-up for further homogeneous expansion.

Feeder Cell- and Undefined Component-Based Culture Conditions

Optimal culture medium (which composed of growth factors) and a suitable microenvironment are essential for the maintenance of the pluripotency and the characteristic of PSCs. The original method for PSCs culture was developed in 1998 and based on their adherence to feeder cells or ECM. Mouse embryonic fibroblast (MEF), which is irradiated or treated with mitomycin-C, was used as a feeder layer for PSC cultures to maintain their self-renewal and proliferation capacities. Other sources of feeder cells include STO or SNL cell lines cultured using fetal bovine serum (FBS) or a serum replacement. MEF provide essential proteins and growth factors, including ECM proteins, vitronectin, laminin, and transforming growth factor β (TGF-β), and form a basic feeder layer in combination with growth medium (Dulbecco's modified Eagle's medium [DMEM]) supplemented with fetal bovine serum (FBS), non-essential amino acids, glutamine, and β-mercaptoethanol. However, MEF and FBS represent xenogeneic-based nutritional sources. The use of animal-derived serum and feeder layers leads to microbial infection and contamination with animal-derived antigens (*N*-glycolneuraminic acid and sialic acid) that induce immune reactions upon the transplantation of stem cells. Collectively, the application of animal-originated culture components represents a challenge for the transplantation of stem cells in human for the clinical application.

Amit et al. developed an alternative defined culture system for hESCs that avoided the use of animal-derived feeder layers and maintained hESC pluripotency, even after a long culture period.This culture system employed a fibronectin matrix by using 15% serum replacement (SR) that was supplemented with growth factors, including transforming growth factor β1 (TGF-β1), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF).

In 2006, TeSR1 was developed, a defined media for ESCs culture that consisted of TGF-β1, lithium chloride, pipecolic acid, and γ-aminobutyric acid.

In 2007, Watanabe et al. showed that Y-27632, a Rho-associated kinase (ROCK) inhibitor, potently suppressed the apoptotic changes that arose from cell dissociation in ESCs cultured in serum-free suspension and promoted cell survival and differentiation.

In 2008, Miyazaki et al. identified for the first time the capacity of various isoforms of the recombinant human laminin in maintaining the pluripotency as well as the undifferentiated state of hESCs after a long time of passaging via their specific binding to integrins.

In 2010, Mei et al. developed a high-throughput method to prepare novel engineered culture substrates for the culture of hPSCs with defined and xeno-free culture conditions. These novel engineered substrates are composed of high acrylate-contained monomers and possess unique physical and chemical properties. In 2010, Rodin et al. successfully maintained the self-renewal property, normal karyotype, and the stemness of hESCs cultured on human recombinant laminin-511-coated culture plate.

In 2011, Irwin et al. synthesized novel engineered hydrogel interface-based culture approach, which is a cost-effective method, scalable, and enable the long-term culture of hESCs in defined culture conditions.
In 2012, Miyazaki et al. proved the capacity of the recombinant prepared recombinant laminins (laminins E8) in promoting the adhesion of hPSCs and maintaining their undifferentiated status under defined culture condition.
In 2013, Zhang et al. potently cultured hESCs for long culture period with a chemically defined, synthetic, thermoresponsive, and 2-(diethylamino) ethyl acrylate-based hydrogels. This novel adhesion method not only maintains the pluripotency and stemness characteristics of hESCs, but also allows the passaging via the thermal modulation (from 37 to 15 °C for 30 min) that avoids the cell damage from using other conventional cell pasaging method

Stem Cell Niche-Associated Adhesion Molecules

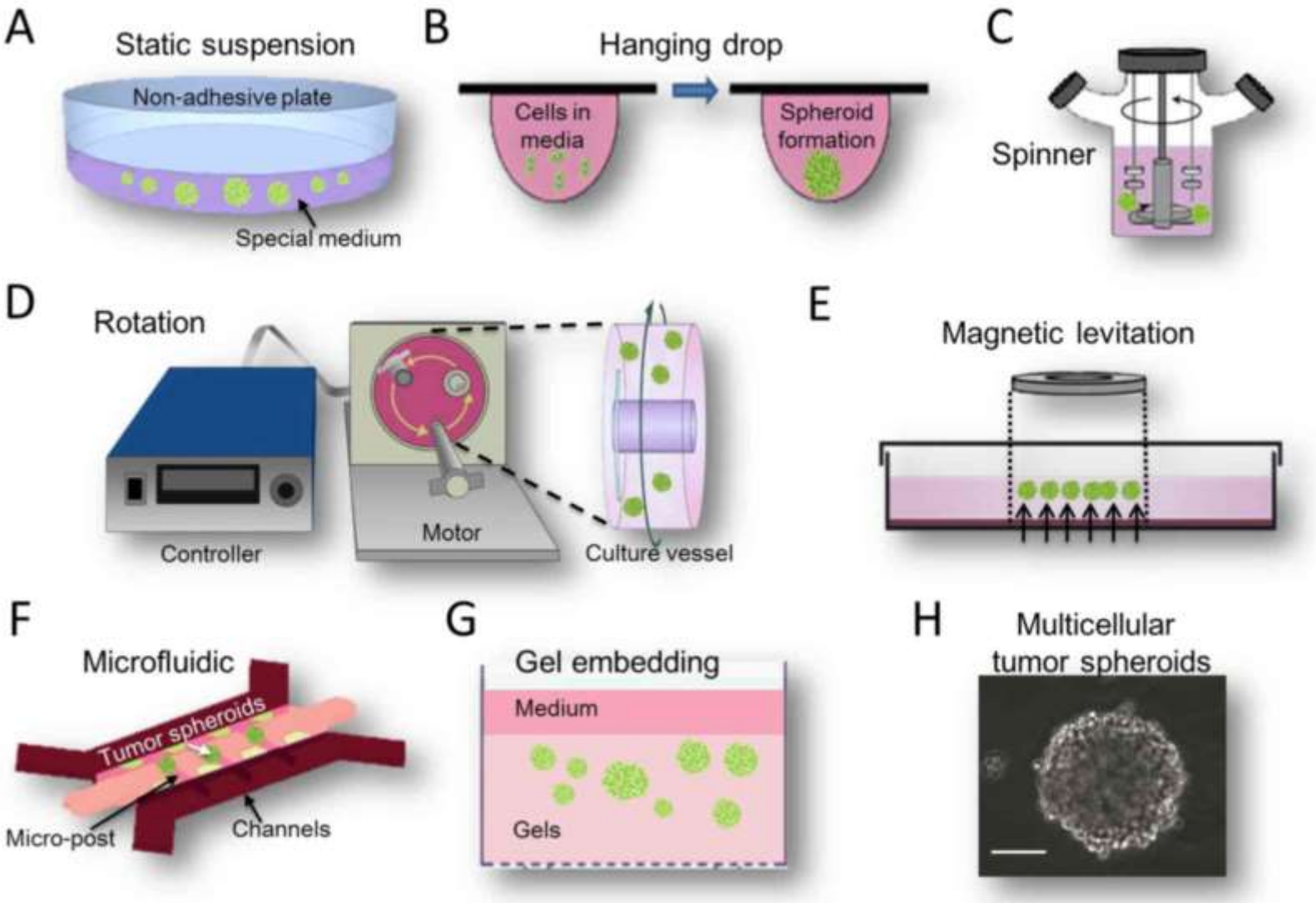
The niche is a unique anatomical slot in which cell–cell and cell–ECM interactions occur, which are mediated by CAMs, and activated growth factors or signaling molecules are found. CAMs are cell-surface proteins, which are involved in homophilic interaction (between the same molecules) or heterophilic interaction (between different molecules).

Cadherins, integrins, CAMs of the immunoglobulin superfamily, and selectins are considered the foremost CAMs classes, with a well-characterized mechanism of action

ECM-Derived Adhesion Molecules

Glycoproteins such as fibronectin, laminin, and vitronectin, elastin, collagens, and proteoglycans are considered to be the main components of ECM

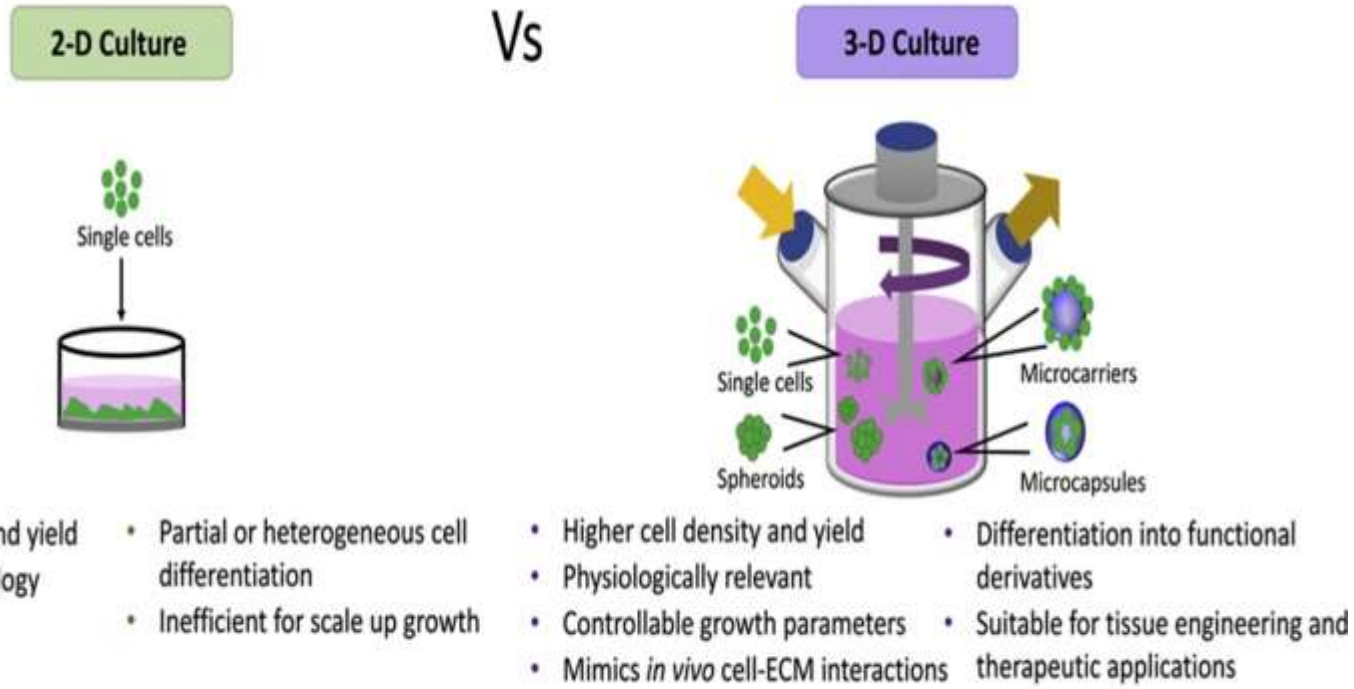
The past decades have witnessed significant efforts toward the development of three-dimensional (3D) cell cultures as systems that better mimic in vivo physiology. Today, 3D cell cultures are emerging, not only as a new tool in early drug discovery but also as potential therapeutics to treat disease



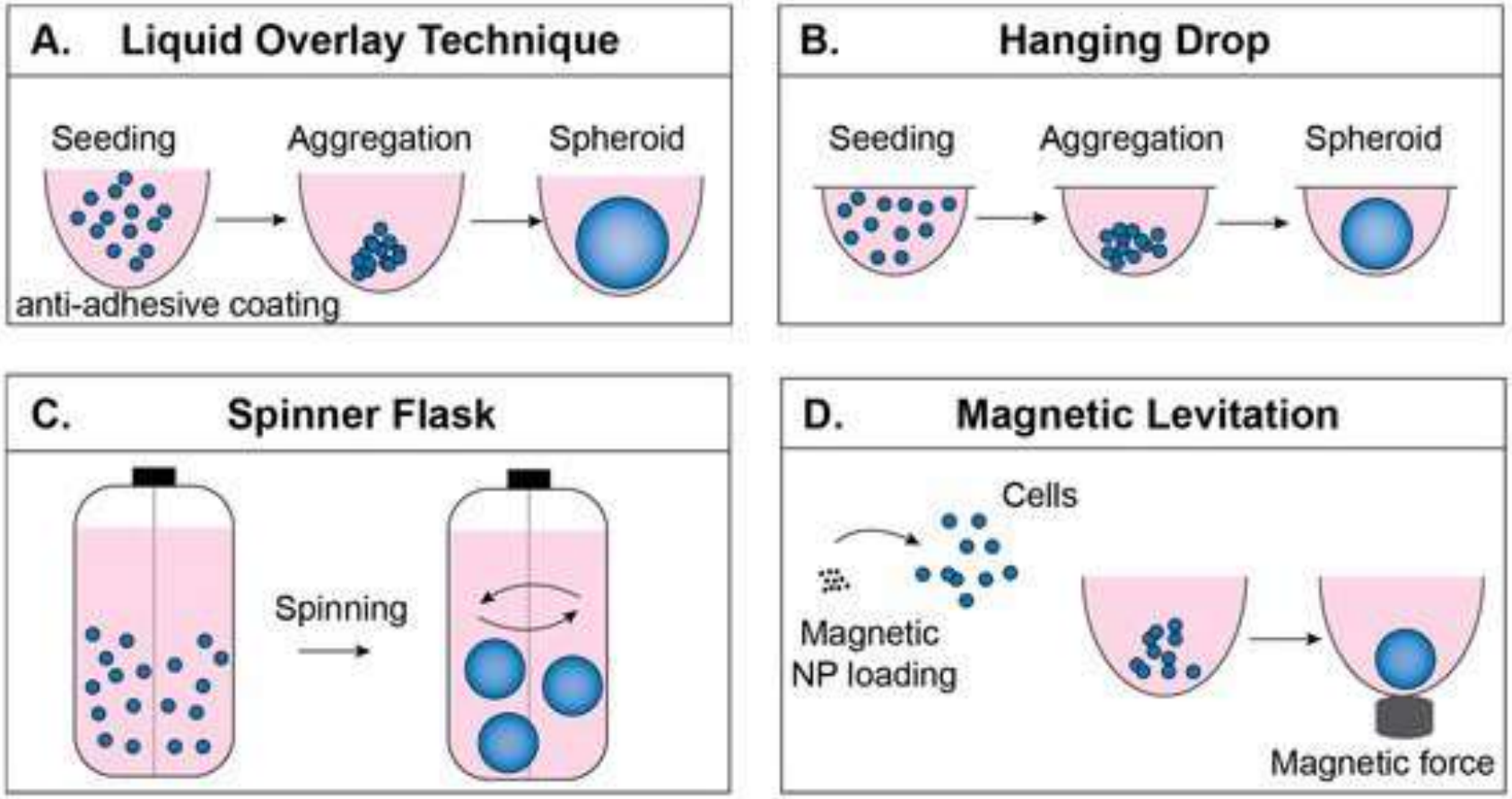
Methods available for multicellular tumor spheroids(MCTS) formation. These methods include (A) static suspension; (B) hanging drop methods; (C) spinner bioreactor; (D) rotational bioreactor; (E) magnetic levitation; (F) microfluidic system; and (G) gel embedding. (H) A classic MCTS was observed by inverted phase contrast microscope (scale bar, 100 μm).

Lack of scalability of 2-D culture systems.

- Physiologically relevant human cell culture techniques.
- Techniques mimicking native microenvironment for SC growth and differentiation.
- Higher density and billion fold expansion using 3-D culture systems.
- Challenges and advantages of 3-D culture systems.



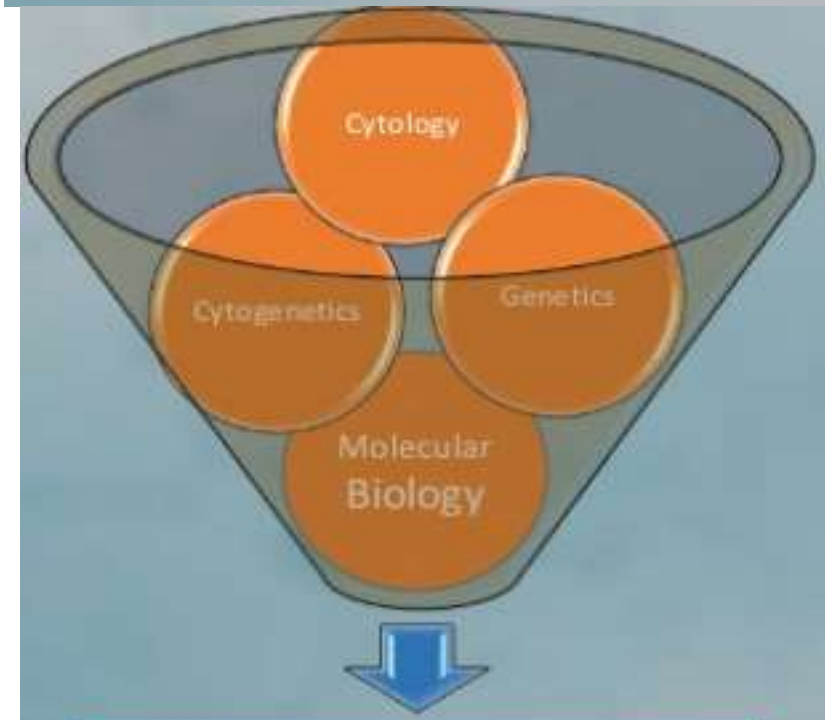
Cell Cultures	Advantages	Disadvantages	Applications (References)
Hydrogel Matrix	Cell–ECM interactions, easy to incorporate growth factors, *in vivo*-like microenvironments, long-term culture, uniform spheroid	Cumbersome to dispense cells in hydrogels and change growth media, thus low throughput, difficult to retrieve cells after 3D formation	*In vitro* angiogenesis and drug testing [57,58]; Drug response study [14,59]; Cancer research [60]
Hanging Droplet	Simple spheroid formation by gravity, homogenous spheroids that are easily accessible	Labor intensive and time consuming, no cell-ECM interaction, difficult to change growth media, transferring of spheroids for analysis required, sensitive to mechanical shocks	Hepatotoxicity testing with HepaRG cells [61,62]; Target identification and validation using RNAi [63]
Liquid Overlay	Simple to use, inexpensive, long-term culture	Labor intensive and time consuming, low throughput due to the centrifugation step involved, heterogeneous spheroids, difficult to mass produce	Evaluation of therapeutic response of anticancer drugs [58]; Identification of anticancer drugs [55]; Hepatotoxicity testing with iPSC-derived hepatocytes [64]


A. Liquid Overlay Technique
Seeding
Aggregation
Spheroid
anti-adhesive coating
B. Hanging Drop
Seeding
Aggregation
Spheroid
C. Spinner Flask
Spinning
D. Magnetic Levitation
Cells
Magnetic
NP loading
Magnetic force

MOLECULAR CYTOGENETICS

(Graphics are collected from internet)

Cytogenetic – The branch of genetics which deals with the study of cell and structure and function of the chromosomes is called as cytogenetic.



Molecular Cytogenetics

It involves the combination of molecular biology and cytogenetic. In general this involves the use of a series of techniques in which DNA probes are labeled with different colored fluorescent tags to visualize one or more specific regions of the genome.

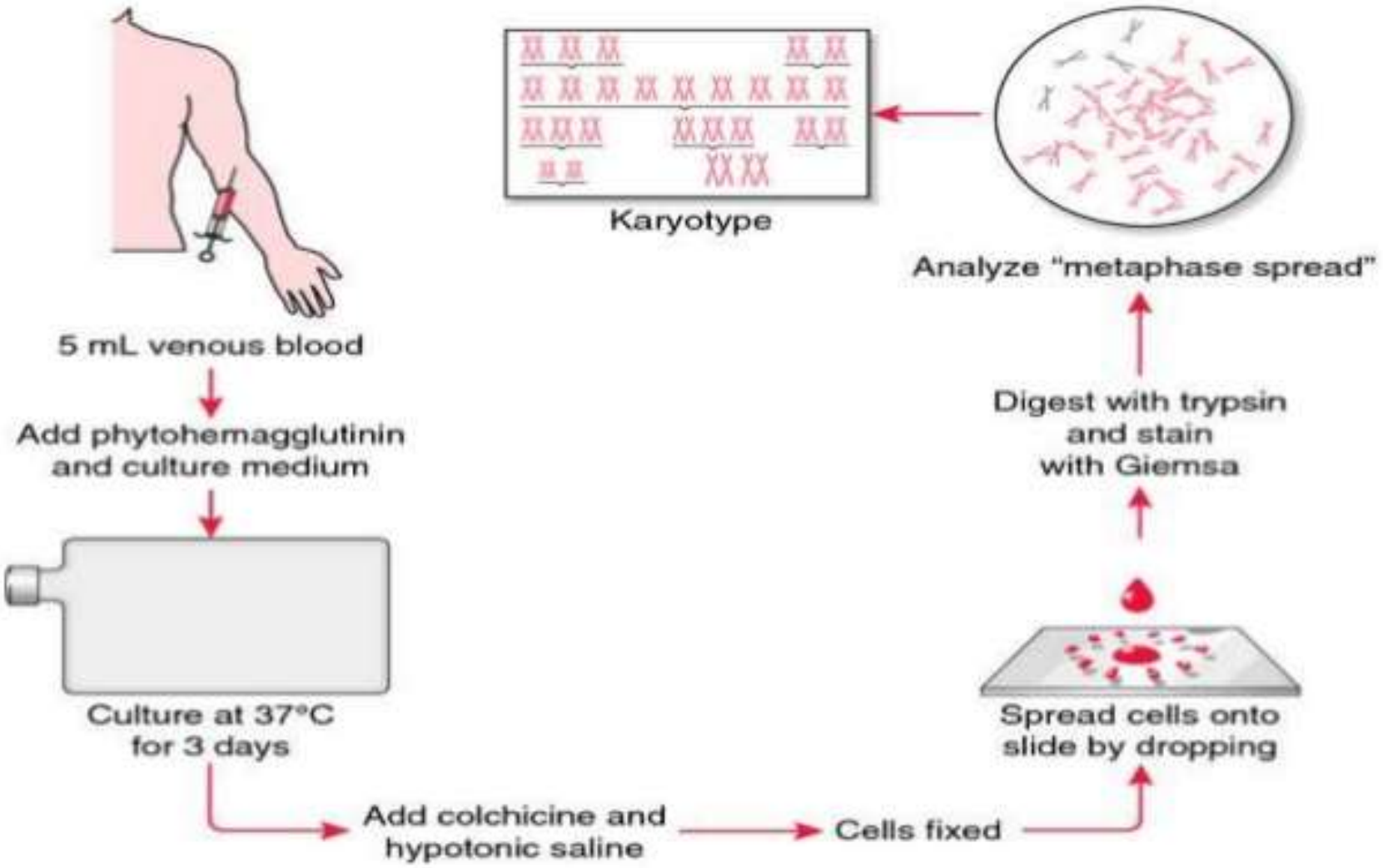
Which Are The Molecular Cytogenetic Techniques…..?

What karyotyping does…..?

- These techniques produce a characteristic pattern of contrasting dark and light transverse bands on the chromosomes.
- Banding makes it possible to identify homologous chromosomes by visualization of chromosomes.
- Banding of homologous chromosomes allows chromosome segments and rearrangements to be identified.
- it helps to detect the chromosomal abnormalities and chromosomal alterations.
- The most widely used banding methods are G-banding (Giemsa-banding) and R-banding (reverse-banding).

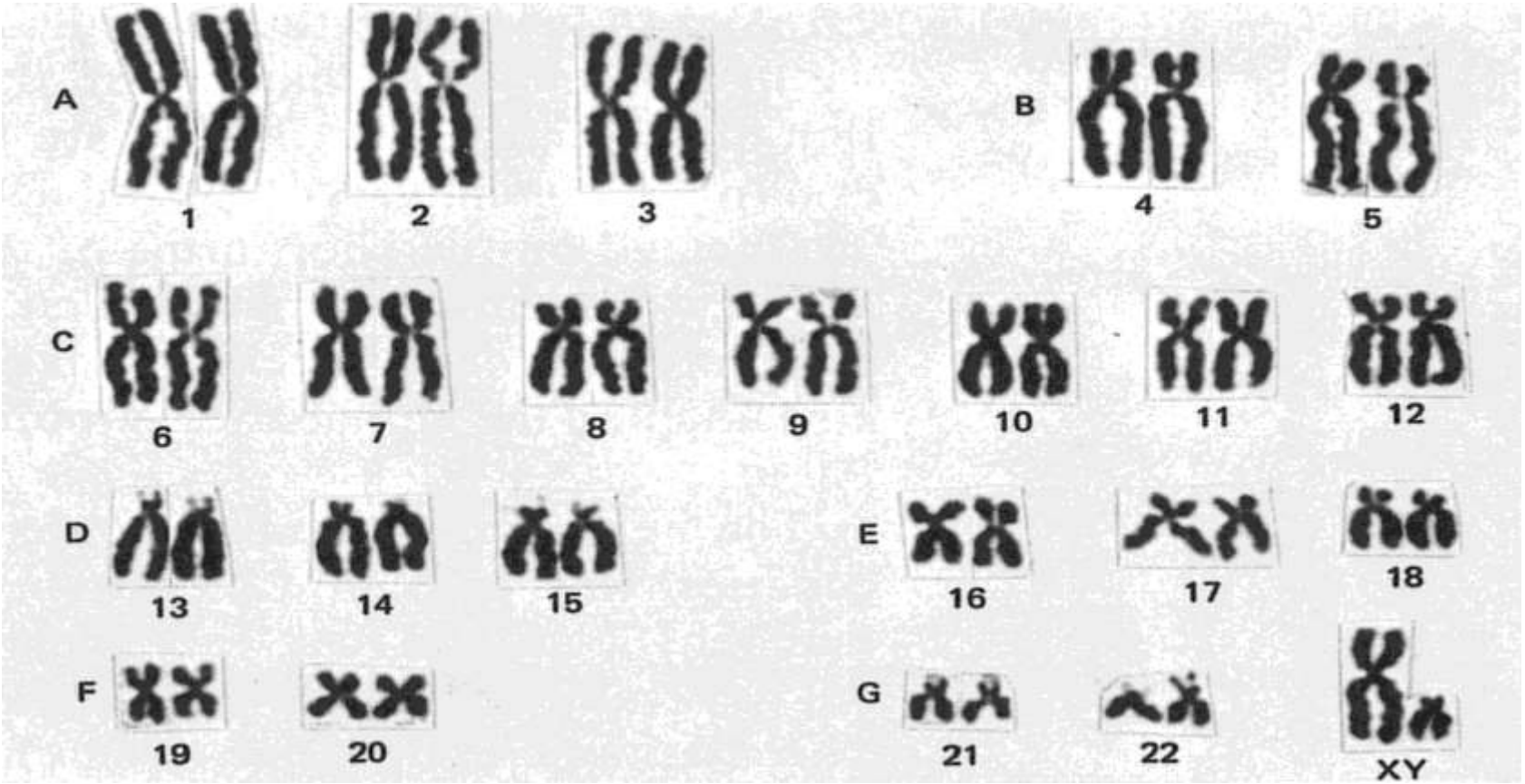
How To Do Karyotyping……?

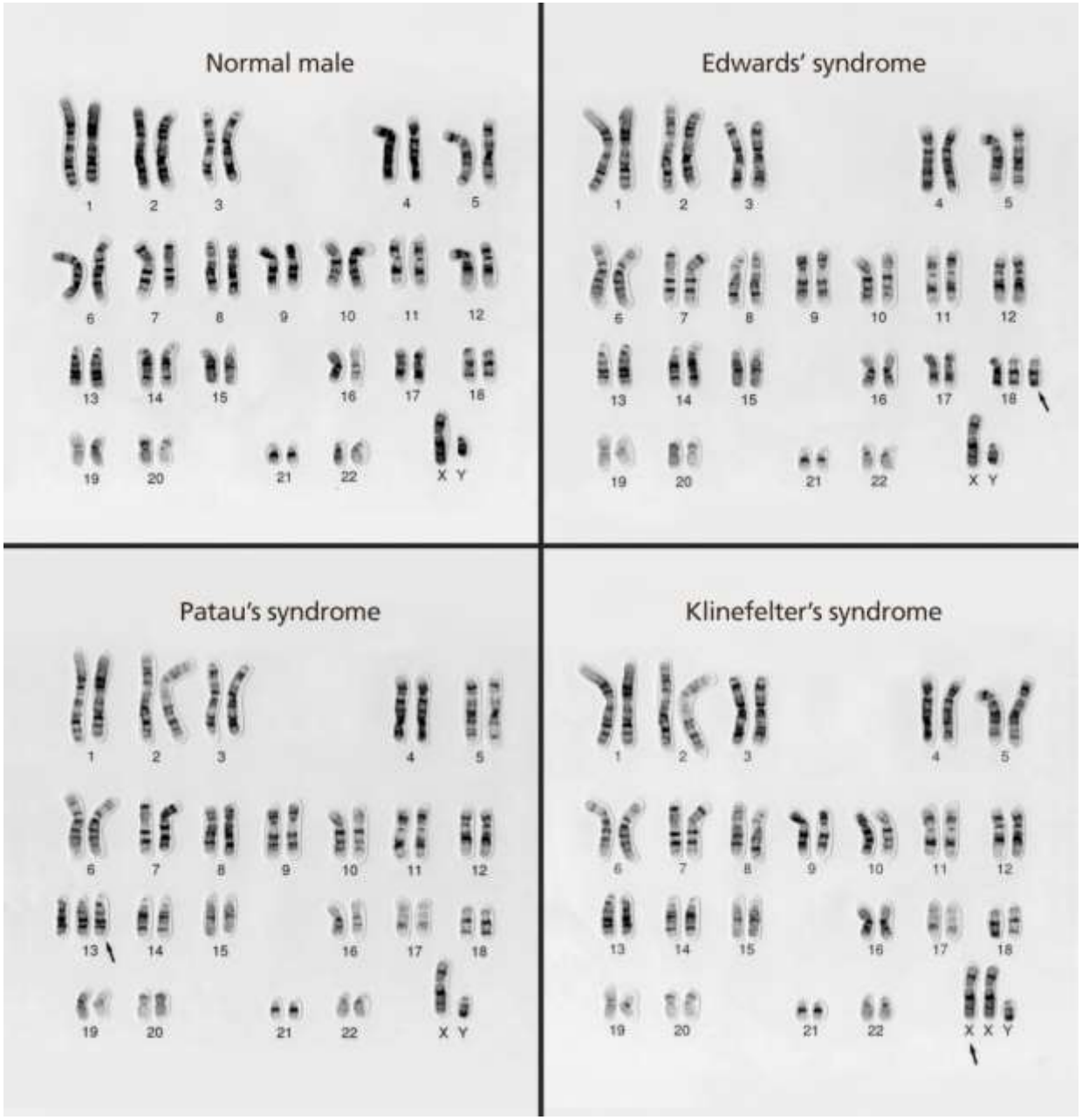
Procedure of karyotyping



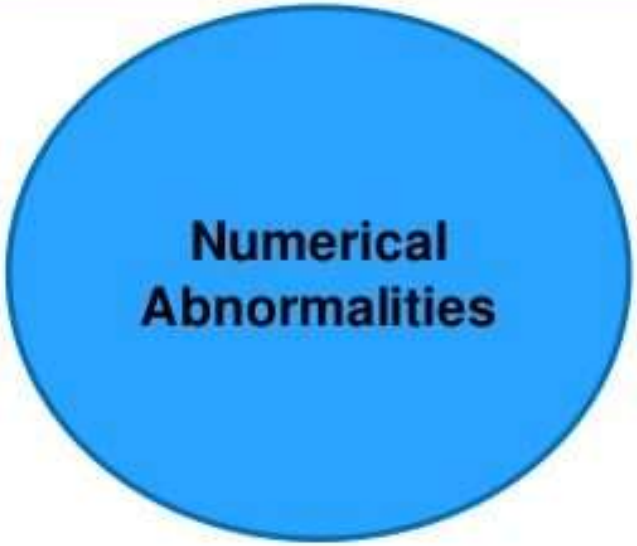
Karyotyping is a test to examine chromosomes in a sample of cells. This test can help identify genetic problems as the cause of a disorder or disease

Karyotypes can reveal changes in chromosome number associated with aneuploid conditions, such as **trisomy 21** (**Down syndrome**). Careful analysis of karyotypes can also reveal more subtle structural changes, such as **chromosomal** deletions, duplications, translocations, or inversions.



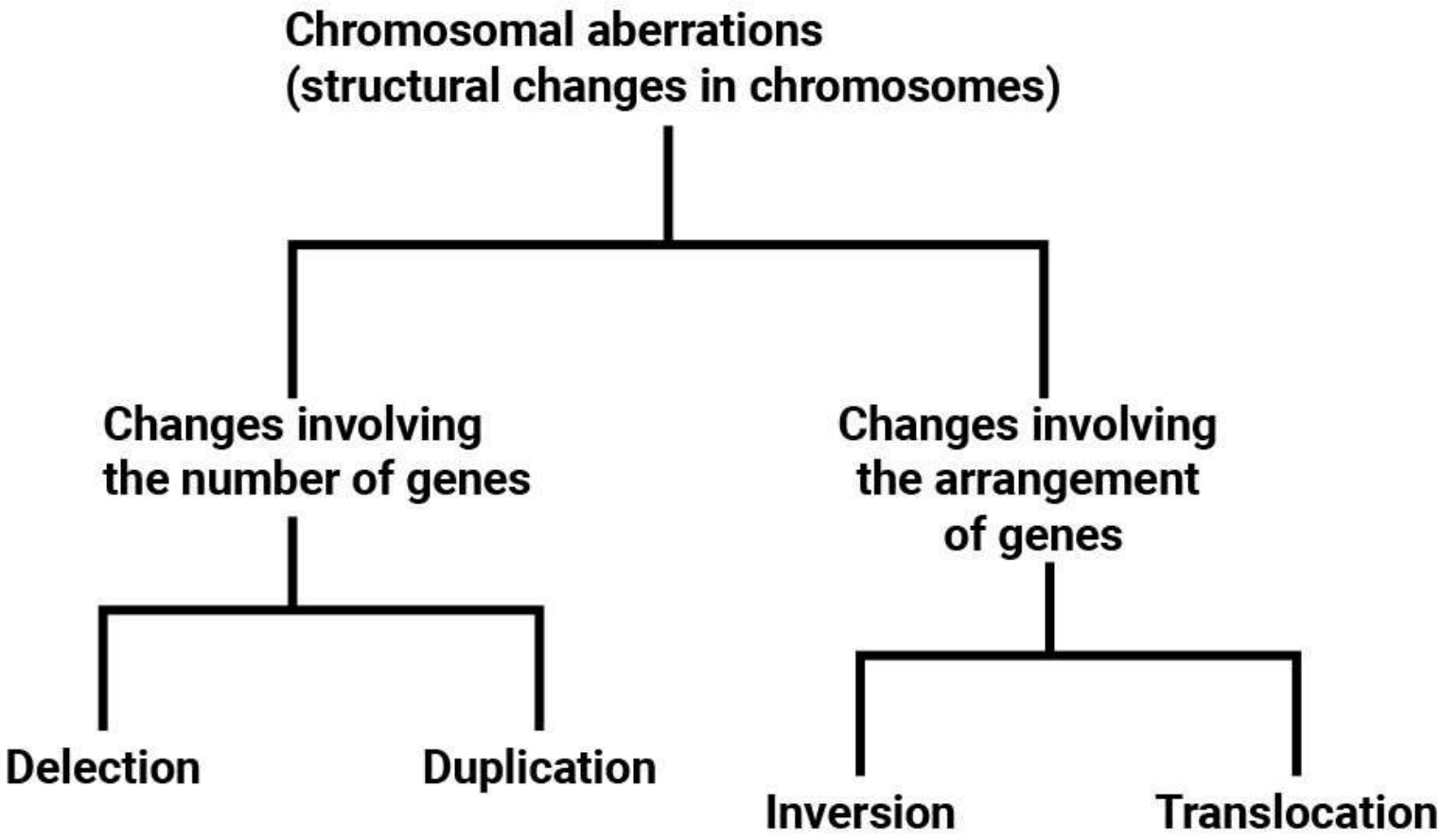

Normal male
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
Edwards' syndrome
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
Patau's syndrome
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
Klinefelter's syndrome
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X X Y

Types of Chromosomal Abnormalities



- aneuploidy
- polyploidy

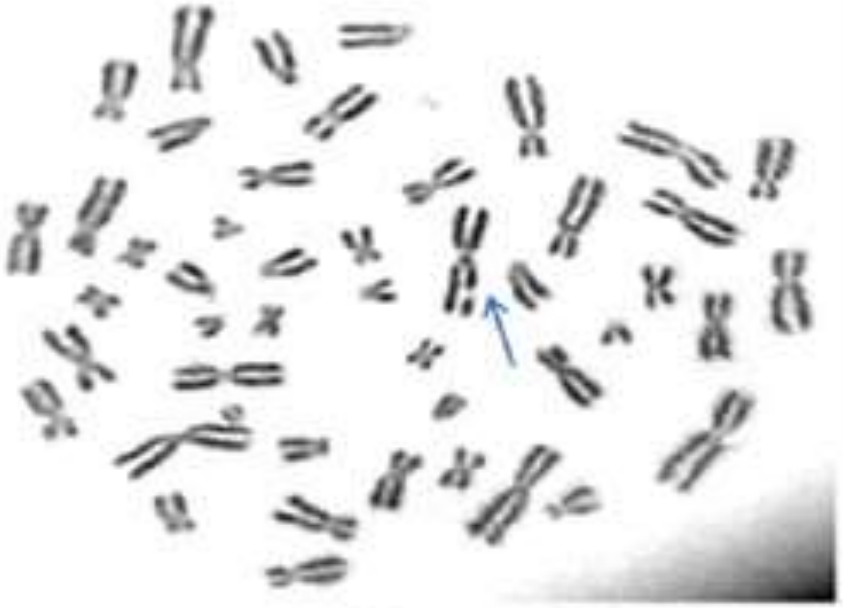
- deletion
- duplication
- inversion


Chromosomal aberrations
(structural changes in chromosomes)
Changes involving
the number of genes
Changes involving
the arrangement
of genes
Delection
Duplication
Inversion
Translocation

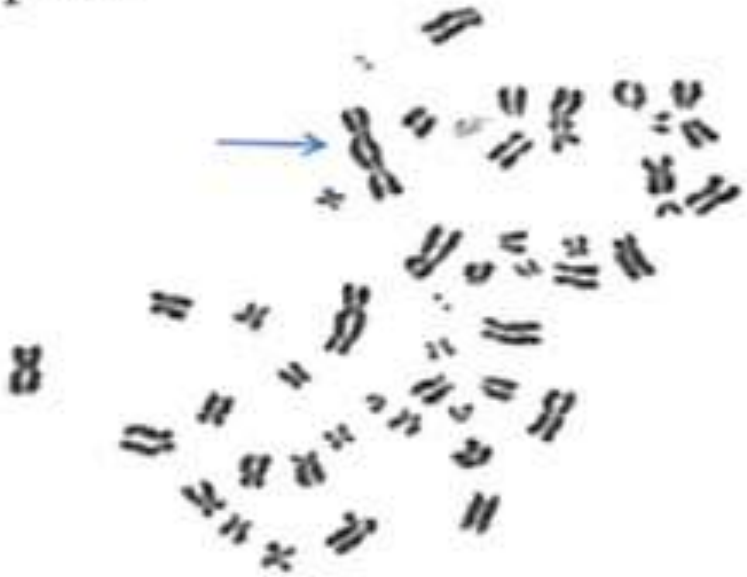
Examples of 2-lesion *Chromosome-type* aberrations

INTERCHANGE	INTER-ARM INTRACHANGE	INTRA-ARM INTRACHANGE
 dicentric	 centric-ring	 interstitial deletion
 reciprocal translocation	 pericentric inversion	 paracentric inversion

Normal metaphase



Chromatid break



Dicentric chromosome

G-banding

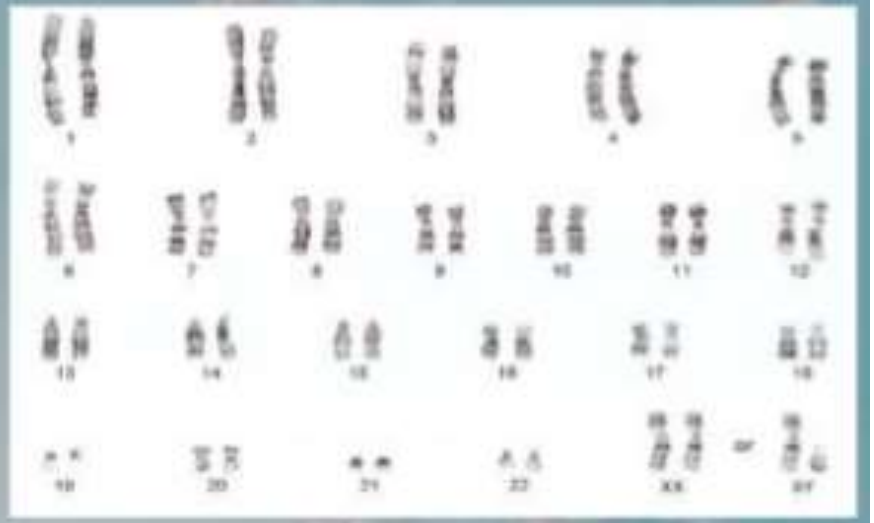
- **G banding** is obtained with Giemsa stain following digestion of chromosomes with trypsin.
- It yields a series of lightly and darkly stained bands — The dark regions tend to be heterochromatic, late-replicating and AT rich.
- The light regions tend to be euchromatic, early-replicating and GC rich.
- This method will normally produce 300–400 bands on chromosome.

R-banding

- is the reverse of G-banding (the R stands for "reverse").
- The dark regions are euchromatic (guanine-cytosine rich regions)
- and the bright regions are heterochromatic (thymine-adenine rich regions).

Arrangement of chromosomes

- Picture shows abrupt arrangement haploid chromosome.
- Then scientist with experience cut out each chromosome and arrange them in order according to their size and pair.



Applications Of Karyotyping

- Karyotype show the chromosomal makeup of an individual.
- Knowing the number of chromosomes is essential for identifying chromosomal variations that cause genetic disorders.
- Correct number of chromosomes and chromosomal abnormality in numbers can be detect.
- Correct size and shape of chromosomes is visualize in this technique.
- We can identify Gender of unknown sample by this technique.

Limitations Of Karyotyping

- Expert technician is require to arrange the chromosome.
- Structural abnormalities can not be detected by this technique.
- By observing karyograph defects like deletion, duplication and many other can not be detected.

What is FISH…..?

- **Fluorescence in situ hybridization (FISH)** is a cytogenetic technique that uses fluorescent probes that bind to only those parts of the chromosome with a high degree of sequence complementarity
- It is used to detect and localize the presence or absence of specific DNA sequences on chromosome .
- FISH is often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification

What are probes

- **probe** is a synthesized fragment of DNA or RNA of variable length which can be radioactively labeled
- The size may be varies from 100-1000 bases long.
- The probe thereby hybridizes to single-stranded nucleic acid (DNA or RNA) whose base sequence allows probe-target base pairing due to complementarity between the probe and target.
- the probe is tagged (or "labeled") with a molecular marker of either radioactive or fluorescent molecules.

Fluroscent In Situ Hybridization

- **Step I – Denaturation**
  - Conversion of double stranded dna in to single stranded dna

- **Step II – Hybridization**
  - Application of probe DNA to slide & overnight incubation at 37°C
  - Binding of probe DNA to trarget DNA.

- **Step III – Post hybridisation washing & detection**
  - Washing of unbound probe DNA.
  - Application of counter stain &

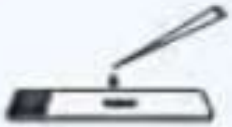
- **Step IV – counter stain**
  - Application of counter stain.

- **Step V – Visualization**
  - visualization using fluorescence microscopy.

FISH protocol

1. Mark hybridizing area

2. Apply 10µl FISH probe for 22mm x 22mm area



3. Cover with cover glass and seal with rubber cement

4. Denature

75℃ 5 min

Hybridization

1. Incubation

Humidified box
37℃ 16 ~ 72 hrs

Wash procedure

Remove rubber cement
Slide into 2X SSC and remove cover glass

2X SSC	2X SSC	2X SSC
Room temp.	/0.3% NP-40	Room temp.
5 min	73~75℃	1 min
	1-2min	

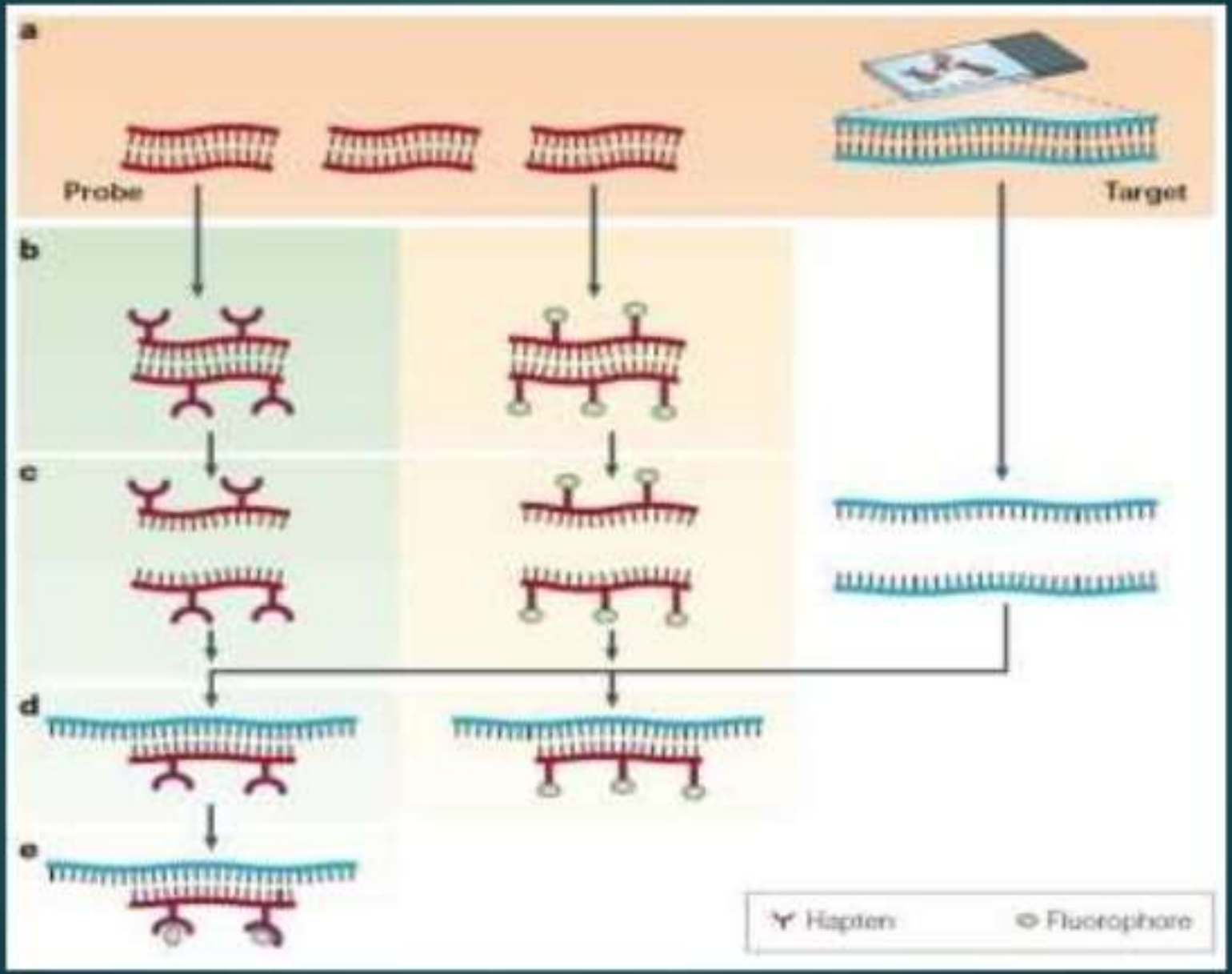
Counter stain

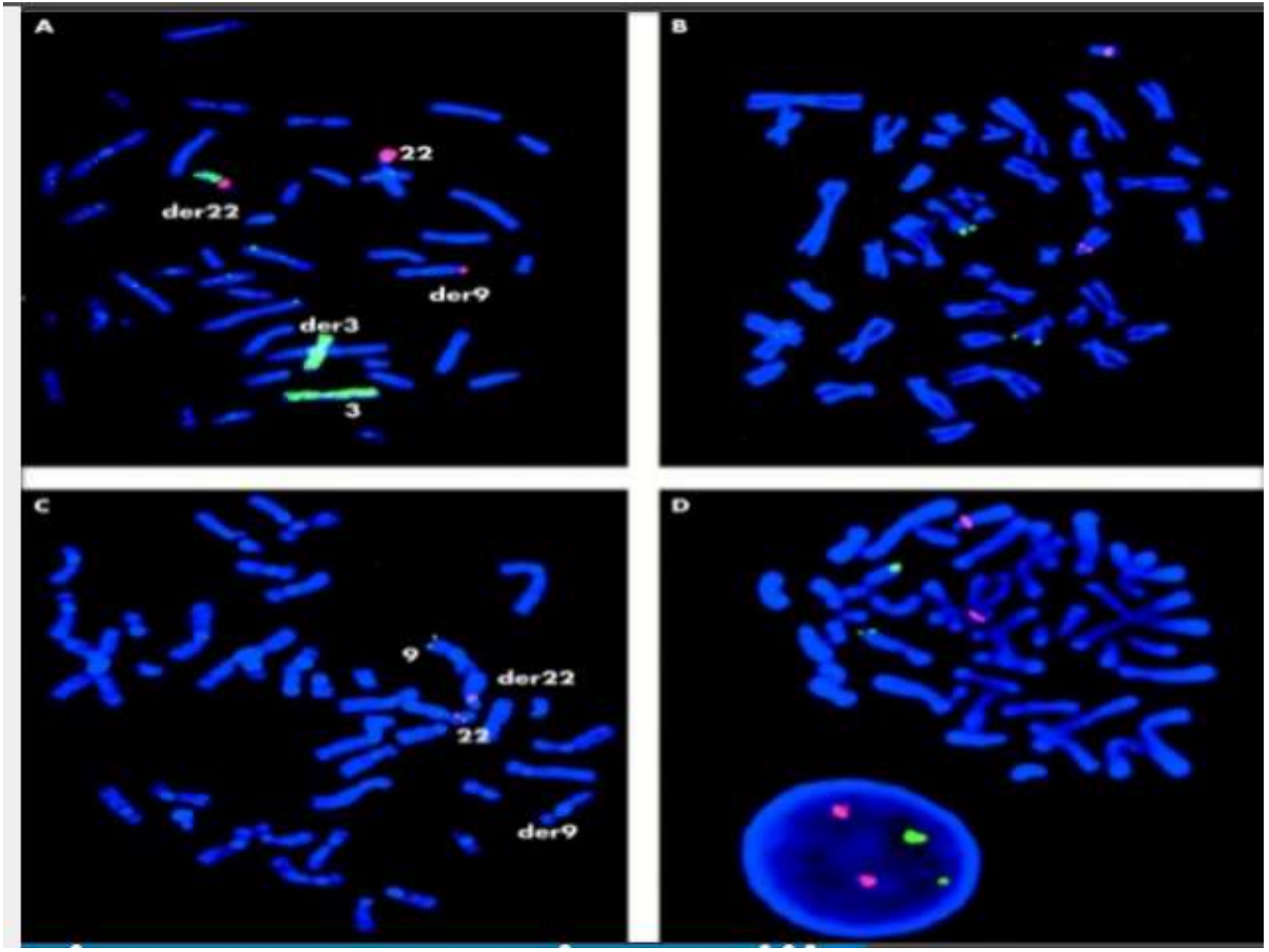
1. Apply 10µl DAPI Solution to target area

*DAPI Paraffin embedded tissue 1500ng/ml

2. Put on cover glass Seal with manicure

Examine


a
Probe
Target
b
c
d
e
Hapten
Fluorophore


A
22
der22
der9
der3
3
B
C
9
der22
22
der9
D

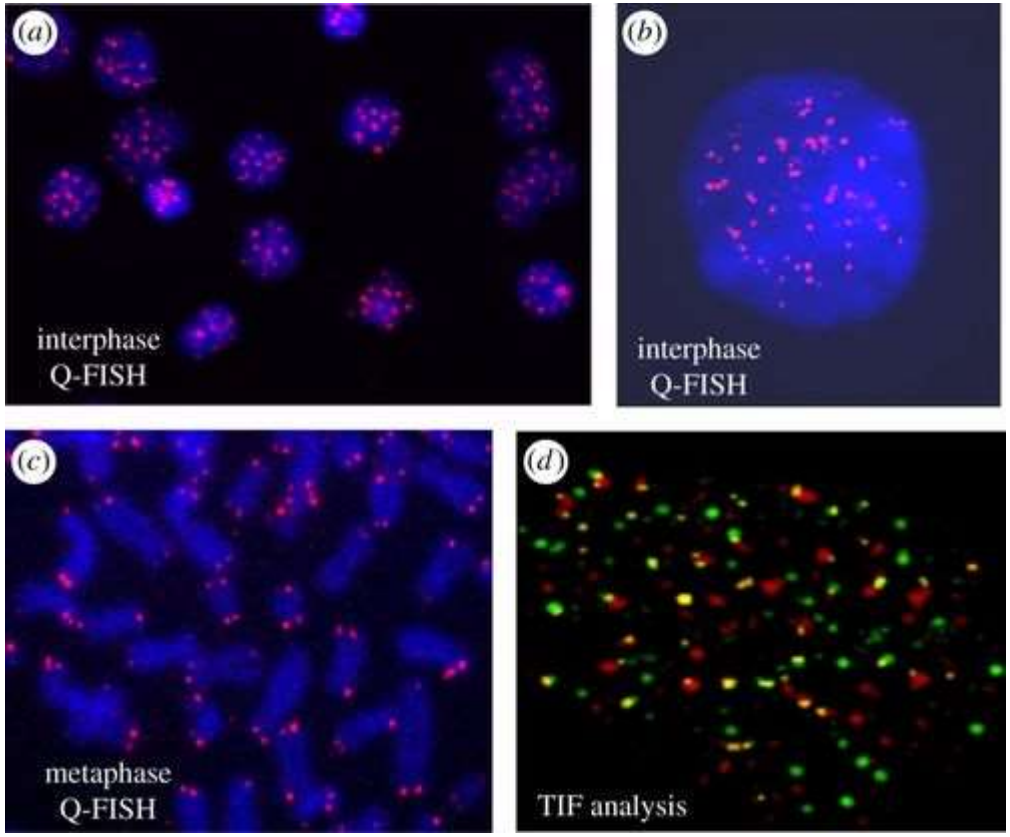
Flow FISH is a telomere measurement technique that combines the use of fluorescent in situ hybridization (**FISH**) with **flow** cytometry. White blood cells (leukocytes) are isolated from a blood sample and mixed with fluorescent peptide nucleic acid (PNA) probes that bind specifically to the telomere repeats (TTAGGG)

Quantitative Fluorescent *in situ* hybridization (Q-FISH) is a cytogenetic technique based on the traditional FISH methodology. In Q-FISH, the technique uses labelled (Cy3 or FITC) synthetic DNA mimics called ***peptide nucleic acid (PNA) oligonucleotides*** to quantify target sequences in chromosomal DNA using fluorescent microscopy and analysis software. Q-FISH is most commonly used to study telomere length, which in vertebrates are repetitive hexameric sequences (TTAGGG) located at the distal end of chromosomes. Telomeres are necessary at chromosome ends to prevent DNA-damage responses as well as genome instability. To this day, the Q-FISH method continues to be utilized in the field of telomere research.

RNA FISH

Featured snippet from the web

Fluorescent in situ hybridization targeting ribonucleic acid molecules (**RNA FISH**) is a methodology for detecting and localizing particular **RNA** molecules in fixed cells. This detection utilizes nucleic acid probes that are complementary to target **RNA** sequences within the cell



Q-FISH generally uses a TTAGGGn PNA labelled probe to hybridize to interphase cells (*a*,*b*). Alternatively, the same probe can be hybridized to metaphase chromosomes (*c*). With the help of a skilled cytogeneticist, this permits determination of which chromosome ends have longer (stronger signals) or shorter (weaker signals) telomeres. Chromosome ends that do not show fluorescence signals are reported as signal-free ends when they actually still have canonical telomeric repeats that are not sufficiently long to hybridize with probes. When telomeres become critically short, they are recognized as DNA damage. The commonly used technique Telomere dysfunctional Induced Foci (TIFs) indicates this damage by co-localization of a DNA damage recognition antibody (such as gamma H2AX or 53BP1) with a telomere-specific labelled probe or a shelterin antibody such as TRF2 (*d*).

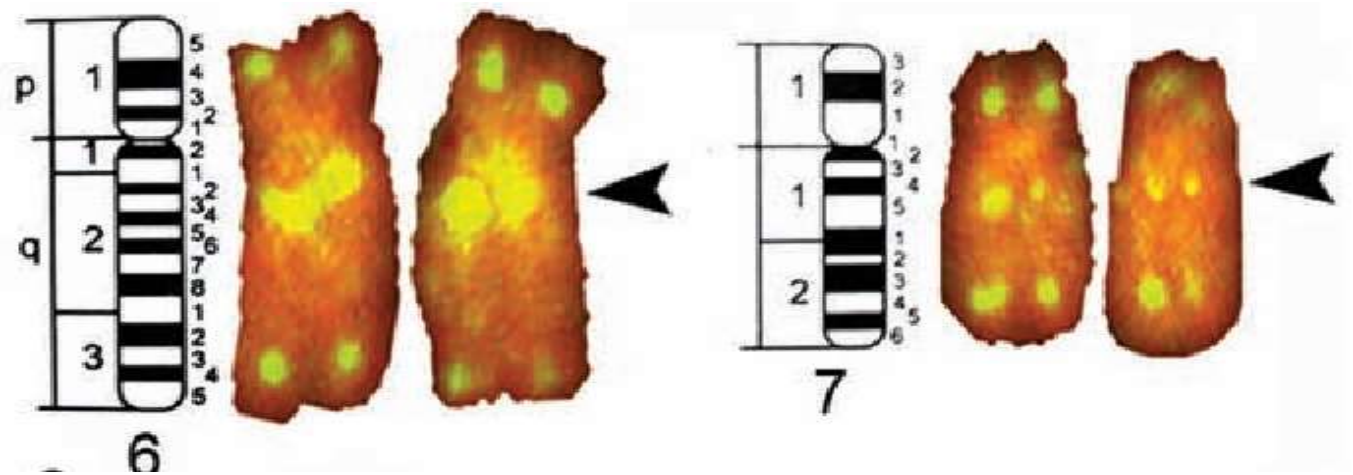
Primed in **situ labeling** (Pellestor et al., 1995; Tharapel and Wachtel, 2006) is a method of target DNA sequence detection and localization, which combines features of fluorescence in **situ** hybridization (FISH) and polymerase chain reaction (PCR), and was developed as a chromosome **labeling** technique based on repetitive alpha satellite sequences. The primed in situ labeling (PRINS) technique uses oligonucleotide primers that are annealed and extended on chromosome preparations on microscope slides in the presence of labeled nucleotides. After extension with Taq DNA polymerase, the relevant sequences are visualized by fluorescence microscopy

Primed *in situ* labeling (PRINS) is a fast and sensitive technique for sequence specific *in situ* detection of DNA. An unlabeled oligonucleotide probe is hybridized and used as primer for chain elongation *in situ* catalyzed by a DNA polymerase. Thus, the oligonucleotide primer binds by sequence-specific base pairing to its target sequence, which is subsequently labeled when labeled nucleotides are incorporated by the DNA polymerase, using the oligonucleotide as primer and the cellular DNA as template. Using unlabeled probes (primers) in the PRINS reaction means that high concentrations can be used, because probe bound to cell-structures cannot function as a primer and therefore does not give rise to background signals—only probe hybridized correctly to DNA can function as primer for chain elongation. Thus, using high probe concentrations the hybridization is very fast. A PRINS reaction normally runs for only 5–30 min. Owing to the speed of the reaction the morphology of chromosomes is very well preserved, which makes it possible to obtain chromosome banding of good quality after a PRINS reaction.

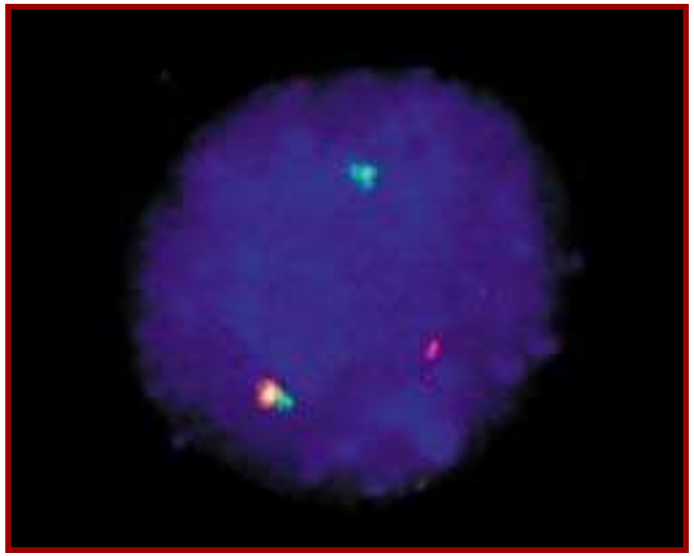
Multiplex in situ hybridization (**M**-**FISH**) is a 24-color karyotyping technique and is the method of choice for studying complex interchromosomal rearrangements. The process involves three major steps.

PRINS-**PR**imed **I**n **S**itu labeling

- Alternative method for the identification of chromosomes in metaphase spreads or interphase nuclei.
- Denatured DNA is hybridized to short DNA fragments, or oligonucleotides followed by primer extension with labeled nucleotides.
- Labeling is detected with a fluorescent conjugated antibody.
- Limited sensitivity
- rapid and low background staining.
- Technique can be coupled with PCR (Cycling PRINS) .



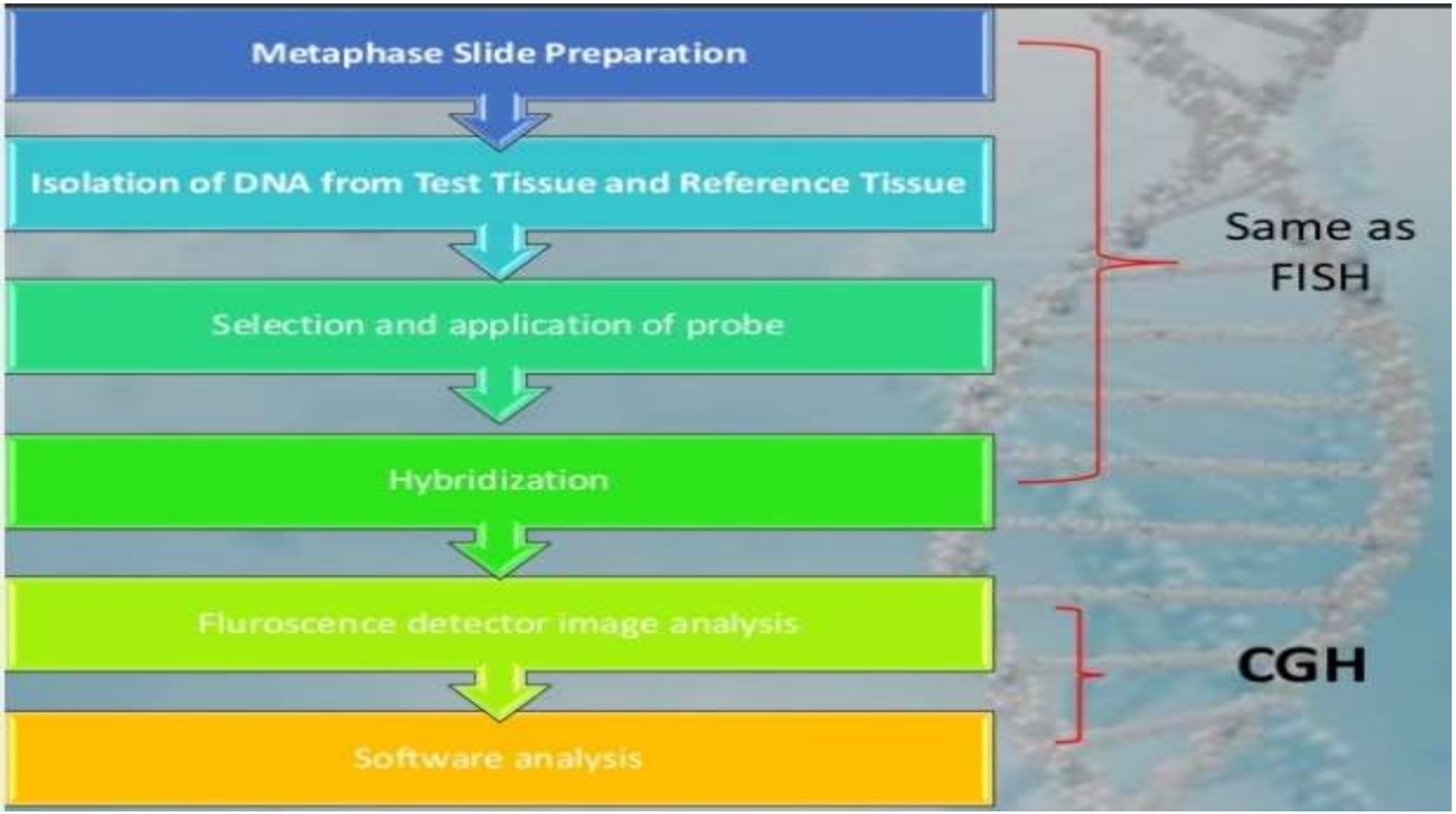
domestic horse metaphase spreads after PRINS with telomere human commercial kit. Arrowhead indicate additional intrachromosomal signal on 6q and 7q chromosomes. Chromosomes of 6 and 7 pairs and idiograms. Arrowheads indicate additional intrachromosomal signal on q arm of both homologous of 6 and 7 chromosomes. Less



bcr/abl Translocation Probe hybridized to an interphase cell. Note the presence of one orange-pink signal, one green signal and one yellow signal (fusion signal) indicates the fusion of the bcr and abl oncogenes

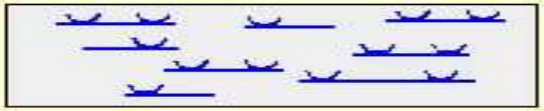
Comparative Genomic Hybridization (CGH)

- **Comparative genomic hybridization** is a molecular cytogenetic method for analyzing copy number variations with the help of hybridization technique.

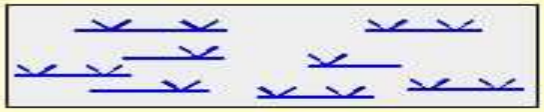


This is achieved through the use of competitive fluorescence in situ hybridization. In short, this involves the isolation of DNA from the two sources to be compared, most commonly a test and reference source, independent labelling of each DNA sample with fluorophores (fluorescent molecules) of different colours (usually red and green), denaturation of the DNA so that it is single stranded, and the hybridization of the two resultant samples in a 1:1 ratio to a normal metaphase spread of chromosomes, to which the labelled DNA samples will bind at their locus of origin. Using a fluorescence microscope and computer software, the differentially coloured fluorescent signals are then compared along the length of each chromosome for identification of chromosomal differences between the two sources. A higher intensity of the test sample colour in a specific region of a chromosome indicates the gain of material of that region in the corresponding source sample, while a higher intensity of the reference sample colour indicates the loss of material in the test sample in that specific region. A neutral colour (yellow when the fluorophore labels are red and green) indicates no difference between the two samples in that location

1. Labeling of genomic tumor DNA and normal genomic control DNA by Nick translation

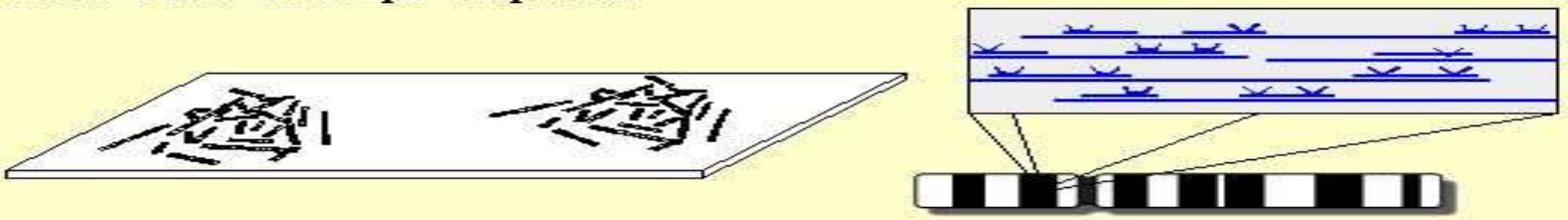


Biotin-labeled tumor DNA

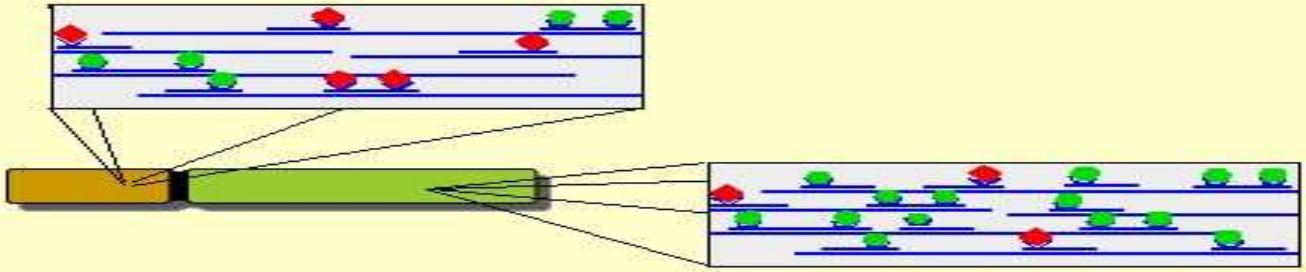


Digoxigenin-labeled control DNA

2. Simultaneous hybridization of differentially labeled tumor and control DNAs to normal human metaphase spreads



3. Fluorescence detection of the hybridized DNAs



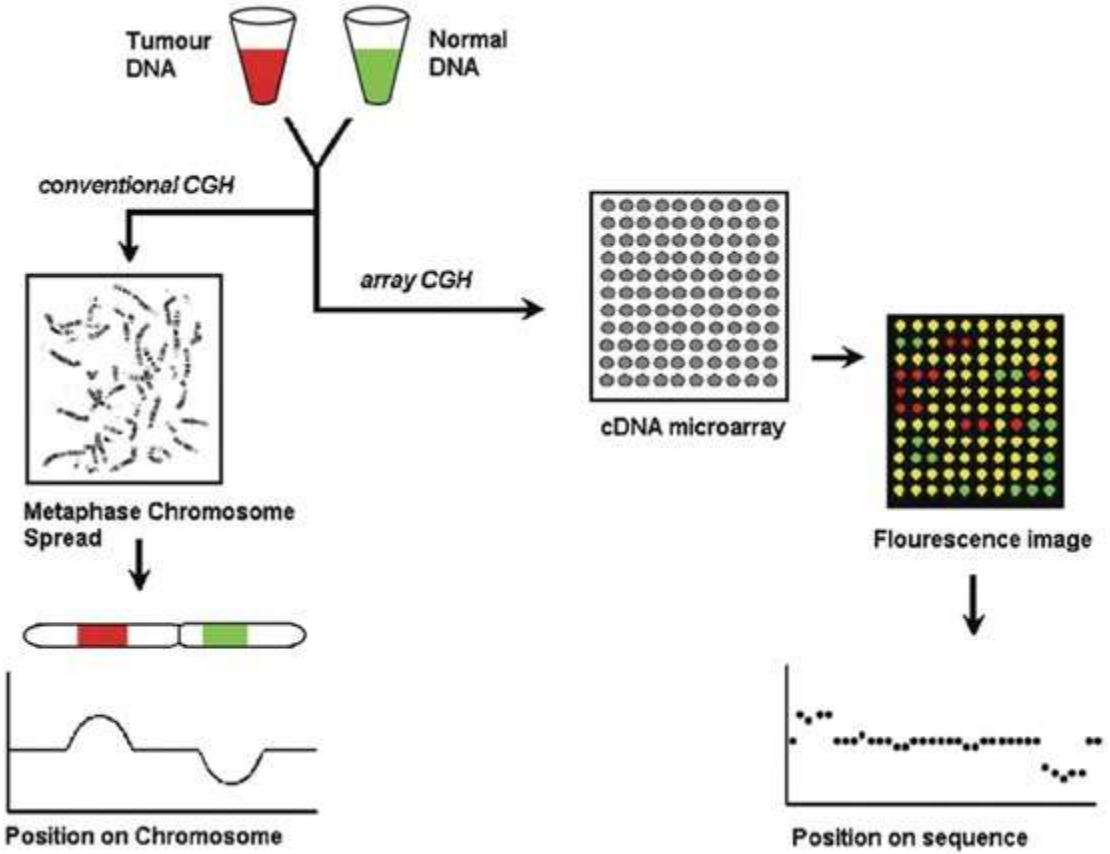
4. Result

balanced DNA content

overrepresentation of the whole chromosome within the tumor DNA

underrepresentation of the long arm within the tumor DNA

high level amplification

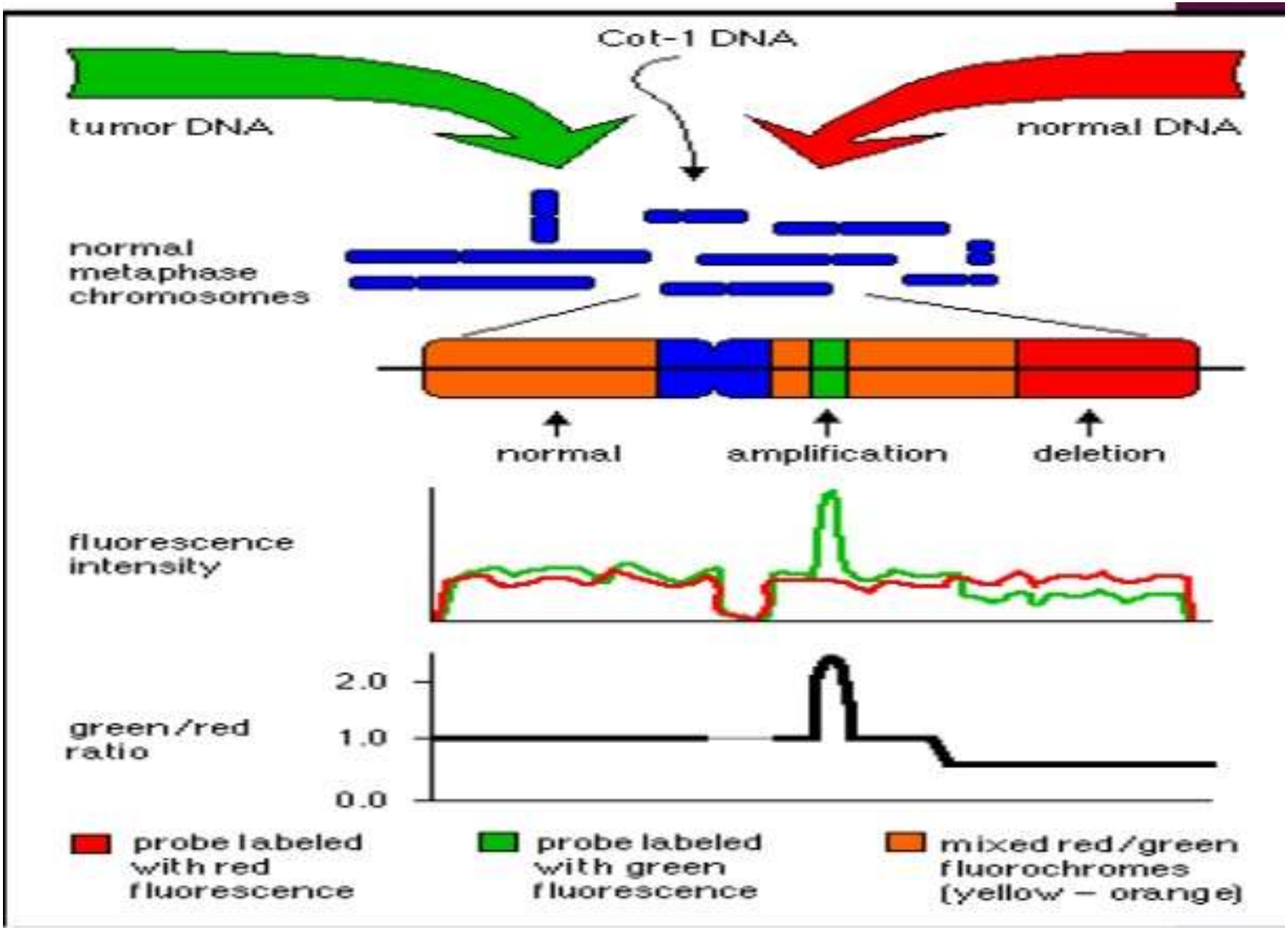


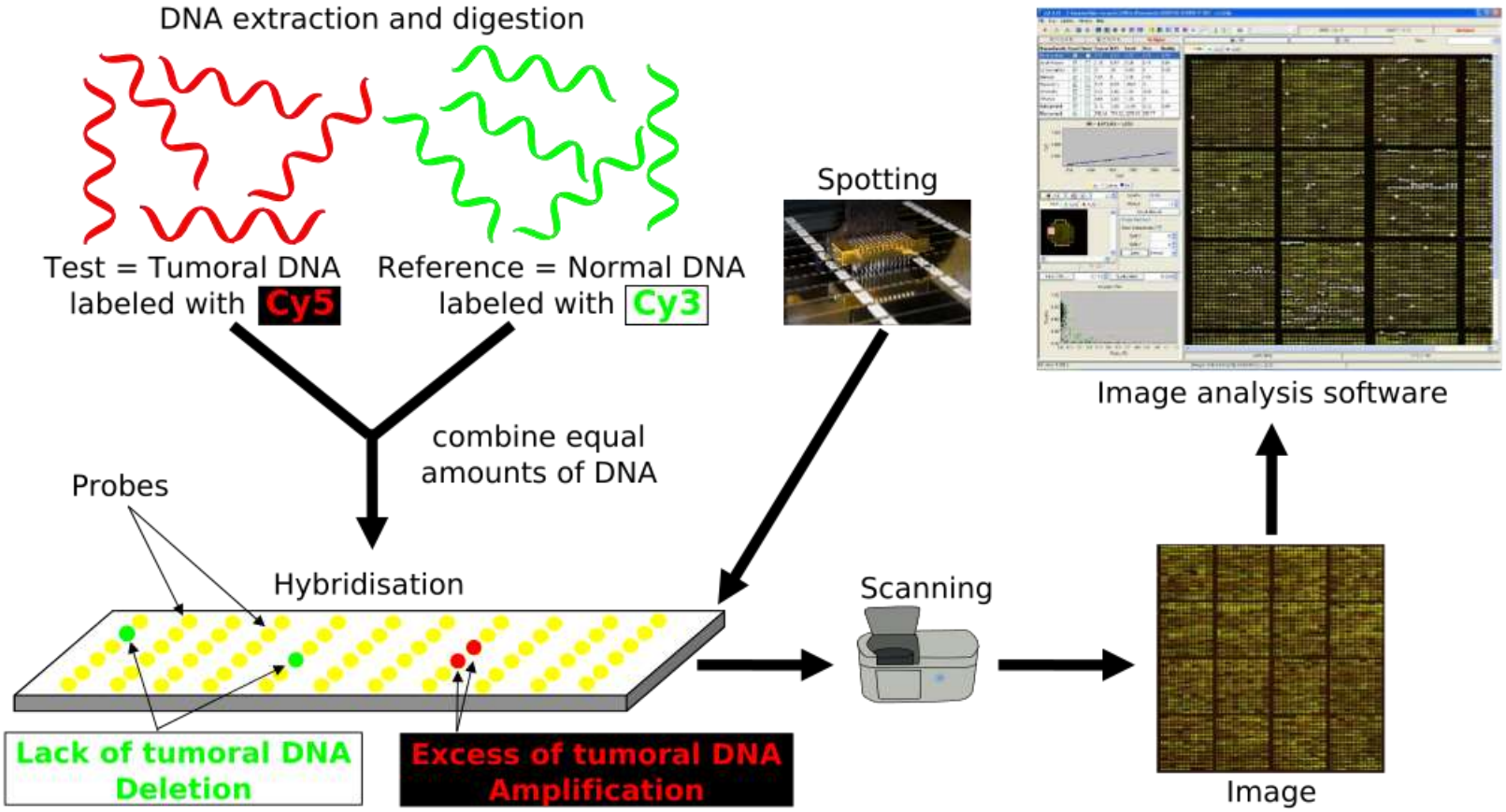
Comparative genomic hybridization. Genomic DNA is isolated from both the tumor sample and the normal reference sample, labeled with different fluorochromes and mixed in the presence of excess Cot-1 DNA to prevent binding of repetitive sequences. In conventional chromosomal CGH, these are hybridized to normal metaphase chromosomes, and the ratio of fluorescence intensities along each chromosome is analyzed. Increased DNA copy number (amplification) in the tumor sample will be detected by increased red fluorescence, whereas decreased copy number in the tumor sample will allow more binding of the normal DNA and increased green fluorescence. On the *right*, a similar hybridization to a cDNA array permits measurement of copy number at a higher resolution. The *red* and *green* spots on the fluorescence image represent increased and decreased copy number changes, respectively

Array comparative genomic hybridization (also microarray-based comparative genomic hybridization, matrix CGH, array CGH, aCGH) is a molecular cytogenetic technique for the detection of chromosomal copy number changes on a genome wide and high-resolution scale.Array CGH compares the patient's genome against a reference genome and identifies differences between the two genomes, and hence locates regions of genomic imbalances in the patient, utilizing the same principles of competitive fluorescence in situ hybridization as traditional CGH.

With the introduction of array CGH, the main limitation of conventional CGH, a low resolution, is overcome. In array CGH, the metaphase chromosomes are replaced by cloned DNA fragments (+100–200 kb) of which the exact chromosomal location is known. This allows the detection of aberrations in more detail and, moreover, makes it possible to map the changes directly onto the genomic sequence.

Array CGH has proven to be a specific, sensitive, fast and highthroughput technique, with considerable advantages compared to other methods used for the analysis of DNA copy number changes making it more amenable to diagnostic applications. Using this method, copy number changes at a level of 5–10 kilobases of DNA sequences can be detected.


Cot-1 DNA
tumor DNA
normal DNA
normal metaphase chromosomes
normal
amplification
deletion
fluorescence intensity
green/red ratio
2.0
1.0
0.0
probe labeled with red fluorescence
probe labeled with green fluorescence
mixed red/green fluorochromes (yellow – orange)


DNA extraction and digestion
Test = Tumoral DNA
labeled with Cy5
Reference = Normal DNA
labeled with Cy3
Spotting
Image analysis software
combine equal
amounts of DNA
Probes
Hybridisation
Scanning
Image
Lack of tumoral DNA
Deletion
Excess of tumoral DNA
Amplification

Application of CGH

- This technology was first developed as a research tool for the investigation of genomic alterations in cancer.
- It allows for a high-resolution evaluation of DNA copy number alterations associated with chromosome abnormalities.
- It provides clinicians with a powerful tool to use in their increasingly sophisticated diagnostic capabilities.
- The use of CGH is considered EXPERIMENTAL AND/OR INVESTIGATIONAL when performed in the absence of symptoms or high risk factors for a genetic disease or when knowledge of genetic status will not affect treatment decisions or screening for the disease.
- Screening for prenatal gene mutations in fetuses without structural abnormalities or testing products of conception after AI.
- Diagnosis of melanoma.
- It helps in detection of balanced rearrangements of chromosomes and for comparison of normal and suspected DNA samples.

Types of probes for FISH

Scientists use three different types of FISH probes, each of which has a different application:

Locus specific probes *bind to a particular region of a chromosome. This type of probe is useful when scientists have isolated a small portion of a gene and want to determine on which chromosome the gene is located, or how many copies of a gene exist within a particular genome.*

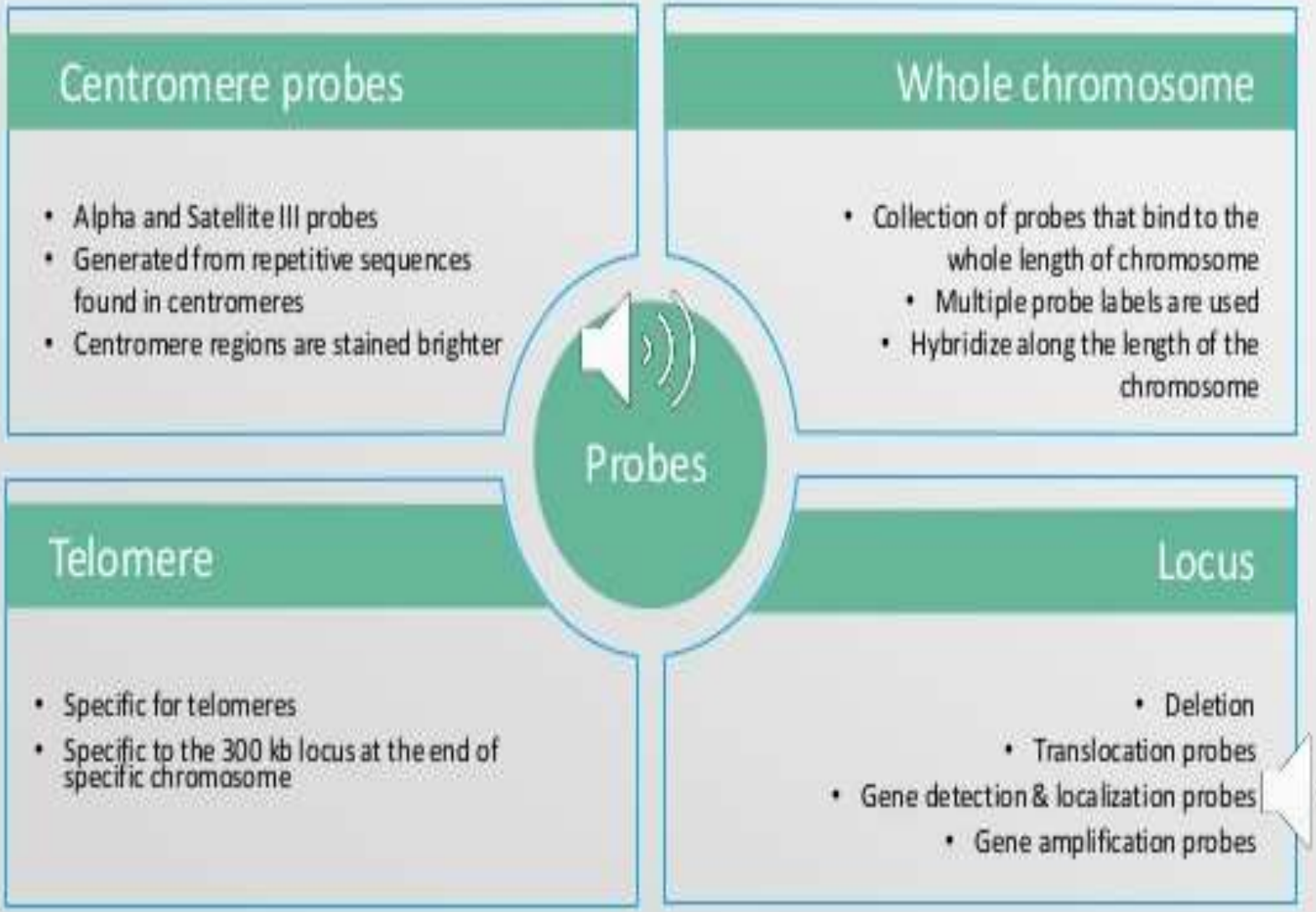
Alphoid or ***centromeric repeat probes*** **are generated from repetitive sequences found in the middle of each chromosome These probes can also be used in combination with "locus specific probes" to determine whether an individual is missing genetic material from a particular chromosome.**

Whole chromosome probes **are actually collections of smaller probes, each of which binds to a different sequence along the length of a given chromosome**

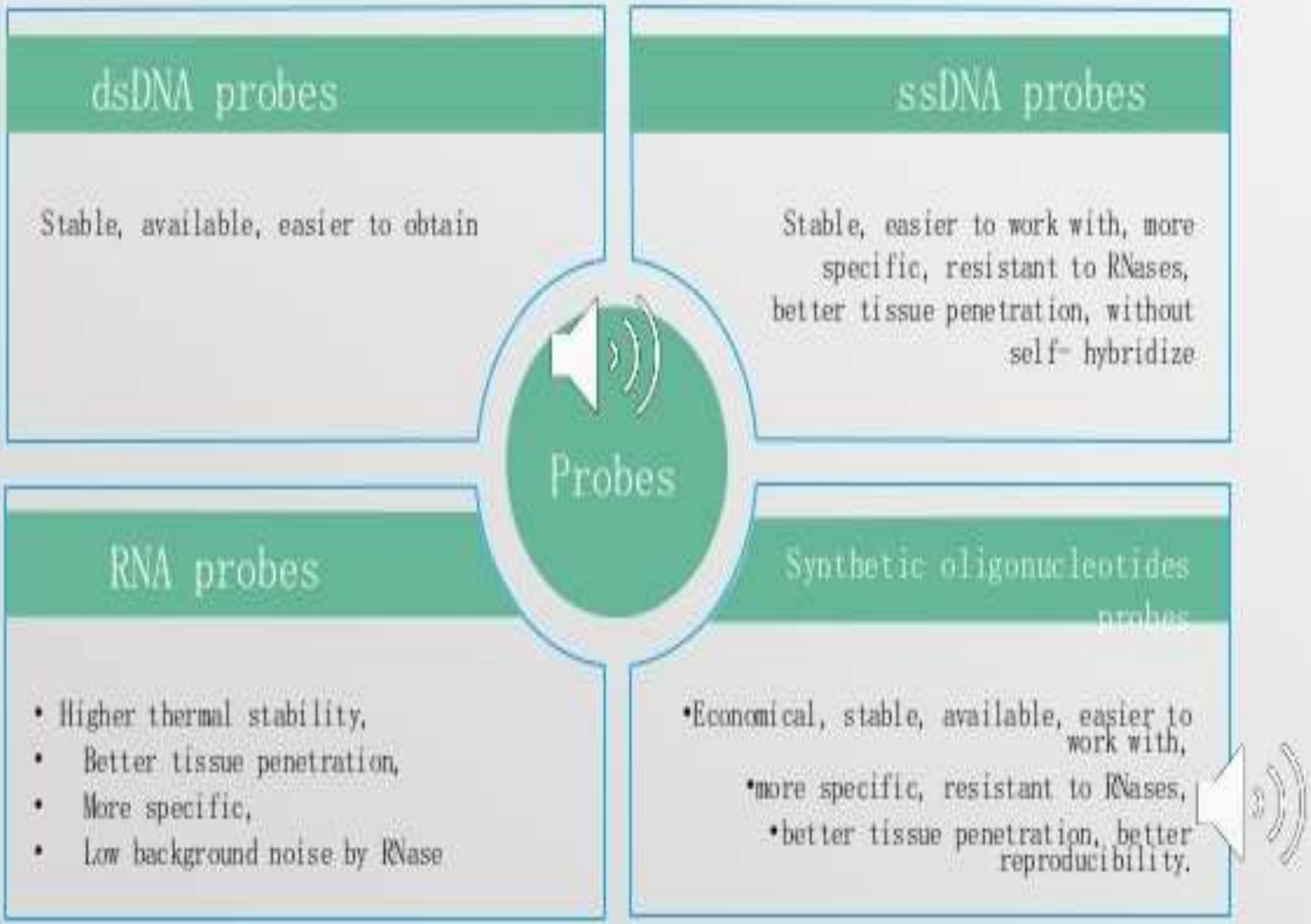
https://www.genome.gov/

Creative Bioarray

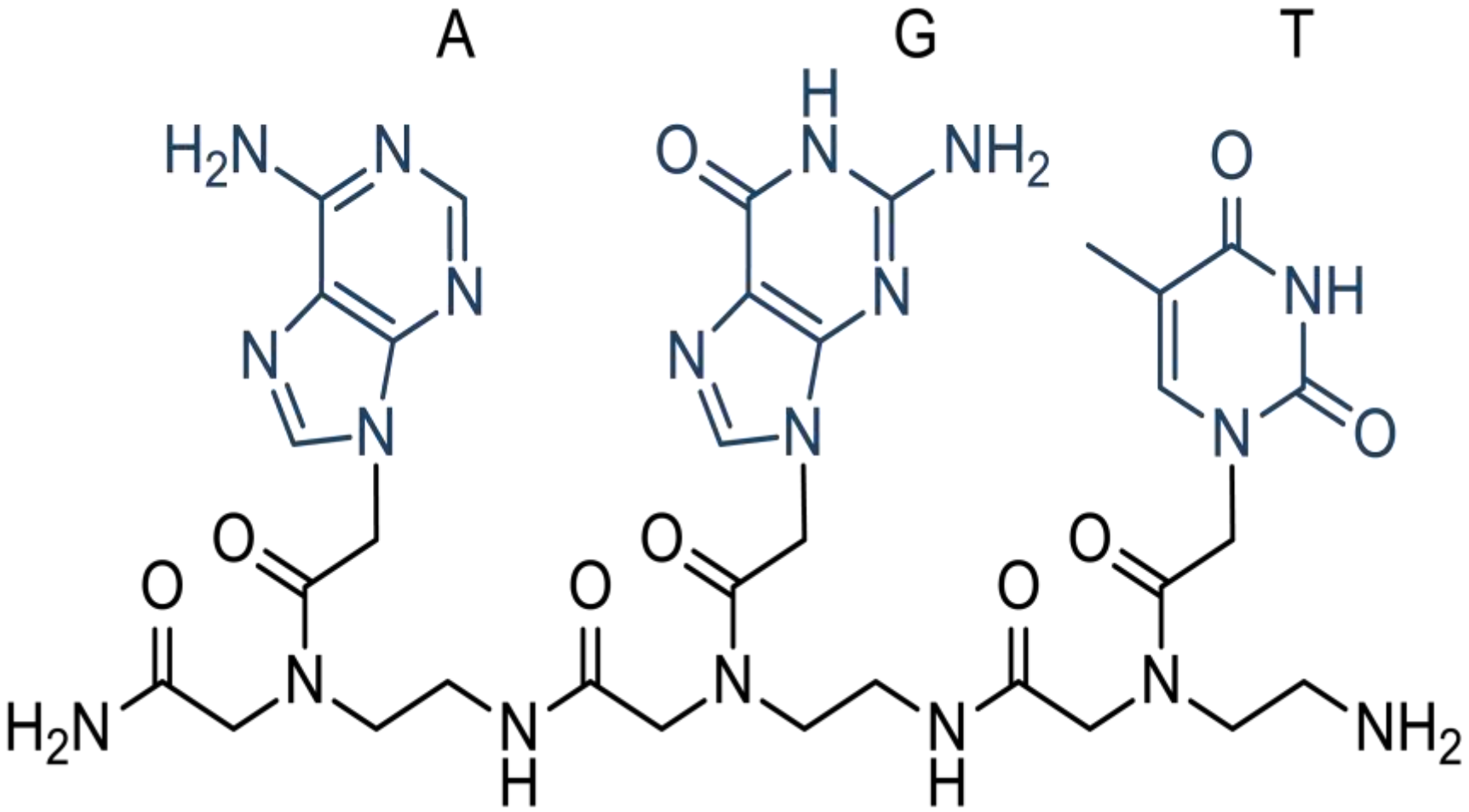
What Kind of Probes Can Be Used?



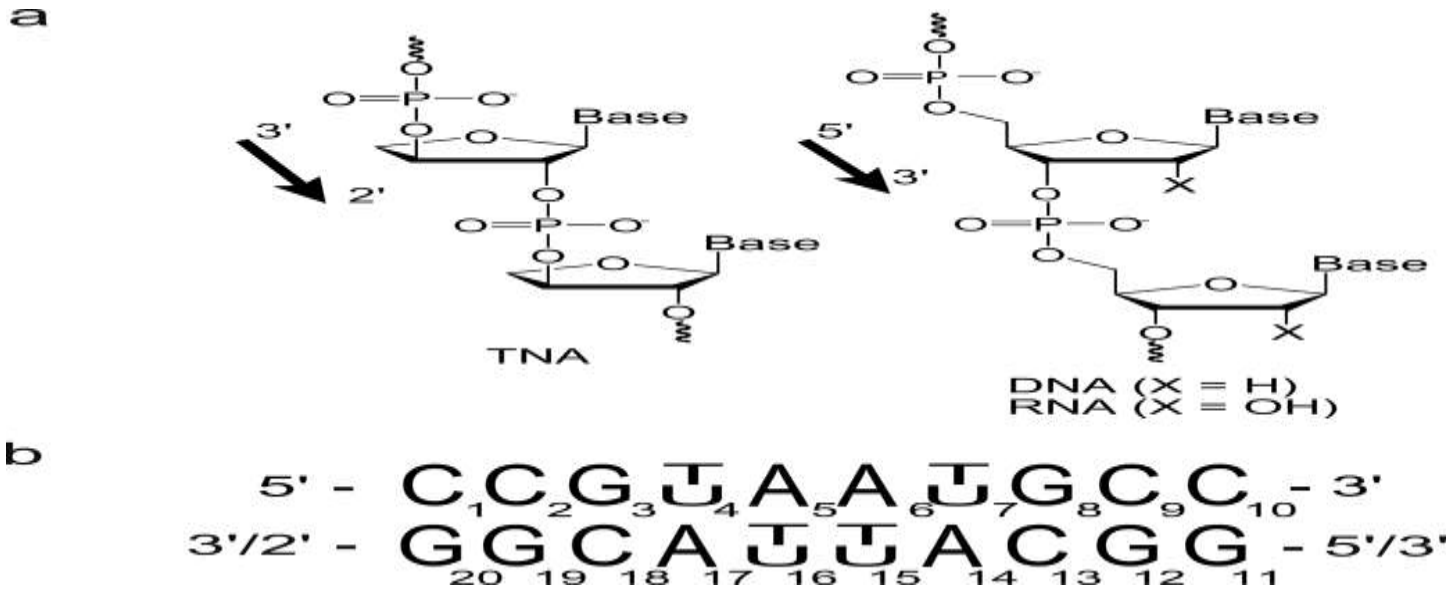
Characteristics of Different Probes Types



Peptide nucleic acids (PNA) are a unique class of oligonucleotide mimics that consist of a peptide-like backbone. Although the idea of replacing the whole sugar-phosphate backbone of DNA with a completely unrelated scaffold such as peptide had been around since the 1970s , it was not until the 1990s that the first PNA system with an *N*-2-aminoethylglycine (aeg) backbone that can recognize its target DNA and RNA was reported . Considering the enormous difference between the two backbones, it is quite surprising that PNA can still retain the ability to recognize natural oligonucleotides having a complementary sequence with high affinity and specificity according to the Watson–Crick base pairing rules . In fact, PNA exhibits an even higher affinity and better discrimination between complementary and mismatched nucleic acid targets than natural oligonucleotides. In addition, the uncharged peptide-like backbone of PNA contributes to several unique properties not observed in other classes of oligonucleotide analogues with negatively charged phosphate groups. These include the relative insensitivity of the PNA–DNA or PNA–RNA hybrids to the ionic strength of the solvent , and the complete stability towards nucleases as well as proteases


A
G
T
H2N
N
N
N
N
H
O
N
NH2
N
N
O
NH
N
O
O
O
O
O
O
O
O
H2N
N
N
N
NH2
H
H

Threose nucleic acid (TNA) is an artificial genetic polymer capable of heredity and evolution that is studied in the context of RNA chemical etiology. Its simplified four-carbon threose backbone replaces the five-carbon ribose in natural nucleic acids.



Backbone structure and nucleotide sequences of TNA, DNA, and RNA. (a) Constitutional structure of the linear backbone and strand directionality of TNA (left panel), and DNA/RNA (right panel). (b) Palindromic nucleotide sequences used in this study. For the chimeric duplexes, the bottom strand was composed of TNA.

Fluorescence *in situ* hybridization (FISH) is a powerful technique that is used to detect and localize specific nucleic acid sequences in the cellular environment. In order to increase throughput, FISH can be combined with flow cytometry (flow-FISH) to enable the detection of targeted nucleic acid sequences in thousands of individual cells. As a result, flow-FISH offers a distinct advantage over lysate/ensemble-based nucleic acid detection methods because each cell is treated as an independent observation, thereby permitting stronger statistical and variance analyses. These attributes have prompted the use of FISH and flow-FISH methods in a number of different applications and the utility of these methods has been successfully demonstrated in **telomere length determination, cellular identification and gene expression, monitoring viral multiplication in infected cells, and bacterial community analysis and enumeration**.

Traditionally, the specificity of FISH and flow-FISH methods has been imparted by DNA oligonucleotide probes.
Recently however, the replacement of DNA oligonucleotide probes with nucleic acid analogs as FISH and flow-FISH probes has increased both the sensitivity and specificity of each technique due to the higher melting temperatures (T_m) of these analogs for natural nucleic acids.
Locked nucleic acid (LNA) probes are a type of nucleic acid analog that contain LNA nucleotides spiked throughout a DNA or RNA sequence. When coupled with flow-FISH, LNA probes have previously been shown to outperform conventional DNA probes and have been successfully used to detect eukaryotic mRNA and viral RNA in mammalian cells

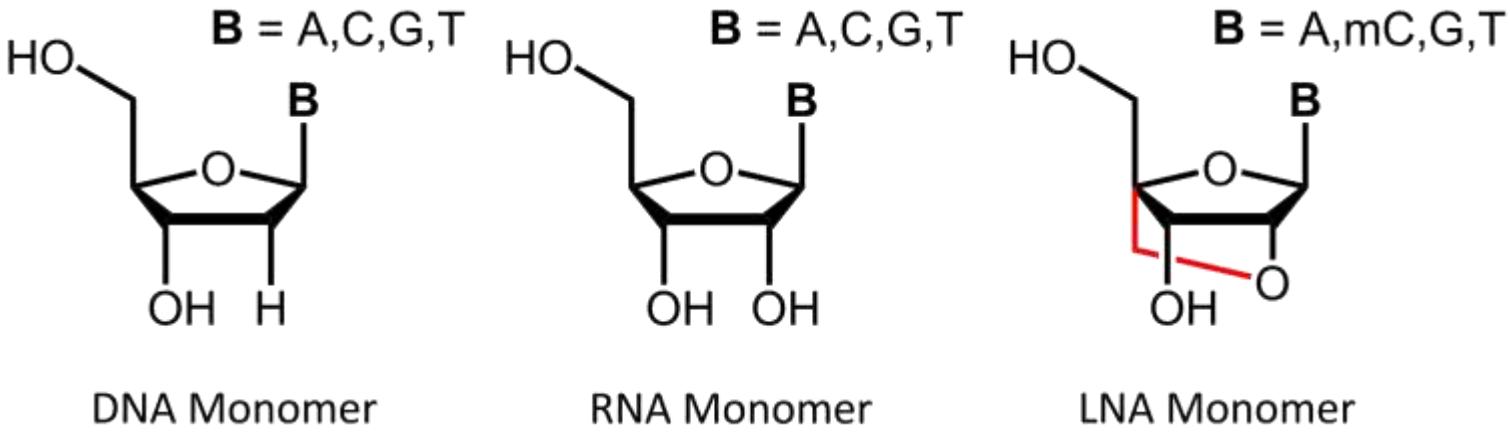
Locked Nucleic Acids (LNAs) constitute a novel class of RNA analogs that have an exceptionally high affinity towards complementary DNA and RNA. Substitution of DNA oligonucleotide probes with LNA has shown to significantly increase their thermal duplex stability as well as to improve the discrimination between perfectly matched and mismatched target nucleic acids.

What is Locked Nucleic Acid?

Locked Nucleic Acid is a type of nucleic acid analog that contains a 2′-0, 4′-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and exceptional biostability.

Duplexes including Locked Nucleic Acid oligonucleotides are considerably more thermally stable than similar duplexes constituted from DNA or RNA oligonucleotides, conferring the following advantages:

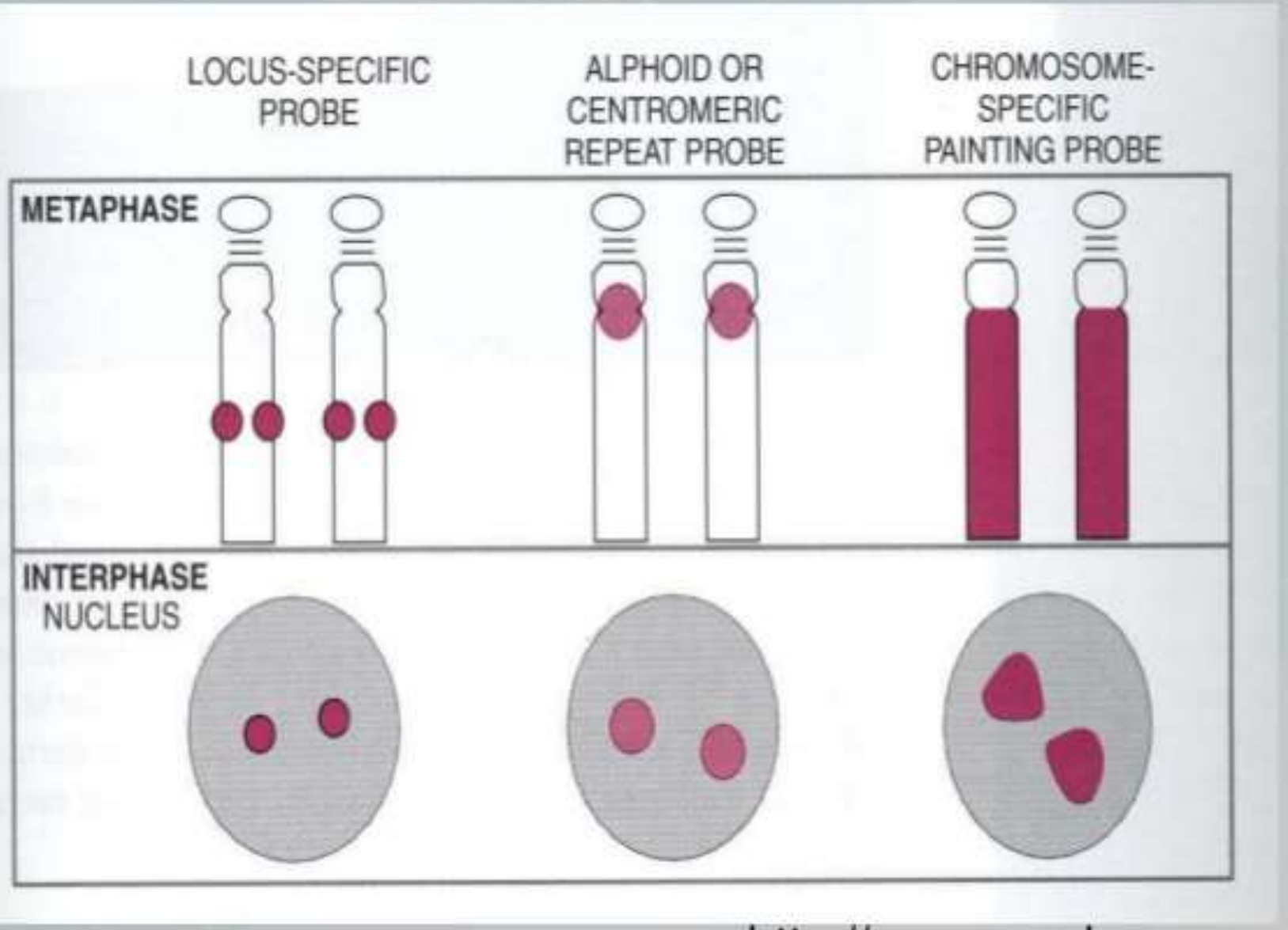
Increased melting temperature of the oligonucleotide duplex - by 3 to 8 °C per incorporated Locked Nucleic Acid nucleoside in the oligomer
Dramatically enhanced affinity towards complementary DNA or RNA
Superior discrimination between matched and mismatched target nucleic acids
Hybridization to complementary nucleic acids, even under low salt conditions and in the presence of chaotropic agents
Increased stability of the oligonucleotide in biological fluids
Strand invasion capability



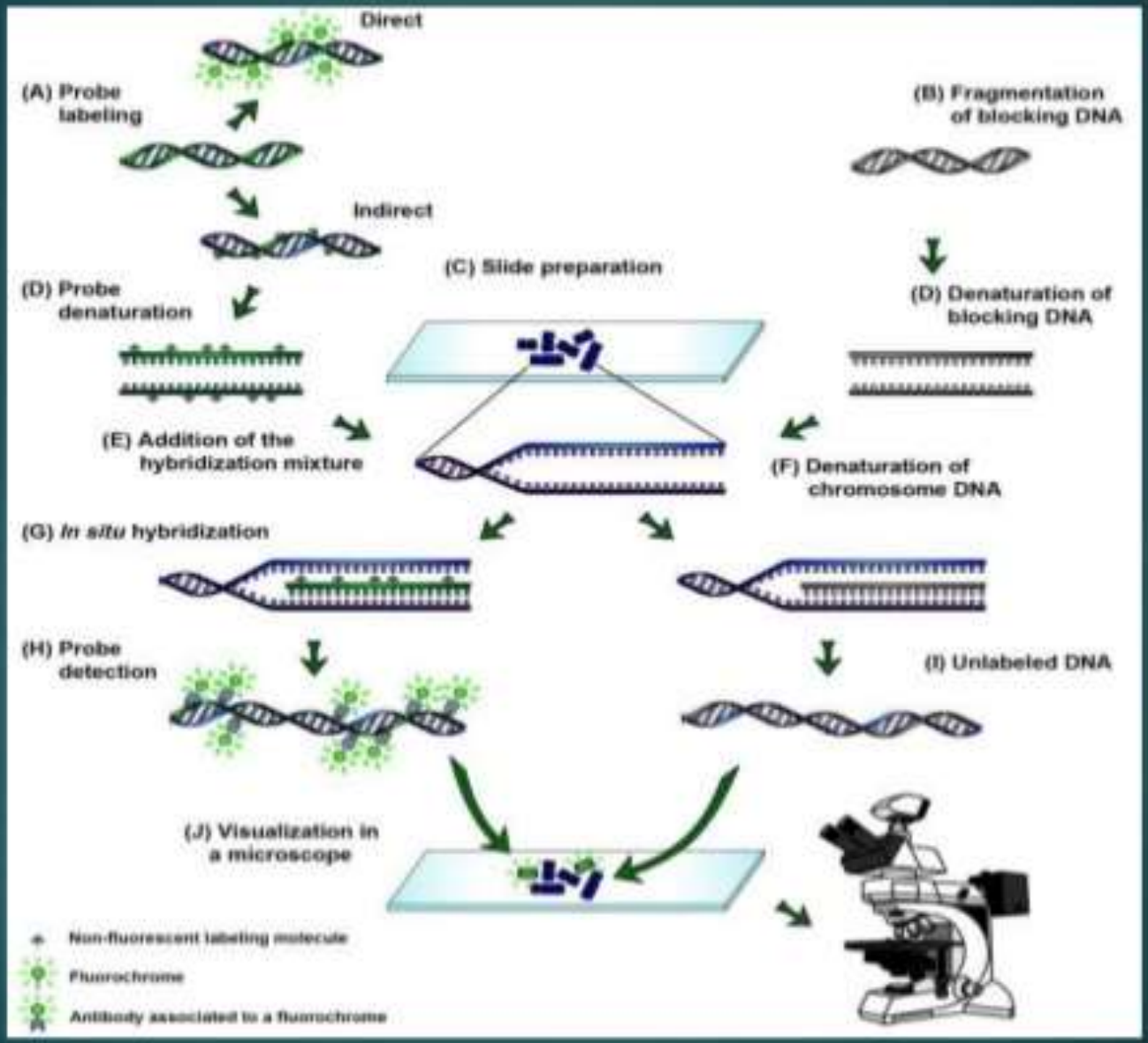
Locked Nucleic Acids (LNA) were first independently described in 1997 by Jesper Wengel[1] and Takeshi Imanishi[2] and have become an important part of hybridisation-based applications.

LNAs are a class of modified RNA-nucleic acid analogues that have an additional methylene bridge. This linkage connects the oxygen atom at the C-2´ of the ribose with the 4´-carbon atom and helps to fix the ribose ring in the 3'-endo conformation ("locked") which leads to the characteristic A-RNA structure and to the exclusive formation of A-duplexes.

TYPES OF FISH PROBES



Types of probe labelling



http://www.intechopen.com

Applications

- Detection of numerical and structural Chromosomal abnormality.
- Identification of marker chromosomes.
- Monitoring the effect of therapy.
- Detection of early relapse or minimal residual diseases.
- Detection gene deletion and gene amplification.

Gozzetti and Beau, 2006

Advantages of fish

- Rapid technique and large number of cells can be scored in a short period.
- Efficiency of Hybridization and deletion is high.
- Sensitivity and specificity is high.
- Cytogenetic data can be obtained from non-dividing or terminally differentiated cells.
- Cytogenetic data can be obtained from poor samples that contain too few cells for routine cytogenetic analysis.
- Methode has been adapted for automated systems.

Gozzetti and Beau, 2006

Limitation of fish

1. Restricted to those abnormalities that can be detected with currently available probe.

2. Only one or a few abnormalities can be assessed simultaneously.

3. Due to Failuer to detect signal FISH is higher sensitive for trisomy but less sensitive foe detecting chromosome loss or deletion.

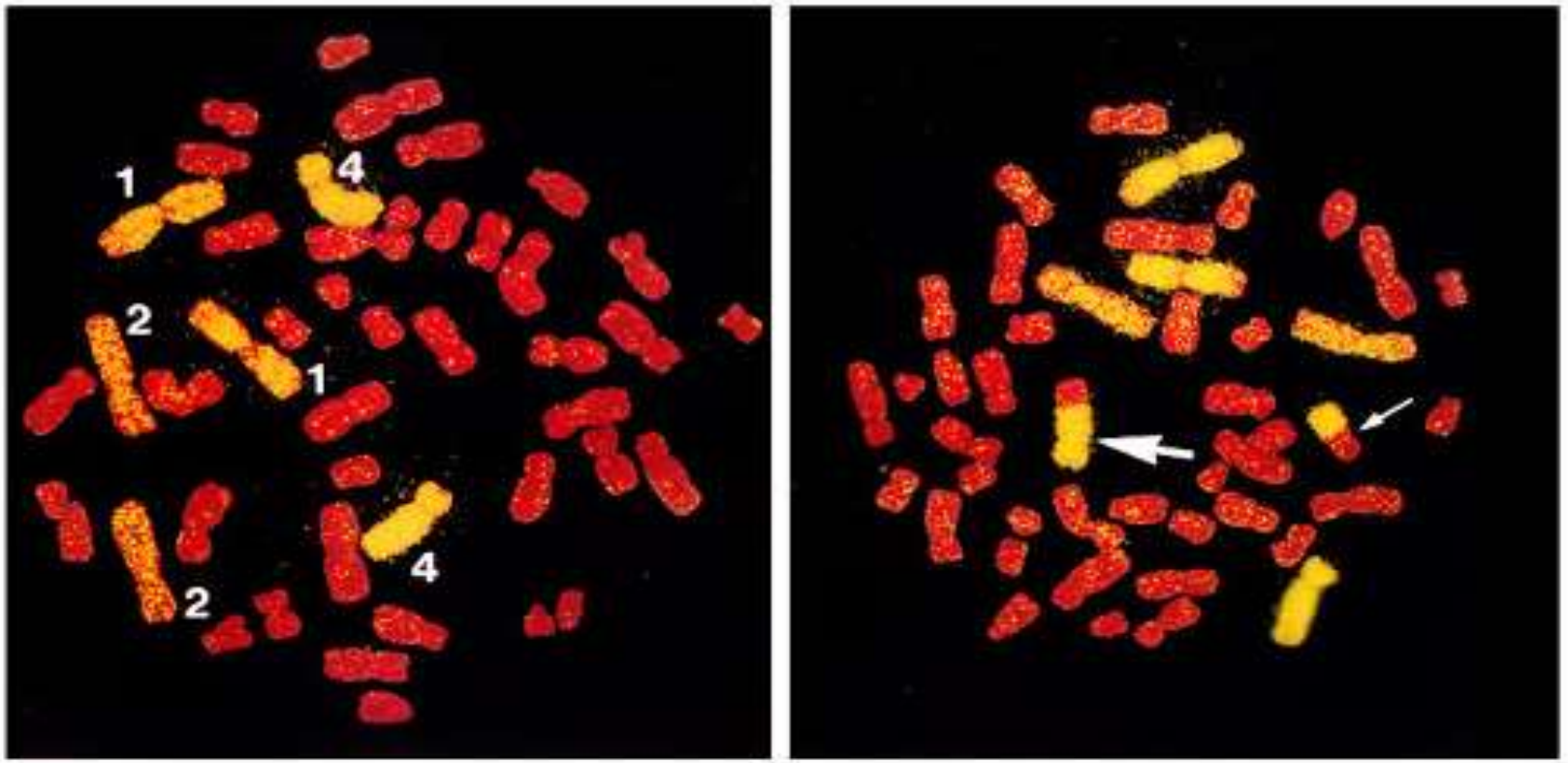
4. Cytogenetic data can be obtained only for the target chromosomes thus FISH is not a good screening tool for cytogenetically heterogeneous disease.

5. Requires fluorescence Microscopy and an image analysis system.

Gozzetti and Beau, 2006

Chromosome painting is a term used to describe the direct visualisation using in situ hybridisation of specific **chromosomes** in metaphase spreads and in interphase nuclei.
The initial approach of fluorescence in situ hybridization (FISH) with the sequences, regions or chromosomes as probe and later, the in situ hybridization with total genome (GISH) as probe laid the foundation for further refinements.

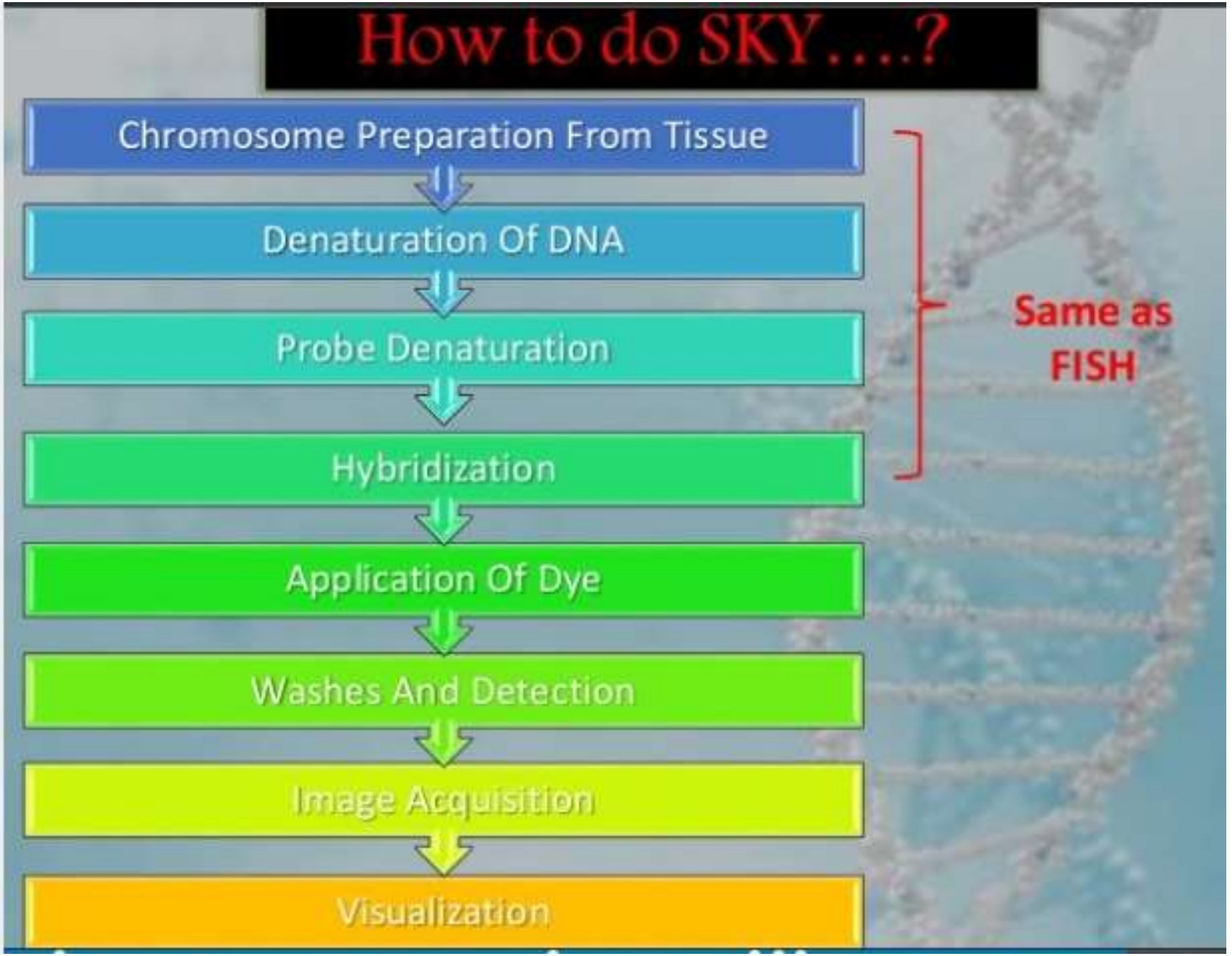
For the development of FISH, it was necessary to isolate each human chromosome. Subsequently, DNA from these chromosomes was fragmented and put into bacterial cells to amplify it (produce many copies). In this way, a large number of copies of DNA from each chromosome can be obtained.
These amplified DNA fragments are labeled with appropriate fluorescent (light-emitting) dyes and allowed to hybridize (attach) to metaphase chromosomes. The fluorescent-labeled DNAs will attach to the analogous chromosomes from which they were derived. (DNA fragments with the same base sequences have the characteristic of attaching to each other.)
In this way, if a part of a painted chromosome (yellow, for example) had undergone an exchange with another, non-painted chromosomes (stained red), it is possible to detect the aberration (termed a reciprocal translocation) because the aberrant chromosome contains both yellow and red segments. Usually, a pair of bi-colored chromosomes can be detected in one metaphase because two chromosomes typically exchange a part of their DNA.



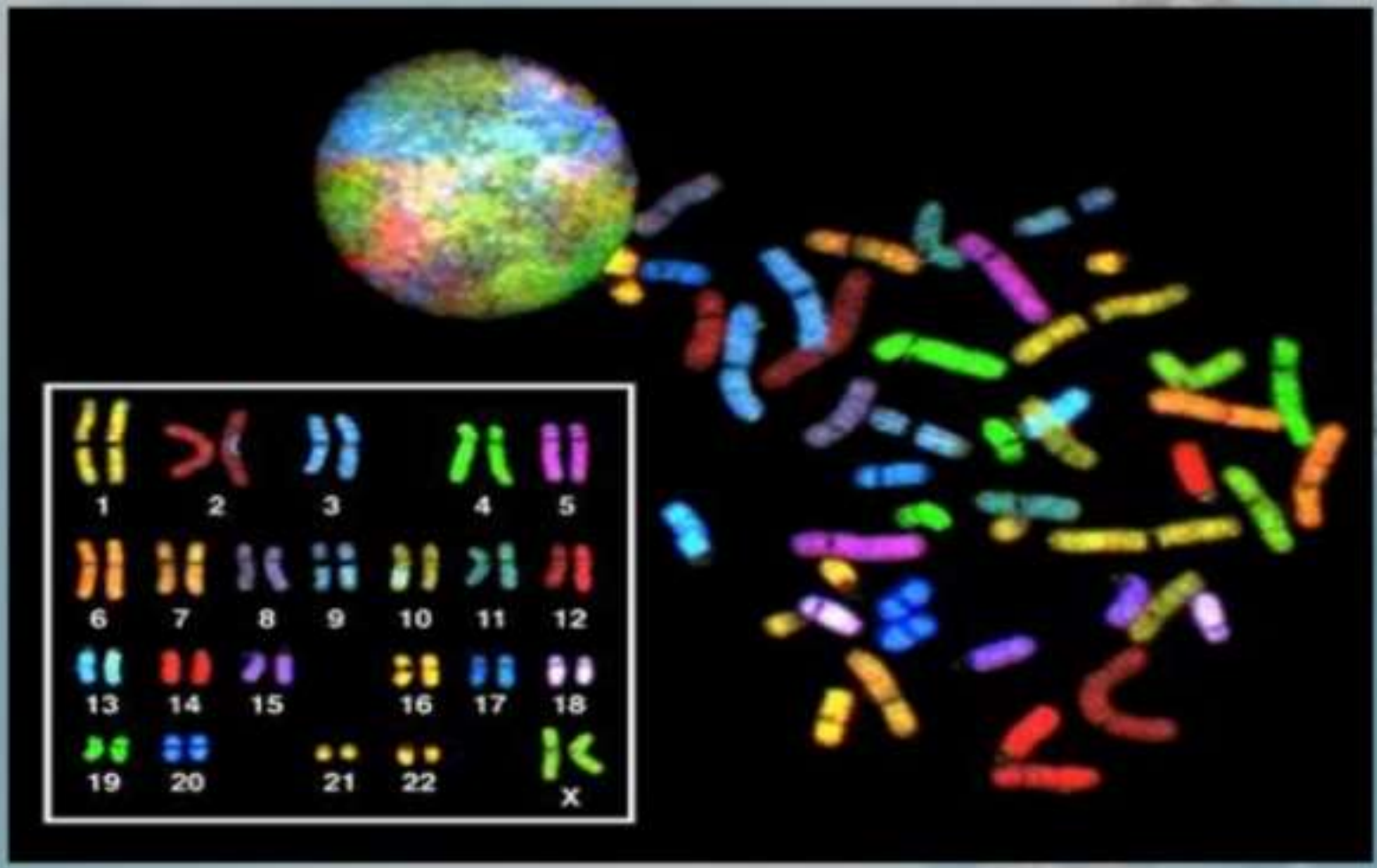
chromosomes 1, 2, and 4 were labeled yellow with FISH and the other chromosomes were stained red. Translocations between yellow and red chromosomes are detected. The left picture represents a normal cell (the numbers in the figure indicate chromosome numbers) and the right picture is an example of reciprocal translocation with two bi-color chromosomes (indicated by two arrows).

What is SKY…?

- Spectral karyotyping is cytogenetical technique used to simultaneously visualize all the pairs of chromosome in an organism in different colors.
- The sky technique is useful for identifying chromosomal abnormalities.
- We can arrange the chromosomes according to their number just by visualization of different colours aquire by the chromosomes.

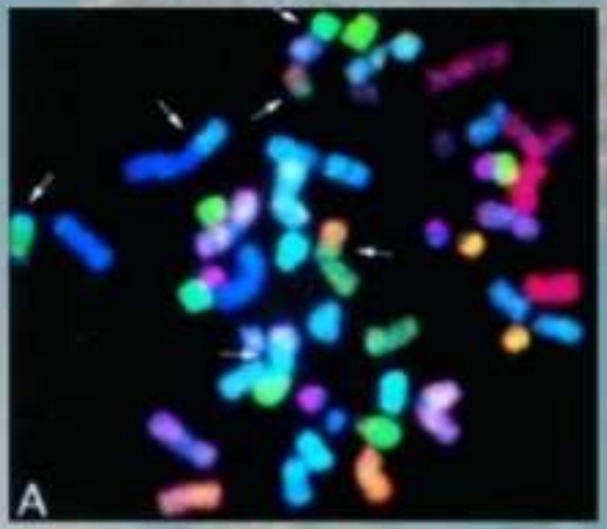

How to do SKY….?
Chromosome Preparation From Tissue
Denaturation Of DNA
Probe Denaturation
Hybridization
Application Of Dye
Washes And Detection
Image Acquisition
Visualization
Same as FISH

Spectral Karyotype Observation



Some Abnormalities Seen In SKY

Translocation



Application of SKY

- This technology allows the isolation of structural chromosome abnormalities
- which then allows determination of the precise molecular address of chromosome breakpoints associated with deletions translocations and insertion.
- SKY can discern the aberrations that can't be detected very well by conventional banding technique and Fluorescent *in situ* hybridization (FISH).
- It allows visualization of all chromosomes in different colours on same platform which is very easy to detect chromosomal abnormalities.

Gene therapy (frominternet)

Medical genetics: Whether a particular human disease has genetic basis----
clinically possible to identify -------Counselling

But genes have many different role – no single biological consequence of mutated gene
may associated with structural protein

Sometime genes of one tissue may have impact on other tissue

EX; Phenylalanaline dehydrolase----Live enzyme convert phelyalaline to tyrosine

May affect neuronal axons

Primary goal of medical research -----to develop treatment
Drug/surgery/diet

Phenylketourea -----phenylalanine free diet

Hemophilia, SCID, Goucher diseases --- Replacement theory/bone marrow/organ transpl
defective protein is replaced by administrating
purified protein

But effective treatment for many genetic disease

---Only when the patient enter a crisis period

---- Live saving but temporary

Also some genetic diseases – affect multiple system and tend to be progressively debilitating –not easy to develop therapy

Above all ---though patients benefied from treatment – most cases suboptimal, recurring , costly, and time consuming

So new form of therapy is sought

Gene transfer --- one strain of bacteria can transfer gene to other and give them new character

Human gene can be transferred into appropriate somatic cell type

In 1980– It was more than a dream

Human somatic gene therapy –with the discovery of

1. Gene isolation technique
2. eukaryotic expression vector
3. Trangenic experiment become more routene

Gene therapy is an experimental technique that uses **genes** to treat or prevent disease. In the future, this technique may allow doctors to treat a disorder by inserting a **gene** into a patient's cells instead of using drugs or surgery

---

- Gene therapy is **the introduction of genes into existing cells** to prevent or cure a wide range of diseases.
- It is a technique for correcting defective genes responsible for disease development.
- The first approved gene therapy experiment occurred on September 14, 1990 in US, when **Ashanti DeSilva** was treated for **ADA-SCID**.

Goal of Gene therapy

A normal gene may be inserted into a **non-specific location within the genome** to replace a non-functional gene. This approach is most common.

An abnormal gene could be **swapped for a normal gene** through homologous recombination.

The **abnormal gene could be repaired through selective reverse mutation**, which returns the gene to its normal function.

The regulation (**the degree to which a gene is turned on or off)** of a particular gene could be altered.

Designing gene therapy:
Preclinical --Phase I--- Phase II ---Phase III
Persimission must be sough from various regulatory authority before preclinical and any of the phase of the study.

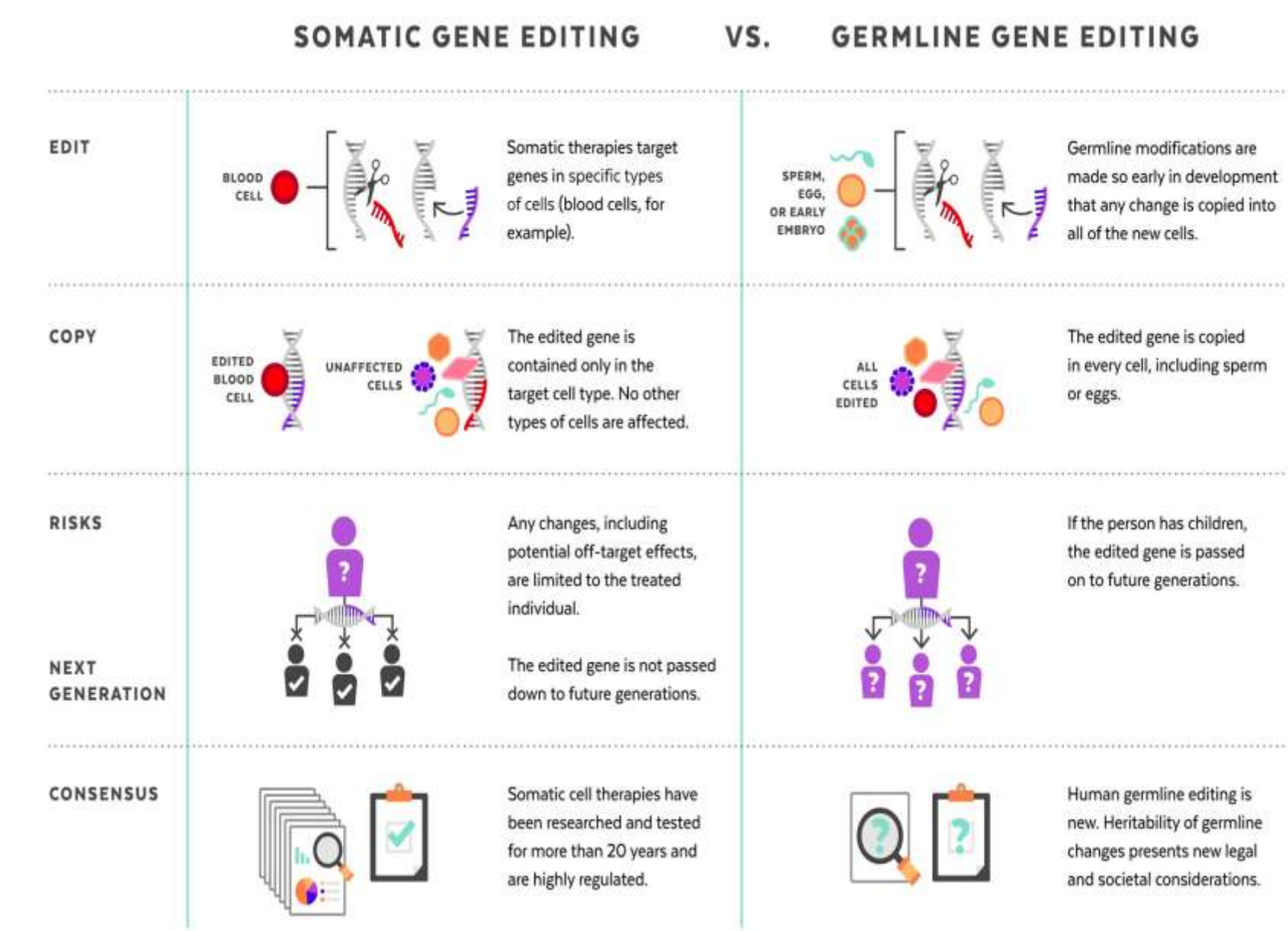
A number of key consideration :

1. Study should be directed for developing treatment for a recognize diseases
2. Risk of patients should be minimal
3. All rules and regularion of the biomedical research should be followed
4. Only somatic genetherapy ; no germ line therapy

CRITICAL BIOLOGICAL CONSIDERATIOBN

a. How will the cells that are to be targeted for correction be accessed
b. How will the remedial gene be delivered
c. What portion of the target cells must acquire the input gene needed to be precisely regulate the diseases
d. Does transcription of the input gene need to be regulated
e. Overexpression of the gene ----alternative physiological problem
f. Input gene will maintain indefinitely or repeated regularly

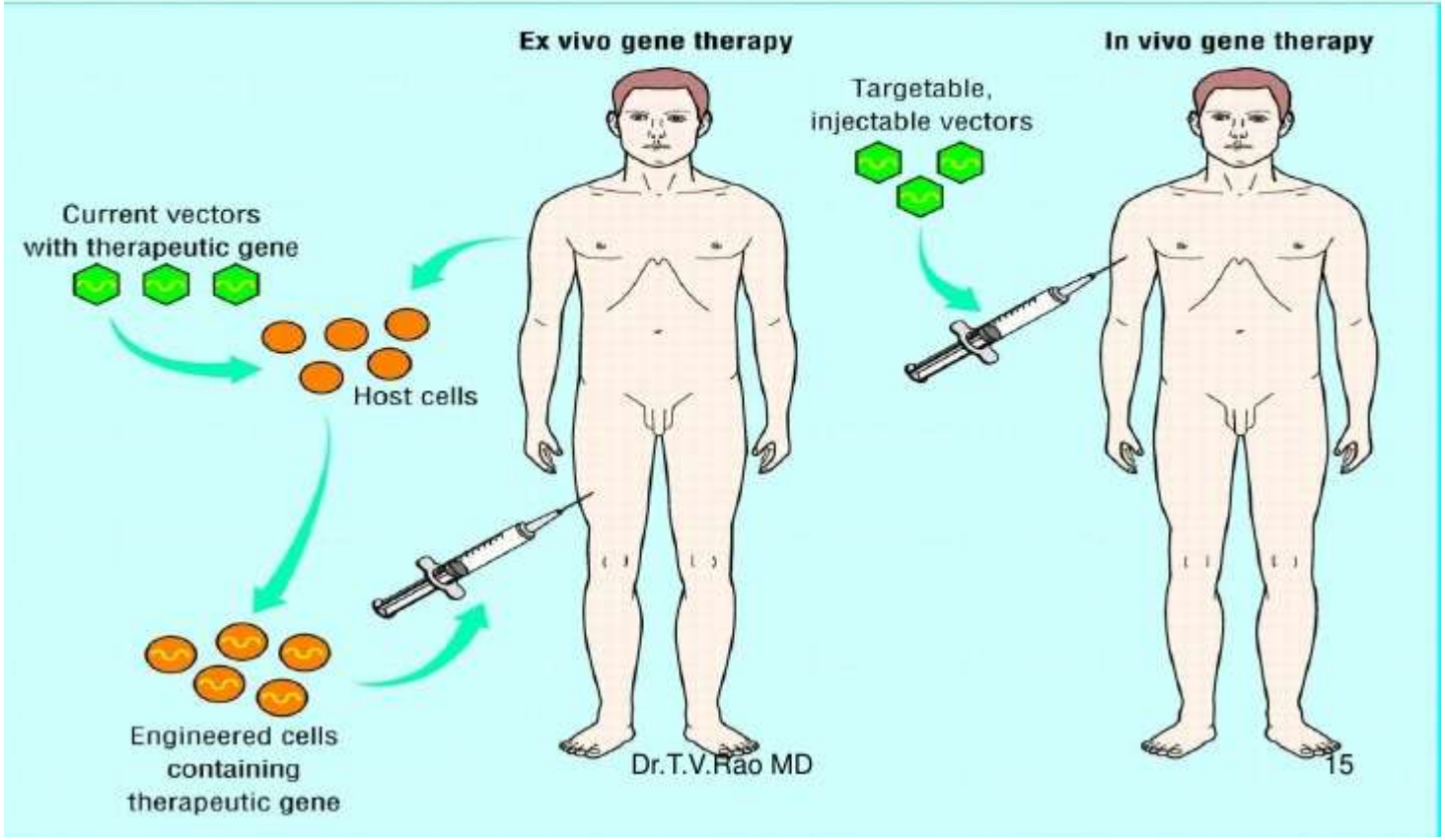
Somatic gene editing compared to germline gene editing.



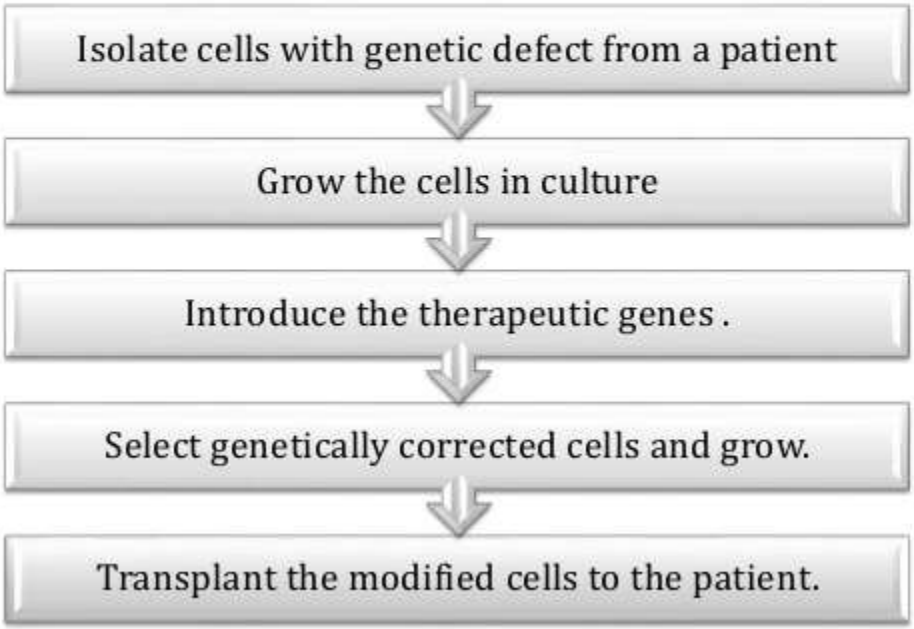
TYPES OF GENE THERAPY

SOMATIC CELL GENE THERAPY	GERM LINE GENE THERAPY
▪ Therapeutic genes transferred into the **somatic cells.**	▪ Therapeutic genes transferred into the **germ cells.**
▪ Eg. Introduction of genes into bone marrow cells, blood cells, skin cells etc.	▪ Eg. Genes introduced into eggs and sperms.
▪ **Will not be inherited** later generations.	▪ **It is heritable** and passed on to later generations.
▪ At present all researches directed to correct genetic defects in somatic cells.	▪ For safety, ethical and technical reasons, it is not being attempted at present.

Vivo to Vitro

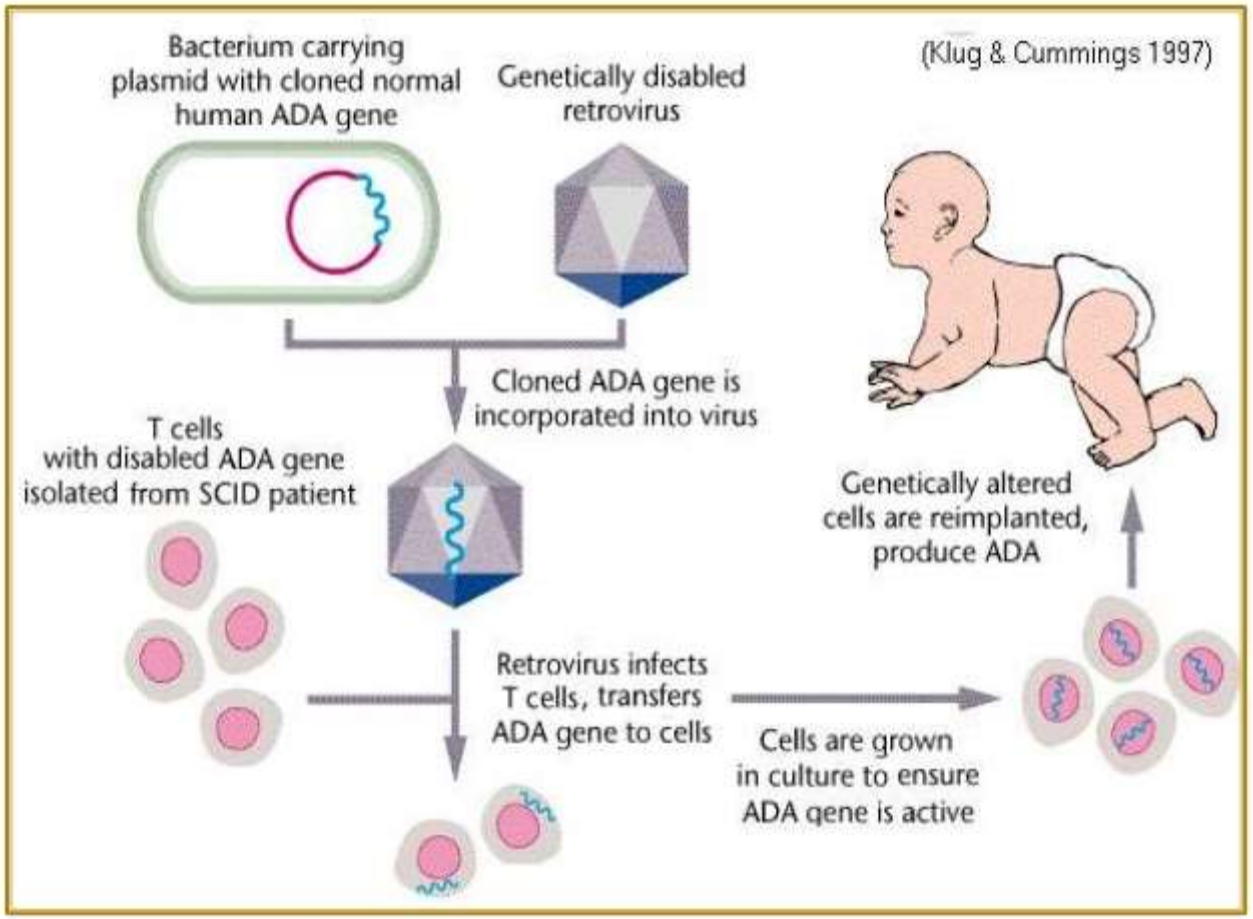


EX VIVO GENE THERAPY



EXAMPLE OF EX VIVO GENE THERAPY

- 1st gene therapy – to correct deficiency of enzyme, **Adenosine deaminase (ADA).**
- Performed on a 4yr old girl Ashanthi DeSilva.
- Was suffering from **SCID- Severe Combined Immunodeficiency.**
- Caused due to defect in gene coding for ADA.
- Deoxy adenosine accumulate and destroys T lymphocytes.
- Disrupts immunity , suffer from infectious diseases and die at young age.



The ability of retroviruses to integrate into the host cell chromosome also raises the possibility of **insertional mutagenesis and oncogene activation**. Both these phenomena are well known in the interactions of certain types of wild-type retroviruses with their hosts

But retrovirus ----------induce cancer
Needed to manipulate

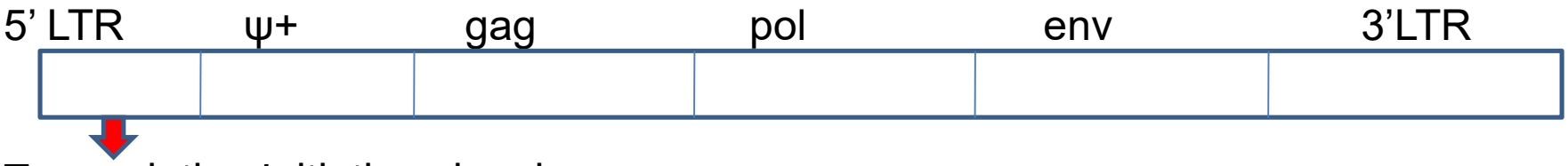
Wild type retrovirus → Two single stranded identical RNA genome that each are organized into six regions

----- 5' LTR

------ A noncoding sequence that is required for packaging the RNA strand inside a virus particle (encapsulation) ψ+ (packaging signal)

----- 3 genes that codes for an internal capsid structure protein (gag), reverse transcriptase and intergrase function (pol) and envelop protein (env)

----- 3' LTR sequences

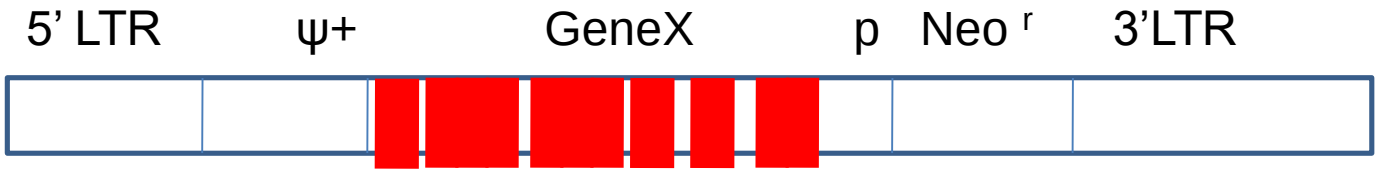


Transcription Initiation signal

Life Cycle

1. **Infection of a target cess**
2. **Production of a DNA copy of the RNA by using Reverse transcriptase**
3. **Transport the Virus DNA into nucleus**
4. **Integration of virus genome into chromosome**
5. **Transcription of viral DNA into mRNA with 5' LTR transcription signal**
6. **Translation in the cytoplasm of gag, pol, env proteins**
7. **Formation and packaging of the virus capsid with two RNA strands and reverse transcriptase molecule**
8. **Shedding of single membrane envelope virus to the exterior of the cells**

Vector



Translation of geneX is driven by sequence within 5'LTR. Internal promoter (p) drives the transcription of selectable markar. The encapsuation region ψ+ retained.

DNA that can be carried by a retroviral vector is ~ 8kb

Although retroviral vector DNA can be used by itself to transform cells, the Efficiencecy of both delivery and intergation is low

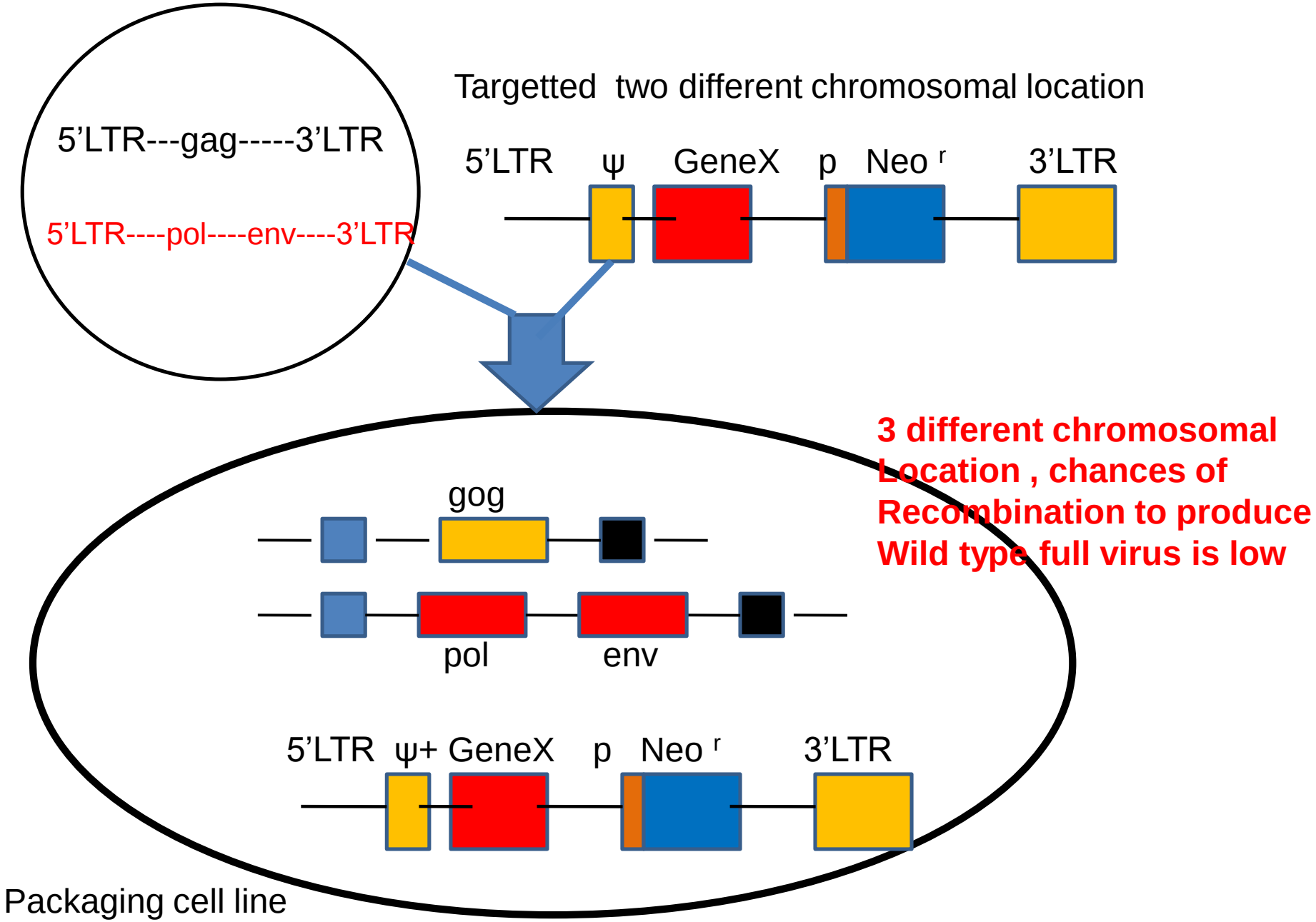
Consequently a methodology was devised to Package retrovirus vector RNA into Virus particles

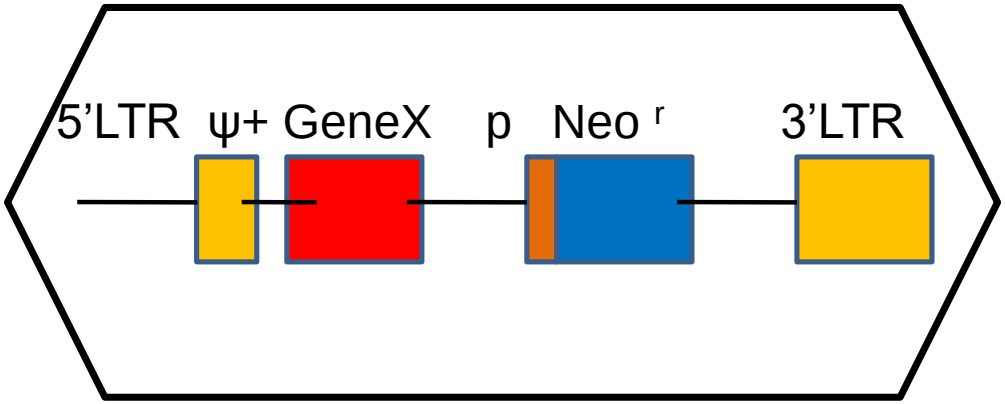
The intact particles deliver the complete vector RNA to host cells at a high frequency
➡ ensuring that its DNA equivalent will be integrated into genome of the host

A cell line was developed which carry ➡

5'LTR---gag-----3'LTR (Δ ψ+) at one chromosomal site

5'LTR----pol----env----3'LTR (Δ ψ) at other chromosomal site


5’LTR---gag-----3’LTR
5’LTR----pol----env----3’LTR
Targetted two different chromosomal location
5’LTR ψ GeneX p Neo r 3’LTR
3 different chromosomal Location , chances of Recombination to produce Wild type full virus is low
gog
pol
env
5’LTR ψ+ GeneX p Neo r 3’LTR
Packaging cell line



No wild type retro virus be produced by packaging cell line – routinely checked

Packaging cell lines provide all the viral proteins required for capsid production and the virion maturation of the vector. These **packaging cell lines** have been made so that they contain the gag, pol and env genes.

Characteristics
The remidial gene product is synthesized
Replication competent retrovirus are not produced
The retrovirus vector DNA has not been inserted into a site that either alter growth properties of the cells or interfere with normal cellular function

Bone marrow transplantation---------Candidate for exvivo gene therapy

Totipotent stem cells → genetically engnirred--------either infuse or transplant

Human adenosine deaminase delivery

Adenosine and deoxyamenase → damage T and B cells

Totipotent stem cells are used
But stem cells are not always available , umbilical cord blood
Rich in stem mcells---- collected during birth

Low LDL-receptor—liver cells

Circulating LDL , arterial blockage or heart diseases

15% Liver is removed → grown in culture → cDNA for LDL receptor → introduce to the patient → sucess

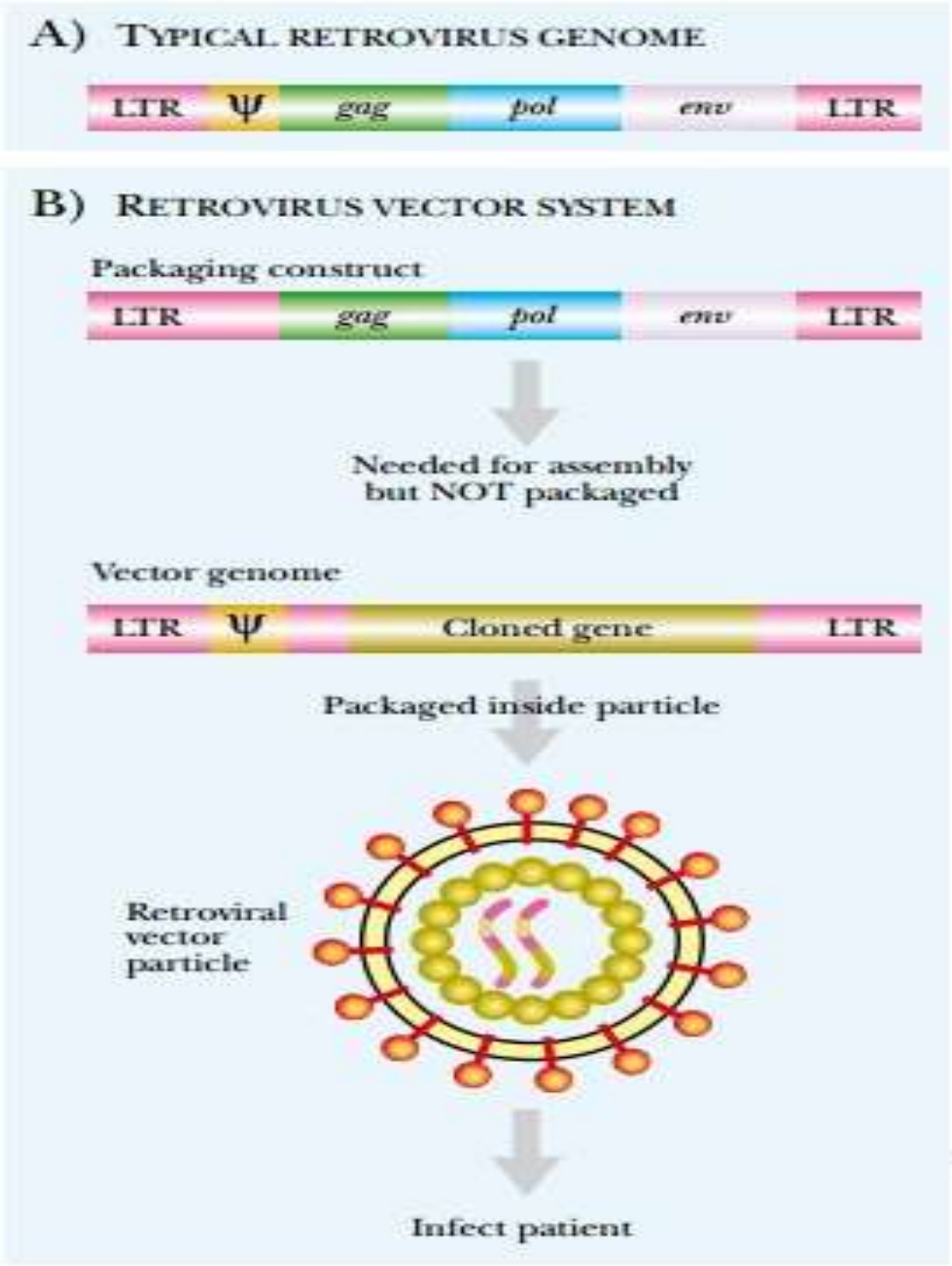
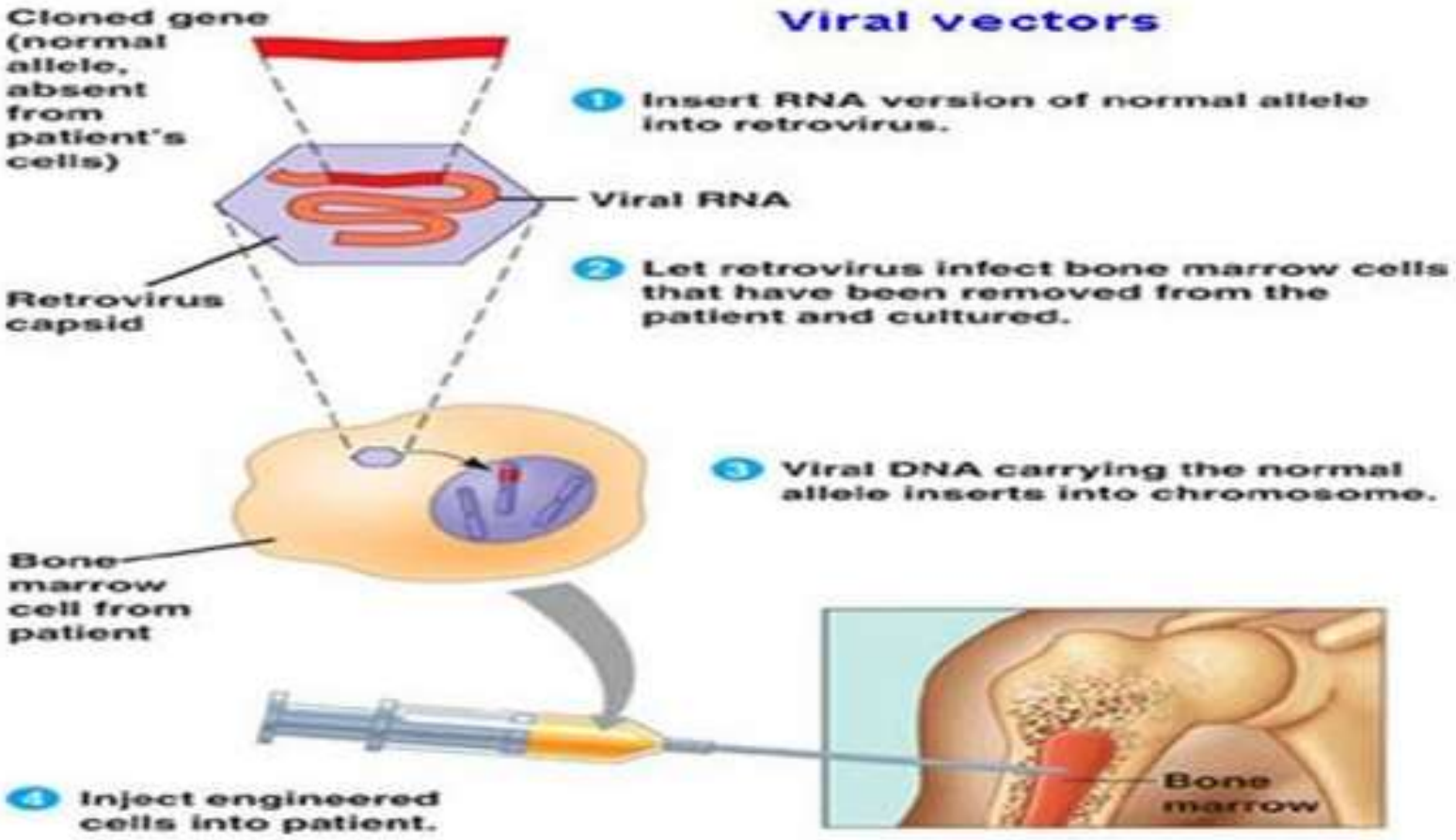


FIGURE 17.7

Retrovirus Genome and Vector System

(A) The retrovirus genome has a packaging signal (ψ) and the genes *gag*, *pol*, and *env* flanked by two direct repeats known as LTRs. (B) Retrovirus gene therapy uses two virus constructs. The therapeutic vector carries the cloned gene and packaging signal flanked by two LTRs. The packaging vector has the three genes necessary for virus assembly and packaging: *gag*, *pol*, and *env*. Because the packaging vector does not carry the packaging signal, it is never packaged and does not infect the patient. When both constructs are present, the therapeutic vector plus cloned gene is packaged into the capsid.

Therapy for Adenosine Deaminase Deficiency



Therapy for Hemophilia:

Hemophilia is a genetic disease due lack of a gene that encodes for clotting factor IX. It is characterized by excessive bleeding. By using a retroviral vector system, genes for the synthesis of factor IX were inserted into the liver cells of dogs. These dogs no longer displayed the symptoms of hemophilia.

Ex Vivo Gene Therapy with Non-Autologous Cells:

The ex vivo gene therapies described above are based on the transplantation of genetically modified cells for the production of desired proteins. However, there are several limitations in using the patient's own cells (autologous cells) for gene therapy. These include lack of enough cells from target tissues, defective uptake of genes and their inadequate expression. To overcome these problems, attempts are on to develop methods to use non-autologous cells (i.e., cells from other individuals or animals). The outline of the procedure is briefly described below.

Tissue-specific cells capable of growing in culture are selected. These include fibroblasts from skin, hepatocytes from liver, and myoblasts from muscle and astrocytes from brain. These cells are cultured and genetically modified with the therapeutic gene. **They are then encapsulated in artificial membrane composed of a synthetic polymer (e.g., polyether sultone, alginase-poly L-lysine-alginate).**

The polymeric membranes are non-immunogenic, therefore the patient can accept non-autologous encapsulated cells. Further, being semipermeable in nature, these membranes allow the nutrients to enter in, and the encoded protein (by the therapeutic gene) to pass out.

Experiments conducted in animals have shown some encouraging results for using non-autologous cells in gene therapy. The encapsulated cells were found to proliferate and produce the required protein. However, the success has been very limited in human trials.

Murine leukemia viruses in gene therapy:

This is a retrovirus that causes a type of leukemia in mice. It can react with human cells as well as the mouse cells, due to a similarity in the surface receptor protein. Murine leukemia virus (MLV) is frequently used in gene transfer.

AIDS virus in gene therapy?

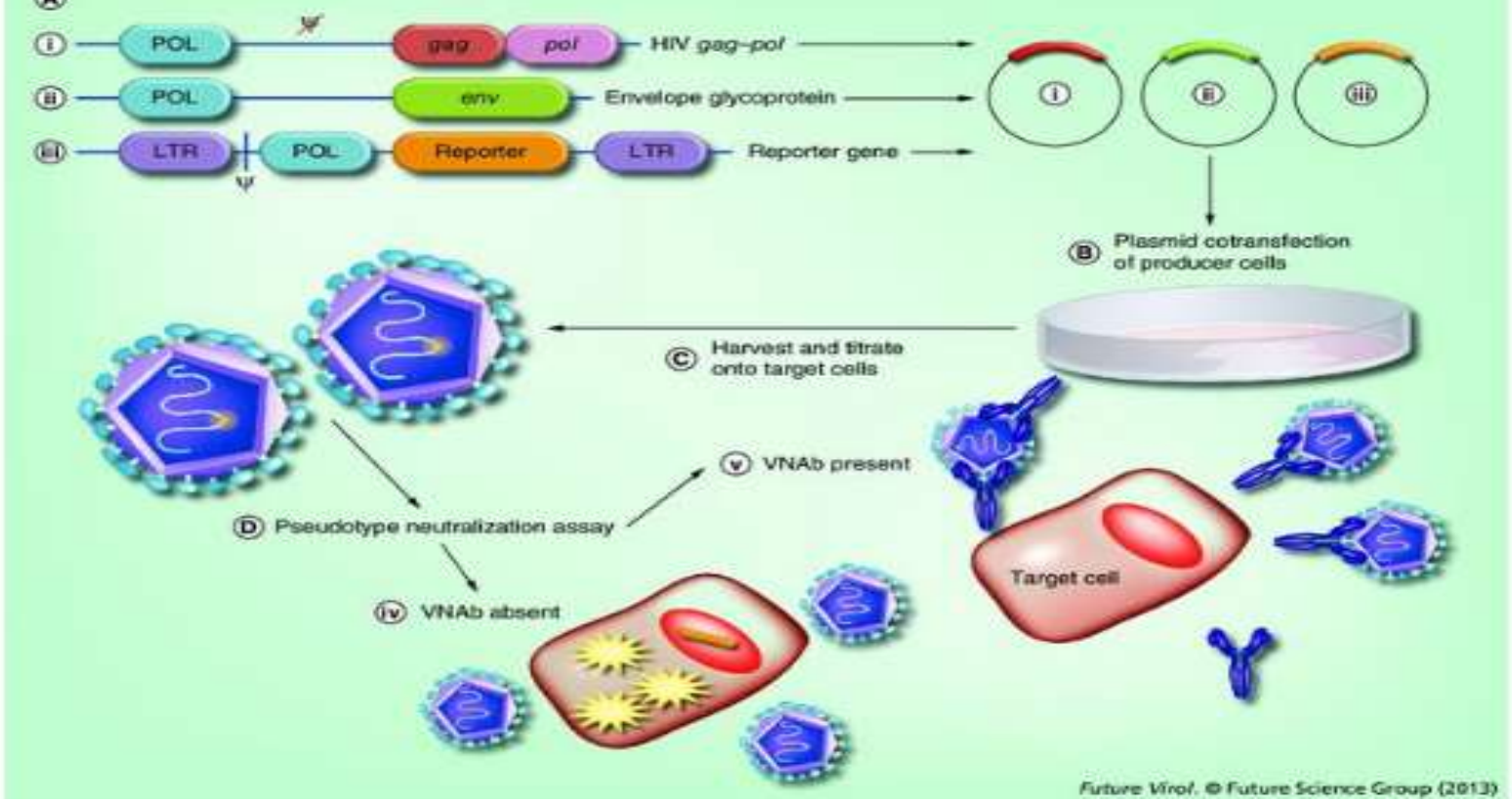
It is suggested that the human immunodeficiency virus (HIV) can be used as a vector in gene transfer. But this is bound to create public uproar. Some workers have been successful in creating a harmless HIV (crippled HIV) by removing all the genes related to reproduction. At the same time, the essential genes required for gene transfer are retained. There is a distinct advantage with HIV when compared with MLV. **MLV is capable of bringing out gene transfer only in dividing cells. HIV can infect even non-dividing cells (e.g., brain cells) and do the job of gene transfer effectively.** However, it is doubtful whether HIV can ever be used as a vector.

Plasmoviruses are plasmids capable of expressing all the viral genes required for generating infectious particles and packaging a defective genome containing a transgene. Transfected as plasmids, plasmoviruses transform the transduced cells into packaging cells that release infectious replication-defective retrovirus vectors (RV) containing a transgene, which are capable of infecting nearby cells

such a vector can efficiently "propagate" the transgene after transfection. Here we examine in greater detail the different steps of plasmovirus replication in vitro in human

5’LTR---p-gag-pol

CMV promoter-TG-env



Three-plasmid cotransfection method for pseudotype virus production. (A) Conventional plasmid DNA expression vectors bearing (i) the HIV gag-pol gene, (ii) the envelope glycoprotein from the virus of interest or (iii) a reporter gene (e.g., luciferase) are generated. (B) All three plasmids are transfected into 'producer' cells (e.g., HEK293 T cells). (C) Supernatants are harvested at 48 h post-transfection and produced pseudotype viruses (PVs) are titrated onto target cells expressing receptors recognized by the envelope protein in order to ascertain a relative transduction titer. (D) PVs can be subsequently employed as surrogate viruses in pseudotype neutralization assays to quantify VNAb responses. Titrated patient samples are preincubated with a fixed titer of PV before addition to target cells. (iv) In the absence of VNAbs, the envelope protein of the virus of interest enables entry of the PV into the target cell and the reporter gene is integrated and expressed. (v) Binding of the envelope protein by specific antibodies in the sample blocks entry of the PV into the target cell, thus preventing expression of the reporter gene. As for traditional plaque reduction neutralization tests, the titer of antibody can be expressed as the highest dilution of sample that inhibits expression by 50 or 90%.

LTR: Long tandem repeat; VNAb: Virus-neutralizing antibody.

Envelope protein pseudotyping of viral vectors

The viral vectors described above have natural host cell populations that they infect most efficiently. Retroviruses have limited natural host cell ranges, and although adenovirus and adeno-associated virus are able to infect a relatively broader range of cells efficiently, some cell types are resistant to infection by these viruses as well. Attachment to and entry into a susceptible cell is mediated by the protein envelope on the surface of a virus. Retroviruses and adeno-associated viruses have a single protein coating their membrane, while adenoviruses are coated with both an envelope protein and fibers that extend away from the surface of the virus. The envelope proteins on each of these viruses bind to cell-surface molecules such as heparin sulfate, which localizes them upon the surface of the potential host, as well as with the specific protein receptor that either induces entry-promoting structural changes in the viral protein, or localizes the virus in endosomes wherein acidification of the lumen induces this refolding of the viral coat. In either case, entry into potential host cells requires a favorable interaction between a protein on the surface of the virus and a protein on the surface of the cell

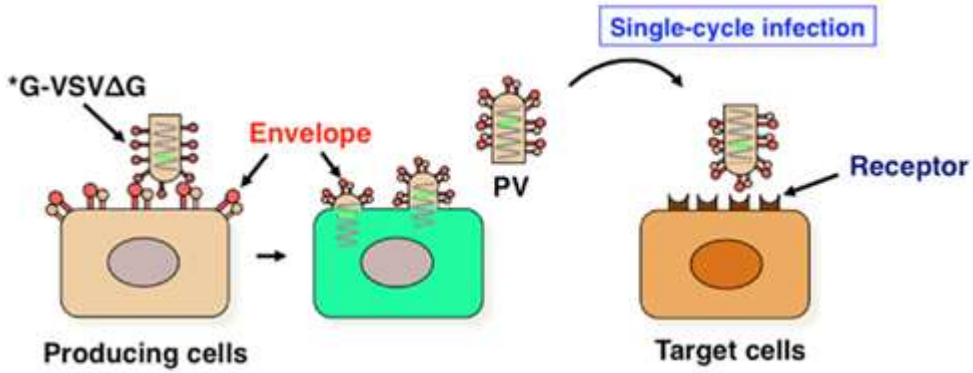
For the purposes of gene therapy, one might either want to limit or expand the range of cells susceptible to transduction by a gene therapy vector. To this end, many vectors have been developed in which the endogenous viral envelope proteins have been replaced by either envelope proteins from other viruses, or by chimeric proteins. Such chimera would consist of those parts of the viral protein necessary for incorporation into the virion as well as sequences meant to interact with specific host cell proteins. Viruses in which the envelope proteins have been replaced as described are referred to as pseudotyped viruses. For example**, the most popular retroviral vector for use in gene therapy trials has been the lentivirus Simian immunodeficiency virus coated with the envelope proteins, G-protein, from Vesicular stomatitis virus. This vector is referred to as VSV G-pseudotyped lentivirus, and infects an almost universal set of cells**. This tropism is characteristic of the VSV G-protein with which this vector is coated. Many attempts have been made to limit the tropism of viral vectors to one or a few host cell populations. This advance would allow for the systemic administration of a relatively small amount of vector. The potential for off-target cell modification would be limited, and many concerns from the medical community would be alleviated. **Most attempts to limit tropism have used chimeric envelope proteins bearing antibody fragments. These vectors show great promise for the development of "magic bullet" gene therapies**

A recombinant retro virus genome--- package in the env protein of another virus – binding specificity and **infection** spectrum increase--- **pseudotype virus**

Depends on env protein

Pseuotype virus----- co transfecting a cell line that produce gag and pol gene product with a recombinant retrovirus vector and a second virus vector that express end gene of another virus

Thus using env protein ----- Infection spectrum can be limited to specific cell type
engnireed to include DNA element , so that it
bind specific receptor
transgene is under control of cell specific promoter



Characteristics of pseudotype virus (PV)

- Viral entry can be examined because of single-cycle infection.
- Can be operated with safety.
- Constructed easily, but only in cells that exhibit high competency of transfection.

Vesicular stomatitis virus (VSV) is an excellent candidate for development as a pseudotype vector. **A recombinant VSV lacking its own envelope (G) gene has been used to produce a pseudotype or recombinant VSV possessing the envelope proteins of heterologous viruses**

The infectivity of rVSV-ΔG pseudotypes is restricted to a single round of replication the analysis can be performed using biosafety level 2

SCHEMATIC REPRESENTATION OF THE PRODUCTION OF PSEUDOTYPE VSV. Producer cells were transfected with an expression plasmid encoding foreign envelope genes and then infected with a VSV G-complemented pseudotype virus (*G-VSVΔG). The pseudotype virus released from the producer cells infected target cells but was not able to produce infectious progeny viruses.

Pseudotyping is the process of producing viruses or viral vectors in combination with foreign viral envelope proteins. The result is a pseudotyped virus particle, also called a pseudovirus. With this method, the foreign viral envelope proteins can be used to alter host tropism or increase or decrease the stability of the virus particles. Pseudotyped particles do not carry the genetic material to produce additional viral envelope proteins, so the phenotypic changes cannot be passed on to progeny viral particles. In some cases, the inability to produce viral envelope proteins renders the pseudovirus replication incompetent. In this way, the properties of dangerous viruses can be studied in a lower risk setting.

Pseudotyping allows one to control the expression of envelope proteins. A frequently used protein is the glycoprotein G (VSV-G) from the Vesicular stomatitis virus (VSV) which mediates entry via the LDL receptor. Envelope proteins incorporated into the pseudovirus allow the virus to readily enter different cell types with the corresponding host receptor.

ADENO VIRUS VECTOR SYSTEM

- Adenoviral DNA does not integrate into the genome and is not replicated during cell division.
- Humans commonly come in contact with adenovirus, majority of patients have already developed neutralizing antibodies which can inactivate the virus.
- Target- **non dividing, dividing cells.**

Characteristics of the commonly-used viral vectors.

Viral system	Adenovirus (Ad5)	AAV	Retrovirus	Lentivirus	HSV-1	Baculovirus
Genome material	dsDNA	ssDNA	RNA	RNA	dsDNA	dsDNA
Genome size	36 kb	8.5 kb	7–11 kb	8 kb	150 kb	80–180 kb
Enveloped	No	No	Yes	Yes	Yes	Yes
Biosafety level	BSL-2	BSL-1	BSL-1/2	BSL-2/3	BSL-2	BSL-1
Insert size	8–36 kb	5 kb	8 kb	9 kb	30–40 kb	No limit known
Max titer (particles/mL)	1×10^{13}	1×10^{11}	1×10^{9}	1×10^{9}	1×10^{9}	2×10^{8}
Tropism	Broad, low for blood cells	Broad, low for blood cells	Broad (pan or psuedo-typed)	Broad (pan or psuedo-typed)	Neurons	Some mammalian cells

Infectivity	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells
Transgene expression	Transient	Transient or stable	Stable	Stable	Transient	Transient or stable
Vector genome form	Episomal	Episomal (>90%), site-specific integration (<10%)	Integrated	Integrated	Episomal	Episomal or integrated
Inflammatory potential	High (low for HC-AdVs)	Low	Low	Low	High	High
Advantages	High titers; extremely efficient transduction of most cell types and tissues	Safe transgene delivery; non-inflammatory; non-pathogenic	Persistent gene transfer in dividing cells	Persistent gene transfer in most tissues	Large packaging capacity; strong tropism for neuronal cells	Large cargo sizes; high level of gene expression
Drawbacks	Capsid mediates a potent inflammatory response (eliminated in HC-AdVs)	Small packaging capacity; requiring helper AdV for replication and difficult to produce pure viral stocks	Only transduces dividing cells; integration might induce oncogenesis in some applications	Integration might induce oncogenesis in some applications	Inflammatory; no expression during latent infection; transient gene expression in non-neuronal cells	Limited mammalian host ran

Adenovirus has received tremendous attention as an effective gene delivery vector and was in fact the first DNA virus to enter rigorous therapeutic development, largely because of its well-defined biology, its genetic stability, its high gene transduction efficiency and its ease of large-scale production

Compared with other viral gene delivery systems, adenoviral vectors offer significant advantages.

First, adenovirus is the most effective means of delivering genes *in vivo* as most human cells express the primary adenovirus receptor and the secondary integrin receptors. Thus are easily infected with adenovirus vectors and consequently yield high levels of the transgene expression.

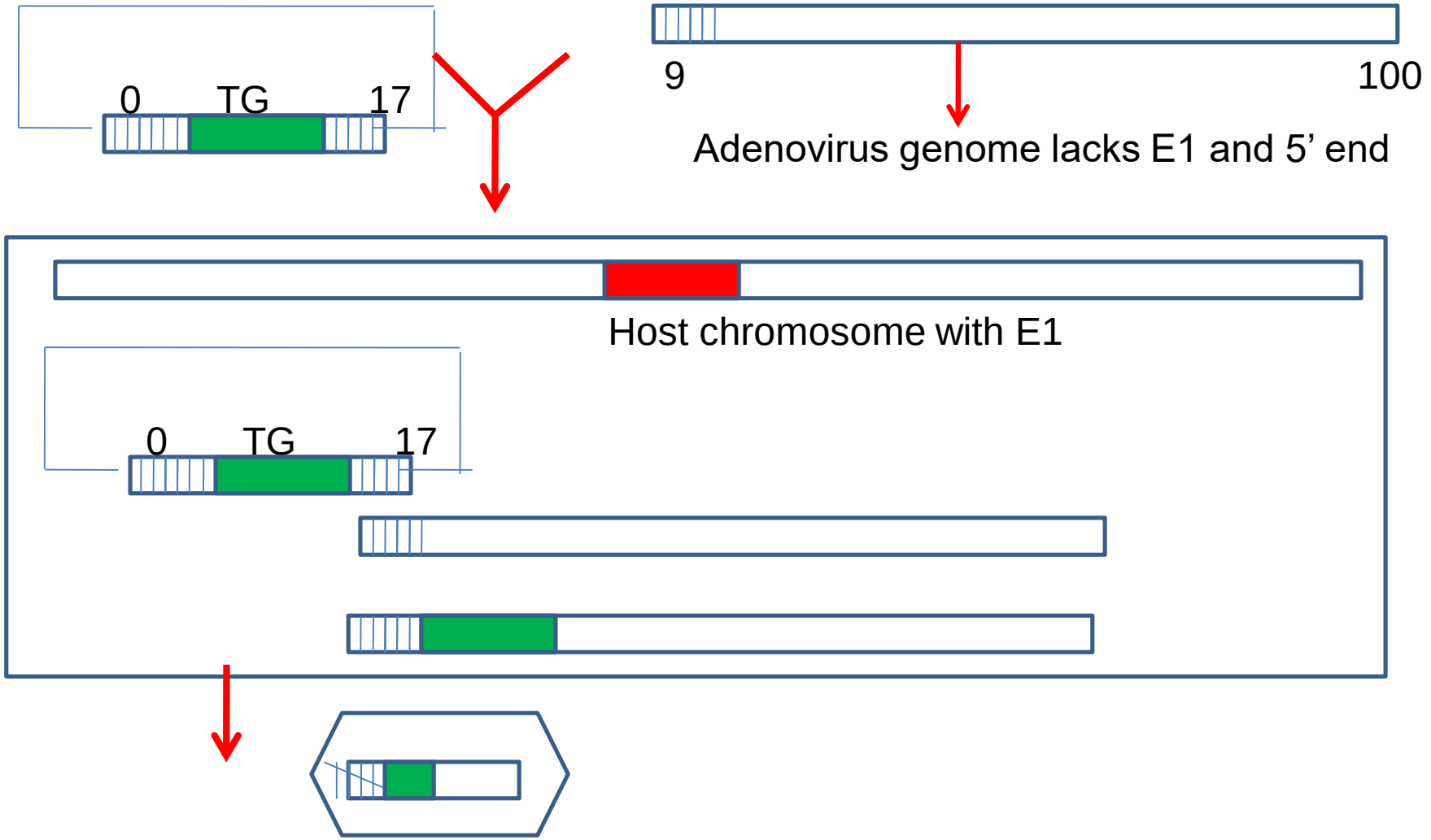
Second, the development of gutless adenoviral vectors allows us to circumvent anti-adenoviral vector immunity.

Third, despite the concern over safety of their use, there has been extensive experience with adenovirus vectors in many different clinical applications, and the safest dosing and routes of administration are now well established. In fact, adenovirus vectors are the most common vector used in clinical trials worldwide and account for >20% of all gene therapy trials

Fourth, adenovirus vectors offer a versatile platform for developing strategies to modify viral capsids in order to enhance therapeutic properties animmunity against the cancer or directly killing the cancer cell is the goal. Furthermore, the combined immunity against the adenovirus together with the short time of expression is ideal for using the adenovirus as a platform for developing vaccines. Interestingly, some of the inherited shortcomings of adenovirus, such as immunity evoked against the adenovirus capsid and low-level expression of adenovirus genes, may now prove beneficial for the development of anticancer immunotherapies, where inducing

Disadvantages to using the Adenovirus as the vector in gene therapy include **non-integration, immunogenicity, replication competence, no targeting, and small insert size**.

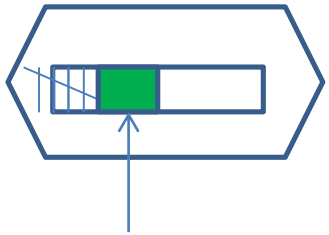
Cell lines that produce adeno virus E1 gene product which is necessary or adenovirus replication
Co-transfect with a plasmid TG in place of E1
And a DNA segment of adeno virus share homology with plasmid
Recomibation occur



DNA cloning capacity is 7.5 kb
If the recombination does not occur transfected DNA is too small to be package
DNA construct pass nucleus and express

Does not integrate to host chromosome, so periodic administration is required.

Trial CFTR-



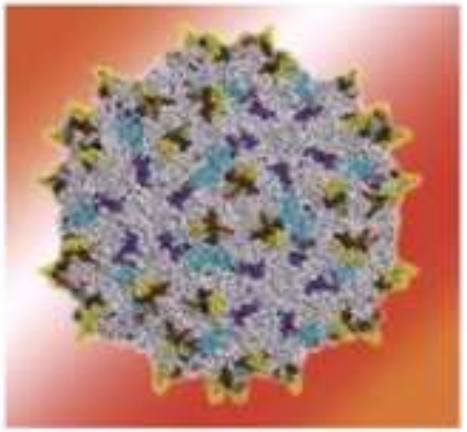
CFTR

Imunological response in patients
Some adenovirus gene product may be responsible

In E.coli – linearized plasmid containing TG ligated with DNA of adenovirus that contain origin of replication, packaging signal, terminal sequence
Construct is co-transfected with adenovirus genome lack E1 and packaging signal
Only the ligated DNA will be replicated and package into virus particle ---others
Can be separated by centrifugation.

Why AAV over adenovirus??

- The adeno-associated viruses are **small and relatively simple** viruses that have greater potential as vectors for gene therapy.
- It is a **single-stranded**, **non-enveloped DNA** virus of 4.5 kb in size.



Why AAV?

- the viruses' apparent lack of pathogenicity.
- It can also infect non-dividing cells (like hematopoietic progenitor cells and post-mitotic neurons) and has the ability to stably integrate into the host cell genome at a specific site (designated AAVS1) in the human chromosome 19.

Relatively easy purification and concentration of the virus due to the stable, naked Icosahedral structure

AAV is one of the most common vectors used in gene therapy. One of the primary reasons for using AAV is that AAV has a long-term and efficient transgene expression in various cell types in many tissues such as liver, muscle, retina, and the central nervous system (CNS)

•The feature makes it somewhat more predictable than retroviruses, which present the **threat of a random insertion and of mutagenesis,** which is sometimes **followed by development of a cancer.**

•The AAV genome integrates most frequently into the site mentioned, while random incorporations into the genome take place with a negligible frequency.
•Random integration of AAV DNA into the host genome is low but detectable.

What to do?

•Development of AAV's as gene therapy vectors, however, has eliminated this integrative capacity by **removal of the *rep* and *cap* from the DNA of the vector.**

Some of the qualities of AAV desirable for gene therapy include-

- **Non-pathogenicity** in humans
- **Broad host range** of AAV should allow delivery of foreign DNA to a wide range of mammalian cells.
- **Stable expression of the transgene** as shown in animal models.
- **Low risk of insertional mutagenesis**.

AAV genome structure

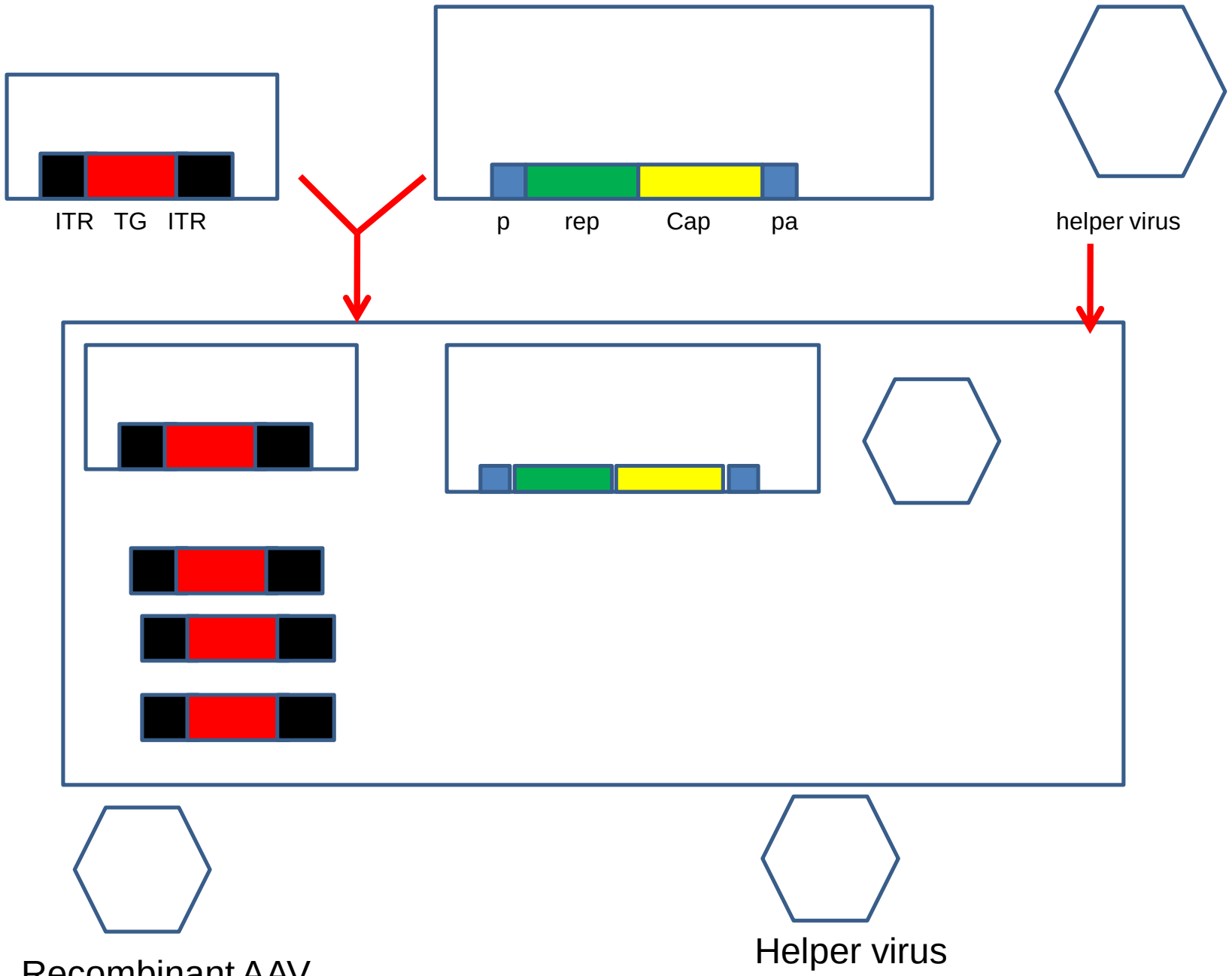
The AAV genome is built of **ss DNA**, either **positive or negative** sensed of about 4.7 kb.

- The genome comprises of inverted terminal repeats
- Two orf's: rep and cap.

1. ITR (inverted terminal repeat)

❖They have the ability to form a hairpin, which contributes to self-priming that allow primase-independent synthesis of the second strand.

The ITR shown to be required for both integration of AAV DNA into the host Cell genome and rescue from it

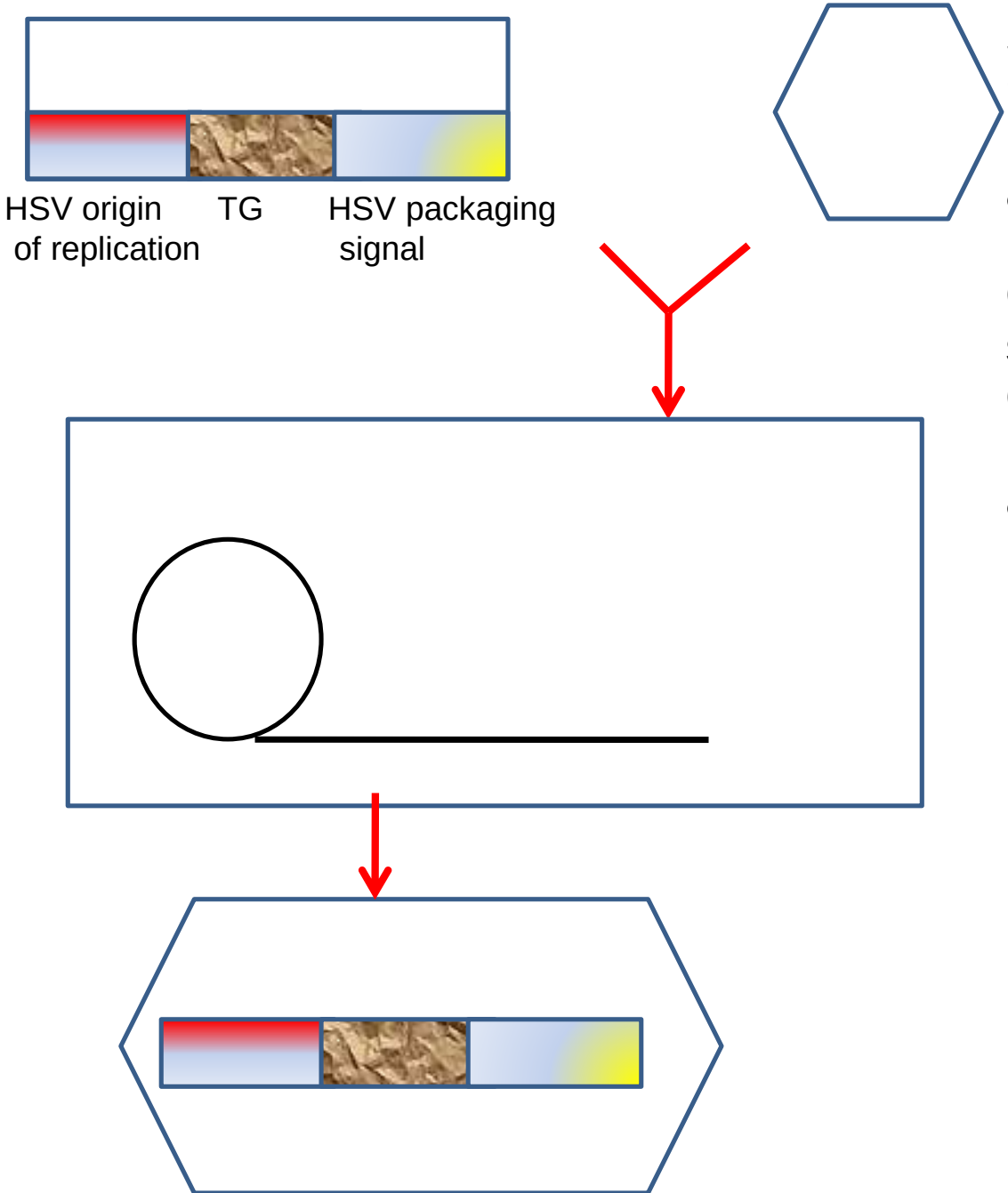

ITR TG ITR
p rep Cap pa
helper virus
Recombinant AAV
Helper virus

Herpes simplex viruses

dsDNA viruses that infect a neurons. Cold sores virus

- HSV vectors are tropic for the central nervous system (CNS) and can establish lifelong latent infections in neurons.
- They have a comparatively large insert size capacity (>20 kb) but are non integrating and so long-term expression of transferred genes is not possible.
- Their major applications are expected to be in delivering genes into neurons for the treatment of neurological diseases, such as Parkinson's disease, and for treating CNS tumors.

- HSV genome is large, measuring 152 kb.
- It is possible to insert additional genes of ~10 kb in size into the intact viral genome.
- There are three main classes of HSV-1 genes, namely immediate-early (IE or a) genes, the early (E or b) genes and the late (L or g) genes.
- After various non-essential DNA sequences have been removed it is possible to insert or *'package' ~30 kb of foreign genetic material into the virion.*
- Can live in neurons in a latent state that does not appear to affect normal cellular physiology
- This has sparked interest in this virus as a potential vector in the treatment of neurological disorders.



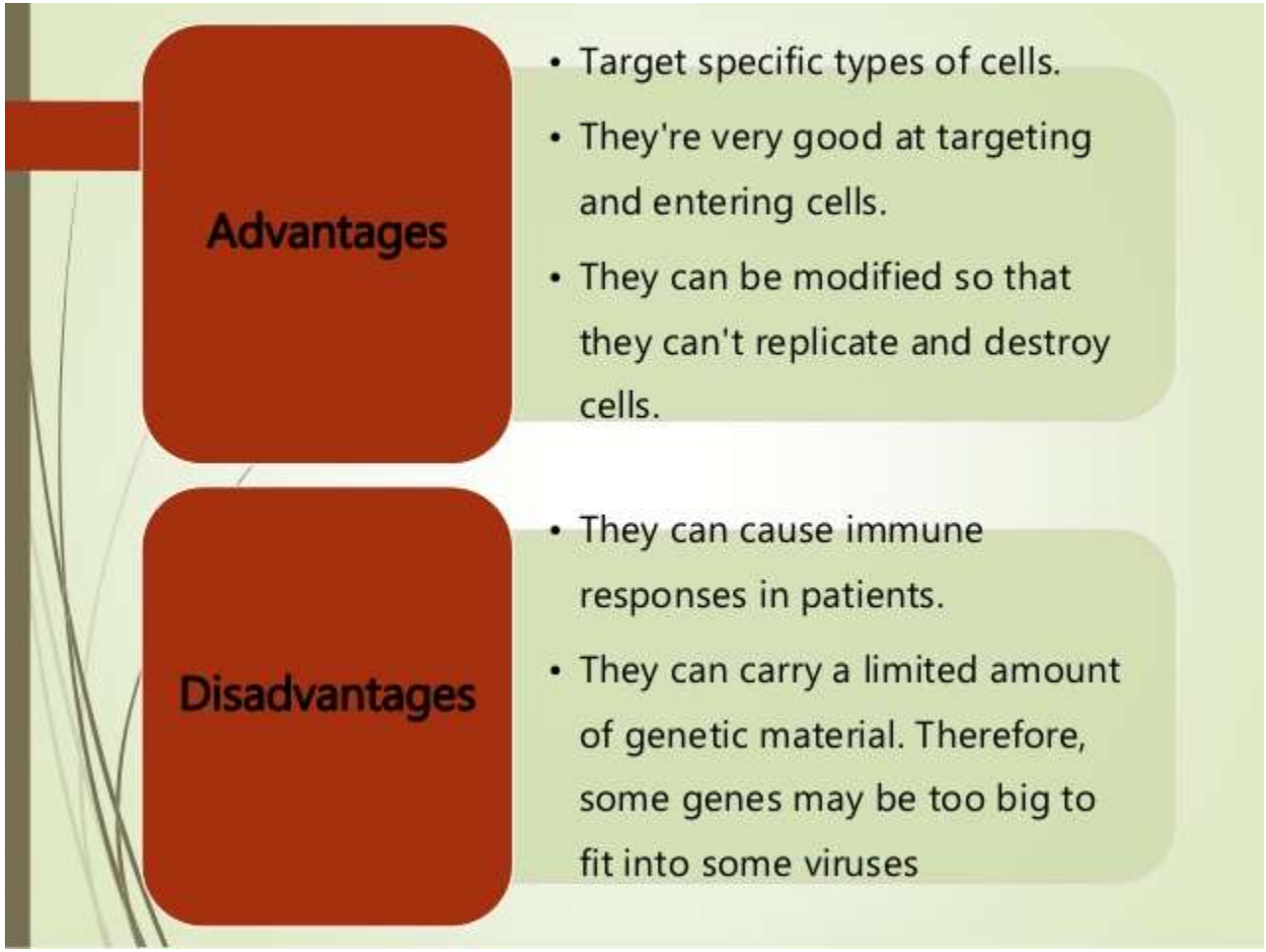
HSv helper virus
Supply the viral proteins for Replication and assembly
For viral components
Genome is modified so that it
Cannot be package
No infectious particle are produced

HSV vector problem

- The direct introduction of HSV into the brain as would be required for testing genes or in gene therapy procedures will result in a lethal encephalitis due to viral replication.

- During the onset of latent infection the virus shut down

  - Shut down of gene expression also occurs for any exogenous genes, other virus promoters such as the immediate early promoter of cytomegalovirus or a variety of cellular promoters.
  - This results in expression of the foreign gene being observed for only a few days at the most.


Advantages
• Target specific types of cells.
• They're very good at targeting and entering cells.
• They can be modified so that they can't replicate and destroy cells.
Disadvantages
• They can cause immune responses in patients.
• They can carry a limited amount of genetic material. Therefore, some genes may be too big to fit into some viruses

Nonviral methods

- Physical methods
- Chemical methods

Chemical methods

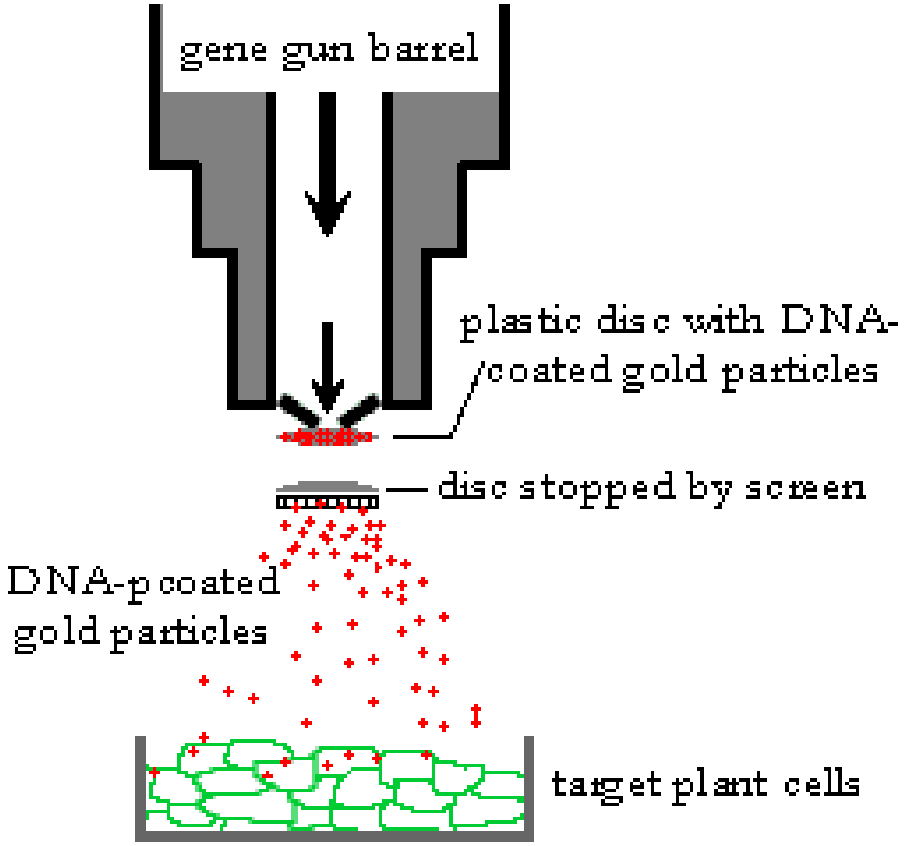
- Encapsulation
- Lipoplexes
- Dendrimers
- polyplexes

Physical methods

- Gene guns
- Electroporation
- Ultrasound
- polymers

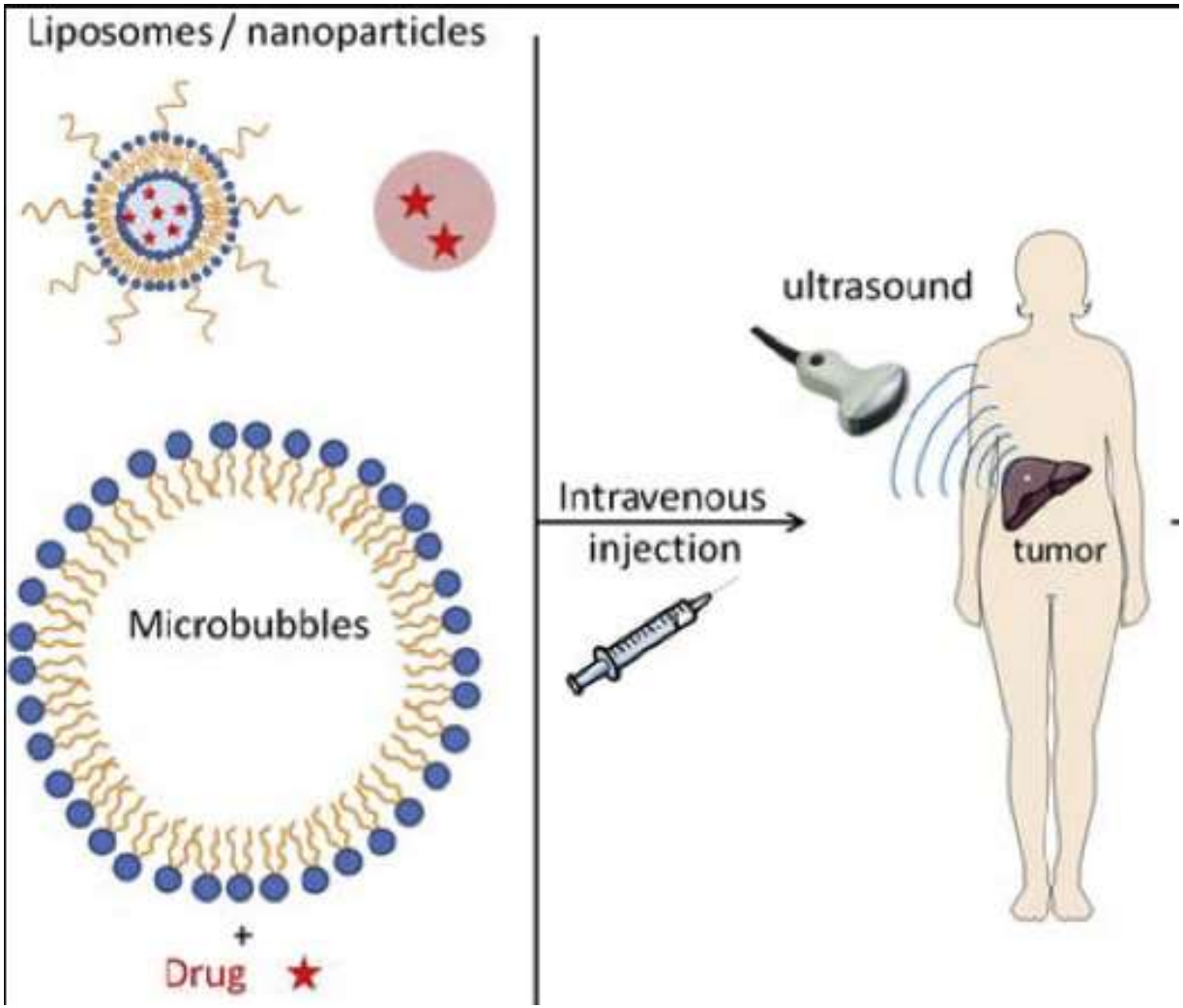
Gene gun

- Delivery with gene gun method is also termed ballistic DNA delivery or DNA-coated particle bombardment, and was first used for gene transfer to plants in 1987.
- This method is based on the principle of delivery of DNA-coated heavy metal particles by crossing them from target tissue at a certain speed
- Generally, gold, tungsten or silver microparticles were used as the gene carrier
- Gene-gun-based gene transfer is a widely tested method for intramuscular, intradermal and intratumoral genetic immunization.


gene gun barrel
plastic disc with DNA-coated gold particles
disc stopped by screen
DNA-p coated gold particles
target plant cells

Ultrasound

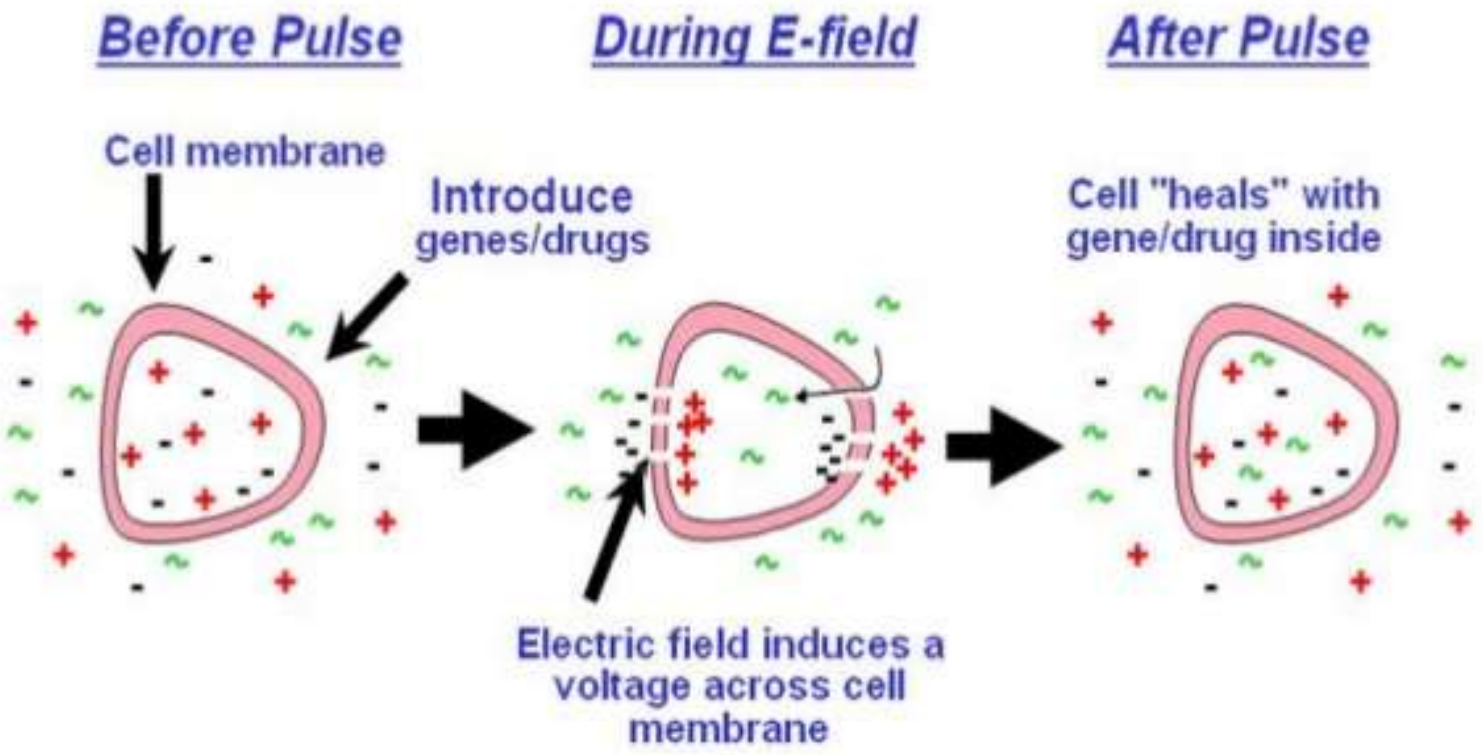
- Ultrasound has many clinical advantages as a gene delivery system, due its easy and reliable procedure
- Microbubbles or ultrasound contrast agents decrease cavitation threshold with ultrasound energy.
- Mostly perfluoropropane-loaded albumin microbubbles were used.
- The transfection efficiency of this system is based on frequency, time of ultrasound treatment, the plasmid DNA mount used, etc


Liposomes / nanoparticles
Microbubbles
+
Drug
Intravenous injection
ultrasound
tumor

Electroporation

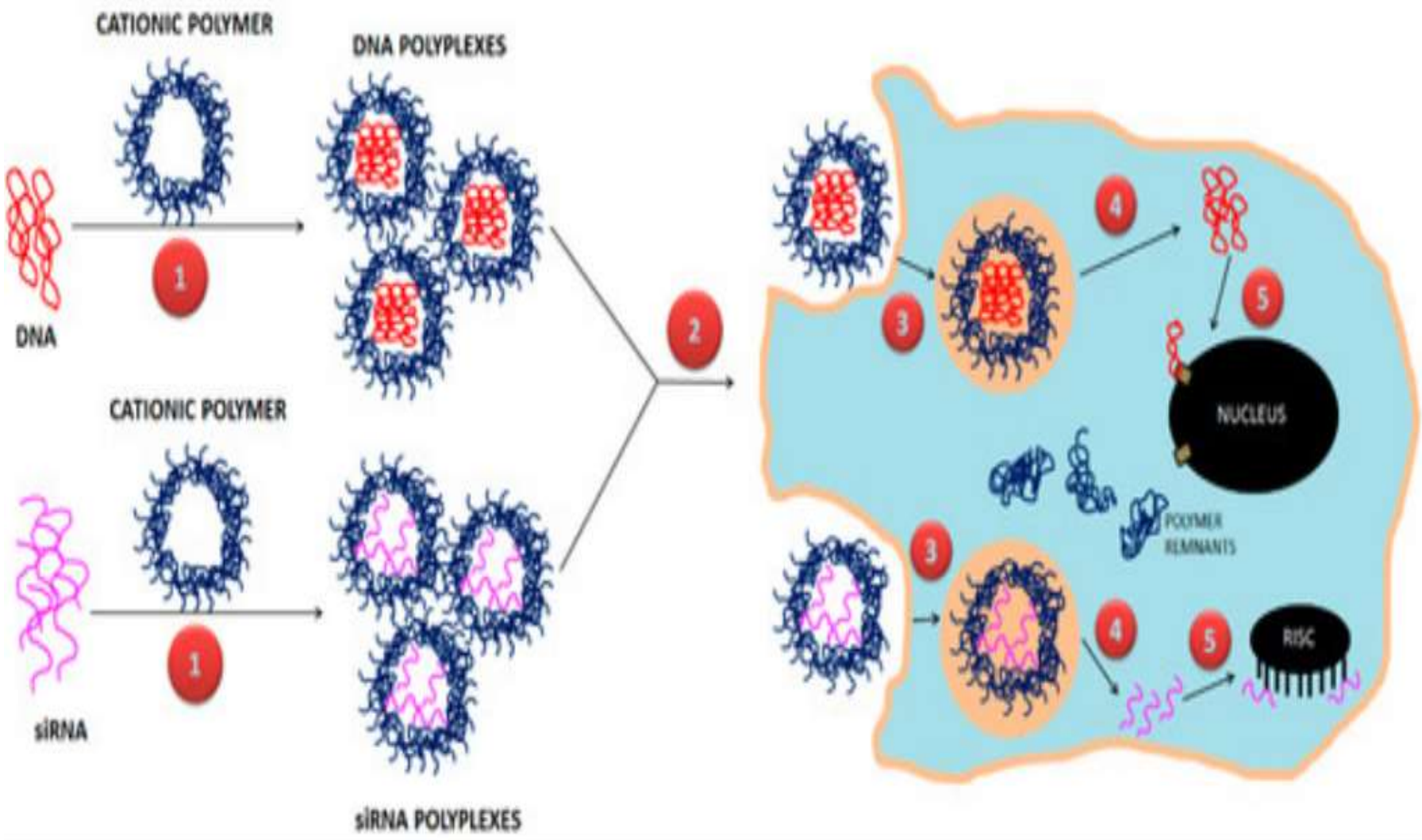
- Electroporation includes controlled electric application to increase cell permeability
- Electroporation introduces foreign genes into the cell by electric pulses. In this method, pores are formed on the membrane surface to enable the DNA to enter the cell.
- If the molecule is smaller than the pore size , it can be transferred to the cell cytosol through diffusion
- loaded molecules and ions can be transported from the membrane via electrophoretic and electro-osmotic means via the effect of electric regions

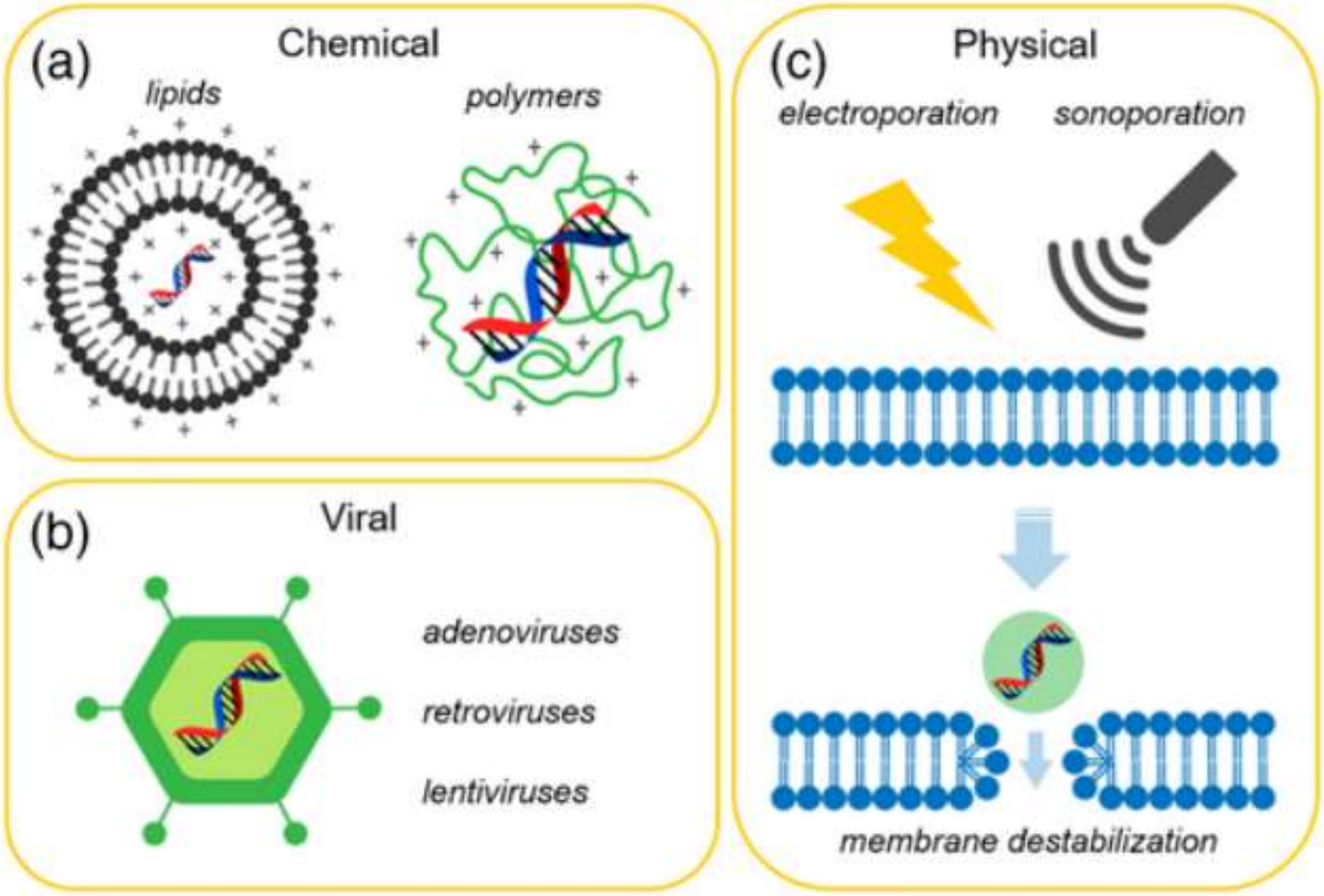
Electroporation Cell Process



polymers

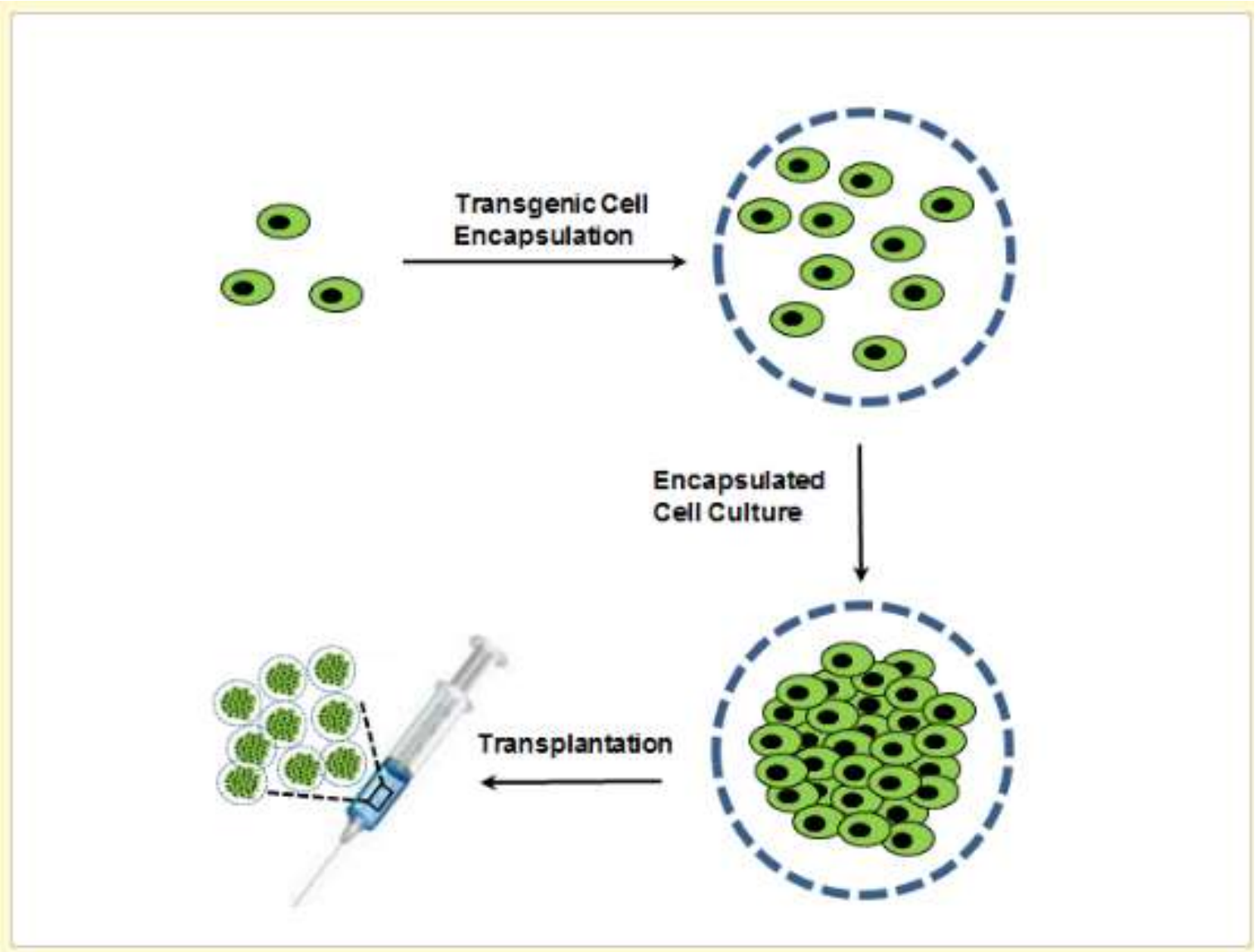
- Polymers are long-chained structures composed of small spliced molecules called monomers.
- Polymers that are composed of a repeated monomer are called homopolymers, while those composed of two monomers are called copolymers.
- Biodegradable polymers are non-water soluble, and undergo chemical or physical change in biologic environment.
- Polyamides, dextran, and chitosan are examples of biodegradable polymers
- non-biodegradable polymers are not degraded in biological environments;
- hydrophilic polymers are hydrogels, which are non-water soluble and swell in water, while hydrophobic polymers are non-water soluble and do not swell


CATIONIC POLYMER
DNA POLYPLEXES
DNA
1
2
CATIONIC POLYMER
siRNA
1
siRNA POLYPLEXES
3
4
5
NUCLEUS
POLYMER REMNANTS
3
4
5
RISC


(a)
Chemical
lipids
polymers
(b)
Viral
adenoviruses
retroviruses
lentiviruses
(c)
Physical
electroporation
sonoporation
membrane destabilization

Encapsulation

- An alternative to electrostatic condensation of DNA is encapsulation of DNA with a biodegradable polymer.
- Polymers that have an ester linkage in their structures (like polyesters) are hydrolytically degraded to short oligomeric and monomeric compounds, which are more easily discharged from the body.
- The degradation mechanism and DNA release can be controlled by changing the physicochemical characteristics and composition of the polymer.
- DNA is protected from enzymatic degradation by encapsulation.



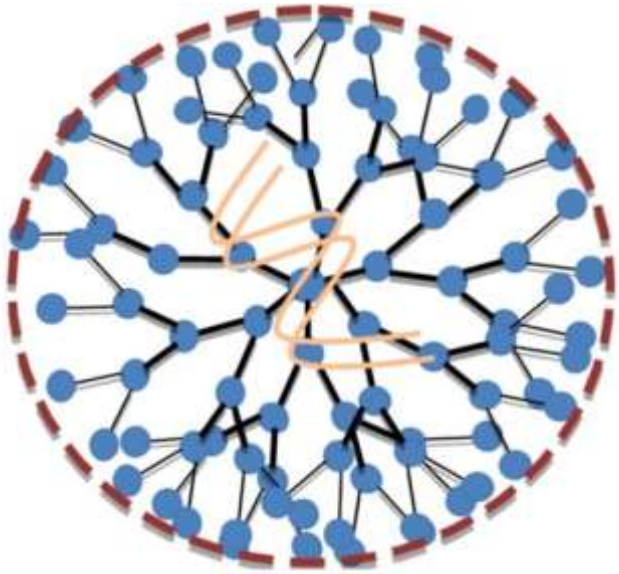
A conceptual schematic demonstrating cell encapsulation for gene therapy.

Oligonucleotides

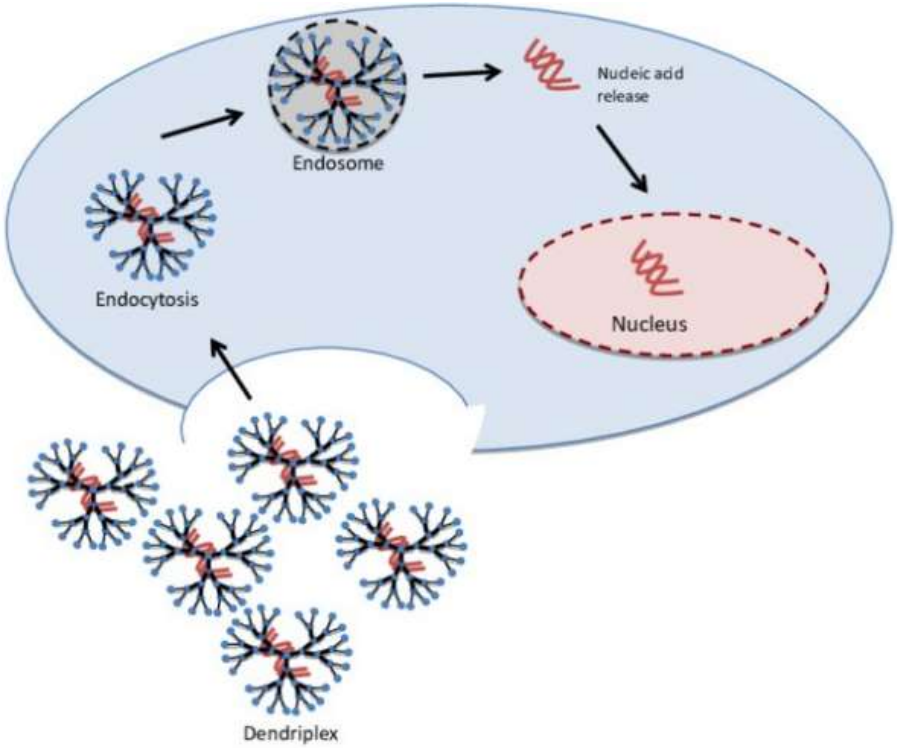
- Deactivate the genes involved in the disease process
- Uses antisense specific to the target gene to disrupt the transcription of the faulty gene
- Double stranded oligodeoxynucleotides as a decoy for the transcription factors that are required to activate the transcription of the target gene
- The oligonucleotide is designed to anneal with complementarity to the target gene with the exception of a central base, the target base, which serves as the template base for repair.

Dendrimers

- Highly branched macromolecule with spherical shape
- Cationic dentrimers associate with nucleic acid
- the lack of ability to transfect some cell types, the lack of robust active targeting capabilities, incompatibility with animal models, and toxicity of cationic lipids are absent in dendrimers
- Dendrimers offer robust covalent construction and extreme control over molecule structure, and therefore size
- Producing dendrimers has been aslow and expensive process



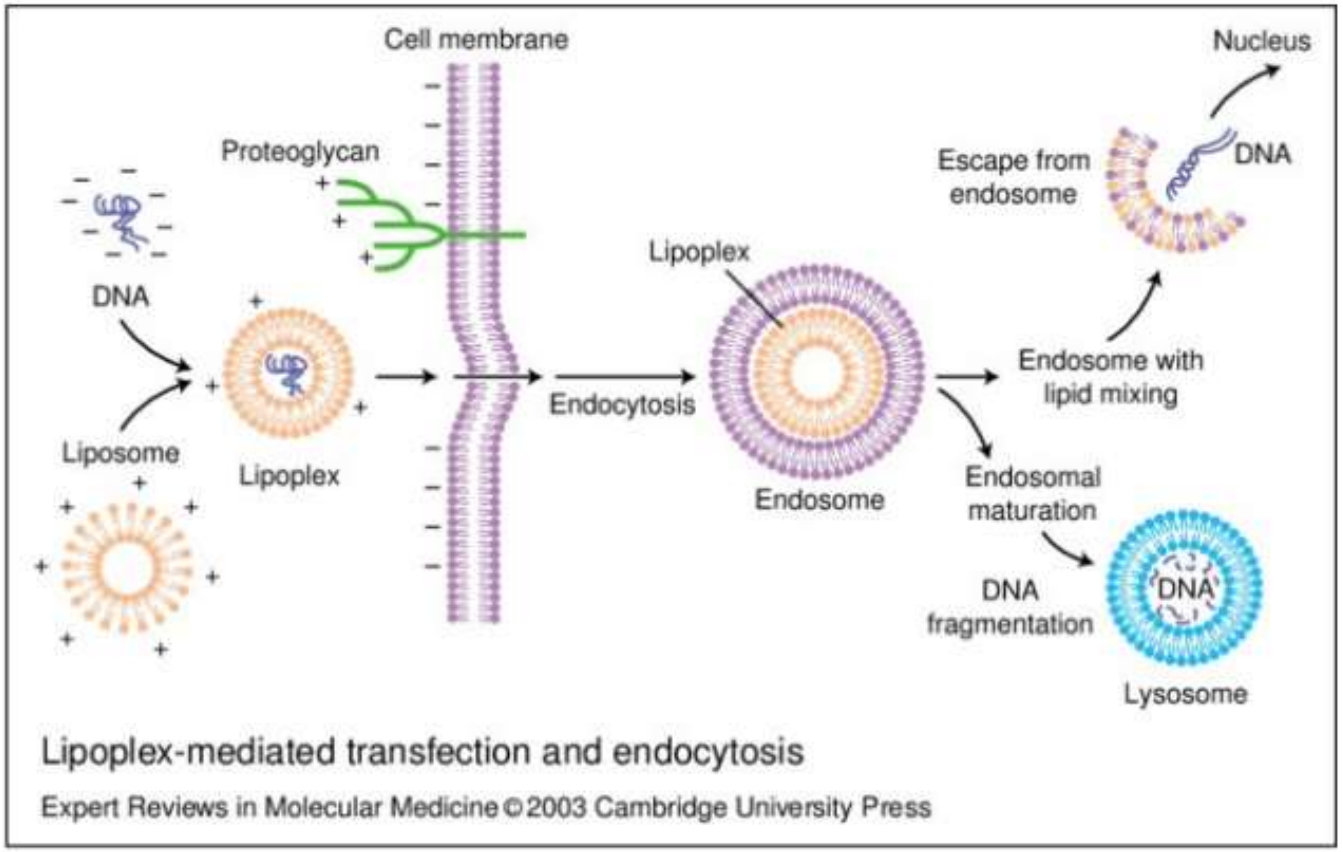
Structure of Dendrimers.



Dendrimers-mediated gene delivery.

lipoplexes

- Anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors
- Cationic lipids, due to their positive charge, were first used to condense negatively charged DNA molecules so as to facilitate the encapsulation of DNA into liposomes
- Helper lipids (usually electroneutral lipids, such as DOPE) were added to form lipoplexes, much higher transfection efficiency was observed
- The most common use of lipoplexes has been in gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell and decrease the activity of oncogenes



Lipoplex-mediated transfection and endocytosis. Cationic lipids forming micellar structures called liposomes are complexed with DNA to create lipoplexes. The structures fuse with the cell membrane, at least sometimes after interactions with surface proteoglycans. The complexes are internalised by endocytosis, resulting in the formation of a double-layer inverted micellar vesicle. During the maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained DNA into the cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression. Alternatively, DNA might be degraded within the lysosome

POLYPLEXES

- Complexes of polymers with DNA are called polyplexes.
- polyplexes cannot directly release their DNA load into the cytoplasm.
- low toxicity, high loading capacity, and ease of fabrication, polycationic nanocarriers.
- co-transfection with endosome-lytic agents such as inactivated adenovirus must occur.
- Use of polymers and copolymers help in the ease of controlling the size, shape, surface chemistry of these polymeric nano-carriers gives them an edge in targeting capability and enhanced permeability.

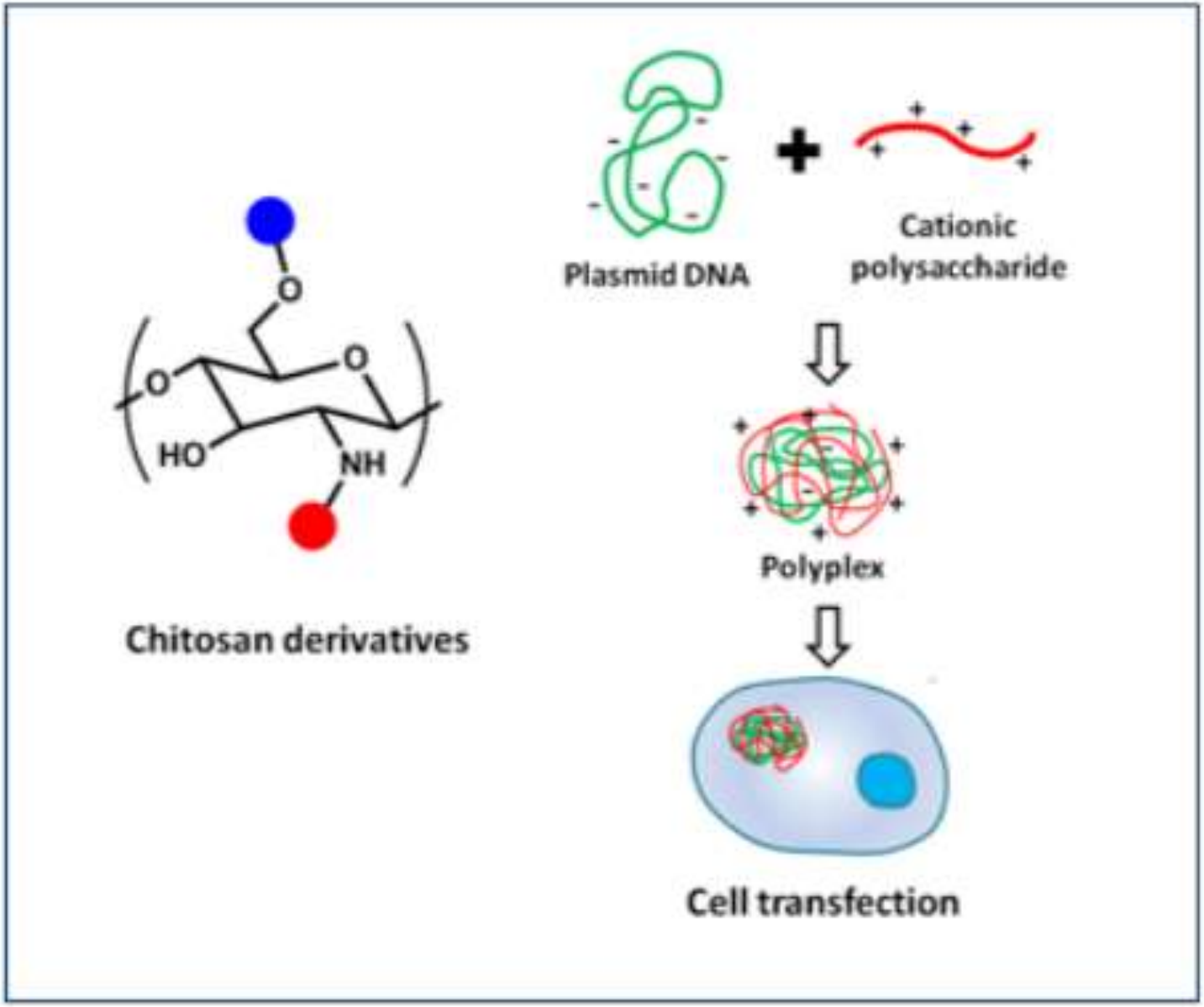


Fig.1 Schematic diagram of chitosan as a gene delivery vector

Poly (lactic-co-glycolic acid) and poly lactic acid

Poly (lactic-co-glycolic acid) (PLGA) and poly lactic acid (PLA) consist of lactic acid and glycolic acid. Biodegradable PLGA and PLA provide sustained gene delivery by bulk hydrolysis and increase stability.

Ethical issues

- To decide what is normal and what is disability
- To decide whether somatic gene therapy is more or less ethical than germ line therapy
- Will the therapy only benefit the wealthy due to its high cost?
- Could the widespread use of gene therapy make the society less accepting of people who are different?
- Should people be allowed to use gene therapy to enhance basic human traits such as height, intelligence, or athletic ability?

Disadvantages

- The genetic testing, screening and research in finding the availability of certain gene is very controversy.
- May increase rate of abortion if prenatal test regarding baby with genetic disease is done.
- The cost is very high and the patient might need an insurance to cover the treatment.
- Cosmetic industry may monopolized this gene therapy if it is used in enhancing beauty and in vanishing the aging effect, rather than used for treatment of a disease.
- Problems with viral vectors

Microbes as sources of antibiotics and therapeutic agents (Collected from internet)

What are Antibiotics A substance, such as penicillin or streptomycin, produced by or derived from certain fungi, bacteria, and other organisms, that can destroy or inhibit the growth of other microorganisms.

How do antibiotics work? Although there are a number of different types of antibiotic they all work in one of two ways: A bactericidal antibiotic kills the bacteria. Penicillin is a bactericidal. A bactericidal usually either interferes with the formation of the bacteriums cell wall or its cell contents. A bacteriostatic stops bacteria from multiplying.

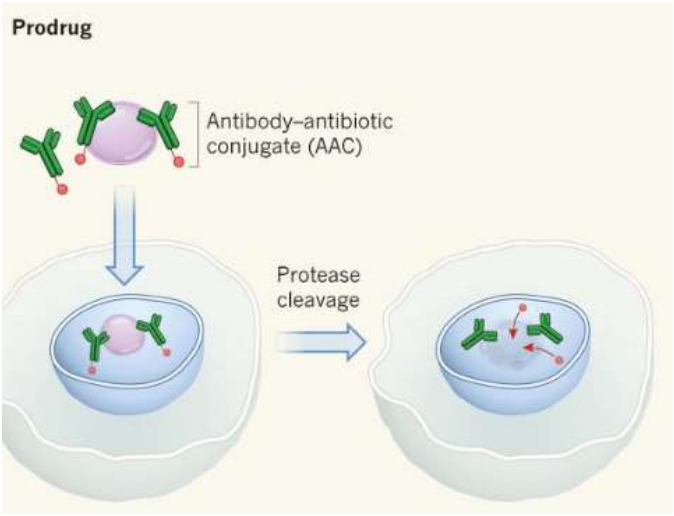
Penicillins Oldest class of antibiotics. **Penicillins are generally bactericidal, inhibiting formation of the cell wall**

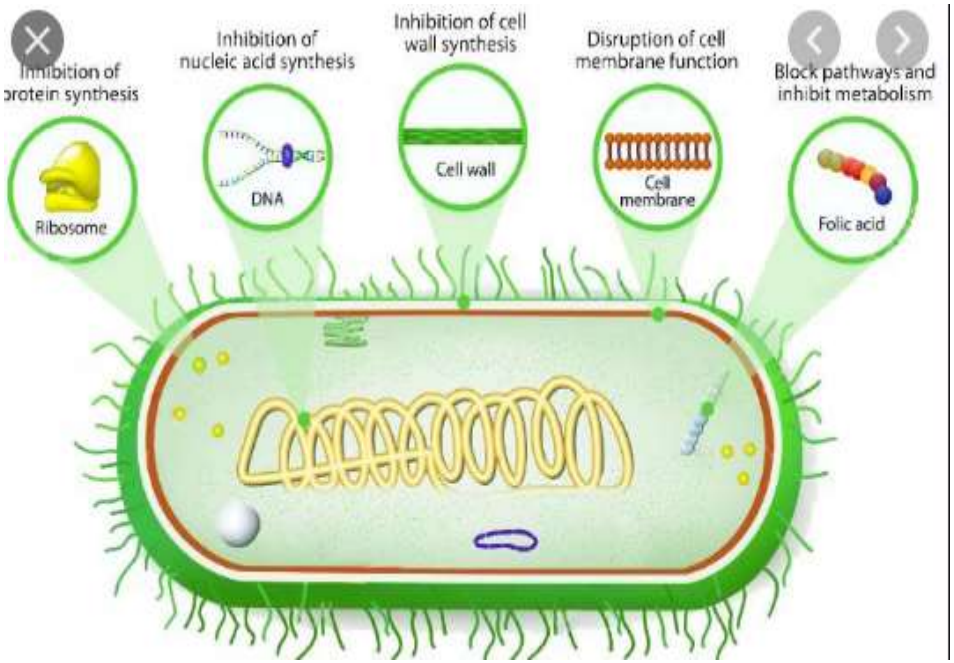
Cephalosporins Produced by the mould Cephalosporium acremonium. Cephalosporins are used to treat pneumonia, various types of skin infections, gonorrhea, urinary tract infections

Tetracyclines : Streptomyces aureofaciens, S. ramosus and Nocardia sulphurea are the actinomycetes which form tetracyclines. For example, Aureomycin. Tetracyclines act as bacterial protein synthesis inhibitor

Macrolides:Derived from Streptomyces bacteria. Macrolides act as bacterial **protein synthesis inhibitors**. Bind to the bacterial ribosome, preventing addition of amino acids to polypeptide chains. Examples, erythromycin, azithromycin.

Aminoglycosides :Derived from various species of Streptomyces. Example, streptomycin, which was greatly used against Mycobacterium tuberculosis. Aminoglycosides act as bacterial protein synthesis inhibitors. other examples include: gentamicin, kanamycin, etc.





Antibiotic covalently linked to an antibody that binds to components of the *S. aureus* cell wall. This prodrug coats the bacterial cell surface but remains inactive until the bacteria enter the host cell. There, protease enzymes cleave the linker region, releasing the active antibiotic, which then kills the bacteria.

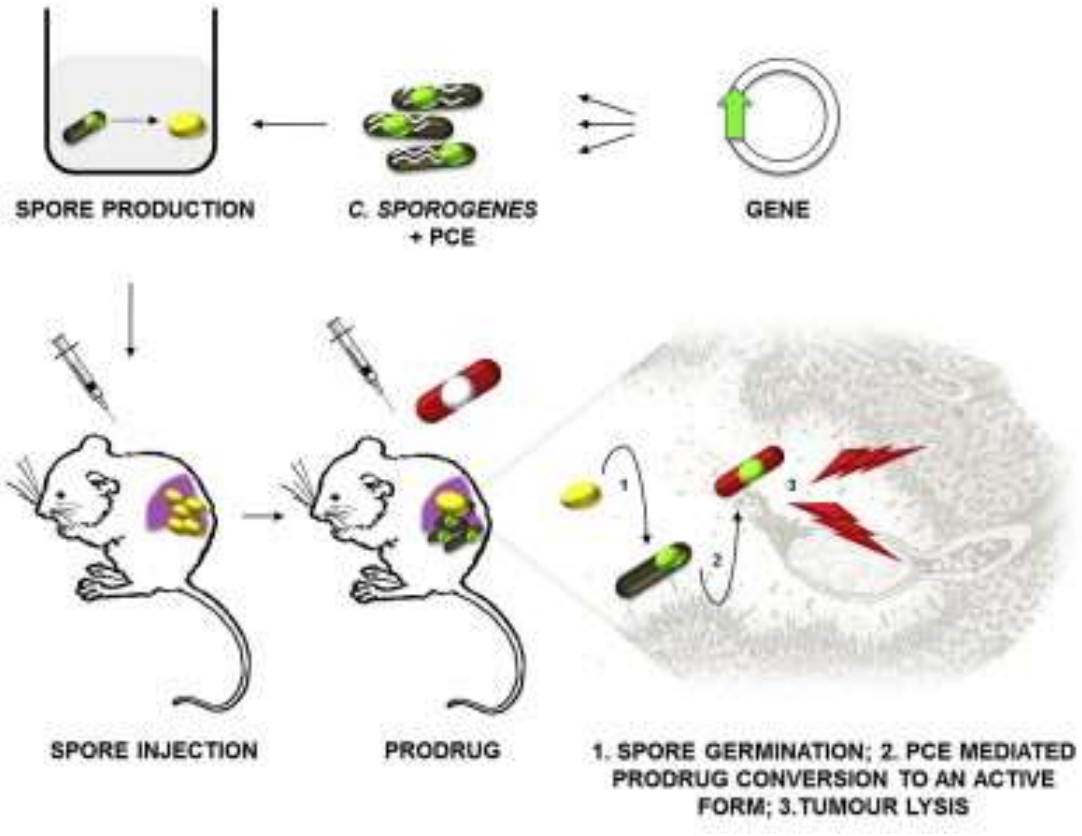
The **clostridia** produce more protein **toxins** than any other bacterial genus and are a rich reservoir of **toxins** for research and medicinal uses. Research is underway to use **clostridial toxins** or **toxin** domains for drug delivery, prevention of food poisoning, and the treatment of cancer and other diseases

General characteristics:

- **anaerobic, gram-positive spore-forming rods**
- **ubiquitous in soil, water and sewage, and some are found in gi tract of humans and animals**
- **many are harmless saprophytes while some are human pathogens**
- **ability to produce disease is due to their ability to**
  - **i. Survive adverse environments (due to spore formation)**
  - **ii. Grow rapidly in enriched, oxygen-deprived environments**
  - **iii. Produce numerous histolytic toxins, enterotoxins and neurotoxins (depending on strains)**

The presence of tissue with significantly reduced levels of oxygen and necrosis are unique to solid tumours. This can be exploited as a niche environment for cancer therapies. Certain novel strategies involve the use of hypoxia-activated prodrugs or development of HIF-1 inhibitors. A particularly promising option is the use of anaerobic bacteria. An oxygen-free environment creates ideal conditions for certain microbes, such as *Clostridium* spp., to colonise and target a necrotic/hypoxic growth. Such a therapy could potentially be used to treat relatively small tumours, preventing further development of hypoxic cancer tissue and the subsequent metastatic progression.

Following administration of *Clostridium* spores to tumour-bearing organisms, these spores can only germinate within the hypoxic/necrotic regions of solid tumours, proving their exquisite selectivity. Recombinant clostridia producing prodrug-converting enzymes or cytokines resulted in the production of such proteins solely within the tumour, and where applicable, could convert the prodrug in a toxic compound

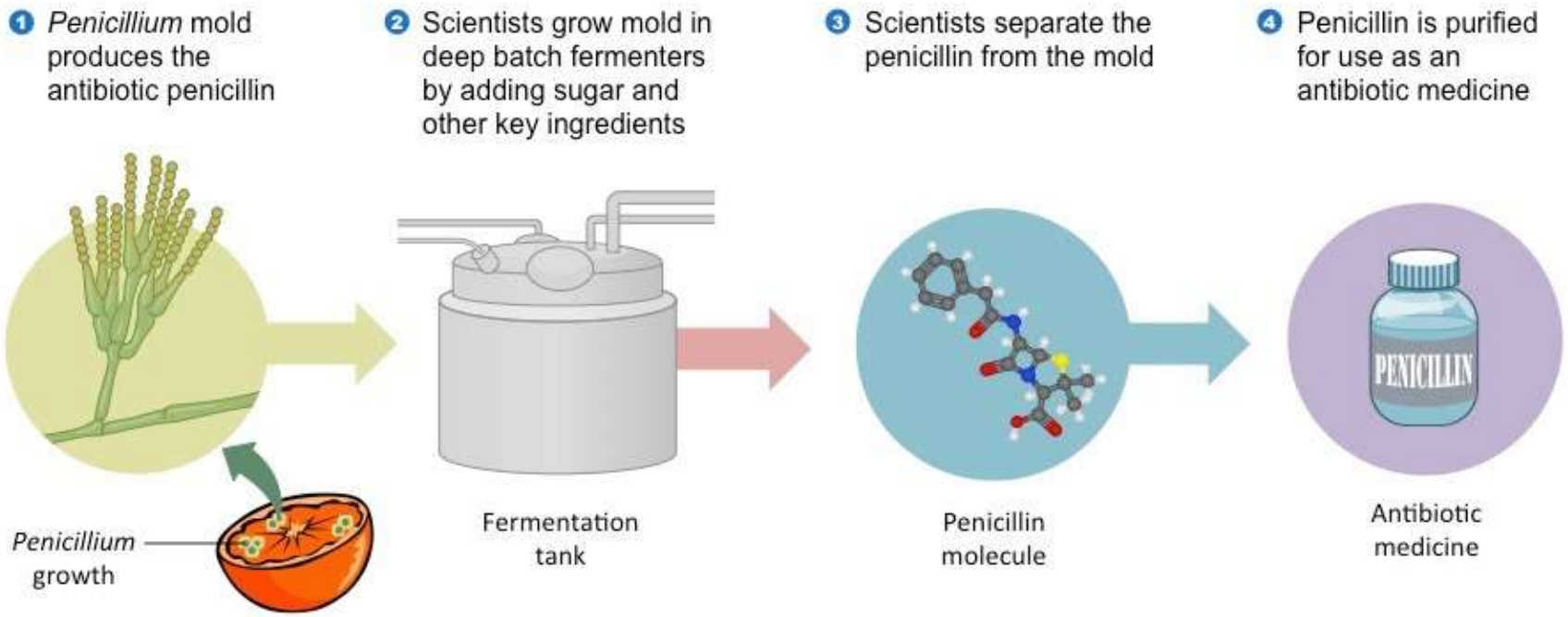


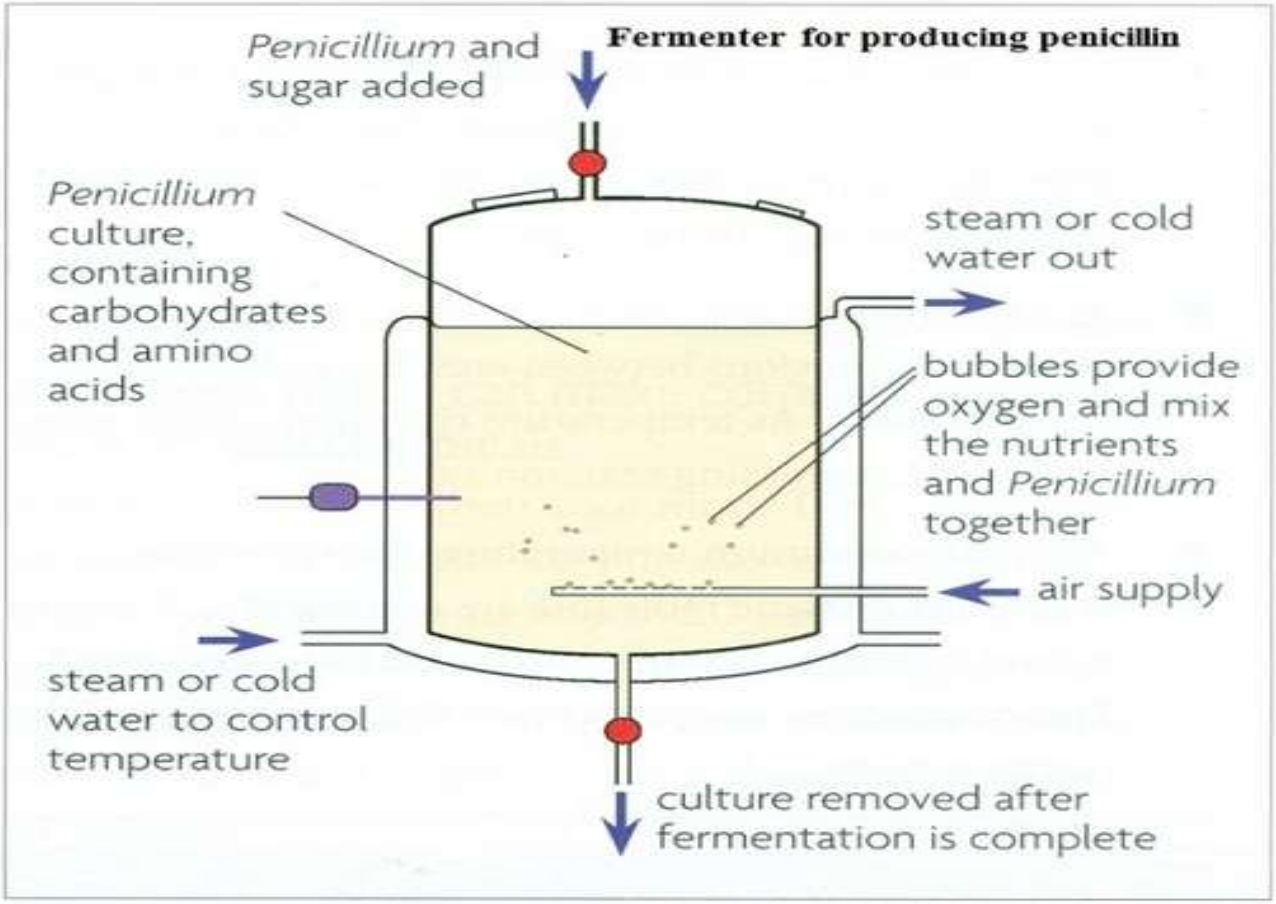
prodrug convertin g enzymes (**PCEs)**

Antibiotic Production: Antibiotics are generally produced in stainless steel fermenters used in the batch or fed-batch mode. A high yielding strain is a prerequisite for antibiotic production The wide spread method of increasing product yield is still random mutation by treatment with mutagenic agents .

Antibiotic Production :Since antibiotics are secondary metabolites, the production medium is so designed that ***a key nutrient becomes limiting at a critical stage to initiate the secondary metabolism in the organism***.The nutrient to be made limiting depends on the process and the antibiotic produced e.g., ***glucose for penicillin production and phosphate for several antibiotics produced by Streptomyces***

Production of Penicillin via Batch Fermentation





For first **15-24 h**, the fungus just grows. After that it begins to secret **penicillin**. Rate of production depends on how much sugar is available:
A lot of sugar à not much penicillin
No sugar à no penicillin
So **small amount** of sugar have to be fed all the time that the fungus is producing penicillin.

The culture is kept going until the **rate** of production is so **slow** that is not worth waiting more (often after a week). Then it is **filtered**, and the liquid is treated to **concentrate** the **penicillin** in it.

- Production of citric acid in continuous fermenters by *Aspergillus niger* for use as a preservative or flavouring

Citric acid (citrate) is widely used as a flavour enhancer, a preservative in manufactured foods and an antioxidant

- It is produced as an intermediate of the Krebs cycle under aerobic conditions

Citric acid is mass produced by continuous fermenter systems from cultures of the fungus *Aspergillus niger*

- Carbohydrates are continuously introduced into the fermenter in order to maintain the citric acid production
- Iron (Fe^{2+} ions) is excluded from the mixture in order to slow the further conversion of citric acid within the Krebs cycle
- As citric acid accumulates it is extracted as part of the medium that is being continuously withdrawn from the fermenter

Production of Citric Acid via Continuous Fermentation

The exploitation of microorganisms to **produce** food ingredients has been going on since antiquity. Microorganisms are being employed, since several decades for the large scale production of a variety of bio-chemicals ranging from alcohol to antibiotics and in processing of foods and feeds. Microorganisms have great potential as natural sources of **drugs** for the treatment and prevention of diseases like cancer, anaemia, diarrhoea, obesity, diabetes, atopic dermatitis, Crohn's disease, et

Bacteria as source of antimicrobial proteins (bacteriocins as biopreservatives)

Microbes as source of antitumor drugs

Microbes as enzyme inhibitors

Biological activities of microbial-derived natural products and biologics.

Name	Origin	Biological activity	References
Antibiotic			
Erythromycin A (1)	*Saccharopolyspora erythraea*	Antibacterial	McGuire et al., 1952; Zhang et al., 2010; Cobb et al., 2013
Tetracycline (2)	*Streptomyces rimosus*	Antibacterial	Chopra and Roberts, 2001; Demain, 2009
Vancomycin (3)	*Amycolatopsis orientalis*	Antibacterial	Geraci et al., 1956; Dasgupta, 2012
Streptomycin (4)	*Streptomyces griseus*	Antibacterial	Schatz et al., 1944; Waksman et al., 1946
Nisin A (5)	*Lactococcus lactis*	Antimicrobial	Li and Vederas, 2009; Gyawali and Ibrahim, 2014
Reuterin (6)	*Lactobacillus reuteri*	Antimicrobial	Talarico et al., 1988; Gyawali and Ibrahim, 2014

Antifungal Agents			
Amphotericin B (7)	*Streptomyces nodosus*	Antifungal	Abu-Salah, 1996; Tevyashova et al., 2013
Ieodoglucomide C (8)	*Bacillus licheniformis*	Antifungal	Tareq et al., 2015
Anticancer and Antitumor			
Bleomycin (9)	*Streptoalloteichus hindustanus*, *Streptomyces verticillus*	Squamous cell carcinomas, Hodgkin's lymphomas and testis tumors	Einhorn and Donohue, 2002; Demain and Vaishnav, 2011
Ddaunorubicin (10)	*Streptomyces peucetius* and various related strains	Acute lymphoblastic or myeloblastic lymphoma	Di Marco et al., 1981; Giddings and Newman, 2013
Immunosuppressant/Anti-inflammatory Agents			
Rapamycin (11)	*Streptomyces rapamycinicus* (formerly, *Strept omyces hygroscopicus* ATCC 29253), *Streptomyces iranensis*, and *Actinoplanes* sp. N902-109	Immunosuppressive, antifungal, antitumor, neuroprotective, neuroregenerative, and lifespan extension activities, growth inhibitory activity against several fungi	Vezina et al., 1975; Mann, 2001; Law, 2005; Pan et al., 2008; Anisimov et al., 2011; Song et al., 2015; Yoo et al., 2017
FK506 (12)	*Streptomyces tsukubaensis* and several *Streptomyces* species	Immunosuppressive, antifungal, anti-inflammatory, neuroprotective and neuroregenerative activities, rheumatoid arthritis treatment	Tanaka et al., 1987; Mann, 2001; Migita and Eguchi, 2003; Demain, 2014; Ban et al., 2016; Yoo et al., 201

Biofilm-Inhibitory Agents			
Cahuitamycins (13)	*Streptomyces gandocaensis*	Inhibitors of *Acinetobacter baumannii* biofilms	Park et al., 2016
Others			
Avermectins (14)	*Streptomyces avermitilis*	Onchocerciasis and lymphatic filariasis	Shen, 2015
Mollemycin A 20 (15)	*Streptomyces* sp. (CMB-M0244)	Gram-positive and Gram-negative bacteria, antimalarial activity	Blunt et al., 2016
Lipstatin (16)	*Streptomyces toxytricini*	Pancreatic lipase inhibitor for obesity and diabetes	

The bacterial and fungal bio-molecules, source, origin and applications

From: Eminence of Microbial Products in Cosmetic Industry

Secondary metabolites	UV and Photo-protective potential, protection from oxidative damage	Mycosporine-like amino acids (MAAs)	*Pseudonocardia* sp. *Actinosynnema mirum* *Streptomyces avermitilis* *Streptomyces lividans* *Corynebacterium glutamicum* *Aurantiochytrium* sp.	Actinomycetes (Actinobacteria)
Pigments	Improves skin, Antioxidant	Astaxanthin	*Paracoccus*, *Agrobacterium aurantiacum*	Bacteria
			Thraustochytrids, *Rhodotorula*, *Phaffia rhodozyma*	Fungi
	UV protectant, antioxidant, skin hydration	Zeaxanthin	*Corynebacterium autotrophicum*	Bacteria
Biosurfactants	Detergent, foaming, emulsifying agent and skin hydrating properties	Viscosin, Rhamnolipids	*Pseudomonas* sp.	Bacteria
		Mannosylerythritol lipid	*Pseudozyma* sp., *Ustilago* sp., *Candida antartica,*	Fungi
		Surfactin	*Bacillus subtilis*, *Bacillus pumilus A*, *B. licheniformis and B. amyloliquefacien*	Bacteria
		Emulsan	*Acinetobacter calcoaceticus*	Bacteria
		Sophorolipid	*Candida* sp.	Fungi

Prior to rDNA technology Our source of therapeutic proteins are limited, very difficult to isolate, purify and make it maketable

rDNAtechnology brings promises to fight against diseases

Epidermal growth factor	burn
Factor VII	Hemophillia
Factor IX	Hemophillia
Growth Hormones	Growth Defects
Serumj Albumin	Insufficient plasma proteins
Tissue plasminogen Activator	Blood clot
Urokinase	Blood clot
Il-2	renal cell carcinoma
Insulin	Diabetics

Recombinant DNA Technology

- Revolutionized biology
- Manipulation of DNA sequences and the construction of chimeric molecules, provides a means of studying how a specific segment of DNA works
- Studies in bacteria and bacterial viruses have led to methods to manipulate and recombine DNA
- Once properly identified, the recombinant DNA molecules can be used in various ways useful in medicine and human biology

Interferon is secreted by cells in response to **stimulation** by a virus or other foreign substance, but it does not directly inhibit the virus's multiplication. Rather, it **stimulates** the infected cells and those nearby to **produce** proteins that prevent the virus from replicating within them.

INTERFERONS USED FOR HCV INFECTION

PRODUCTION OF INTERFERONS

- **Interferon** was named for its ability to interfere with viral proliferation.
- Interferon's are virus-induced proteins produced by virus-infected cells.
- Interferon are antiviral in action and act as first line of defense against viruses causing serious infections, including breast cancer and lymph nodes malignancy.
- Natural interferon is produced in very small quality from human blood cells.
- It is very costly.
- It is now possible to produce interferon by recombinant DNA technology at much cheaper rate.

Techniques

Target protein is isolated ➞ aa sequences ➞ DNA coding sequence ➞ c-DNA library probing

Or

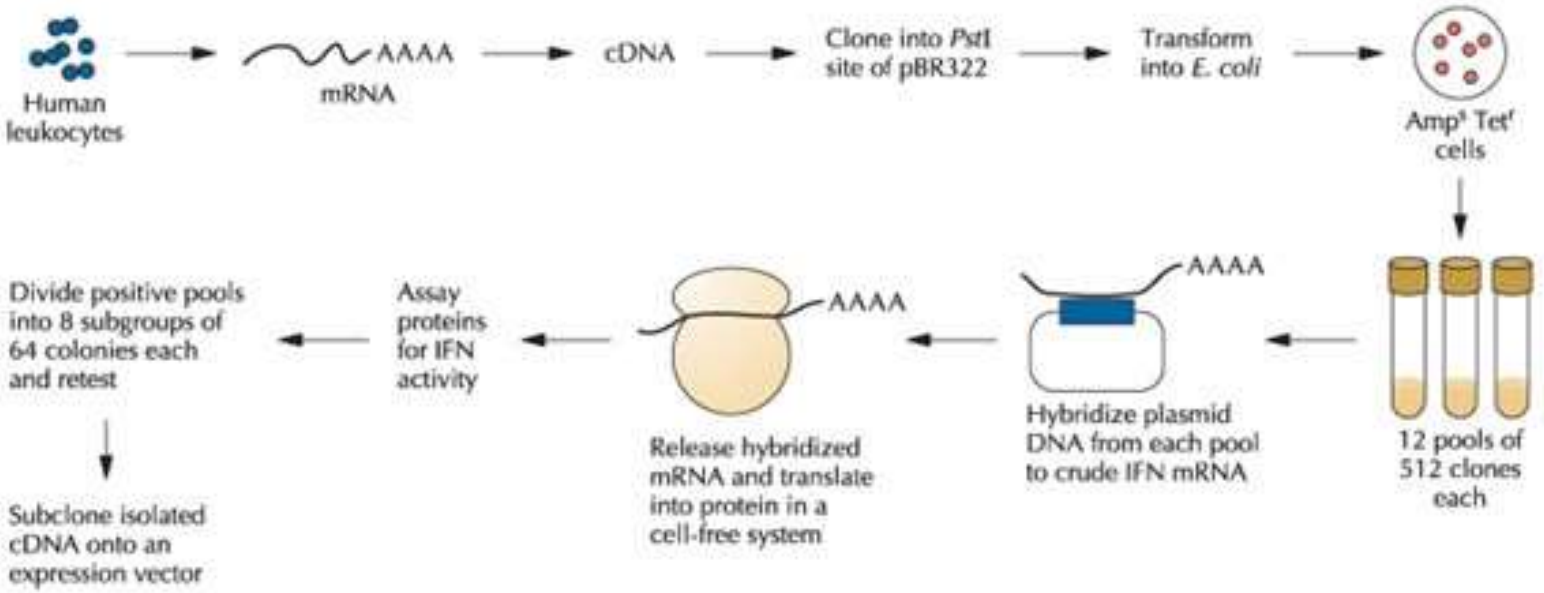
Antibody develop against purified proteins ➞ probe gene expression library

For human proteins ➞ specific tissue cells are enriched with the proteins eg. pancrease (insulin) ➞ mRNA ➞ Protein

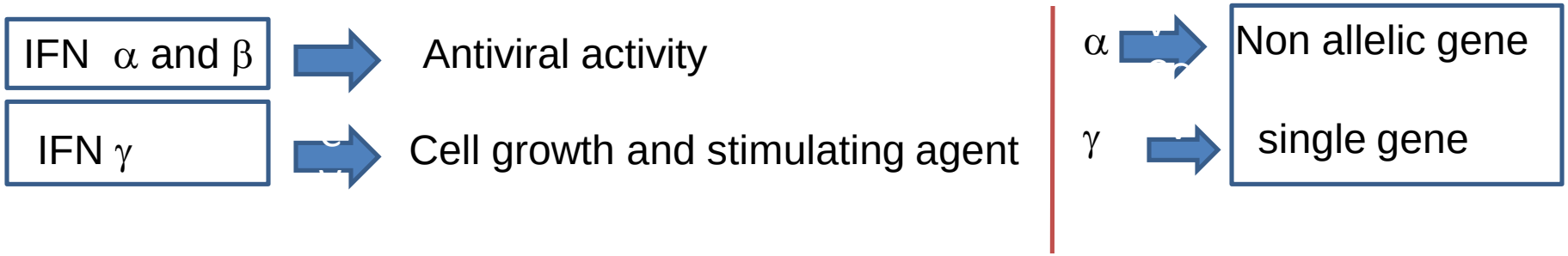
But simple c-DNA enrichment procedure may not be work well for human proteins

Isolation of IFN

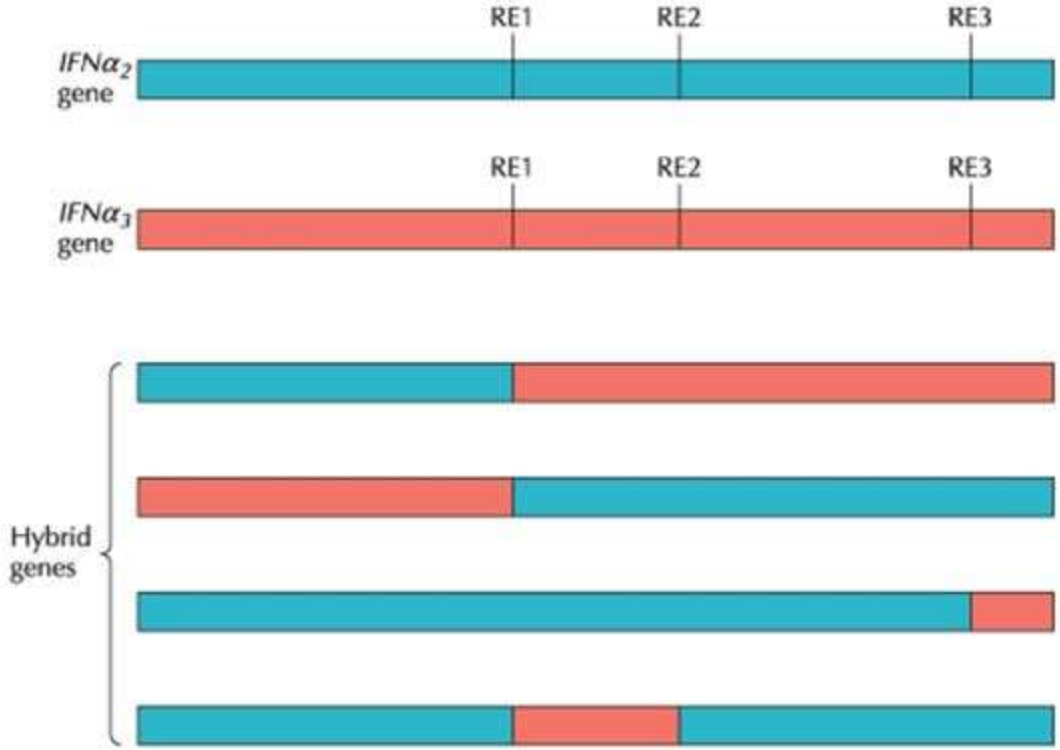
- Size fractionated mRNA fromj human leukocytes
- RT; inserted into pst1 site of plasmid PRB322
- 600 clones grouping
- Hybridize with crude interferon mRNA
- Invitro translation
- Assayed for antiviral activity
- Repeated cloning ➞ complete cDNA for human IFN identified


Human leukocytes
AAAA
mRNA
cDNA
Clone into PstI site of pBR322
Transform into E. coli
Amp^s Tet^r cells
12 pools of 512 clones each
Hybridize plasmid DNA from each pool to crude IFN mRNA
AAAA
Release hybridized mRNA and translate into protein in a cell-free system
AAAA
Assay proteins for IFN activity
Divide positive pools into 8 subgroups of 64 colonies each and retest
Subclone isolated cDNA onto an expression vector

Hybrid interferon



Hybrid products: INF



•Interferons assist the immune response by **inhibiting viral replication** within host cells, activating natural killer cells, increasing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infection

•IFN cDNA isolated early 80s

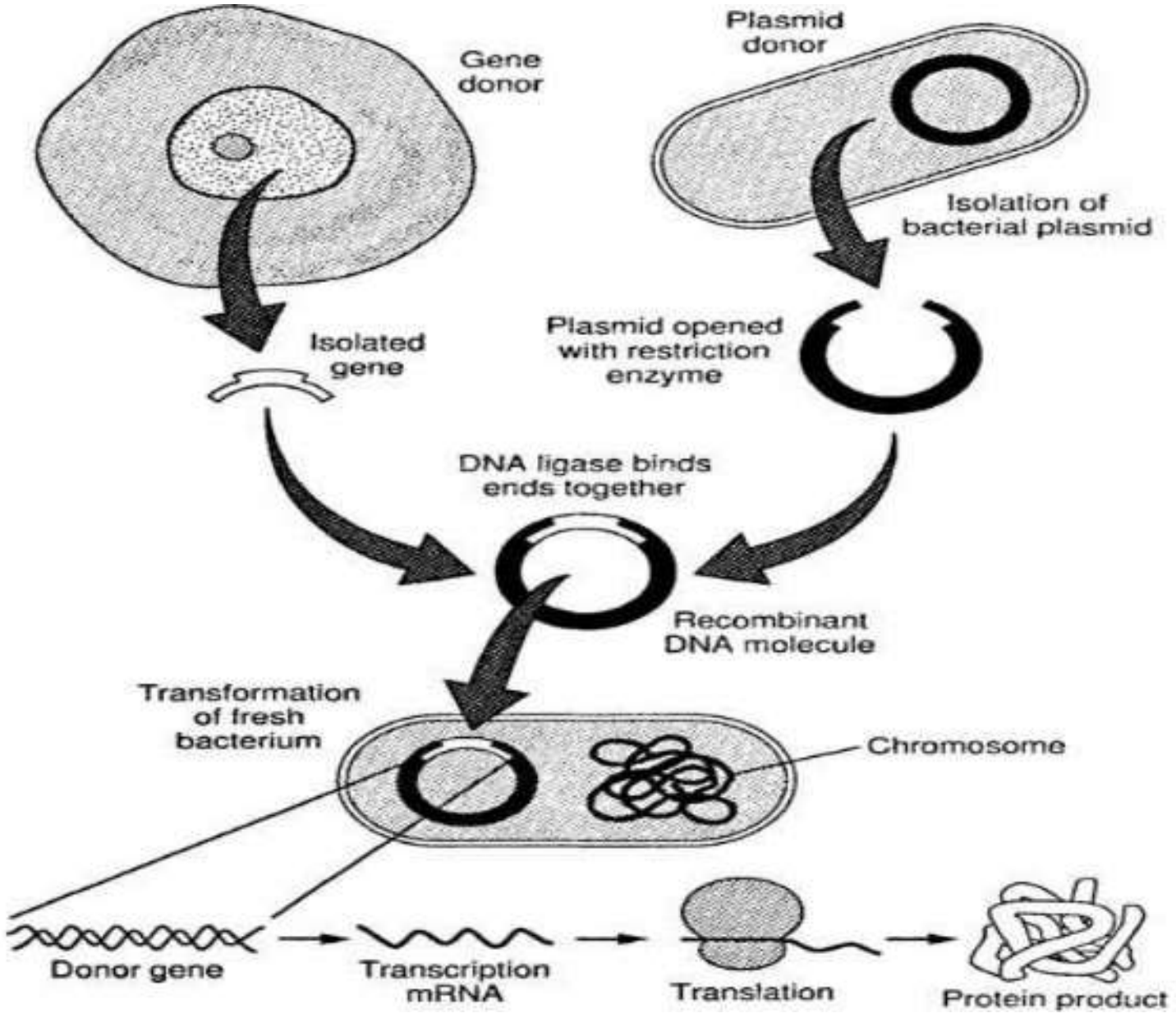
•Now, three groups of IFN genes identified: α, β, γ

- •IFNα family of 13 genes; IFNβ family of 2 genes; IFNγ of 1 genes

•IFN α1 and α2 have common RE sites

•Hybrid INFs demonstrate potential therapeutics by combining functional domains

•Some (2003)- successful clinical trials, approved for use as human therapeutic agents


Gene donor
Plasmid donor
Isolation of bacterial plasmid
Isolated gene
Plasmid opened with restriction enzyme
DNA ligase binds ends together
Recombinant DNA molecule
Transformation of fresh bacterium
Chromosome
Donor gene
Transcription mRNA
Translation
Protein product

Human Growth Hormones

- **Somatostatin and Somatotrophin are two proteins that act in conjunction to control growth processes in the human body, their malfunction leading to painful and disabling disorders such as Acromegaly (uncontrolled bone growth) and Dwarfism.**
- **Somatostatin was the first human protein to be synthesized in *E. coli. Being a very* short protein, only 14 amino acids in length, it was ideally suited for artificial gene synthesis.**

Engineering human growth hormones

Designing protein → **Functional domain shuffling**

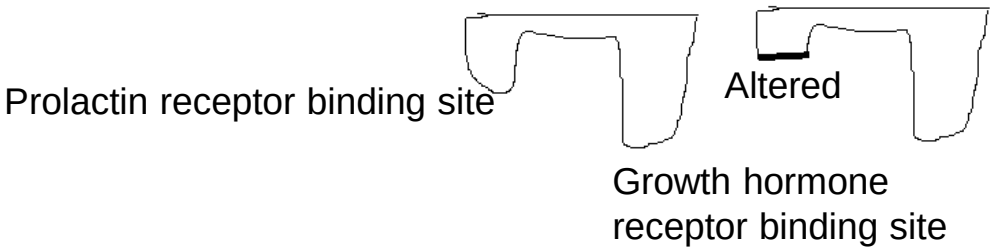
Designing protein → **Directed mutagenesis to augment or constrain mode of action**

human growth hormones → Growth hormones receptor

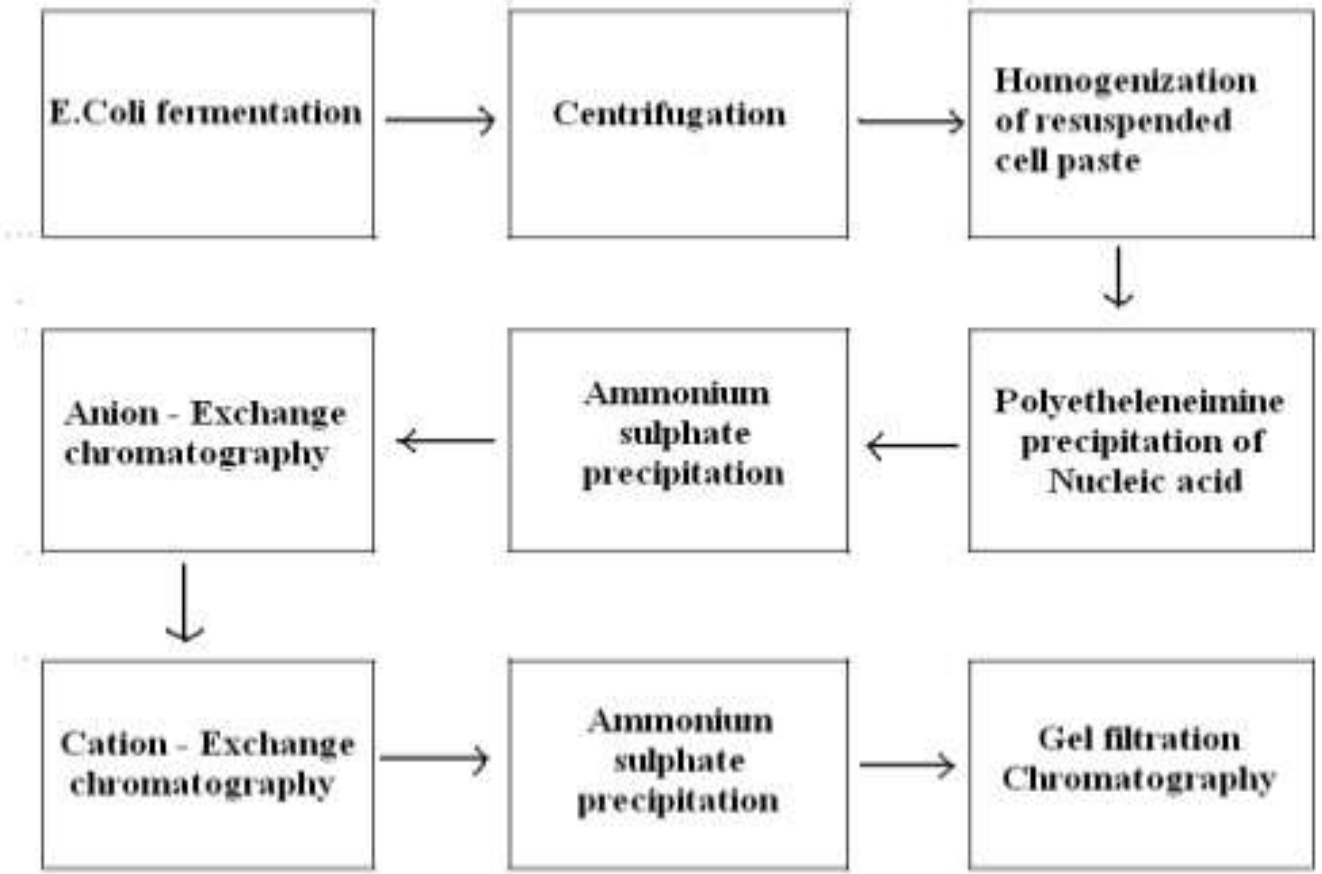
human growth hormones → Prolactin receptor

For therapy it needs to bind with only growth hormone receptor to avoid unwanted side effects; both growth hormone and prolactin receptor binding side of HCH overlap but not identical

So site specific mutagenesis of cloned HCG cDNA is used to change some a that acts asa ligand to Zn ++ ; his 18, his 21 and glu 174 an ion required for the high affinity binding of hGH to prolactin receptor



RECOVERY OF HUMAN GROWTH HORMONE...



Recombinant Pharmaceuticals

- Human Insulin
- Human Growth Hormone
- Human blood clotting factors
- Vaccines
- Monoclonal Antibodies
- Interferons
- Antibiotics & other secondary metabolites

Human Insulin

- Earliest use of recombinant technology
- Modify E.coli cells to produce insulin; performed by Genentech in 1978
- Prior, bovine and porcine insulin used but induced immunogenic reactions
- Also, there were many purification and contamination hassles.
- To overcome these problems, researchers inserted human insulin genes into a suitable vector (E.coli)

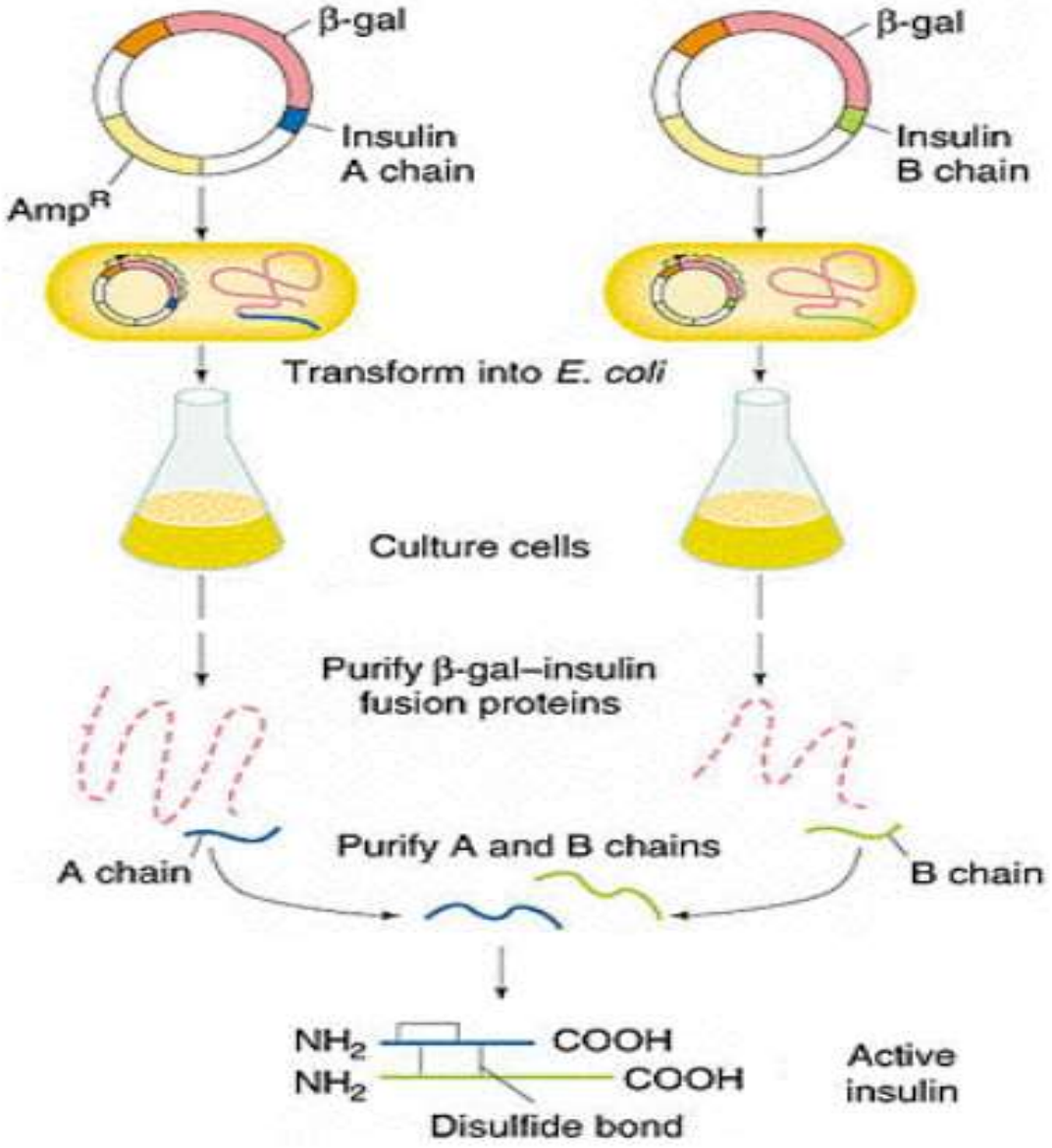
Producing Recombinant Insulin

- First, scientists synthesized genes for the two insulin A & B chains.
- They were then inserted into plasmids along with a strong lacZ promoter.
- The genes were inserted in such a way that the insulin & B-galactosidase residues would be separated by a methionine residue. This is so that the insulin A & B chains can be separated easily by adding cyanogen bromide.

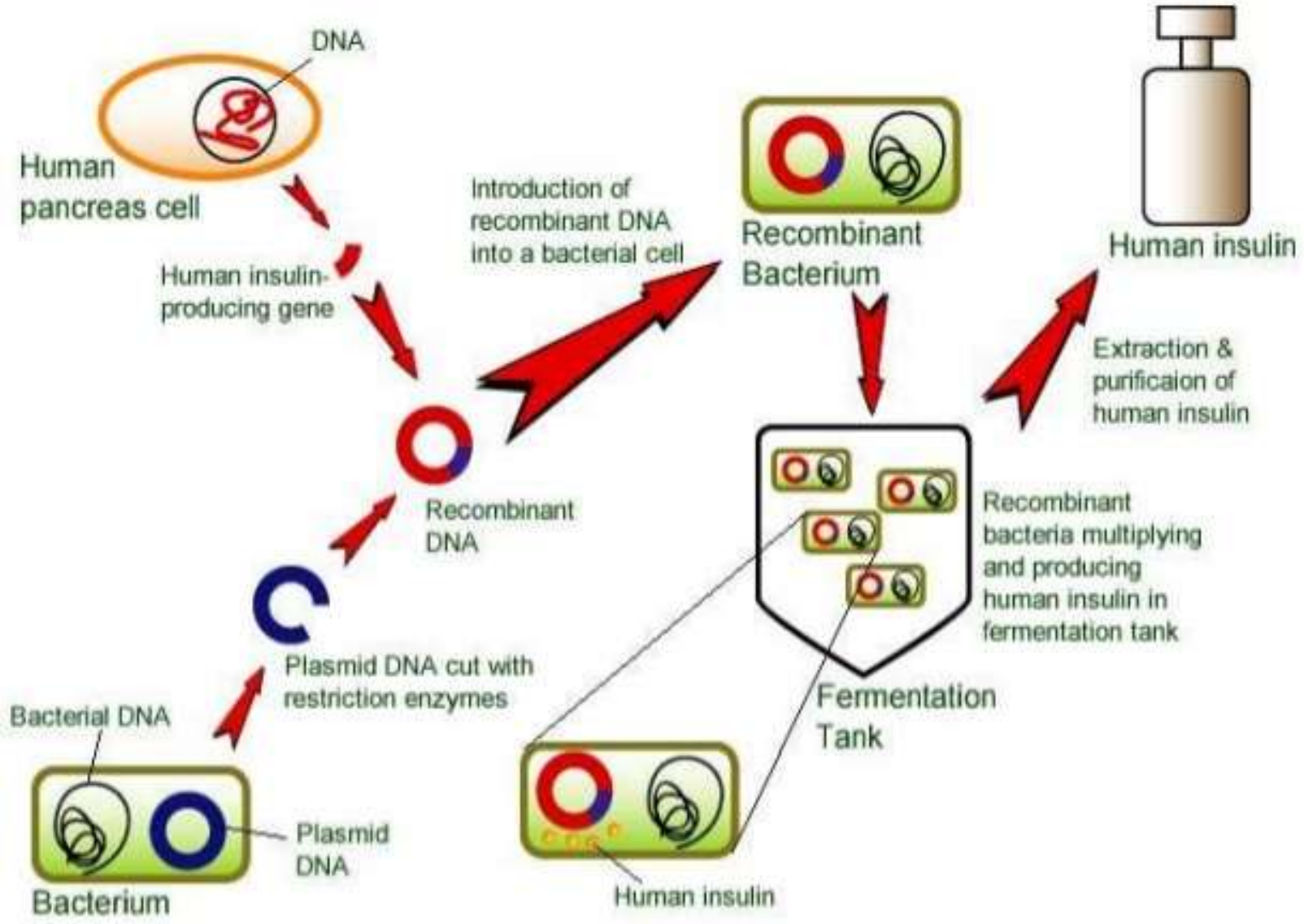
Cyanogen bromide hydrolyzes peptide bonds at the C-terminus of **methionine** residues.

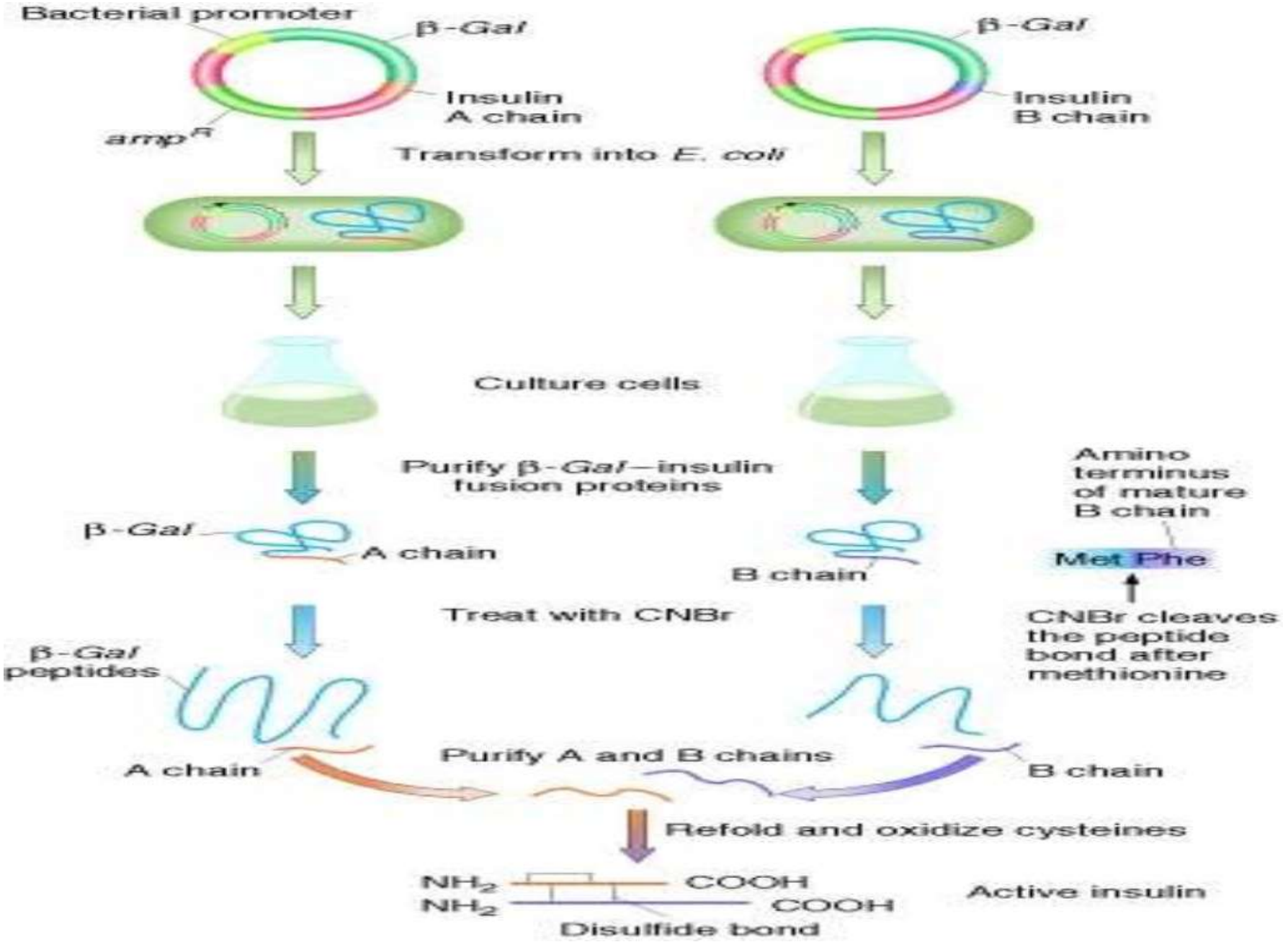
Producing Recombinant Insulin

- The vector was then transformed into E.coli cells.
- Once inside the bacteria, the genes were "switched-on" by the bacteria to translate the code into either the "A" chain or the "B" chain proteins found in insulin
- The purified insulin A and B chains were then attached to each other by disulphide bond formation under laboratory conditions


β-gal
Insulin
A chain
Amp^R
β-gal
Insulin
B chain
Transform into E. coli
Culture cells
Purify β-gal–insulin
fusion proteins
A chain
Purify A and B chains
B chain
NH2
COOH
NH2
COOH
Disulfide bond
Active
insulin

Human Insulin Production

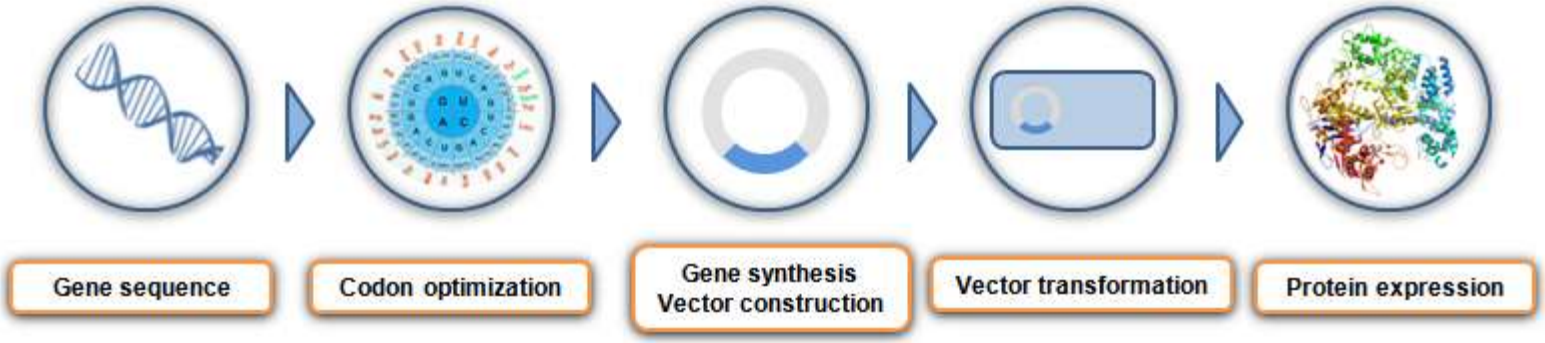



Bacterial promoter
β-Gal
Insulin A chain
amp^R
β-Gal
Insulin B chain
Transform into E. coli
Culture cells
Purify β-Gal—insulin fusion proteins
β-Gal
A chain
B chain
Amino terminus of mature B chain
Met Phe
CNBr cleaves the peptide bond after methionine
Treat with CNBr
β-Gal peptides
A chain
Purify A and B chains
B chain
Refold and oxidize cysteines
NH2
COOH
NH2
COOH
Disulfide bond
Active insulin

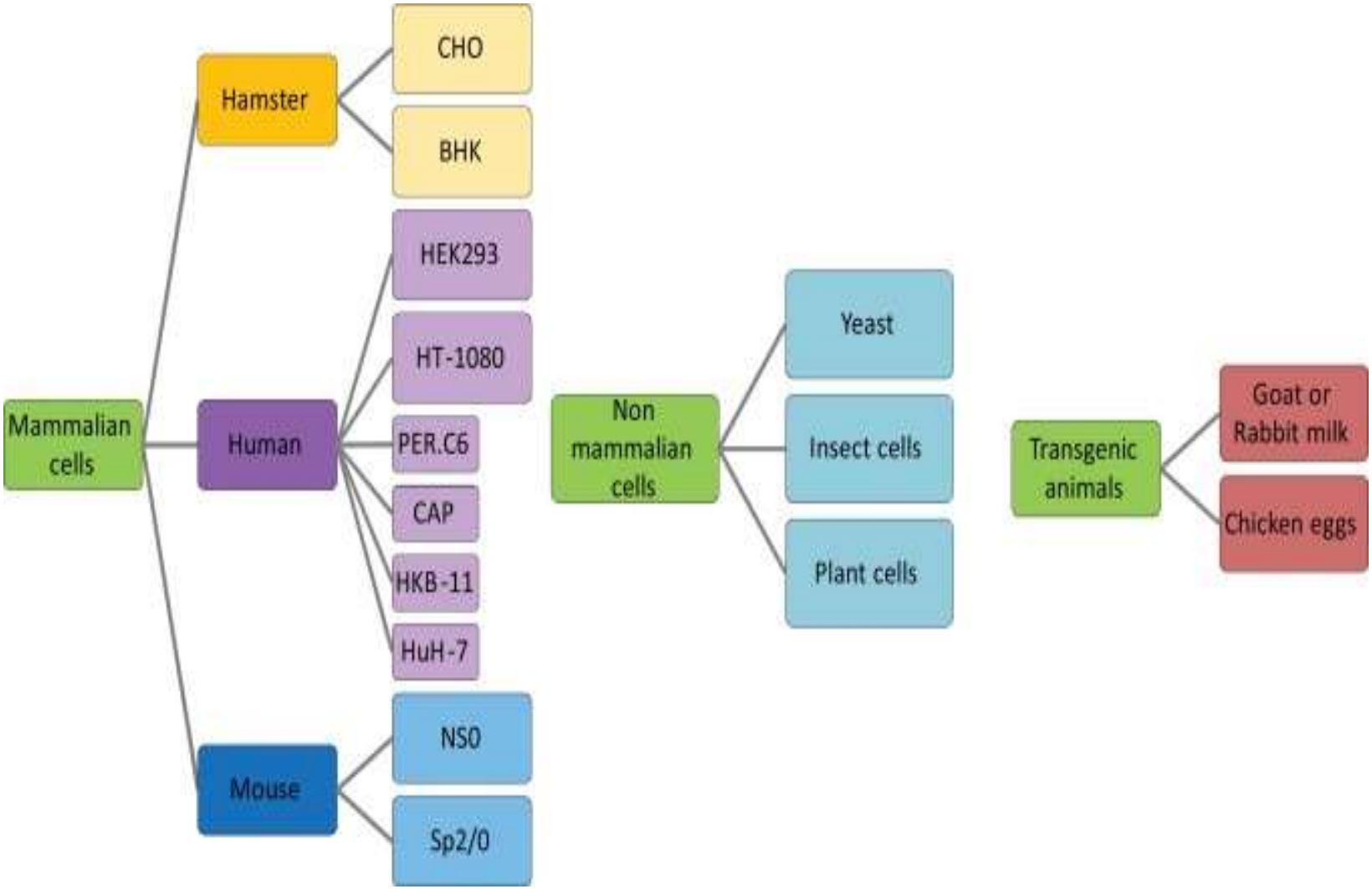
Optimizing gene expression

Authenticated proteins should be synthesized sufficient quantities in either prokaryotic or eukaryotic expression system → preferably prokaryotic low cost And scale up but not all proteins are in functional form, so a comparison is necessary

IL3 → best organism → Bacillus Licheniformis though higher level of expression in E.Coli construct in β Gal , but not acceptable for therapy
Yeast are able to glycosylate the protein (IL3) but expression is low, but glycosylation is not essential for its activity, also glycosylation produces size heterogeneity

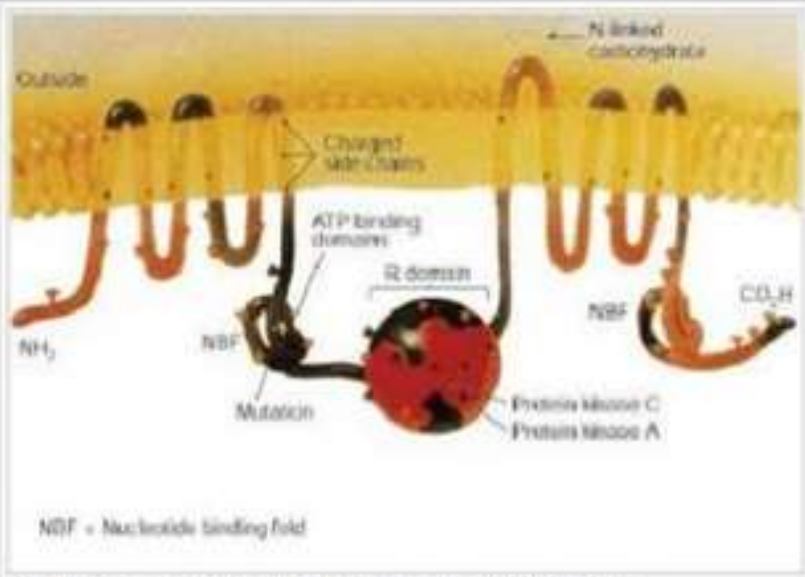


Expression systems used for glycoprotein production by biopharmaceutical industries.



Cystic fibrosis

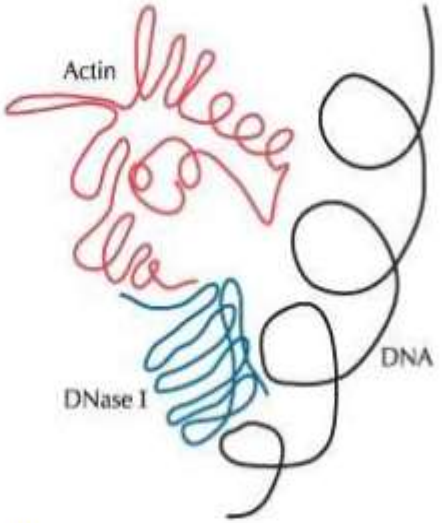
Mutation	Frequency worldwide
ΔF508	66.0%
G542X	2.4%
G551D	1.6%
N1303K	1.3%
W1282X	1.2%



CFTR protein - Molecular structure of the CFTR protein

- Genetic disease affecting lungs and digestive system
- Average life span 37 years, extended and extending
- In US, ~1/3,900; 1/22 are carriers
- Most common in Europeans and Ashkenazi Jews
- Cystic fibrosis transmembrane conductance regulator (CFTR)
- Chloride ion channel, sweat, digestive juices and mucus
- thick, sticky mucus to build up in the lungs and digestive tract
- 7q31.2 -> 180,000 bp gene, 1,480 AAc
- Most common mutation DF508; 1,400 other mutations
- DF508: missense, not folded correctly
- Lungs susceptible to bacterial infection
- Antibiotics treatment results in resistance and combination with DNA from bacteria and leukocytes causes pulmonary problems (mucus)

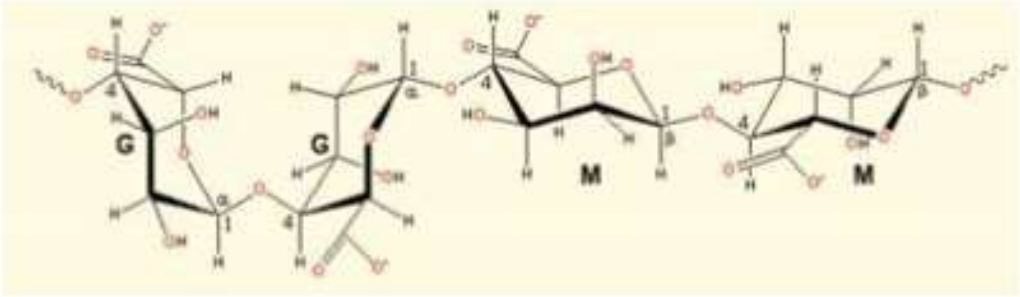
Treatment



- Genentech: hDNase I in CHO cells
- Not a cure, but alleviates symptoms
- Purified protein delivered via aerosol mist to lungs of CF^-
- Approved by FDA in 1994

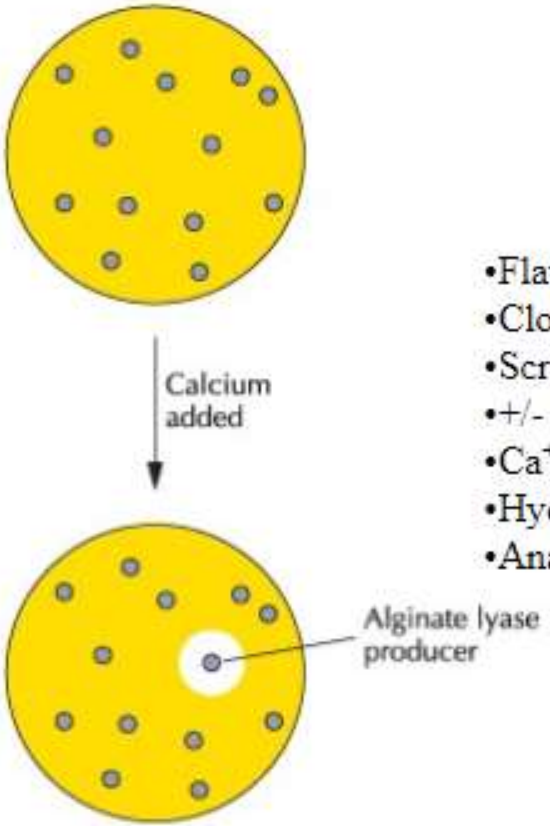
- Another symptom,
- In response to bacteria in lungs,
  leukocytes cluster and lyse bacteria (and leukocytes)
  - Lysed leukocytes release actin
  - Monomeric actin binds DNase I very tightly and inhibits
  - Limits effectiveness
- X-ray structure data suggested Ala-144 required for binding or Tyr-65
- Changing either to Arg decreases actin binding by 10,000x
- Clinical efficacy of mutants to be determined (2003)

Clearing the lungs 2 with alginate lyase



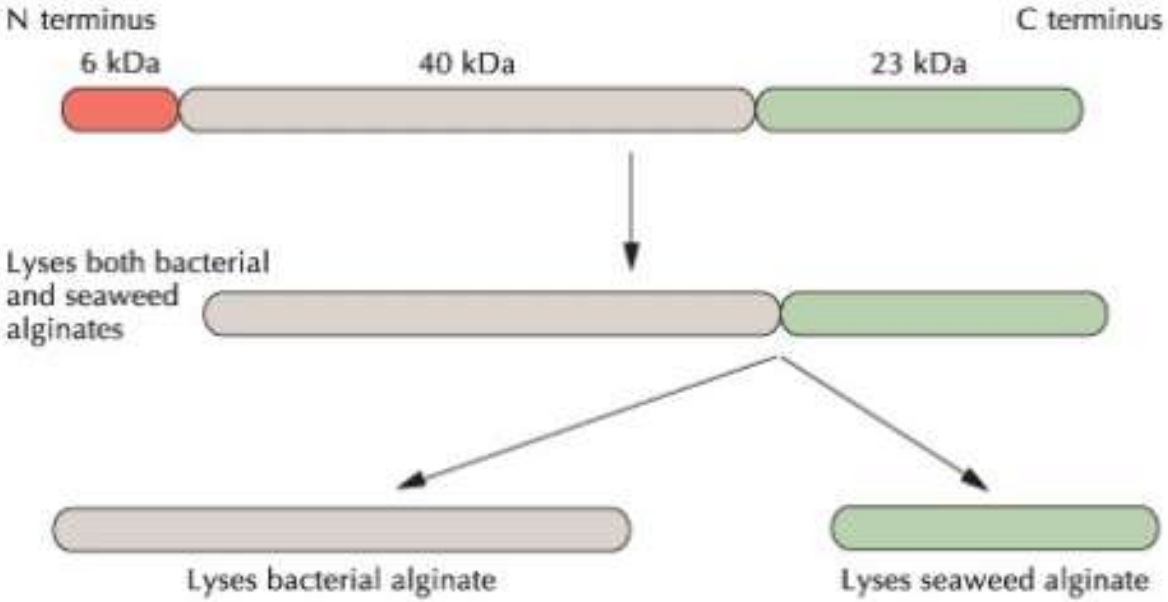
- Alginate produced by seaweeds, soil and marine bacteria
- P. aeruginosa excretion in lungs contributes to viscosity of mucus
- In addition to DNase I treatment, alginate lysate can be used as therapeutic agent

Cloning alginate lyase



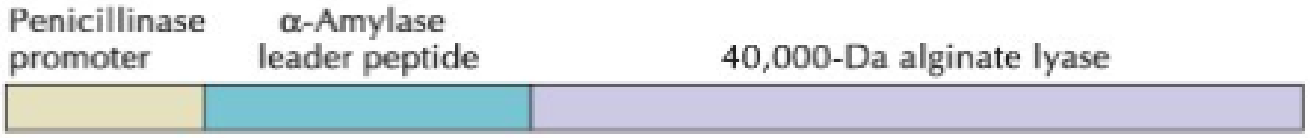
- Flavobacterium sp.
- Clone bank in E. coli
- Screen by plating onto medium plus alginate
- +/- Ca^{++}
- Ca^{++} + alginate = cross-linked opaque
- Hydrolyzed alginate does not cross-link
- Analysis and characterization of clones and alginate lyase

Alginate lyase[s]



- ORF 69,000 Da
- Precursor of three alginate lyases
- -> 3,000 Da + 63,000 Da
- 63,000 Da lyses both bacterial and seaweed alginates
- 63,000 Da -> 23,000 Da seaweed effective+ 40,000 Da bacterial effective
- Clone bacterial activity portion

Optimization of activity



- Increase expression of 40,000 Da protein
- PCR amplify and insertion behind strong promoter
- B. subtilis plasmid, fused to a B. subtilis a-amylase leader peptide, directs secretion and penicillinase gene promoter
- Expressed and assayed for halo phenotype
- Liquifies alginates produced by P. aeruginosa isolated from lungs of CF patients
- 2003, additional trials to determine if effective therapeutic agent

Recombinant enzymes

Cystic fibrosis — fatal hereditary diseases
recurring lung infection
treatment with antibiotic → antibiotic resistance
presence of bacteria (alive or dead) → accumulation of thick mucus
very difficult to breath

Treatment — DNAse I into the aerosol of lung
reduce the viscosity of mucus
easier to breath

It is not treatment but relieve to the patients

Aliginate lyase ; Long polysaccharide polymer produced by wide range of seaweeds soil and marine bact.
composed of chains of sugar β–D- Mannuronate and α-L- gluronate
form inter and intra chain crosslink in presence of Ca^{++}
Crosslinked aliginate polymer forms elastic gel
Excretion of aliginate by mucoid strain of psedomonas aeroginosa
contribute significantly to the viscosity of the mucus of cystic fibrosis
Thus aliginate lyase can liquify the bacterial aliginate

Aliginate lyase genes

Flavobacterium Sp. A gram negative soil bact.

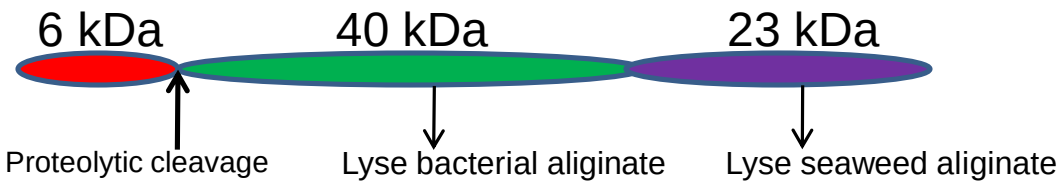
↓

Clone bank in E.coli and tested for aliginate producing clones by plating in medium containing aliginate

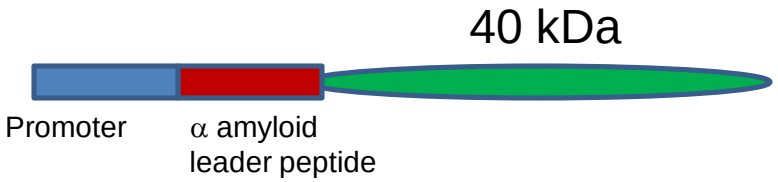
↓

All the colonies which produce aliginate lyase will produce halo when Ca2+ was added to the medium

DNA 69 Kda protein lyse both bacterial and seaweed aliginate



PCR amplification of 40 kDa inserted into bacillus subtilliis fused into B subtilies a amylase to direct secretion under under the transcriptional control of penicilase gene expression



Recombinant Blood Clotting Factors

- **Human factor VIII is a protein that plays a central role in blood clotting.**
- **The commonest form of haemophilia in humans results from an inability to synthesize factor VIII**
- **The factor VIII gene is very large. The mRNA codes for a large polypeptide (2351 amino acids), which undergoes a complex series of post-translational processing events, eventually resulting in a dimeric protein consisting of a large subunit and a small subunit.**

Production of Recombinant Human Blood Clotting Factors

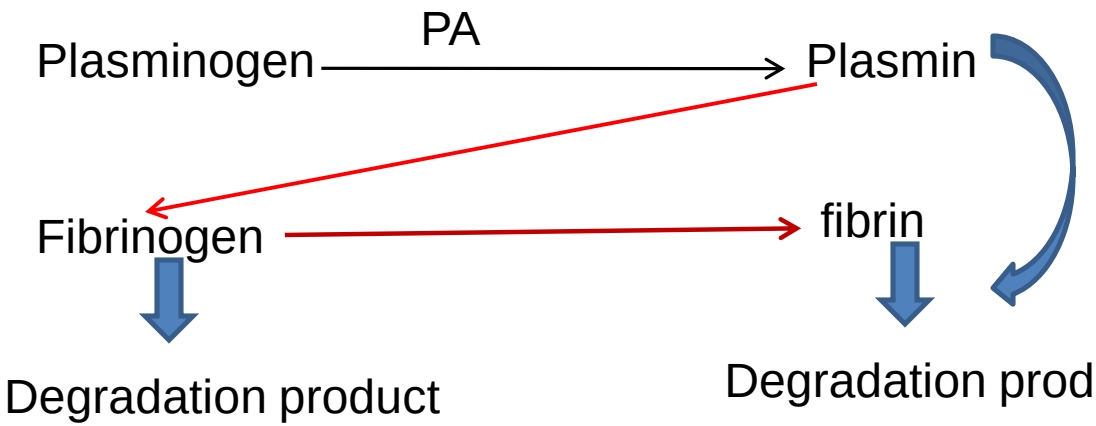
- The two subunits contain a total of 17 disulphide bonds and a number of glycosylated sites. As might be anticipated for such a large and complex protein, it has not been possible to synthesize an active version in *E. coli.*
- Two separate fragments from the cDNA were used. Each cDNA fragment was ligated into an expression vector along with Ag promoter (a hybrid between the chicken b-actin and rabbit b-globin sequences) and a polyadenylation signal from SV40 virus.
- The plasmid was introduced into a hamster cell line and recombinant protein obtained.

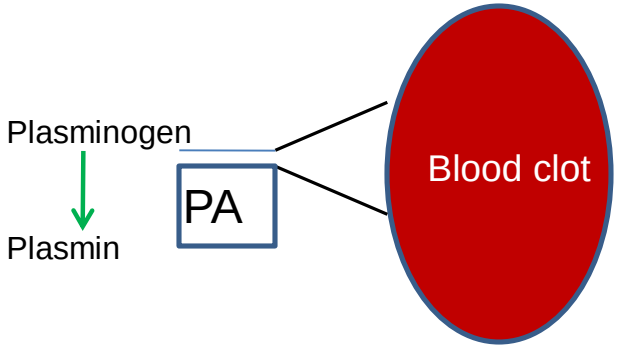
Production of Recombinant Human Blood Clotting Factors

- Alternative method- pharming
- The complete human cDNA has been attached to the promoter for the whey acidic protein gene of pig, leading to synthesis of human factor VIII in pig mammary tissue and subsequent secretion of the protein in the milk.
- The factor VIII produced in this way appears to be exactly the same as the native protein

Blockage of cerebral or coronary artery by blood clot ⟹ Thrombus, a network Of fibrin, a blood clotting agent ➡ defective wall of blood vessel

- Normally, a plasmin, a serine protease, which is produced by plasminogen by plasminogen activator
- Degrade blood clot and make them dissolve
- arterial blockage is occur when this biological system does not work
- So plasmin become an therapeutic agent
- But, Plasmin also degrades fibrinogen, a precursor of fibrin
- So, Plasmin therapy can lead to excessive internal bleeding as a result of depletion of fibrinogen level
- Plasminogen activator coupled with antibody specific for fibrin has developed called tissue plasminogen activator (tPA)
- Antibody has be humanized near future

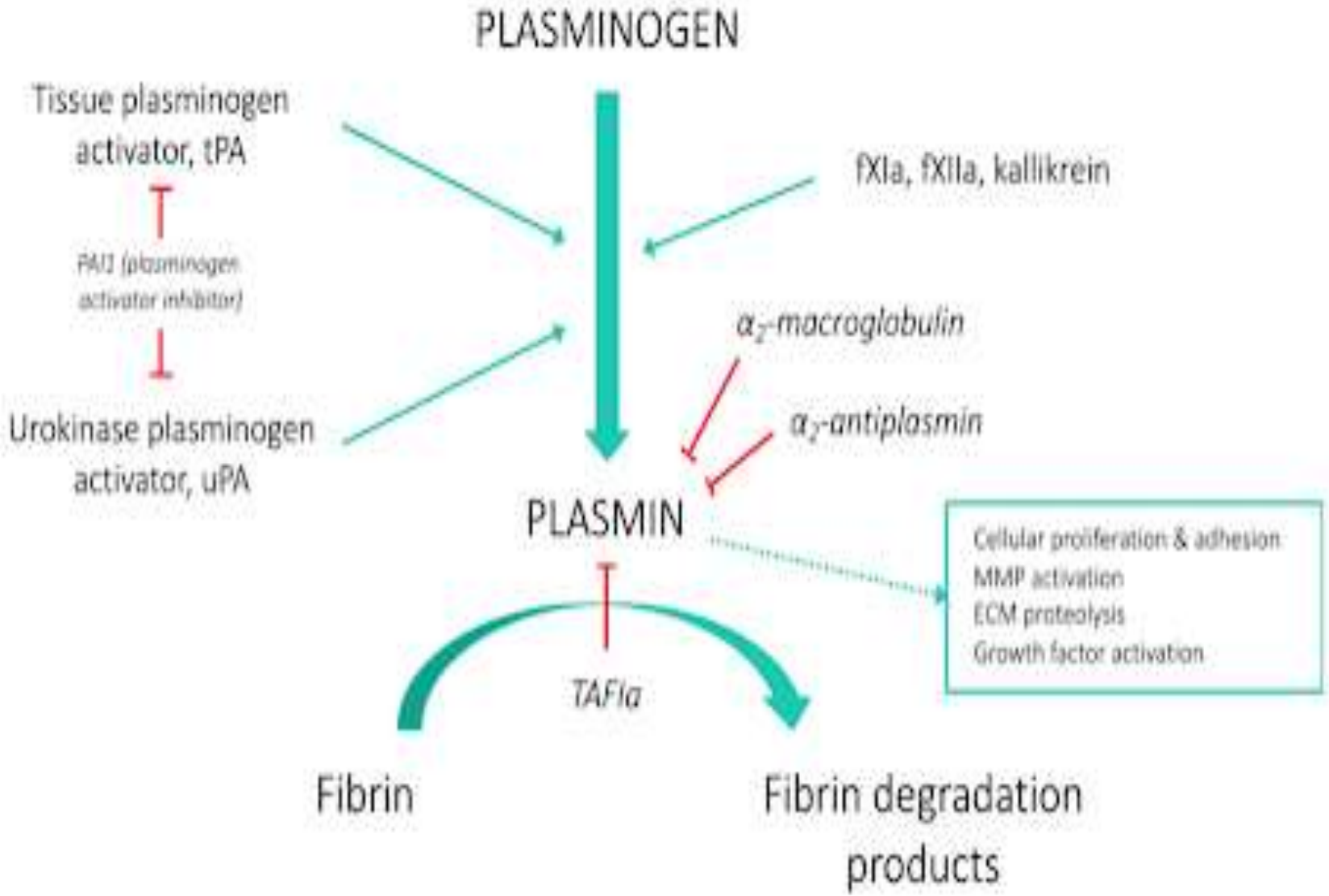




Urokinase is an endogenous activator, which is **produced** by kidney cells and can be recovered from urine. It directly activates plasminogen and is now manufactured with use of recombinant DNA technology.

Urokinase is a physiologic thrombolytic agent that is produced in renal parenchymal cells. Unlike streptokinase, **urokinase** directly cleaves plasminogen to produce plasmin. When it is purified from human urine, approximately 1500 L of urine is needed to yield enough **urokinase** to treat a single patient

Streptokinase, acts with plasminogen to produce an "activator complex" that converts plasminogen to the proteolytic enzyme plasmin.
The **mechanism** by which dissociated **streptokinase** is eliminated is clearance by sites in the liver; however, no metabolites of **streptokinase** have been identified.


PLASMINOGEN
Tissue plasminogen activator, tPA
PAI1 (plasminogen activator inhibitor)
Urokinase plasminogen activator, uPA
fXIa, fXIIa, kallikrein
α2-macroglobulin
α2-antiplasmin
PLASMIN
Cellular proliferation & adhesion
MMP activation
ECM proteolysis
Growth factor activation
TAFIa
Fibrin
Fibrin degradation products

Recombinant Antibodies

- An immunoglobulin which produced because of the introduction of an antigen into the body, and which possesses the ability to recognize the antigen.
- Using recombinant antibody has significant advantages compared with the conventional antibody and there for its use becoming more popular now days.
- The fact that no animals are needed in the manufacturing procedure of the recombinant antibodies, in addition, the manufacturing time is relatively short compared with the conventional method.
- Moreover, the quality of the final product is higher

Production of Recombinant Antibodies

- The production of non-animal recombinant antibodies can be broken down into five steps:

(1) creation of an antibody gene library

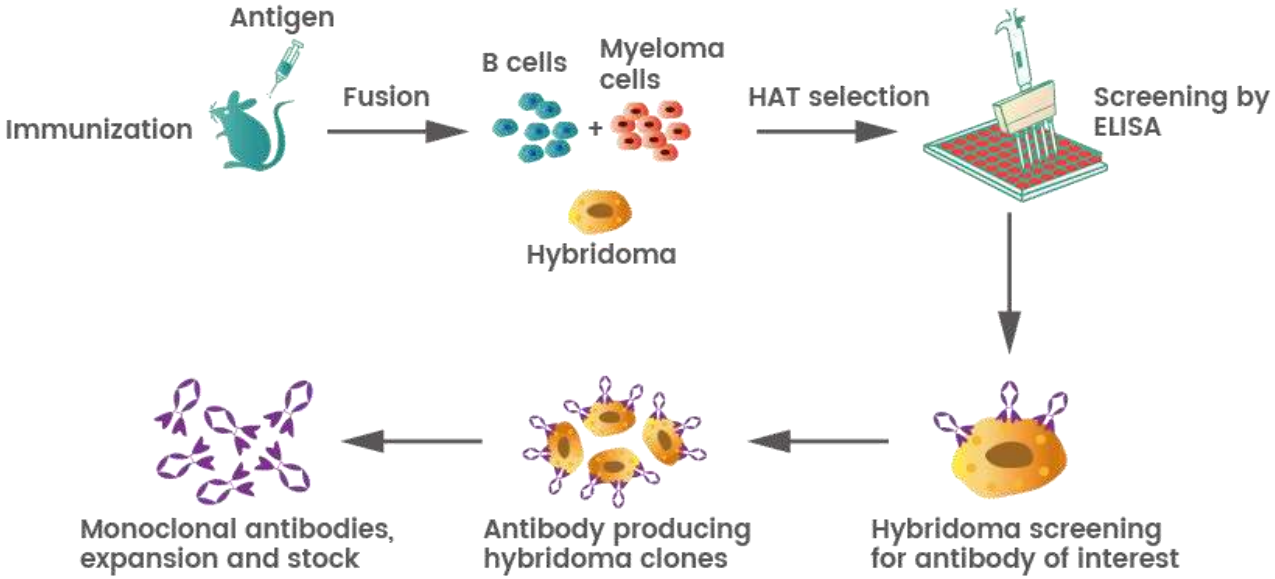
(2) display of the library on phage coats or cell surfaces

(3) isolation of antibodies against an antigen of interest

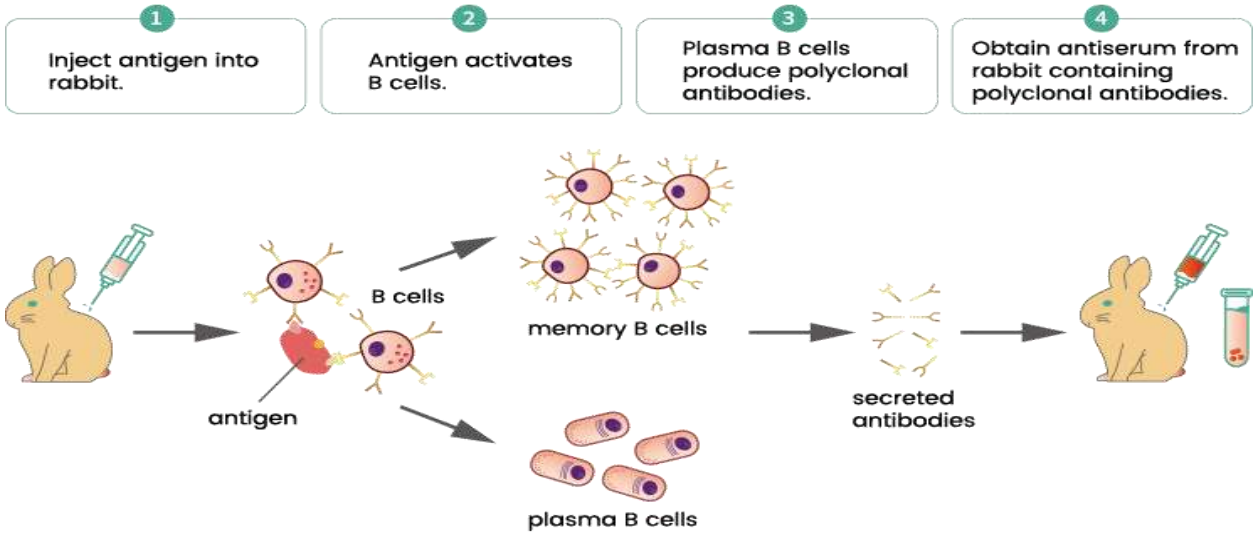
(4) modification of the isolated antibodies and

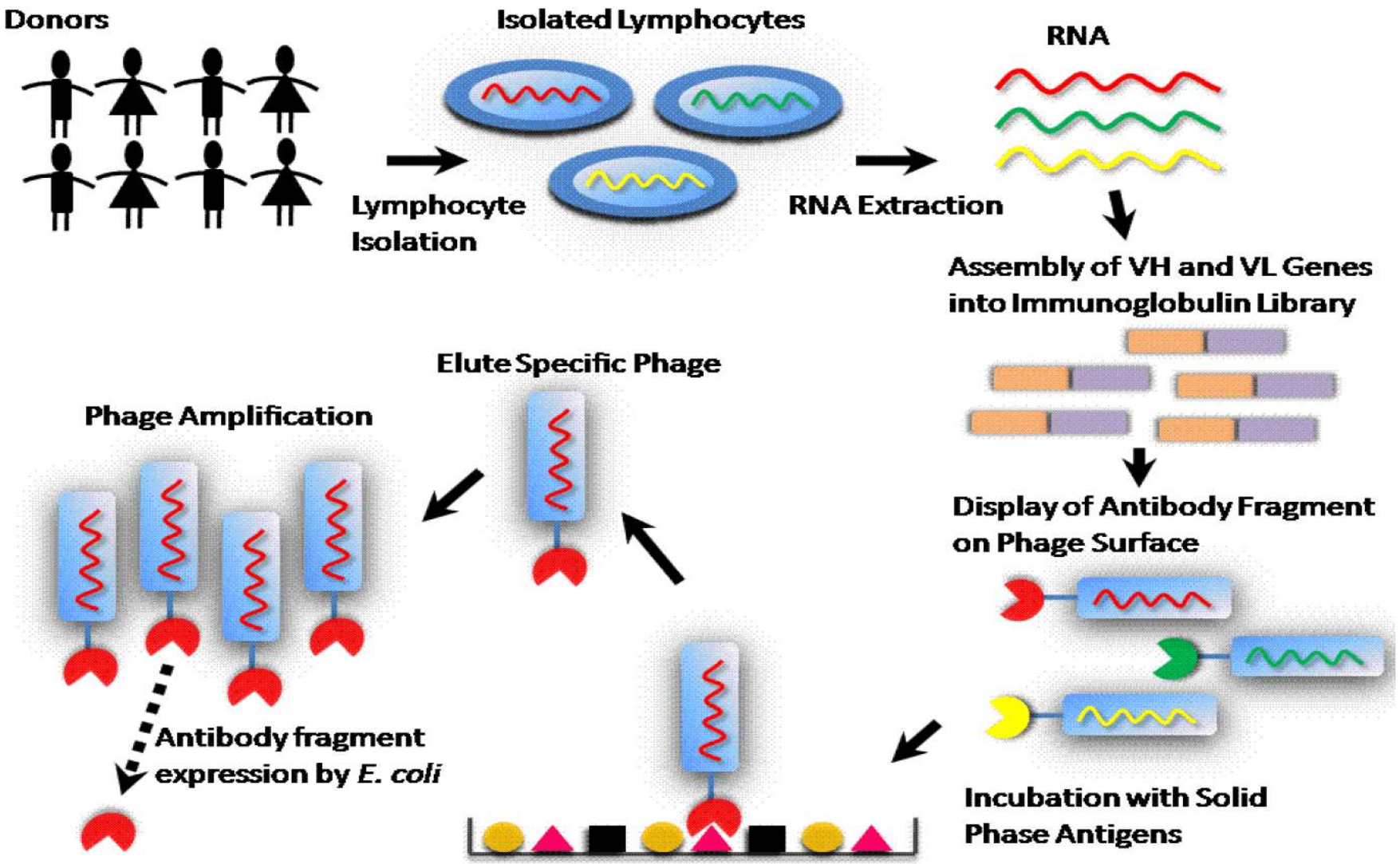
(5) scaled up production of selected antibodies in a cell culture expression system.

Monoclonal antibody production in mice



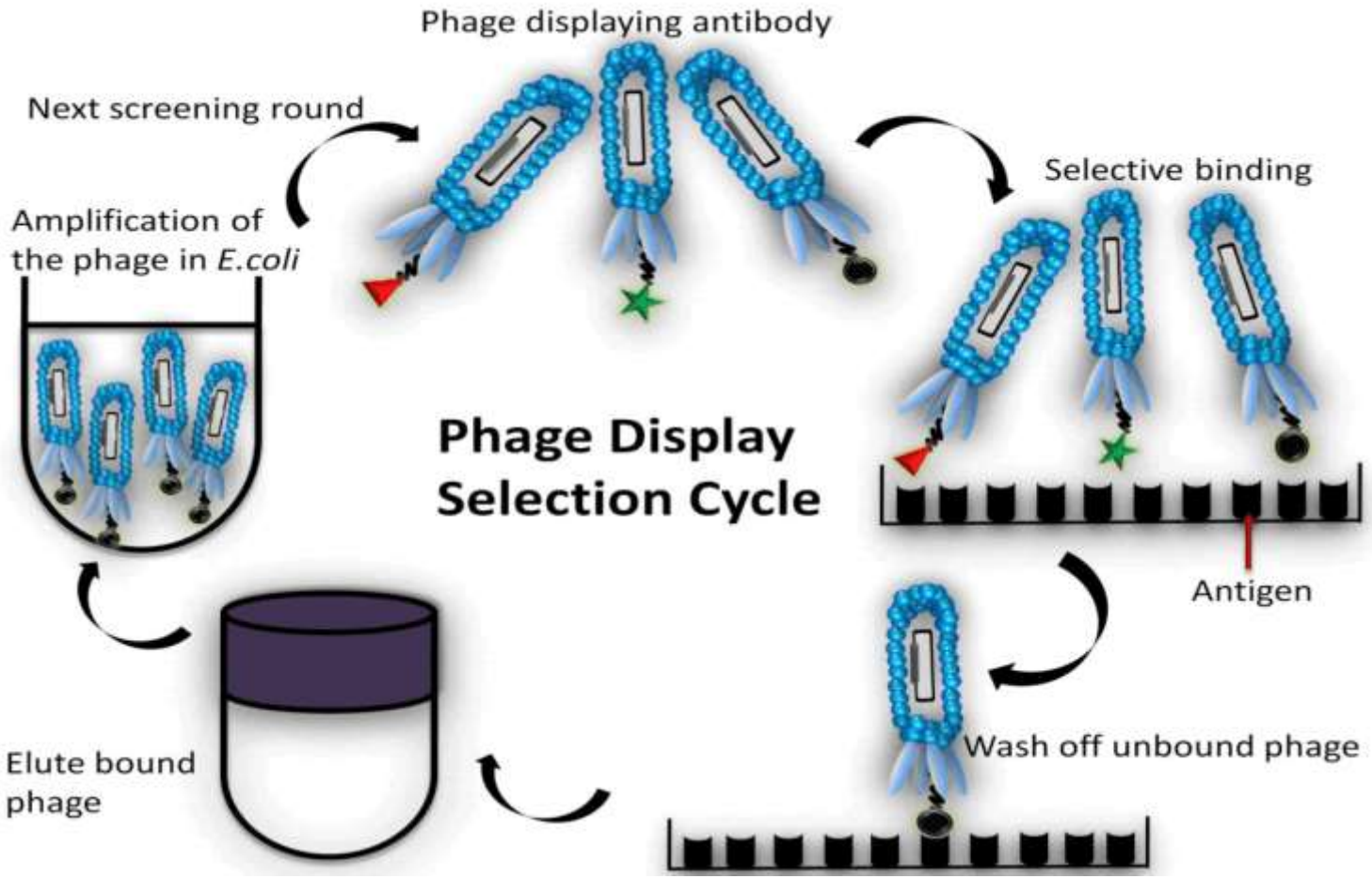
Polyclonal antibody production in rabbits





General scheme for the preparation and selection of a naïve recombinant antibody library. This figure indicates the general steps involved in the construction and selection of a recombinant antibody from a phage display library.

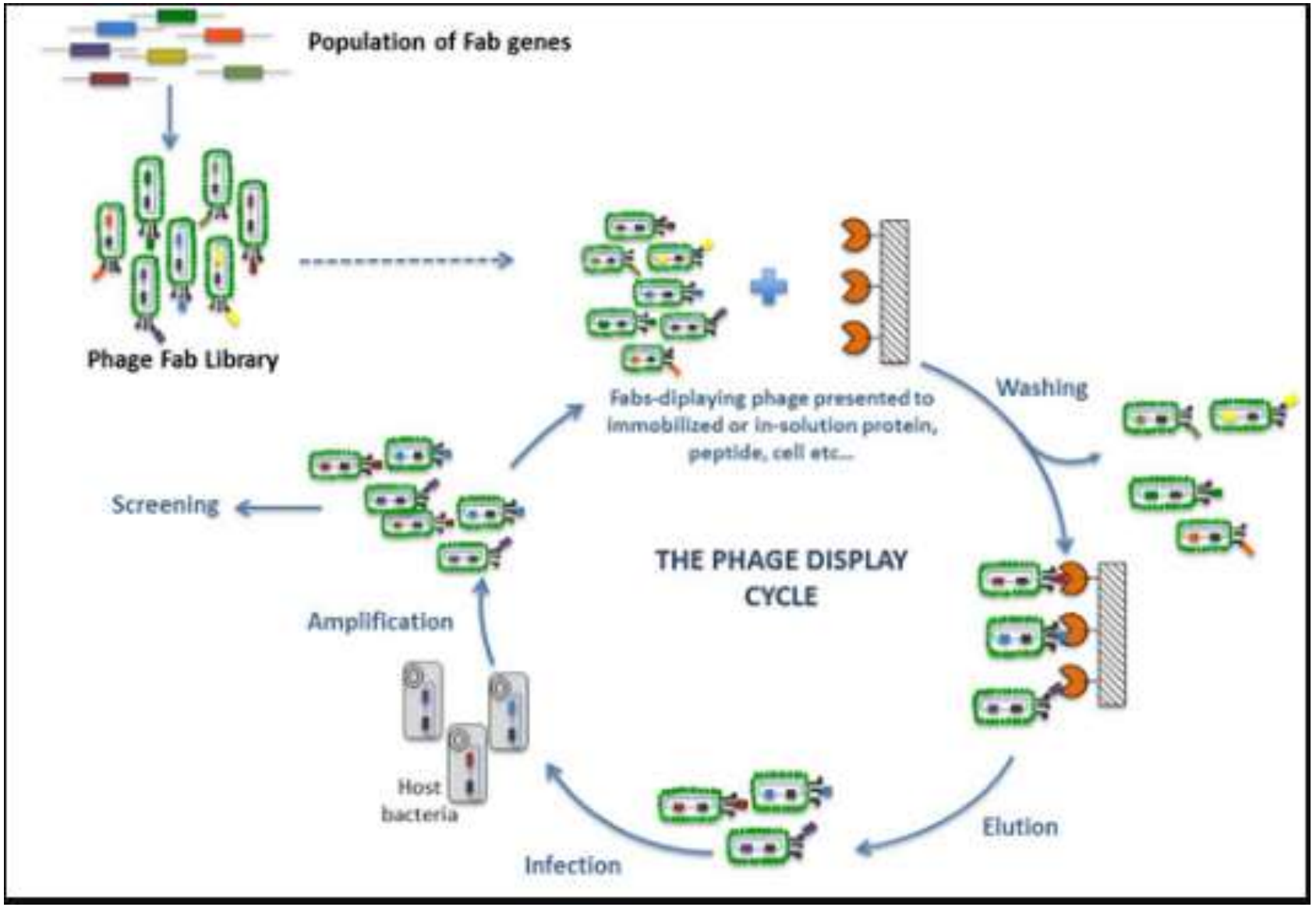
Phage Display

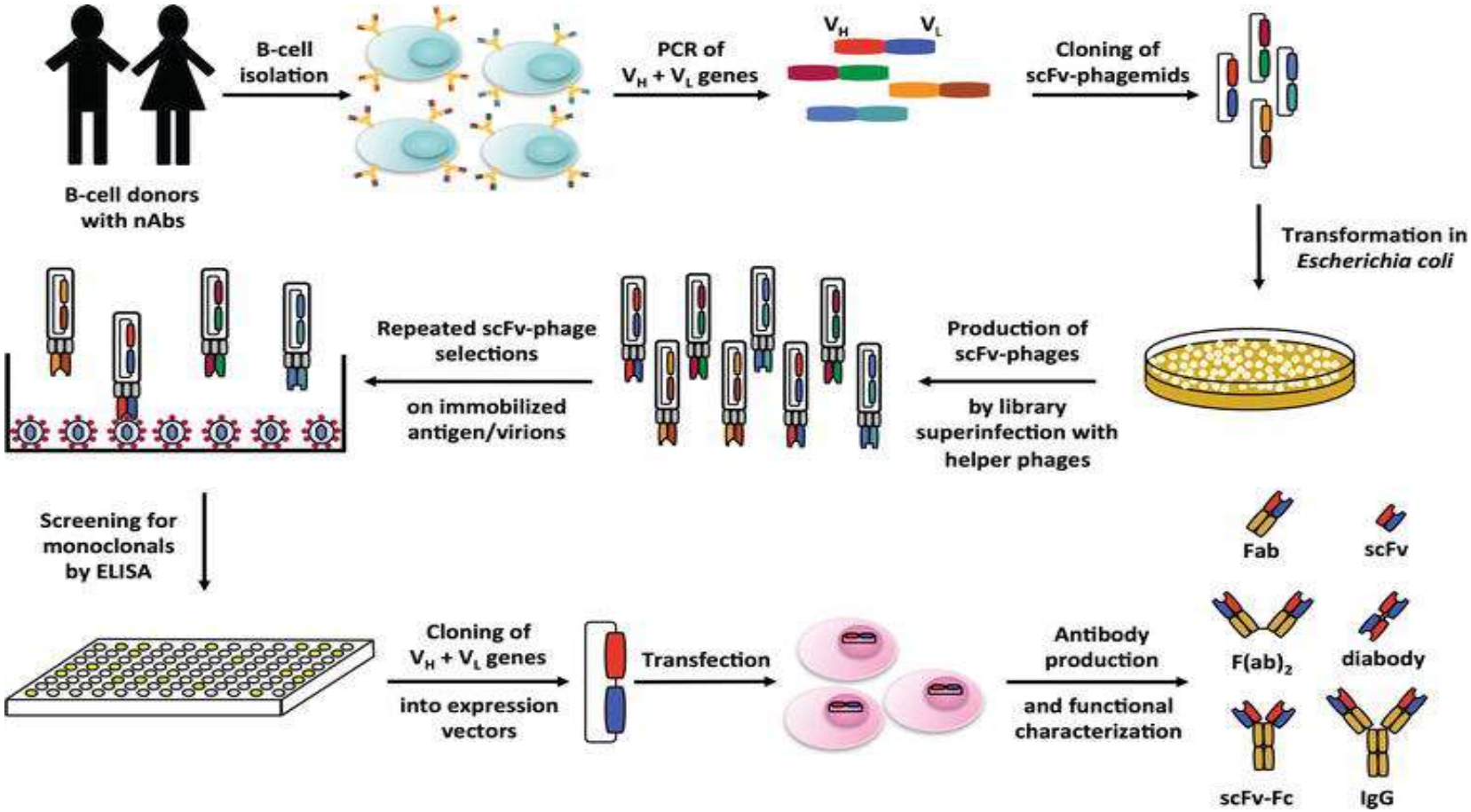


Schematic representation of the phage display bio-panning process. Antibody-presenting phages (top) are incubated with the surface-immobilized analyte of interest (right). Unbound phage particles are washed away (bottom), and analyte-bound phages are eluted (bottom) and amplified by re-infection of Escherichia coli (left). The amplified phages are rescued for the following round of panning. In the final round of panning, the antibody may be cloned out of the phage for retrieval.

Effective display formats for Antibodies(Abs) are scFv, Fabs, immunoglobulin variable fragments (Fvs) with an engineered intermolecular disulphide bond to stabilize the VH–VL pair and diabody fragments. Antibody libraries are constructed by reverse-transcribing and PCR amplifying genes encoding antigen-binding domains of heavy (VH) and light (VL) chains from lymphocyte total RNA. By combining different VH and VL, a process termed chain shuffling, unique antibody fragments are formed. The smaller size of the scFv format makes their libraries genetically more stable than Fab libraries.

Phage display cycle. DNA encoding for millions of variants of certain ligands (e.g., fab,peptides, proteins, or fragments thereof) is batch-cloned into the **phage genome as part of one of the phage coat proteins** (pIII, pVI, or pVIII). Large libraries containing millions of different ligands can be obtained by force-cloning in *E. coli*. From these repertoires, phage carrying specific-binding ligands can be isolated by a series of recursive cycles of selection on Ag(agar plate), each of which involves binding, washing, elution, and amplification.

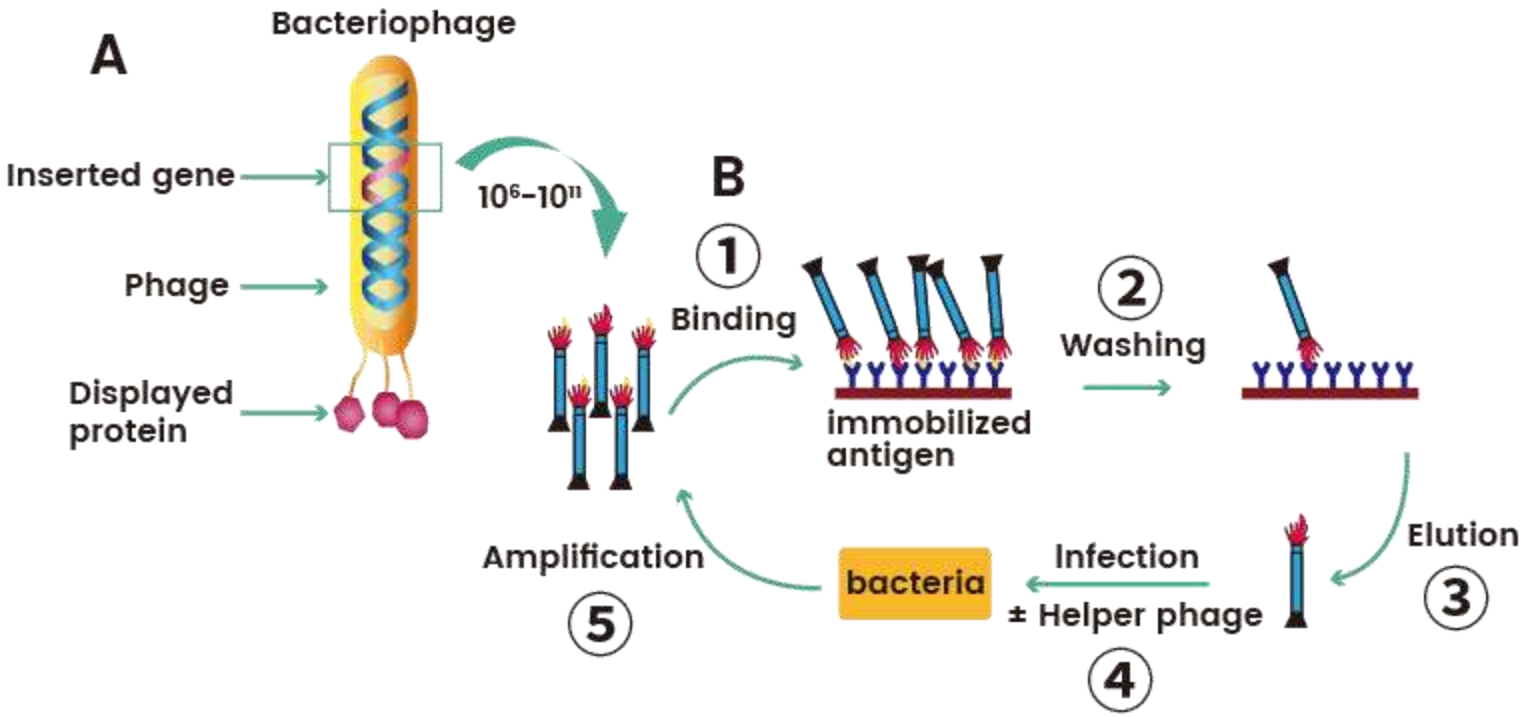

Population of Fab genes
Phage Fab Library
Fabs-diplaying phage presented to immobilized or in-solution protein, peptide, cell etc...
Washing
Elution
Infection
Host bacteria
Amplification
Screening
THE PHAGE DISPLAY CYCLE



Isolation of human neutralizing antiviral antibodies (nAbs) by phage display technique. Lymphocytes comprising B-cells from humans harboring neutralizing antibodies with unique features, e.g., Ebola disease survivors are isolated from blood, spleen, lymph nodes, or bone marrow by standard techniques (e.g., PBMCs by Ficoll density gradient centrifugation). Lymphocytes RNA is prepared and transcribed into single-stranded cDNA that is used as the source for PCR amplification of the variable heavy (VH) and light chain (VL) genes. Variable genes are randomly cloned into phagemid vectors as scFv antibody fragments prior to electroporation of phagemids into E. coli bacteria to produce combinatorial immune libraries. Library glycerol stocks are then used for the generation of a bacterial culture that is superinfected with a helper phage to produce phages presenting different scFvs on their surface. Specific binding scFv-phages are enriched over several selection rounds by stringent washing and elution using antigen/virions immobilized on immunotubes. After screening for monoclonal binders on ELISA plates, the best specific binders are directly produced as monovalent scFvs in bacteria cultures or cloned into appropriate expression vectors for the production of Fab, or various bivalent antibody formats prior to functional analysis (e.g., virus neutralization capacity and affinity).

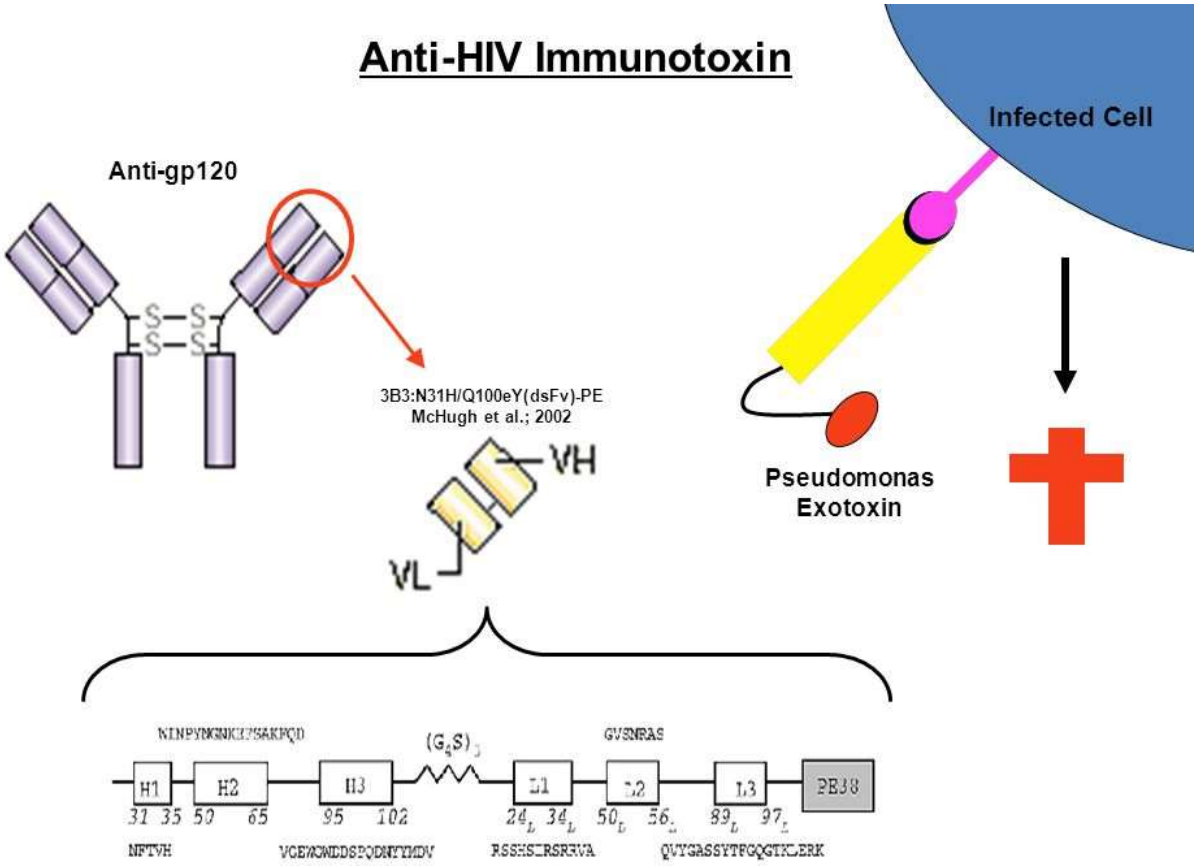
Single chain fragment variable (**scFv**)-**phages**

Since the commercialization of the first therapeutic monoclonal antibody product in 1986, this class of therapeutics has grown significantly . In 2016, 5 of the top 20 pharmaceuticals were therapeutic antibody drugs . The vast **majority of monoclonal antibodies is approved for the treatment of cancers, multiple sclerosis, or rheumatoid arthritis** . However, numerous potent human or humanized antiviral antibodies against H5N1 influenza virus, human immunodeficiency virus (HIV), herpes simplex virus (HSV), human cytomegalovirus (CMV), hepatitis C virus (HCV), Ebola virus, severe acute respiratory syndrome (SARS) virus, and other viral infections are in preclinical development, clinical studies, or even approved for antiviral treatment



The first clinically approved therapeutic antibody obtained with the help of phage display was **adalimumab (marketed as Humira®),** which neutralizes tumor necrosis factor and is mainly used against rheumatoid arthritis. Phage display technology is very helpful for high throughput screening for human therapeutic antibody candidates.

Anti-HIV Immunotoxin



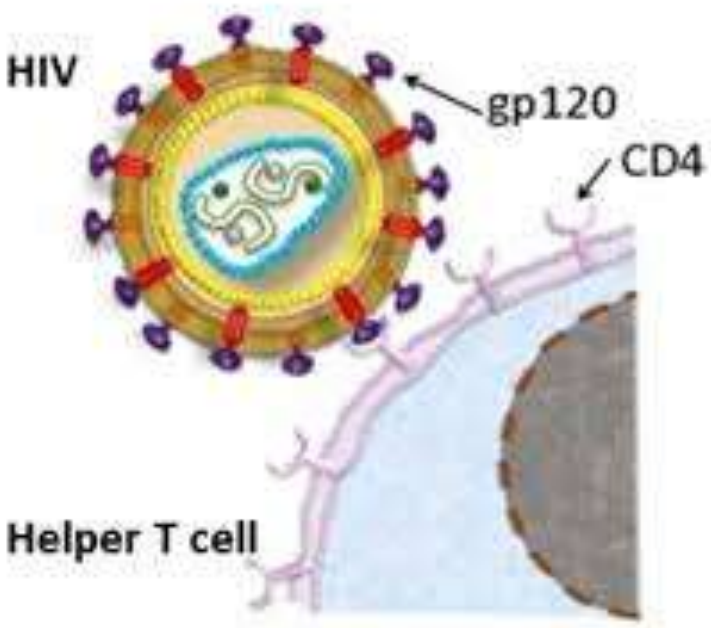
HIV Therapeutic agent

Affects CD4+T cells; Infected cells can be lysed by the production and release of HIV particles

Infected cells act as a production plant for the synthesis of of an HIV glycoprotein (gp120) that leads to destruction of other Th cells

An infected cell can fuse with other Th cells to form a sanctum that incapacitates individual T cell function

Thus an infected cell has gp120 on its surface



Pseudomonas exotoxin : Three domain

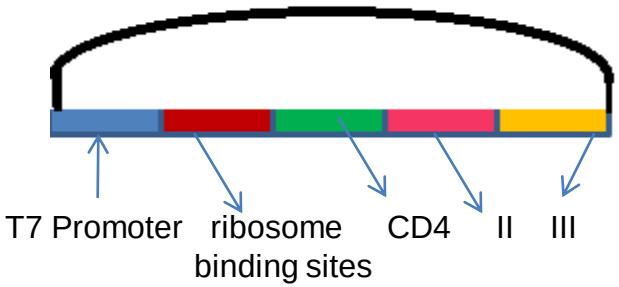
I : Cell binding

II: translocation of proteins into cells

III: ADP ribosylation ➡ Block EF2 ➡ inhibit protein synthesis

A fusion protein of exotoxin where CD4 replace domain I

Express in E.Coli; isolated : Thus CD4 finger and exotoxin is hired killer



The exotoxin may provoke immun responses in Human, the drug should be used with cyclosporine

Other toxin may also be used

- **Classification based on molecular types:**

Antibody based drugs, Fc fusion proteins, anticoagulants, blood factors, growth factors, hormones, interferon, bone morphogenetic proteins, interleukins and thrombolytic.

- **Classification based on molecular mechanism:**

Binding non-covalently to target e.g. –mAbs

Affecting covalent bonds e.g. – enzymes

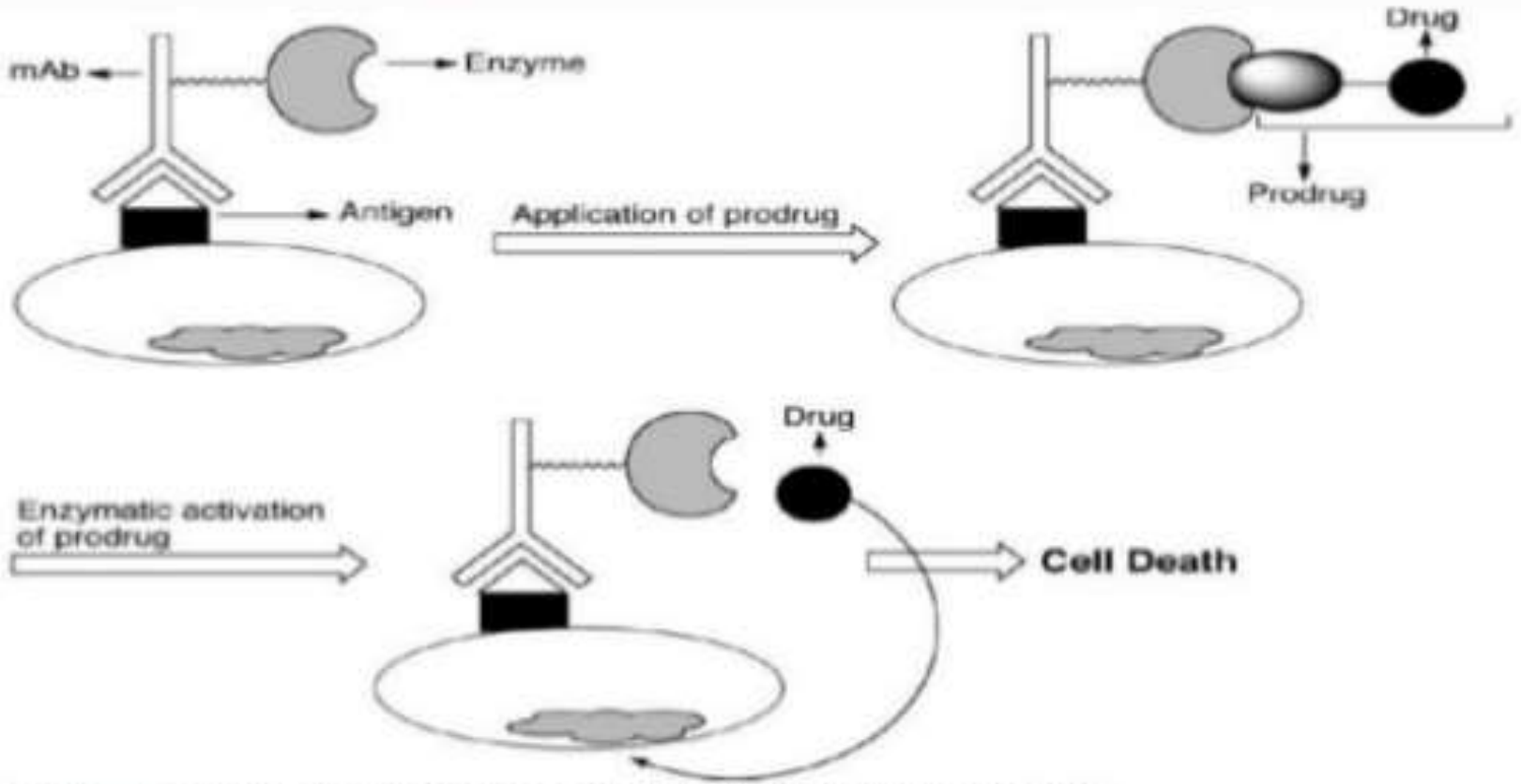
Exerting activity without specific interactions e.g. - serum albumin

- This led to the development of large-scale mammalian cell culture, for example, the use of Chinese Hamster Ovary (CHO) cell cuture bioreactors.

Limitations of microbial bioreactors

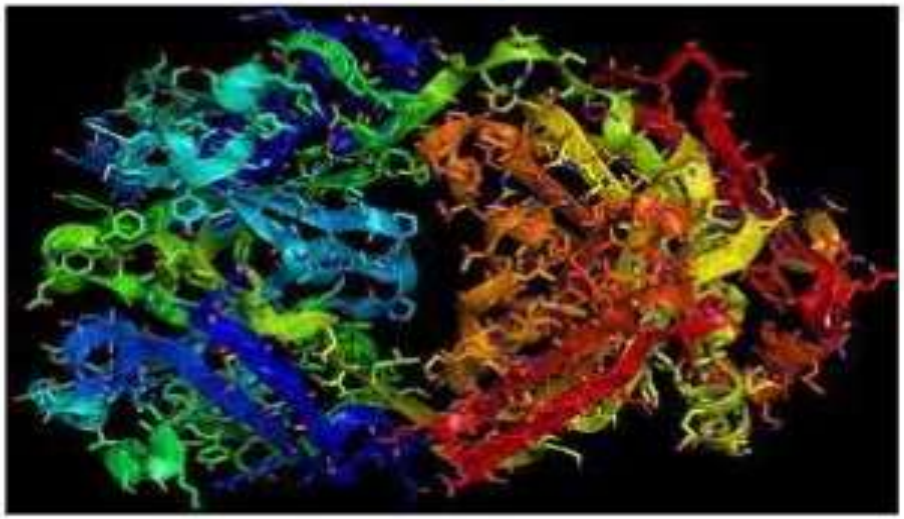
- Bacteria often improperly fold complex proteins, leading to involved and expensive refolding processes and ;
- Both bacteria and yeast lack adequate post-translational modification machinery for mammalian-specific *N*- and *O*-linked glycosylation, γ-carboxylation, and proteolytic processing

Antibody-directed Enzyme Prodrug Therapy (ADEPT)



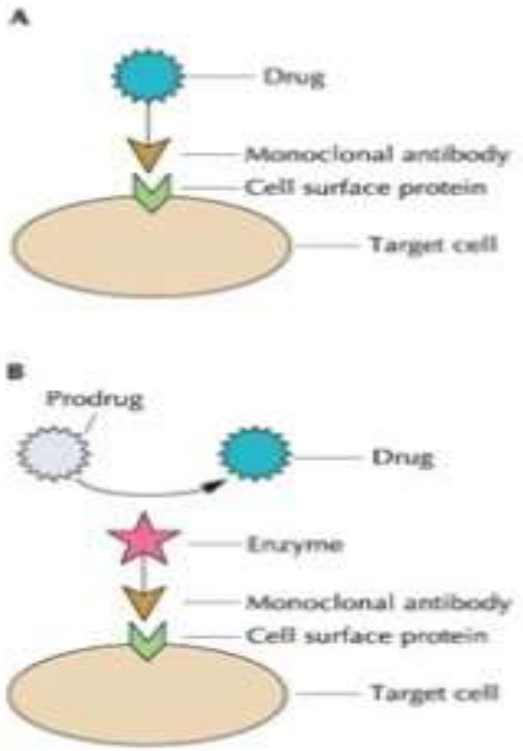
Schematic presentation of antibody-directed enzyme prodrug therapy (ADEPT). mAb-enzyme conjugate is given first, which binds to antigens expressed on tumor surfaces. Prodrug is given next, which is converted to active drug by the pre-targeted enzyme.

Herceptin®



- "Magic bullet"
- Genentech. FDA 9/98; Aullrich/Genentech and DSlamon/UCLA Jonsson Cancer Ctr
- Trastuzumab (trade name Herceptin)
- Humanized monoclonal antibody
- Target is HER2/neu receptor (erbB2)
- HER2-positive metastatic breast cancer
- Anti-cancer therapy in breast cancer, over-expressing erbB2 receptor
- ErbB2 receptor amplification occurs in 25-30% of early-stage breast cancers
- Transmembrane Tyr kinase, activating PI3K/Akt pathway and MAP pathway
- Overexpression promotes invasion, survival and angiogenesis of cells
- Also confers therapeutic resistance to cancer therapies
- Herceptin binds to extracellular domain of erbB2 receptor,
- Arresting cell at G1 phase

Magic bullet: delivery of drug to site



- Binding of mAb requires second step
- 1) delivery of drug
- 2) delivery of enzyme to convert pro-drug

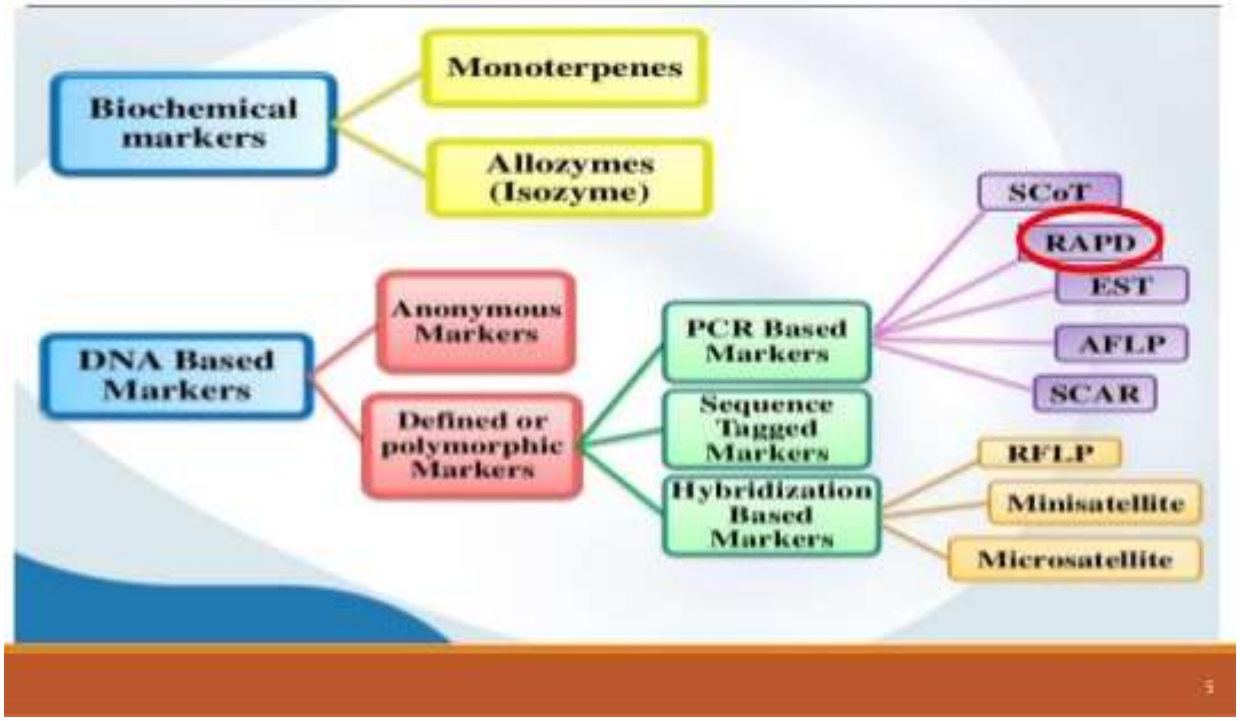
Molecular Markers

(From ineternet)

Markers

Any genetic trait that can be identified with confidence & relative case and can be followed in a mapping population is called as marker.

Genetic/ Molecular marker is a specific location on a chromosome that is defined by a naked eye polymorphism as differences in electrophoretic mobility of specific proteins or as differences in specific DNA sequence.



Specific fragments of DNA that can be identified within the whole genome. More: Molecular markers are found at specific locations of the genome. They are used to 'flag' the position of a particular gene or the inheritance of a particular characteristic

Various types of PCR based markers are utilized to evaluate DNA polymorphism and tag genes, such as Randomly-**amplified polymorphic DNA markers (RAPD)**, Sequence-tagged sites (STS), Allele-specific associated primers (ASAPs), Expressed sequence tag markers (EST), Single strand conformation polymorphism (SSCP),

- **Discovery of potential novel molecular markers of human diseases**
- **Identification of novel molecular markers of human diseases**
- **Utility of molecular markers to develop useful molecular assays for detection, diagnosis, and prediction of disease outcomes**

Markers on the Basis of Inheritance

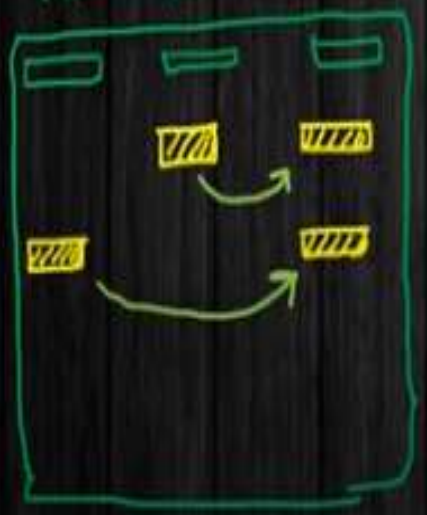
(1) Dominant Marker → ISSR, RAPD, AFLP (+/-)

(2) Co-Dominant Marker → SSR, RFLP, STR (Heterozygote Vs Homozygotes)
**

Co-Dominant

AA aa Aa) → hetero zygote

P1 P2 F1



Dominant

BB

P1 P2 F1

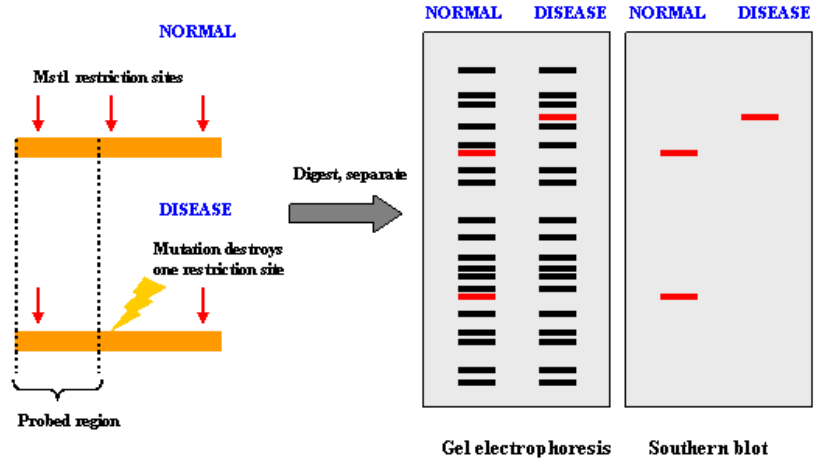
What is RFLP?

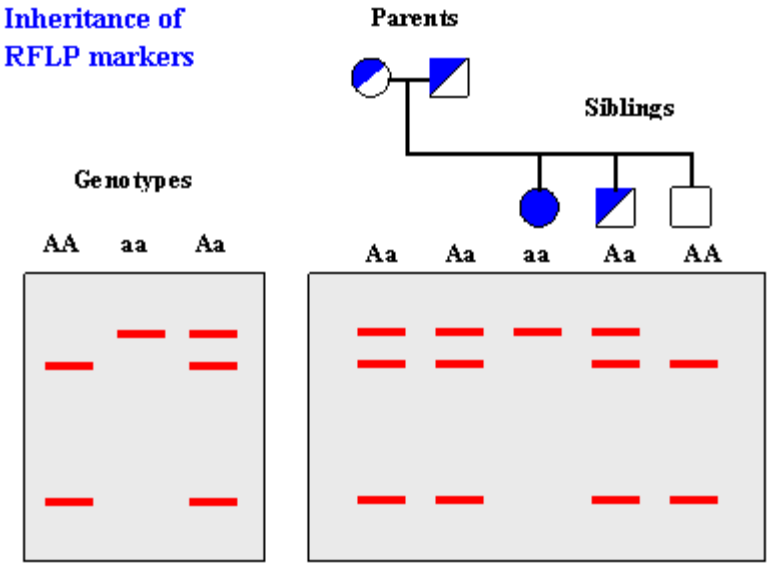
Restriction Fragment Length Polymorphisms (RFLPs) is a technique that is used to detect the genetic variations in homologous DNA sequences. It is the first method developed for DNA profiling. Organisms have unique DNA fingerprints or DNA profiles. RFLP serves as an important tool to analyze the variation between DNA profiles of intraspecific or closely related organisms since homologous sequences have different restriction sites (locations) that are unique to a particular organism. *When homologous DNA are digested with specific restriction endonucleases, it will result in different DNA profiles which are unique to each individual*. Therefore, the principal of this method is the detection of genetic variation among organisms by restricting homologous DNA with specific restriction enzymes and analysis of the fragment length polymorphism via gel electrophoresis and blotting. Blotting patterns are unique to each organism and characterize the specific genotypes.

Steps of RFLP

- **Isolation of sufficient amount of DNA from samples**
- **Fragmentation of the DNA samples with specific restriction endonucleases into short sequence**
- **Separation of the resulted fragments with different lengths by agarose gel electrophoresis.**
- **Transfer of the gel profile into a membrane by Southern blotting**
- **hybridization of the membrane with labelled probes and analysis of the fragment length polymorphism in each profile**

RFLP is a very important technique in detecting disease inheritance and finding the risk of the disease occurrence among family members. RFLP is also frequently used in genome mapping, identifying criminals in forensics, paternity testing, etc. RFLP has several limitations as well. RFLP requires the prior knowledge of sequence data to design probes for hybridization. It also requires isolation of sufficient amount of DNA from the sample to analyze, which is difficult in forensic studies.





SNPsor INDELs can create or abolish restriction endonuclease (RE) recognition sites, thus affecting quantities and length of DNA fragments resulting from RE digestion.

Developing RFLP probes

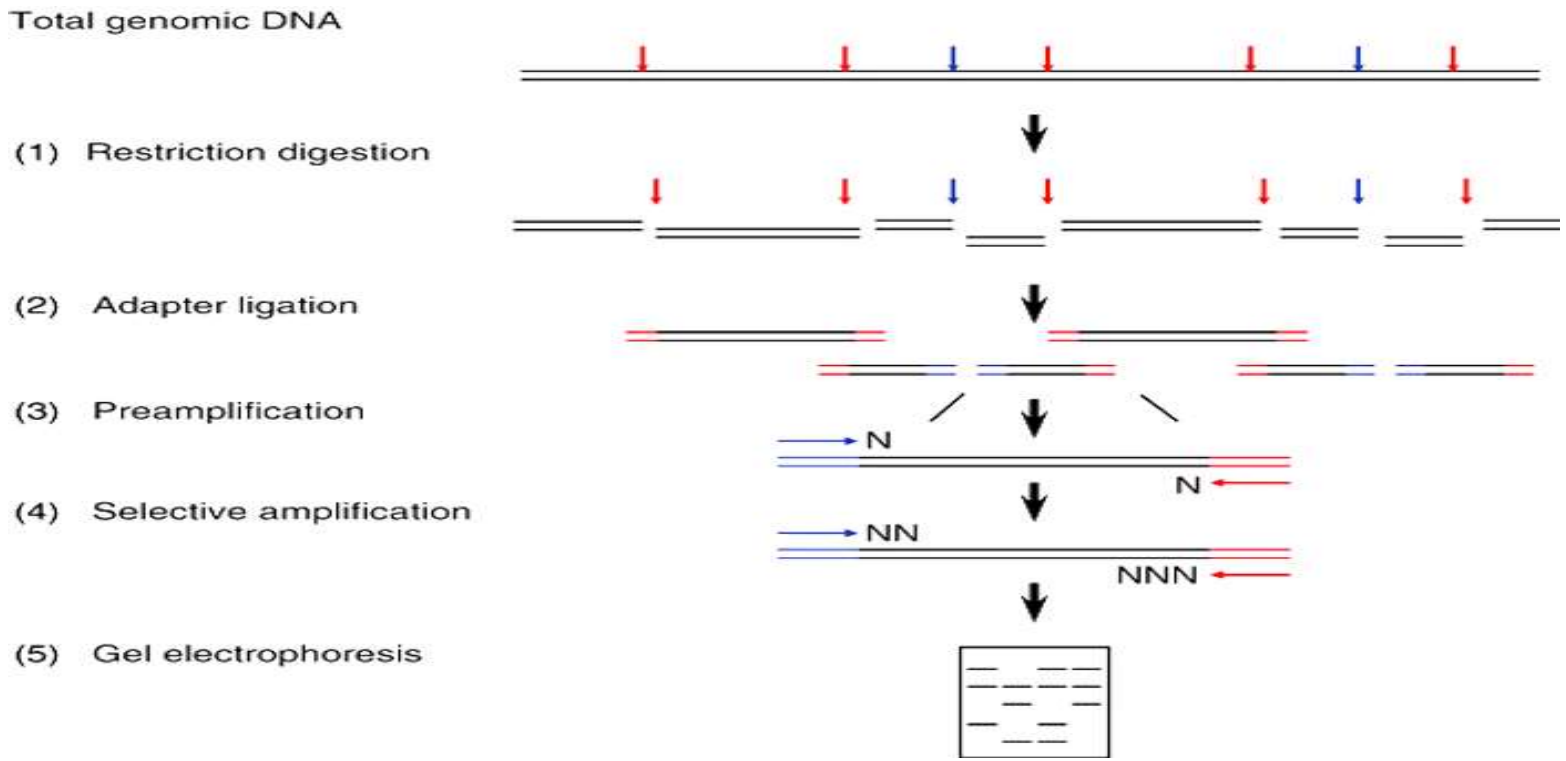
- Total DNA is digested with a methylation-sensitive enzyme (for example, PstI), thereby enriching the library for single- or low-copy expressed sequences (PstI clones are based on the suggestion that expressed genes are not methylated).
- The digested DNA is size-fractionated on a preparative agarose gel, and fragments ranging from 500 to 2000 bp are excised, eluted and cloned into a plasmid vector (for example, pUC18).
- Digests of the plasmids are screened to check for inserts.
- Southern blots of the inserts can be probed with total sheared DNA to select clones that hybridize to single- and low-copy sequences.
- The probes are screened for RFLPs using genomic DNA of different genotypes digested with restriction endonucleases. Typically, in species with moderate to high polymorphism rates, two to four restriction endonucleases are used such as EcoRI


A
5 KB
homologous 1
5 KB
homologous 2
Probe
B
5 KB
homologous 1
2 KB
3 KB
homologous 2
Probe
A
B
5 KB
3 KB
2 KB

AFLP (Amplified Fragment Length Polymorphism) is an important tool in molecular biology and is extensively used in genetic variation analysis. *AFLP is based on the specific PCR amplification of the fragmented genomic DNA and detection of the polymorphism by autoradiographs via gel electrophoresis*. AFLP widely contributes to identifying genetic differences in strains or closely related species of various kingdoms including plants, animals, bacteria and fungi. AFLP can be carried out with small amounts of unknown DNA samples. It does not require prior sequence knowledge and designing of probes.

- **Steps of AFLP**
- **Isolation of DNA**
- **Digestion of the DNA with restriction endonucleases**
- **Ligation of the restricted DNA fragments with adaptors**
- **Selective amplification of the fragments with specific restriction sites**
- **Separation of the PCR products by gel electrophoresis**
- **Visualization of the gel matrix by autoradiograph**

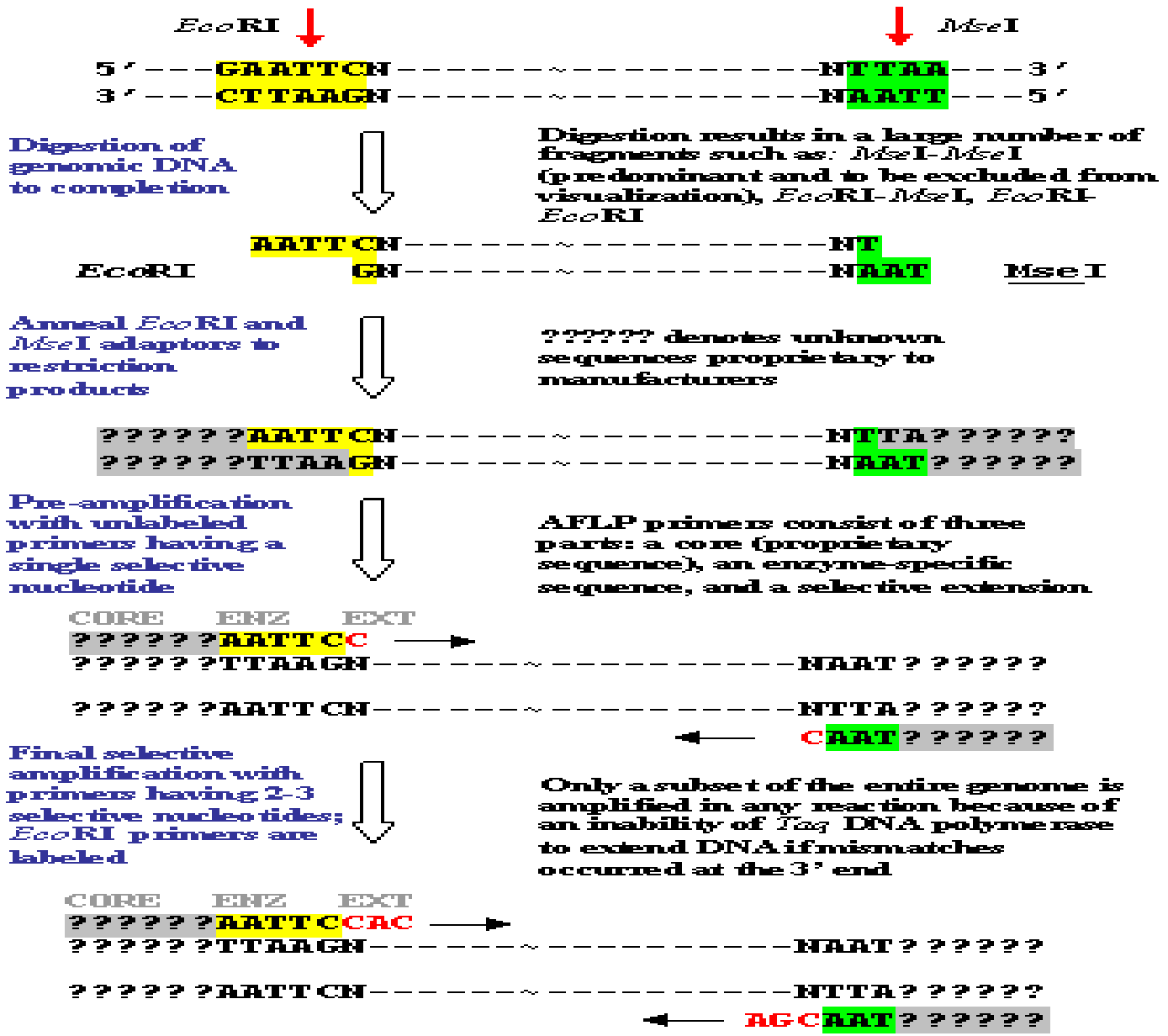
AFLP is a more sensitive and reproducible method which can be used in DNA profiling of several taxa including fungi, bacteria, plants and animal without prior knowledge of DNA sequences. It helps in identifying slight differences among individuals in populations due to its highly sensitive nature. AFLP is also important in genome mapping, forensic studies, parental testing, genotyping, etc.

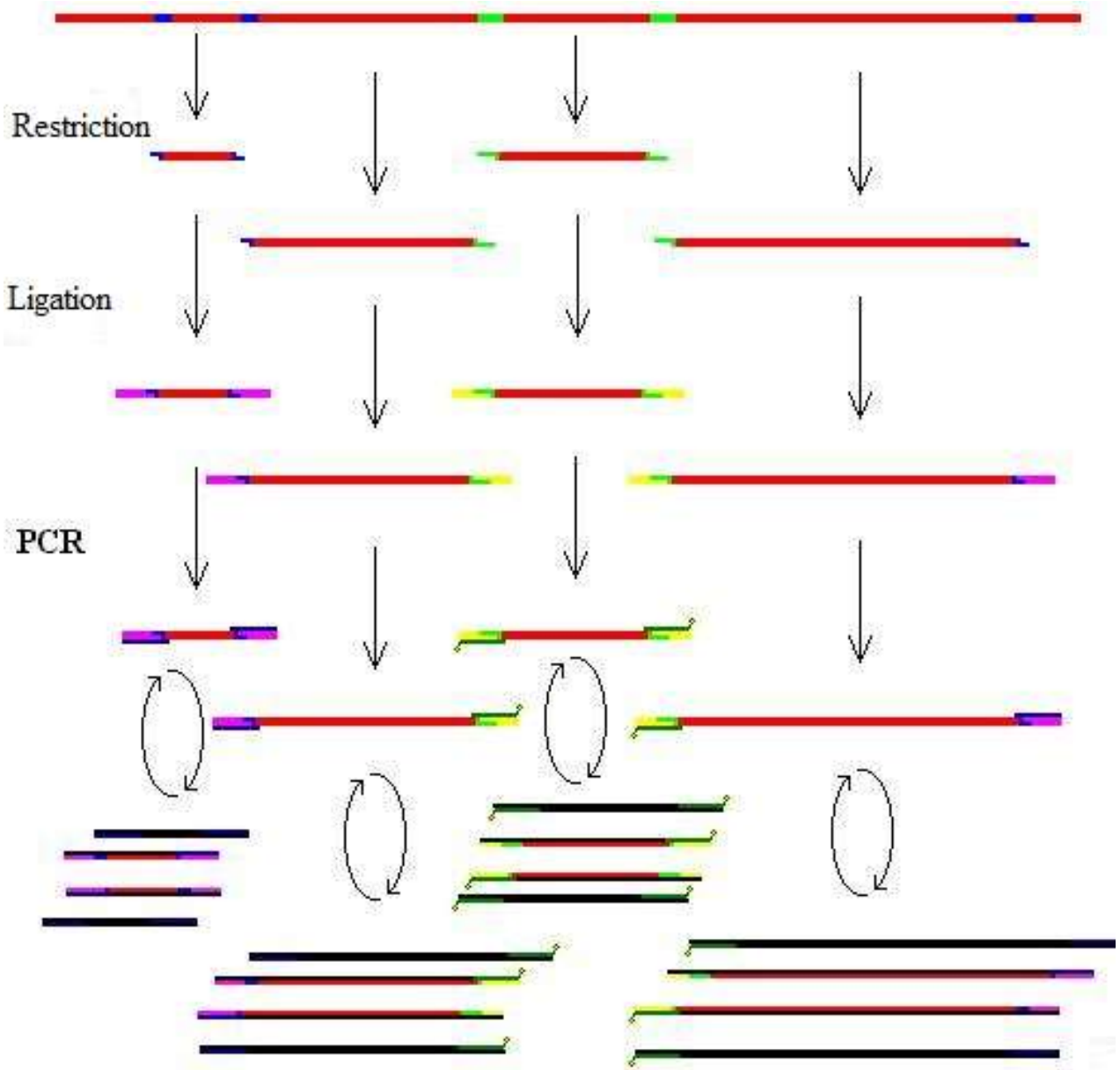


Outline of the AFLP procedure

Template fragments are generated by: (1) digestion of genomic DNA with a combination of the two restriction enzymes EcoRI and MseI (blue and red arrows represent EcoRI and MseI restriction enzyme sites, respectively); (2) ligation of the double-stranded EcoRI- (blue) and MseI- (red) specific adapters to the fragment ends; (3) a pre-amplification step using primers that match the adapter sequences and that carry each one selective nucleotide (represented by N) at their 3 end are used to PCR-amplify subsets of the EcoRI/MseI templates; (4) a final selective PCR-amplification step in which additional selective nucleotides are added to the EcoRI and MseI primers; and (5) the electrophoretic size fractionation and the display on denaturing polyacrylamide gels of the EcoRI/MseI amplification products.

AFLP procedure




Restriction
Ligation
PCR

What is the difference between AFLP and RFLP?

ALFP vs RFLP	
AFLP involves selective PCR amplification of the digested DNA.	RFLP does not involve PCR unless it is PCR-RFLP.
Sequence Knowledge	
Prior sequence knowledge is not required.	Prior sequence knowledge is required to designed RFLP probes.
Reliability	
This is more reliable.	This is less reliable compared to AFLP.
Efficiency in Detecting Polymorphism	
This has a higher efficiency in detecting polymorphism than RFLP.	This is less efficient compared to AFLP.
Cost	
This is a little expensive compared to RFLP.	This is less expensive compared to AFLP.
Applications	
AFLPs have been applied to genome mapping, DNA fingerprinting, genetic diversity studies, paternity testing and forensics	RFLP analysis is an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Random Amplified Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA, or RAPD, marker analysis utilizes short PCR primers consisting of random sequences usually in the size range of 8 to 15 nucleotides in length. Complex patterns of PCR products are generated as these random sequence primers anneal to various regions in an organism's genome. RAPD suffers from poor reproducibility between laboratories largely because of the requirement of consistent PCR amplification conditions including thermal cycler ramp speeds. The complex patterns of RAPD also prevent mixture interpretation and provide challenges in consistent scoring of electrophoretic images even in single-source samples.

- RAPD markers are decamer DNA fragments.
- RAPD is a type of PCR reaction, Segments amplified are Random.
- No knowledge of DNA sequence required. Hence a popular method.
- In recent years, RAPD is used to Characterize, & Trace, the phylogeny of diverse plant & animal species.
- Identical 10-mer primer will or will not amplify a segment of DNA, depending on positions that are complementary to the primer sequence.

How it Works ?

- The principle is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template.
- This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome.
- These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualised by ethidium bromide staining.

- Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites.
- Recently, sequence characterised amplified regions (SCARs) analysis of RAPD polymorphisms showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions.
- In order for PCR to occur:

  1) the primers must anneal in a particular orientation (such that they point towards each other) and,
  2) they must anneal within a reasonable distance of one another.

FINDING DIFFERENCES BETWEEN GENOMES USING RAPD ANALYSIS

- Consider the Figure 2 (genome A). If another DNA template (genome B) was obtained from a different (yet related) source, there would probably be some differences in the DNA sequence of the two templates. Suppose there was a change in sequence at primer annealing site #2

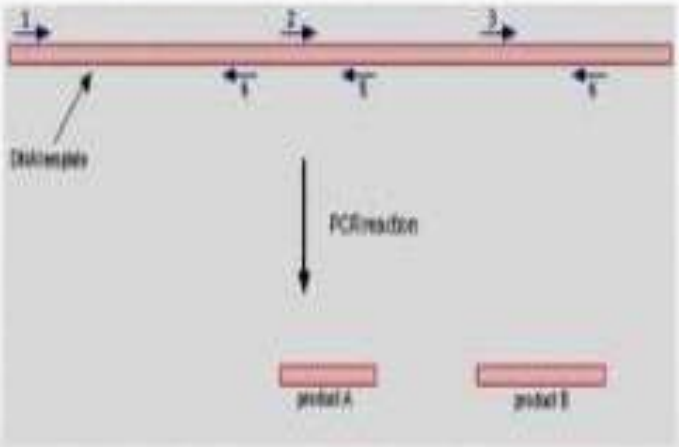


Figure 2 (genome A)

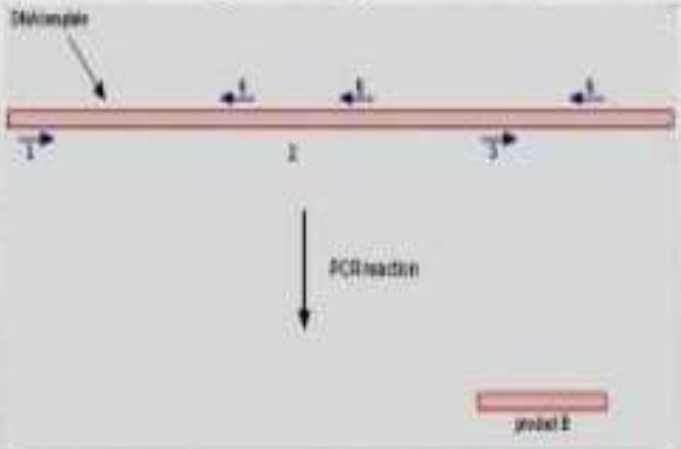
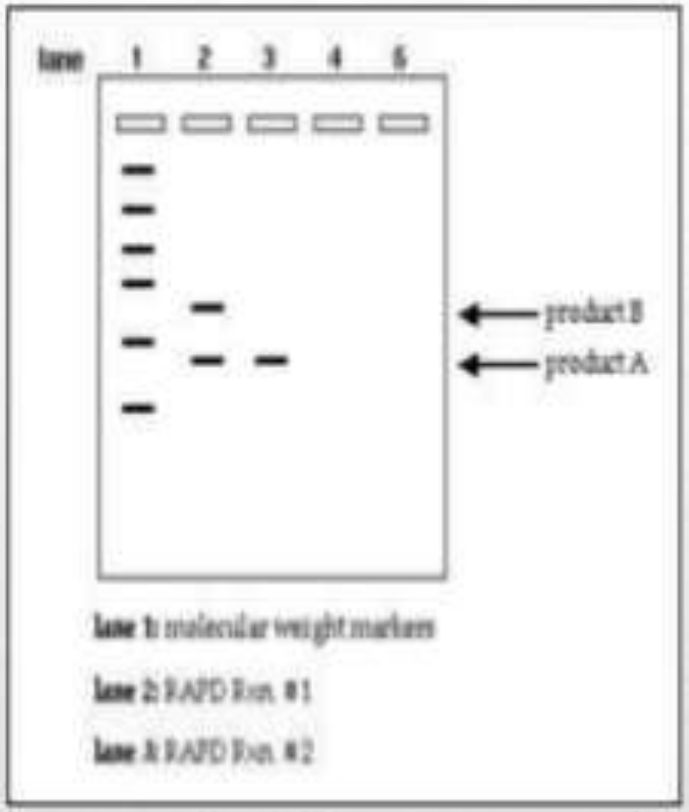


Figure 3 (genome B)

- Genome A and B can represent genomic DNA from two individuals in the same species or possibly from two different species.
- Certain portions of genomic DNA tend to much conserved (very little variation) while other portions tend to vary greatly among individuals within a species or among different species.
- The trick in RAPD PCR analysis is to:

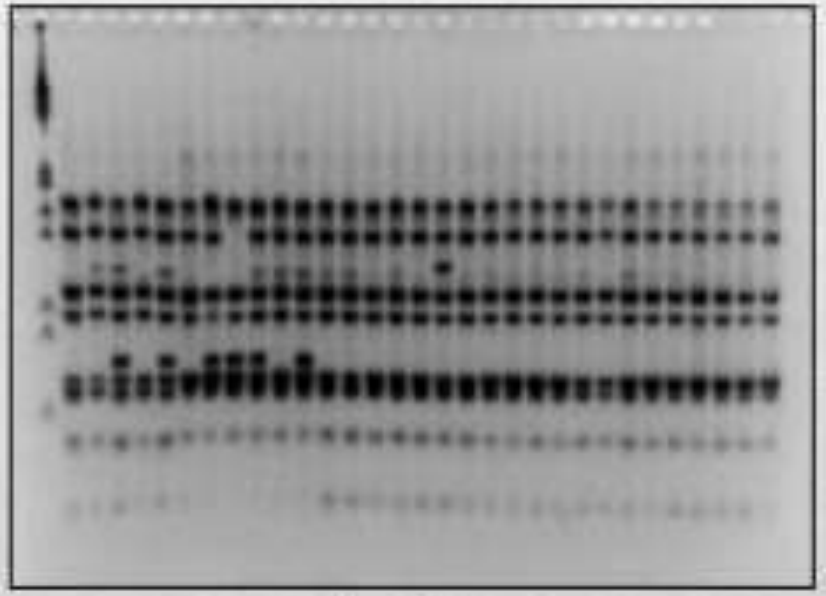
1. Find those sequences which have just enough variation to allow us to detect differences among the organisms that we are studying.
2. find the right PCR primers which will allow us to detect sequence differences.



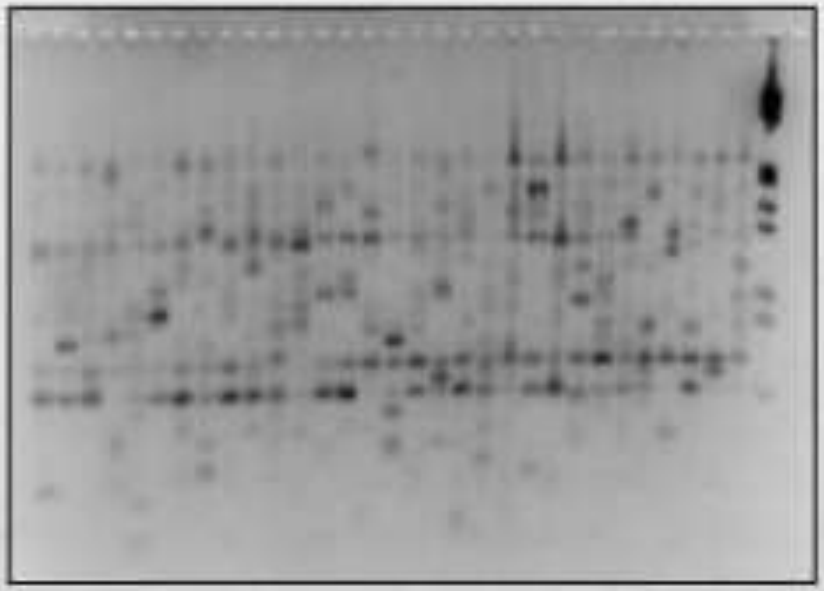
(genomes A and B) on a agarose gel

INTERPRETING RAPD BANDING PATTERNS

- Each gel is analysed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring can be done based on the banding profiles which is clear and transparent (Fig. 5a) otherwise the scoring is very difficult (Fig. 5b).



(Fig. 5a)



(Fig. 5b)

- Criteria for selecting scoring bands:
  1) reproducibility—need to repeat experiments.
  2) thickness 3) size and,
  4) expected segregation observed in a mapping population.
- DNA polymorphism among individuals can be due to:
  1) mismatches at the primer site.
  2) appearance of a new primer site and,
  3) length of the amplified region between primer sites.
- The NTSYS-pc software ver. 2.02 is used to estimate genetic similarities with the Jaccard's coefficient.

Advantages

- It requires no DNA probes and sequence information for the design of specific primers.
- It involves no blotting or hybridisation steps, hence, it is quick, simple and efficient.
- It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated.
- High number of fragments.
- Arbitrary primers are easily purchased.
- Unit costs per assay are low compared to other marker technologies.

Disadvantages

- Nearly all RAPD markers are dominant,
- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome.
- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product.
- Lack of a prior knowledge on the identity of the amplification products.
- Problems with reproducibility.
- Problems of co-migration.

Random amplified polymorphic DNAs (RAPDs) is a method of PCR where arbitrarily chosen 10 base primers are used to search for variation in DNA (Williams et al., 1990). RAPDs data can contain artifacts (Ellsworth et al. 1993), and are not fully reproduceable (Paterson 1996). However, RAPDs have been be used to generate large numbers of genetic markers useful for linakge mapping quickly and cheaply (Antolin et al., 1996).

Paran and Michelmore (1993) were able to separate RAPD fragments on an agarose gel, excise the bands from the gel and reamplify individual bands from gel slices using the original RAPDs primer. They were then able to clone and sequence the PCR products and use sequence data to design PCR primers specific to specific RAPDs fragments, and use PCR to produce specific RAPD's fragments from genomic DNA, which then can function as sequence tagged sites (STSs). This method allows for rapid generation of STSs derived from RAPDs fragments, and eliminates the problems associated with reproduceability associated with RAPD analyses. Reamplification from genomic DNA and subsequent sequencing of the PCR products also allows for the identification of any artifacts.

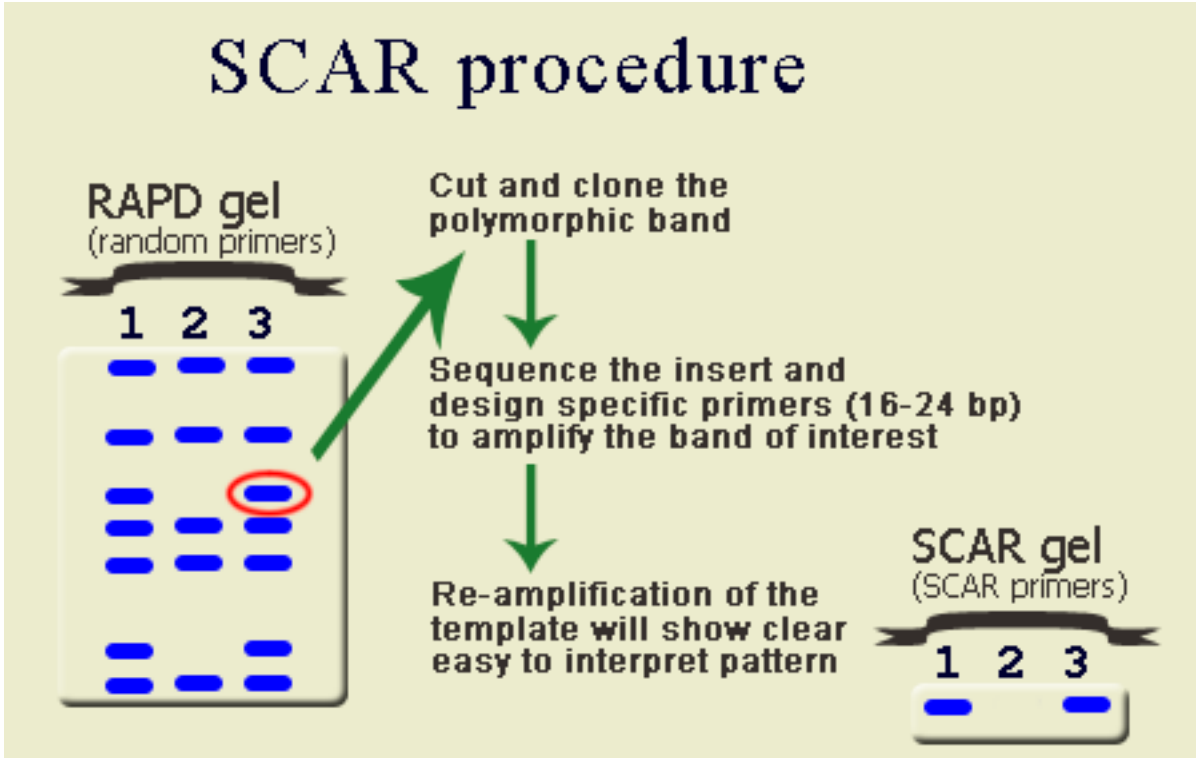
A **sequence-tagged site** (or **STS**) is a short (200 to 500 base pair) DNA sequence that has a single occurrence in the genome and whose location and base sequence are known

Microsatellites, otherwise called Simple sequence repeats (SSRs) or Short Tandem Repeats (STRs), are rehashing sequences of 2-5 base sets of DNA.it is a sort of Variable Number Tandem Repeat (VNTR). **SSR markers** are important in various gene studies

A **minisatellite** is a tract of repetitive DNA in which certain DNA motifs (ranging in length from 10–60 base pairs) are typically repeated 5-50 times. ... Confusingly, **minisatellites** are often referred to as VNTRs, and **microsatellites** are often referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs).

Sequence Characterized Amplified Region (SCAR)

DNA fragments amplified by the Polymerase Chain Reaction (PCR) using specific 15-30 bp primers, **designed from nucleotide sequences established in cloned RAPD** (Random Amplified Polymorphic DNA) fragments linked to a trait of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a co-dominant marker may be an additional advantage of converting RAPDs into SCARs



STS

Expressed sequence tags.	Simple sequence length polymorphs.(SSLPs)	Random genomic sequences.

- **Expressed sequence tags:**
  It is the sequence obtained by the analysing the cDNA.
- Now cDNA is obtained by mRNA,that is by using reverse transcriptase enzyme. there by knowing the gene which codes for a particular proteins.
- Thus coding sequences which are unique hence used as STS.

STSs are **very helpful for detecting microdeletions in some genes**. For example, some STSs can be used in screening by PCR to detect microdeletions in Azoospermia (AZF) genes in infertile men.

↓

when there are no sperm in the ejaculate.

What is EST ???

- ESTs are small pieces of DNA sequence (usually 100 to 800 nucleotides long) generated by sequencing randomly selected cDNA clones from a library

- Expressed Sequence Tags (ESTs) are short , single-pass sequence reads from mRNA (cDNA).

- ESTs are bits of DNA sequence that represent genes expressed in certain cells, tissues, or organs from different organisms and

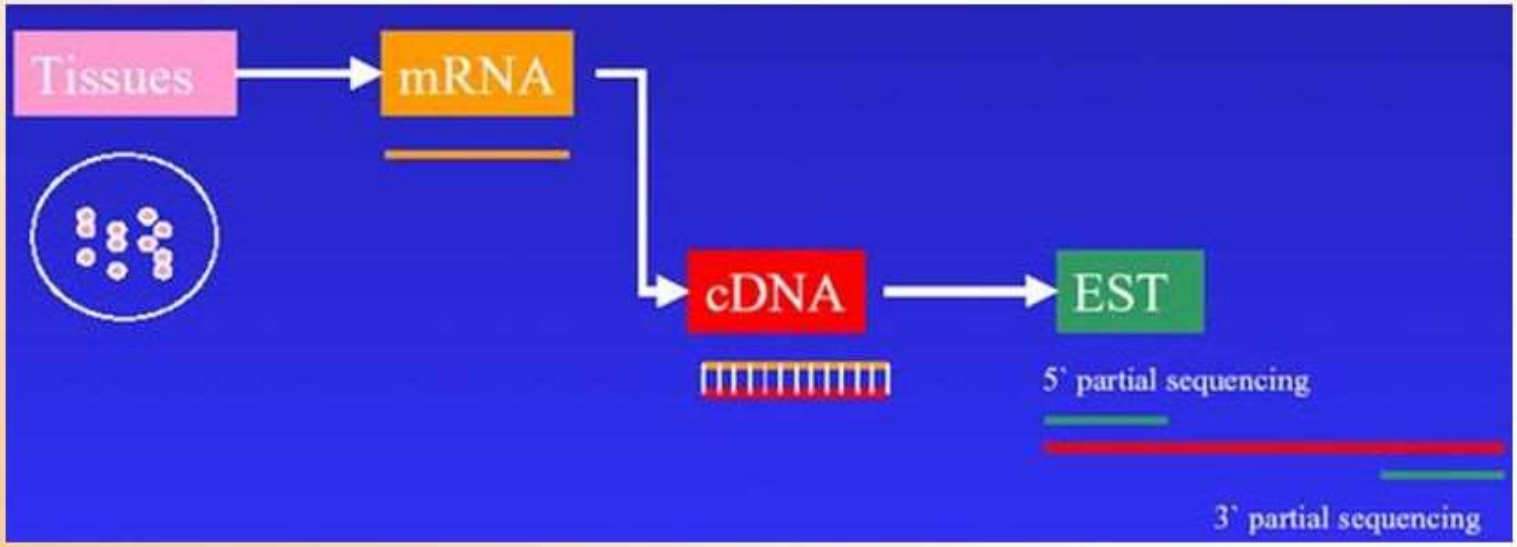
- use these "tags" to fish a gene out of a portion of

Application of EST

- Identify unknown genes and to map their positions within a genome.
- ESTs provide researchers with a quick and inexpensive route for discovering new genes,
- For obtaining data on gene expression and regulation,
- constructing genome maps.
- Economical approach to identify and characterize expressed genes
- EST represent a snapshot of genes expressed in a given tissue and/or at a given developmental stage

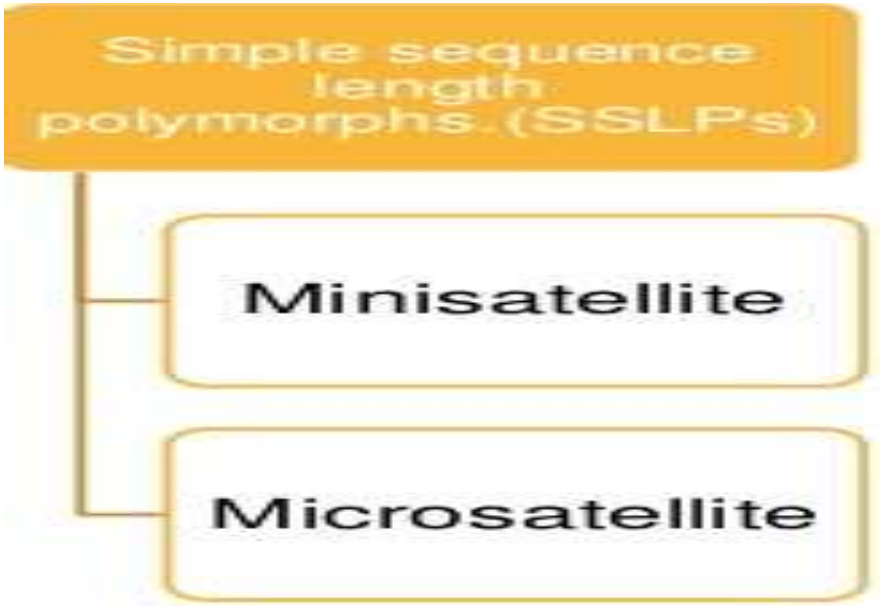
Expressed sequence tag (EST)

- a short sub-sequence of a cDNA sequence
- to identify gene transcripts
- instrumental in gene discovery and gene sequence determination
- approximately 74.2 million ESTs now available in public databases (GenBank 1 January 2013, all species).



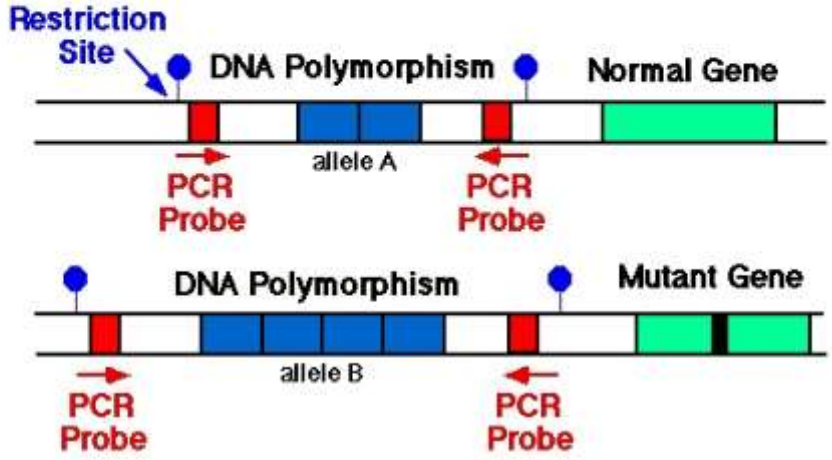
SIMPLE SEQUENCE LENGTH POLYMORPHS.(SSLPS)

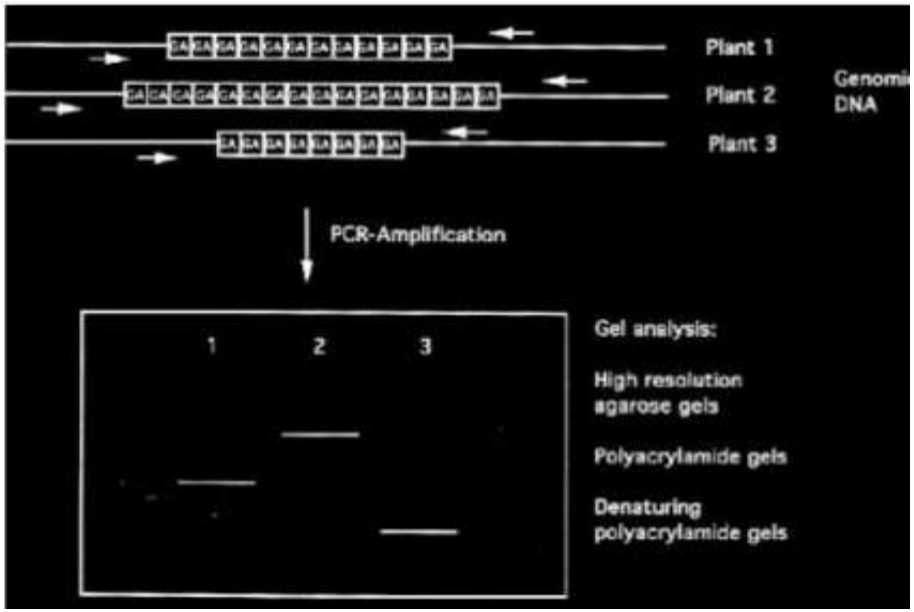
- SSLPs are array of repeating sequences, that display length of variations, different allele containing different numbers of repeat units. Unlike the RFLP SSLPs can be multi allelic as each SSLPs can have a number of different variable length..



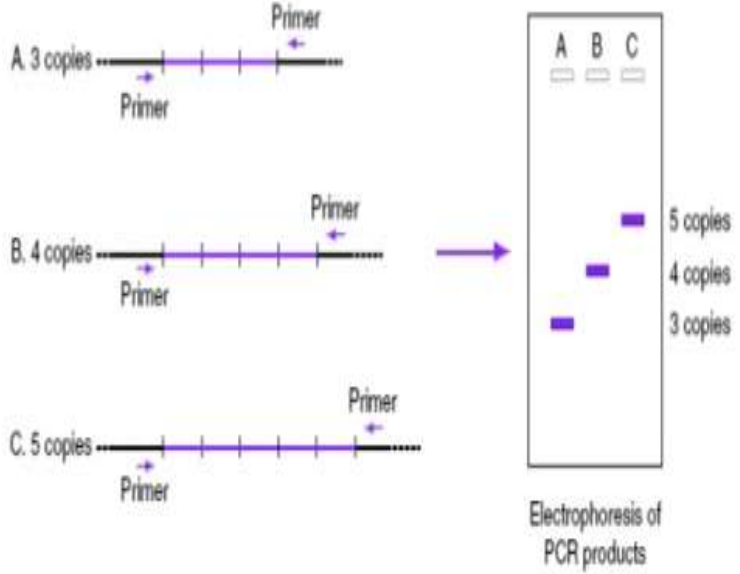
- **Minisatellite** : also known as varaible numbers of tandem repeats.(VNTRs) in which repeat units are upto 25 bp
- **Microsatelite** or simple tandem repeats (STRs): In which repeat units are upto 13bp or less.

MICROSATELLITE.....

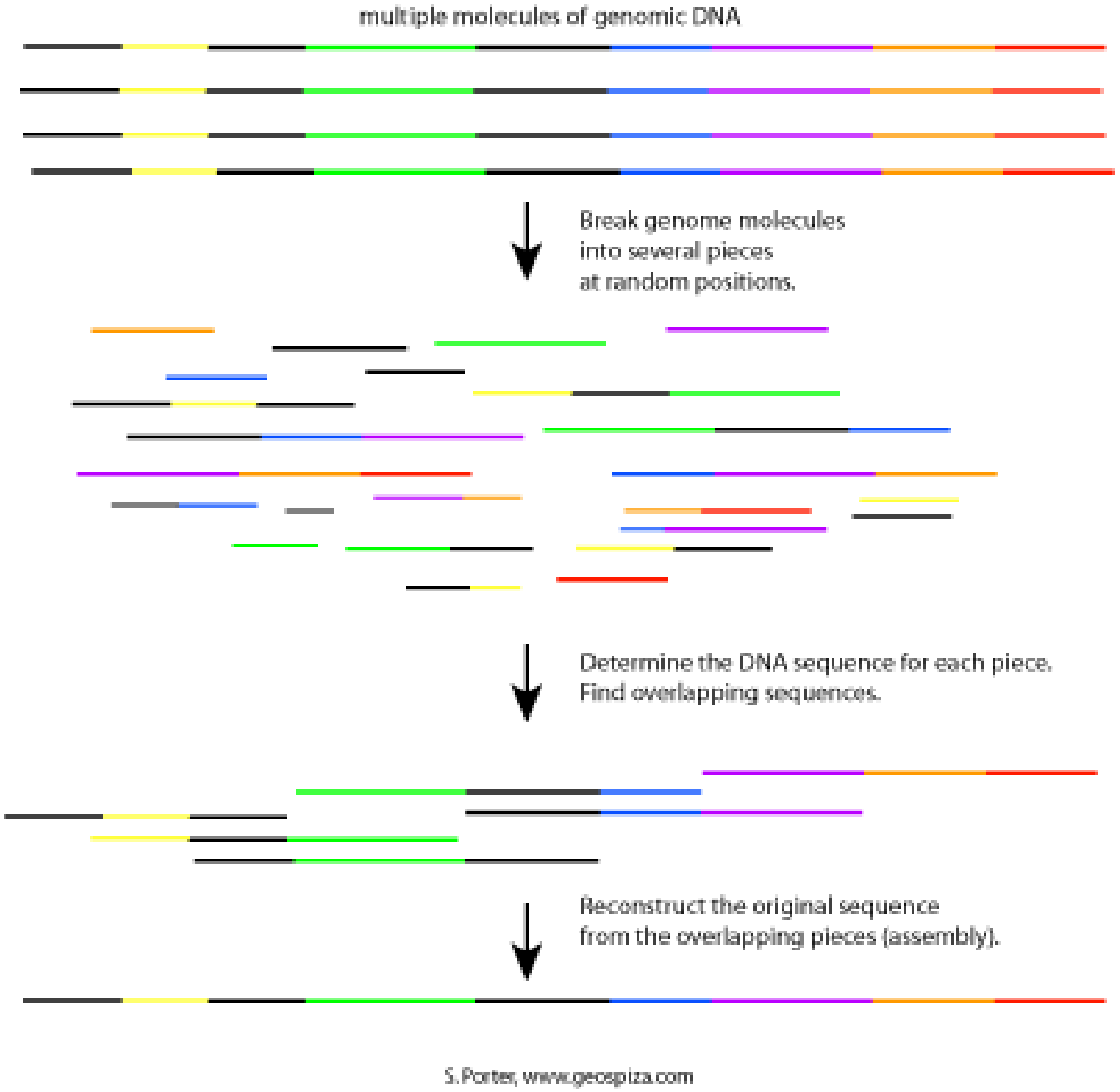




MINISATELLITE.....



Random genome sequence



Inter simple sequence repeat (ISSR)

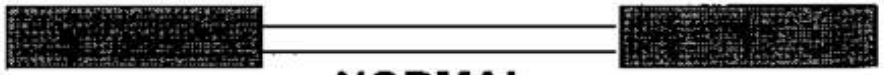
Inter simple sequence repeat (**ISSR**)-PCR is a technique, which involvesthe use of microsatellite **sequences** as primers in a polymerase chainreaction to generate multilocus markers

ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. **ISSRs** are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp).

Inter-simple sequence repeats (**ISSRs**) are regions in the genome flanked by microsatellite sequences. ... **ISSR markers** are easy to use, low-cost, and methodologically less demanding compared to other dominant **markers**, making it an ideal genetic marker for beginners and for organisms whose genetic information is lacking

INTER-SSR PCR

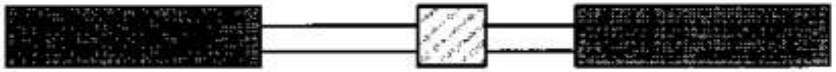
PCR PRODUCT



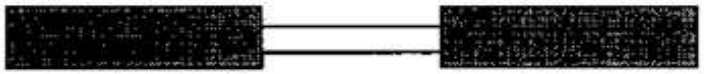
NORMAL

New Band Appearance

INSERTION



INTERNAL DELETION



TRANSLOCATION

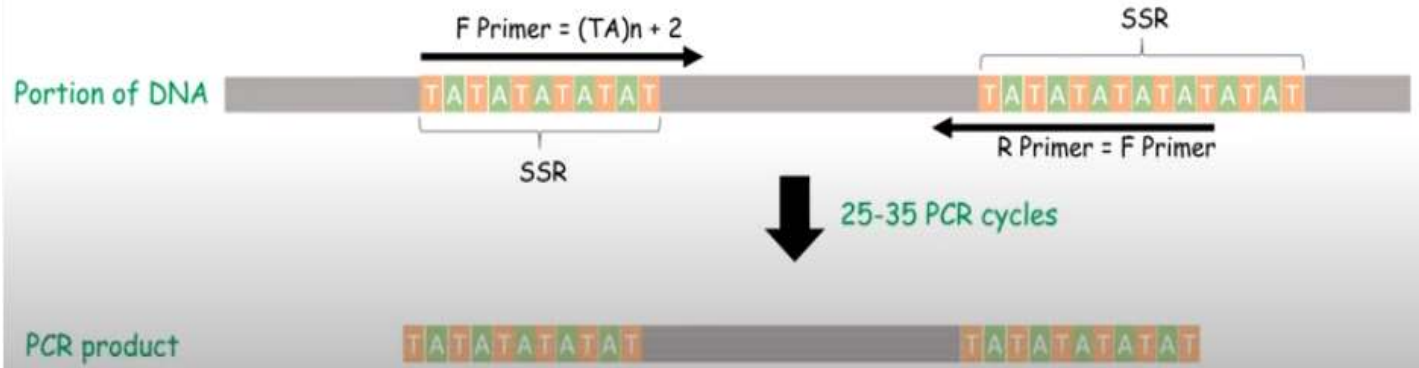
Band Disappearance

PRIMER SITE LOSS

TOTAL/PARTIAL CHROMOSOME LOSS

ISSR (Inter Simple Sequence Repeat)

- SSR are tandem repeats of 1-6 bp
- ISSR is **a single primer designed from SSR region** that act as forward and reverse primers.
- ISSR amplifies region between two SSR repeats using PCR.



ISSR variation

ISSR (Inter Simple Sequence Repeat)

Pros
Sequence information not essential
Random distribution
Environmentally stable
Less DNA
Technically easy

Cons
Dominant
Low Reproducibility
Locus non-specific

Start Codon Targeted (SCoT) Polymorphism analysis

- SCoT is a novel method for generating plant DNA markers.
- This method was developed based on the short conserved region flanking the ATG start codon in plant genes.
- SCoT uses single 18-mer primers in polymerase chain reaction (PCR) and an annealing temperature of 50°C.
- PCR amplicons are resolved using standard agarose gel electrophoresis.
- This method was validated in rice using a genetically diverse set of genotypes and a backcross population.

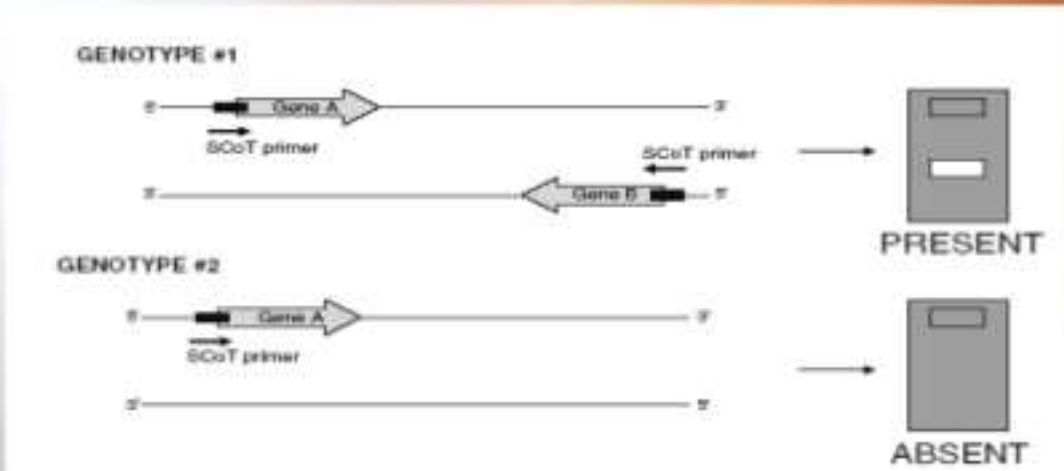


Diagram showing principle of SCoT PCR amplification

Molecular diagnostics

(From internet)

Molecular diagnostics is a collection of techniques used to analyse biological markers in the genome and proteome—the individual's **genetic** code and how their cells express their genes as proteins—by applying **molecular** biology to medical testing.

- **Establishment of a good diagnostic laboratory for rapid and reliable diagnosis to ensure proper preventive and control measures of infectious diseases in livestock is a necessary step.**
- **The most authentic diagnosis of diseases is made by isolation and identification of the pathogen. However, it is time consuming, tedious and sometimes requires living medium.**
- **The development of various immunological and molecular techniques has revolutionized diagnostic procedures by providing specific diagnosis or detailed characterization of any pathogen or host pathogen interactions.**
- **Some of the recent immunological and molecular techniques employed in addition to the conventional ones for the diagnosis.**

Strategy : specific, sensitive and simple

Conventional :False positive/ negative
unable to discriminate pathogen/ non pathogen
Long term culture

Immunological techniques : Conventional immunoasays for the diagnosis of diseases have been based on the detection of antibody to the pathogen of interest, using techniques such as virus neutralization, enzyme-linked immunosorbant assay (ELISA), complement fixation and agar gel immunodiffusion. These assays generally rely on the interaction of serum polyclonal antibodies against the agent of interest, followed by the use of a detection system such as the cytopathic effect, haemolysis or colour change of a reaction medium.

The **serum virus neutralization** (SVN) **assay** is a serological **test** used to detect the presence and magnitude of functional systemic antibodies that prevent infectivity of a **virus**.

Virus neutralization is a specialized type of immunoassay because it does not detect all antigen–antibody reactions. It only detects antibody that can block virus replication. This is important because related groups of viruses may share common antigens, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually based on virus neutralization (although this is not always specified). For example, there are three major poliovirus serotypes (neutralization serotypes). In order to protect against poliovirus infection, a successful vaccine must induce neutralizing antibodies to poliovirus Types 1, 2, and 3.

VIRUS NEUTRALIZATION TEST

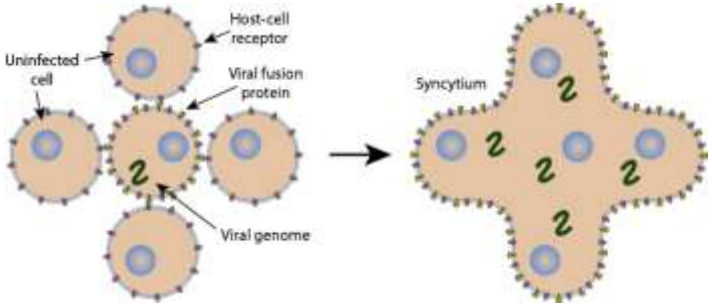
Introduction

- Serological method that detects the presence of viral neutralizing antibodies.
  - The antibodies bind to the viral particles
    - Monoclonal and polyclonal antibodies
- Block viral infectivity-No cell infection

- VNT are conducted by :
  - mixing dilutions of antibodies with standardized amount of virus, incubating them and cultured into cells, eggs or animals to have a clear cytopathic effect observation

- CPE effects:
  - Rounding of cells
  - Change in texture-granular/hyaline/glassy
  - Formation of syncytium

syncytium
a single cell or cytoplasmic mass containing several nuclei, formed by fusion of cells or by division of nuclei.



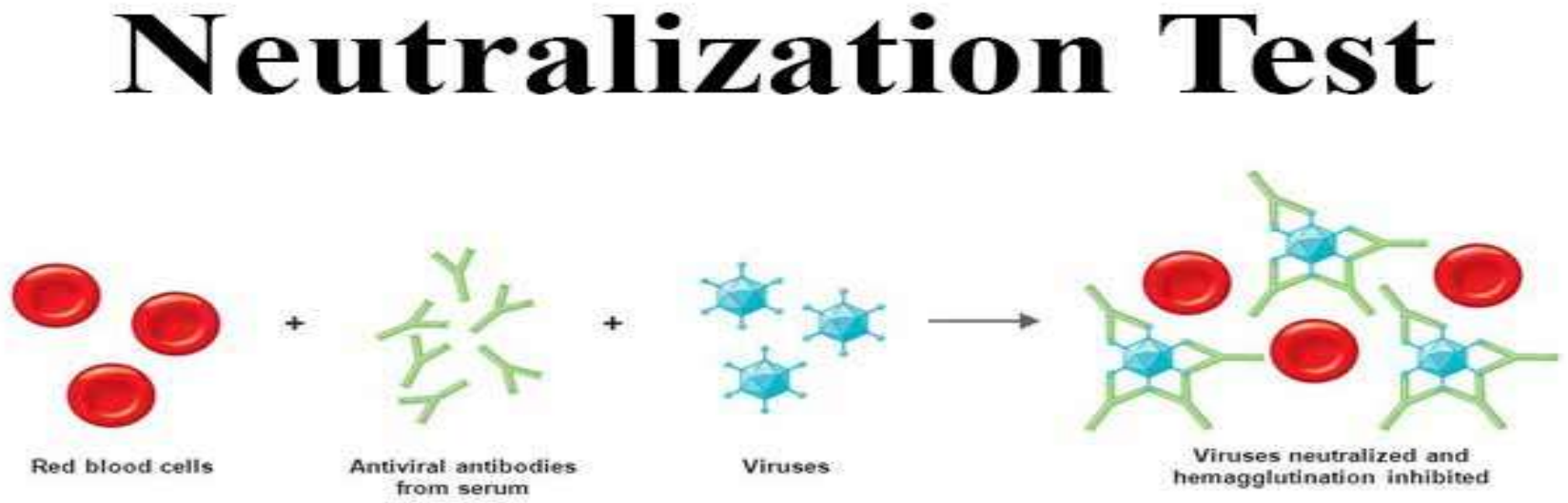
Syncytia is formed by fusion of an infected cells with neighboring cells leading to the formation of multi-nucleate enlarged cells. This event is induced by surface expression of viral fusion protein that are fusogenic directly at the host cell membrane

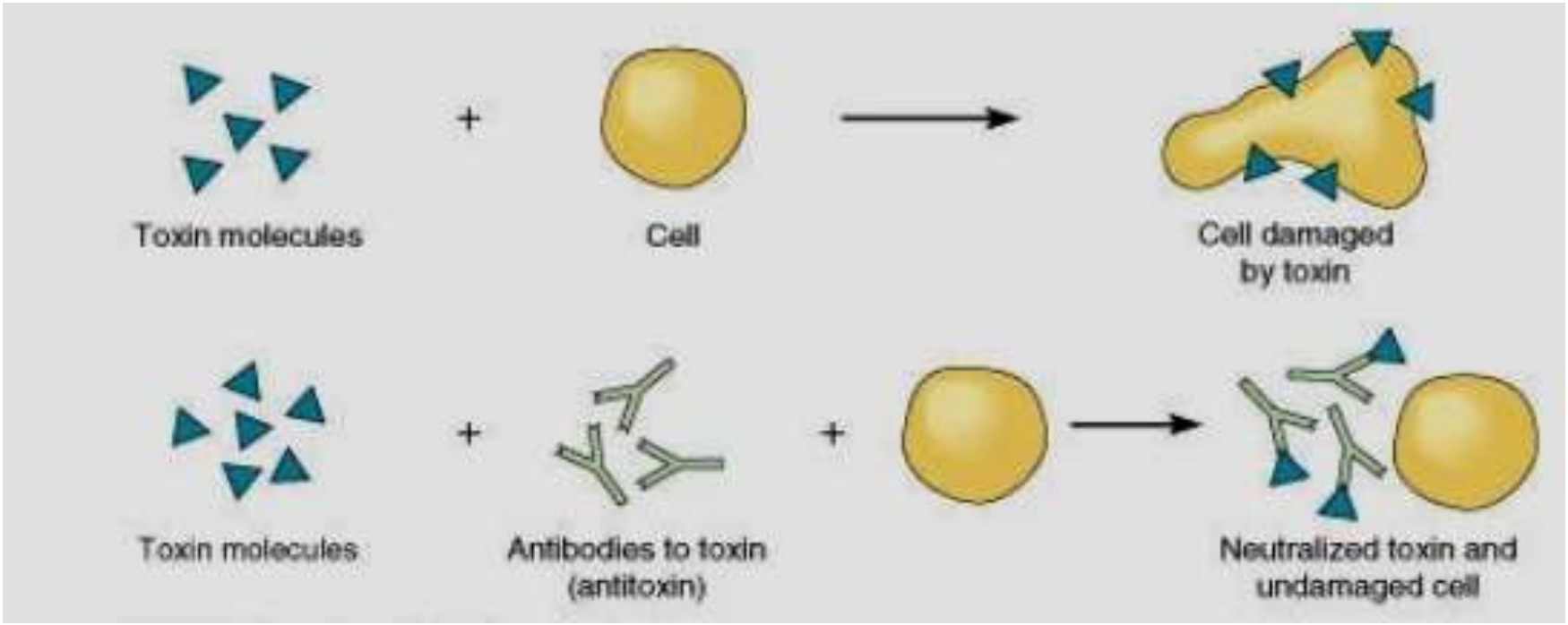
Hyaline: any of several translucent nitrogenous substances related to chitin, found especially around cells, and readily stained by eosin.

What is cytopathic effect of CMV virus?
The early cytopathic effect (ECE) consists of the **rounding of infected fibroblasts**, whereas the late cytopathic effect (LCE) is characterized by the appearance of granular or dense intracytoplasmic and intranuclear inclusion bodies, as well as by an increased cell volume (Gandhi and Khanna, 2004; Sekhon et al., 2004)

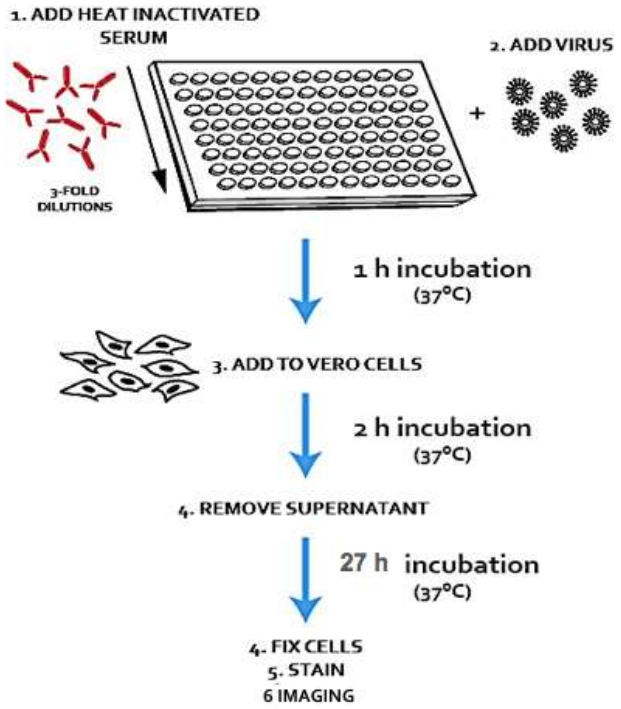
Virus neutralization is a specialized type of immunoassay because it does not detect all antigen–antibody reactions. It only detects antibody that can block **virus** replication.

By binding specifically to surface structures (antigen) on an infectious particle, **neutralizing antibodies** prevent the particle from interacting with its host cells it might infect and destroy




Toxin molecules
+
Cell
Cell damaged by toxin
Toxin molecules
+
Antibodies to toxin (antitoxin)
+
Neutralized toxin and undamaged cell

The microneutralization assay



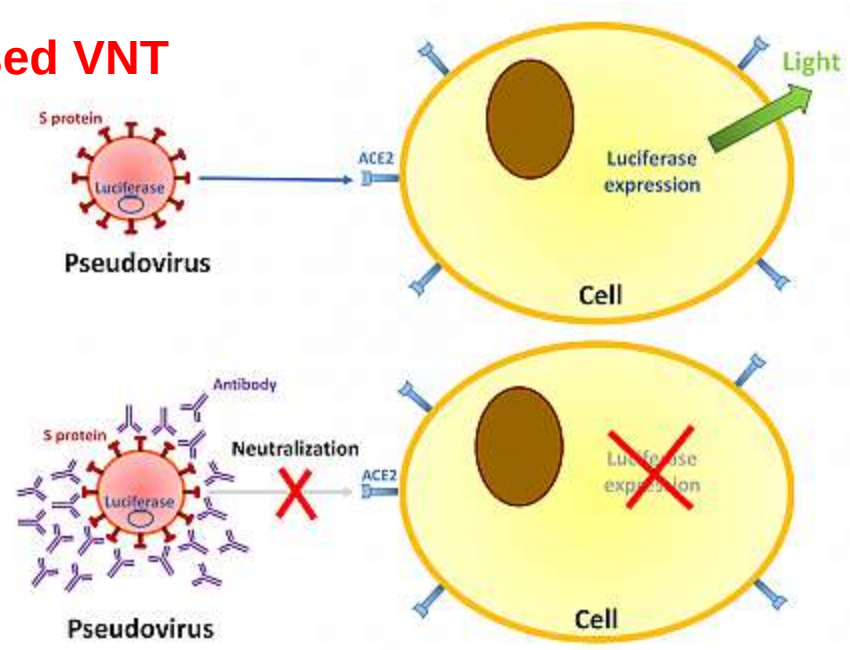
a serial dilution of heat-inactivated serum is mixed with a fixed concentration of virus, and the two are then added to permissive cells for 1 hour before removing. A few days later, the dilution of antibodies that neutralized 50% of the infection is scored, and comparisons can be made between different sera. In these assays it is important that the concentration of virus is kept low enough to be able to detect infection but without saturating the amount of antibodies in the serum.

Research laboratories and pharmaceutical companies are racing to produce antibody tests that can detect COVID-19 infection with sufficient specificity and sensitivity.
There are two types of antibody test one can aim for. The first type is the conventional virus neutralization test (cVNT), which detects neutralizing antibodies (NAbs) in a patient's blood. The cVNT requires handling live SARS-CoV-2 in a specialized biosafety level 3 (BSL3) containment facility and is tedious and time-consuming, taking 2–4 days to complete.

The pseudovirus-based VNT (pVNT), on the other hand, can be performed in a BSL2 laboratory, but still requires the use of live viruses and cells.

A surrogate VNT (sVNT) that detects NAbs, without the need for any live virus or cells, that can be completed in 1–2 h in a BSL2 laboratory. Using purified receptor-binding domain (RBD) from the S protein and the host cell receptor ACE2, our test is designed to mimic the virus–host interaction in an ELISA plate well. This RBD–ACE2 interaction can be neutralized (that is, blocked) by specific NAbs in patient or animal sera, in the same manner as in cVNT or pVNT

The pseudovirus-based VNT



Pseudotypes or pseudotype particles are chimeric “viruses” consisting of a virus core (typically a lentiviral vector) surrounded by a lipid envelope with the surface glycoproteins of another virus (the virus of interest). By using a vector which can’t replicate itself and is hence not pathogenic, viruses can be studied in a safer system. In the case of SARS-CoV-2, the pseudovirus has to express the S glycoprotein, which mediates entry into the host cells by binding to human angiotensin-converting enzyme 2 (ACE2). In addition to the surface glycoproteins of the virus of interest, the pseudotyped virus contains the gene of a luciferase, which is only expressed after entering the cell. The more pseudoviruses enter the cells, the more luciferase is expressed, and the higher the intensity of emitted light.

A surrogate VNT (sVNT)

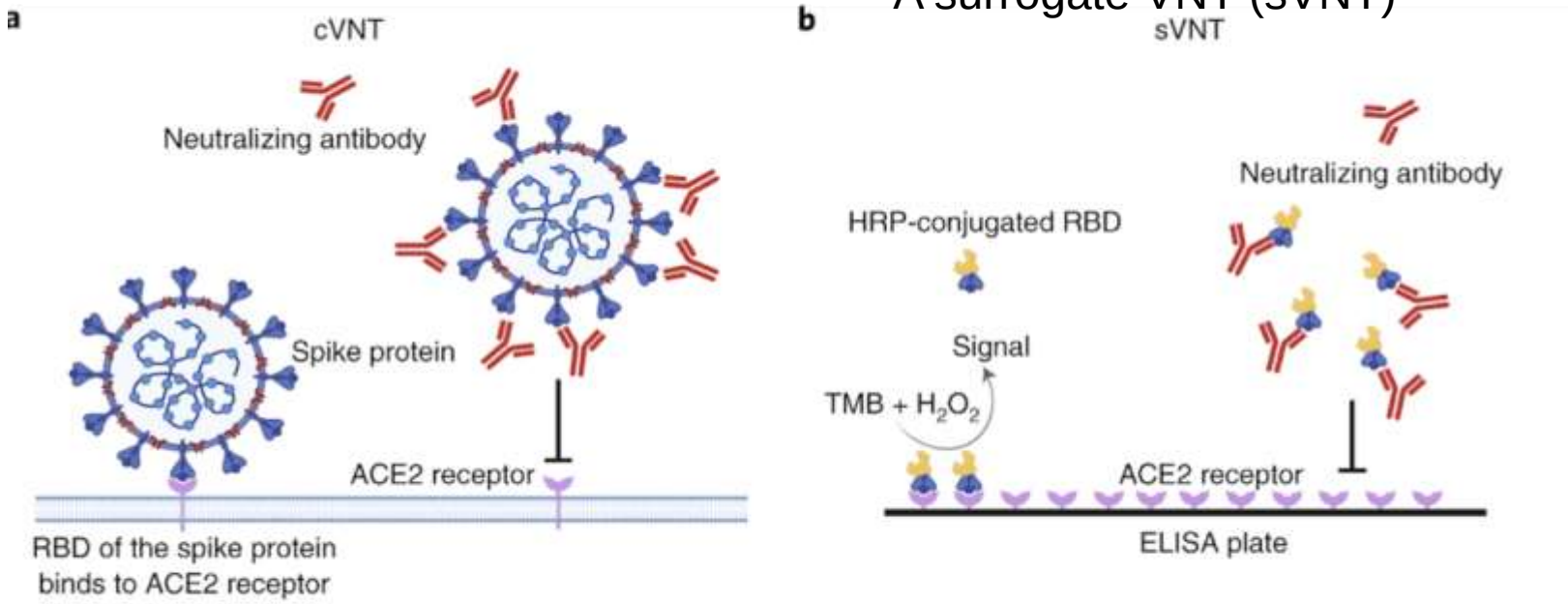


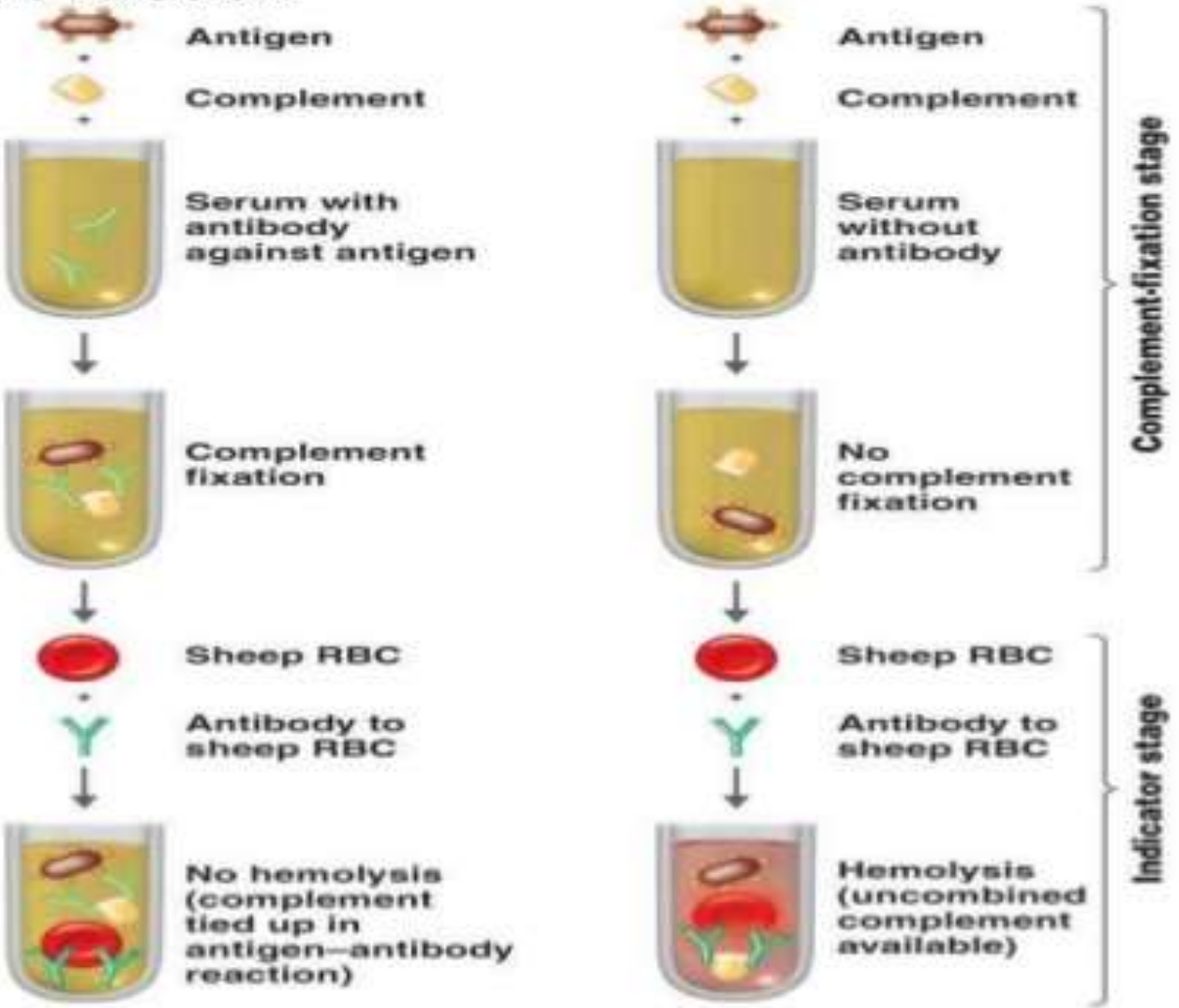
Fig. 1: Principle and initial validation of the SARS-CoV-2 sVNT. a, The mechanism of cVNT. Anti-SARS-CoV-2 neutralizing antibodies block the SARS-CoV-2 spike protein from binding to hACE2 receptor proteins on the host cell surface. b, In the sVNT assay, anti-SARS-CoV-2 neutralizing antibodies block HRP-conjugated RBD protein from binding to the hACE2 protein pre-coated on an ELISA plate. The illustrations were created using BioRender. The data presented are the mean of two independent experiments.

TMB (3,3′,5,5′-Tetramethylbenzidine)

The **complement fixation test** is a blood **test** in which a sample of serum is exposed to a particular antigen and **complement** in order to determine whether or not antibodies to that particular antigen are present. The nature of **complement** is to react in combination with antigen–antibody complexes

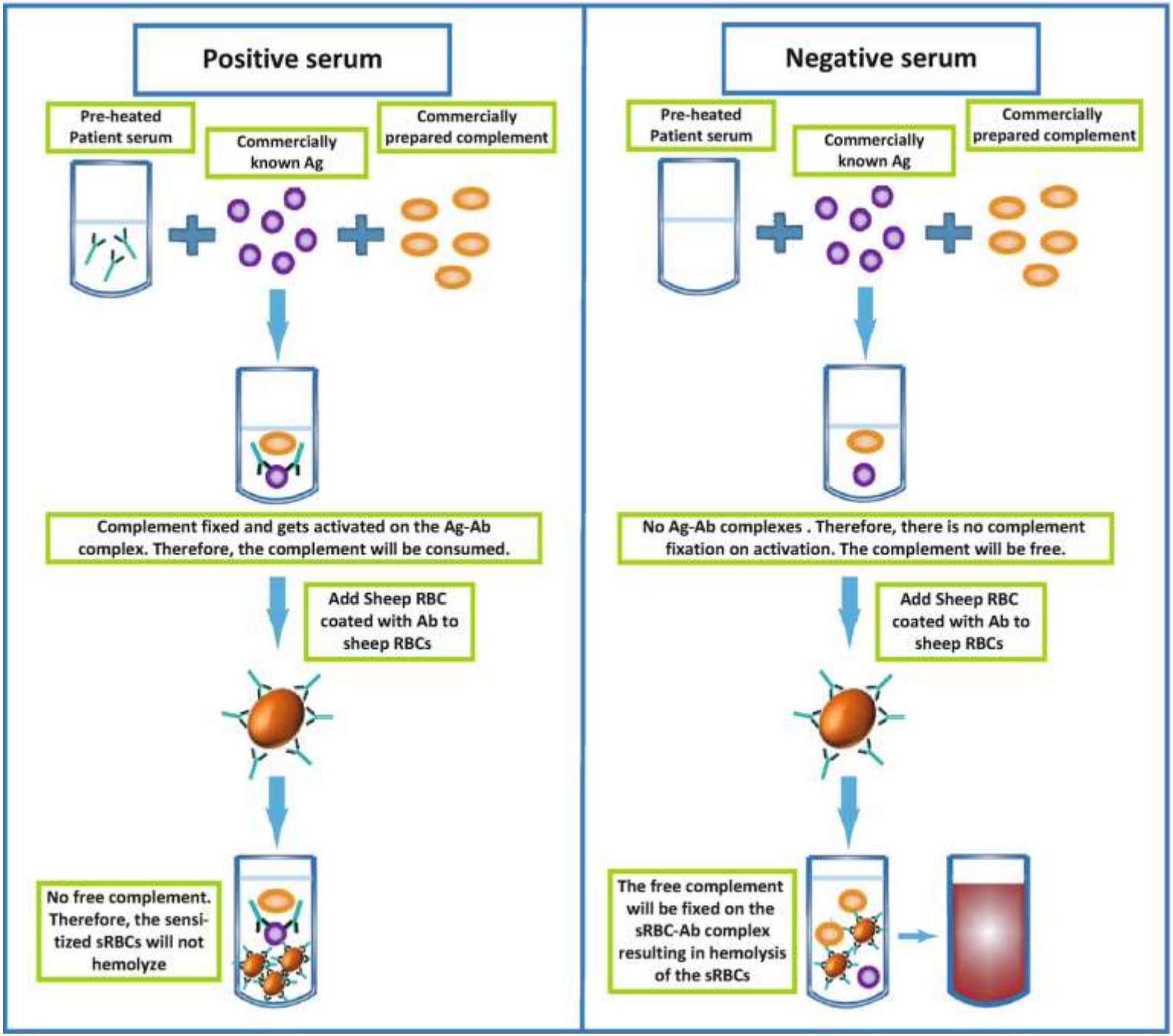
The **complement fixation test** is an immunological medical **test** that can be used to detect the presence of either specific antibody or specific antigen in a patient's serum, based on whether **complement fixation** occurs.

Complement Fixation

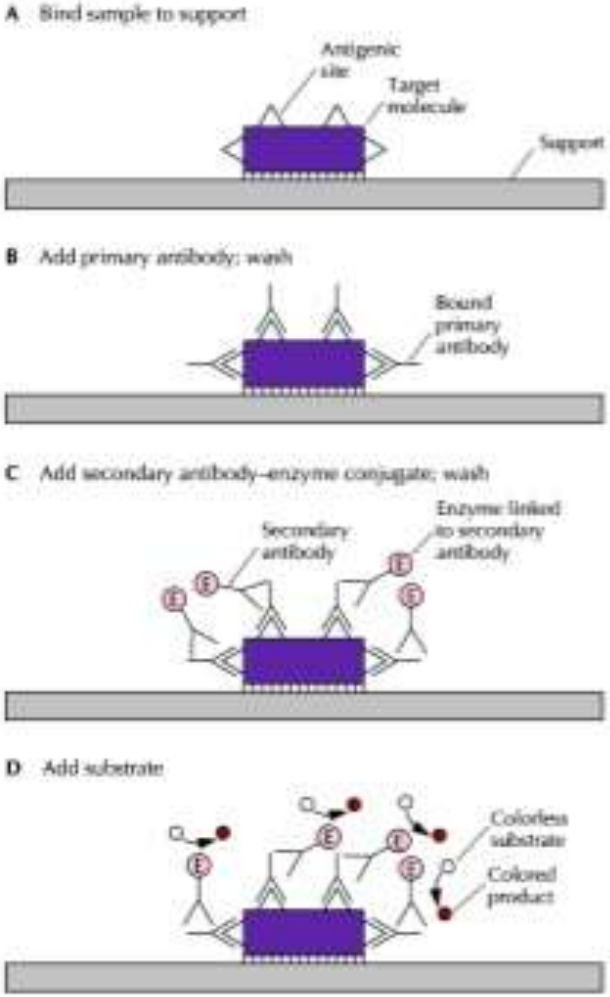


(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

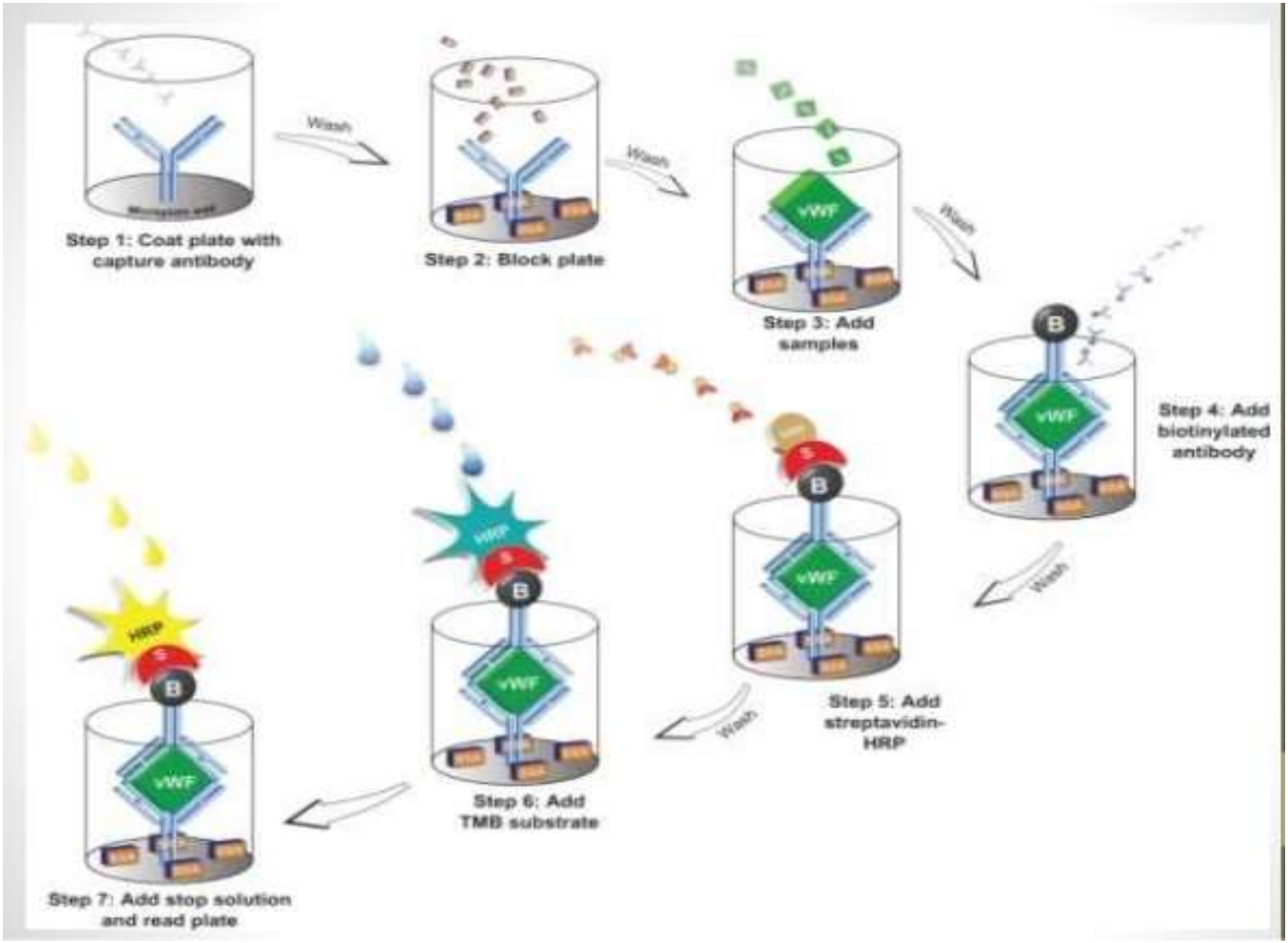

Positive serum
Pre-heated Patient serum
Commercially known Ag
Commercially prepared complement
Complement fixed and gets activated on the Ag-Ab complex. Therefore, the complement will be consumed.
Add Sheep RBC coated with Ab to sheep RBCs
No free complement. Therefore, the sensi-tized sRBCs will not hemolyze
Negative serum
Pre-heated Patient serum
Commercially known Ag
Commercially prepared complement
No Ag-Ab complexes . Therefore, there is no complement fixation on activation. The complement will be free.
Add Sheep RBC coated with Ab to sheep RBCs
The free complement will be fixed on the sRBC-Ab complex resulting in hemolysis of the sRBCs

Immunological Diagnostics Methods - ELISA



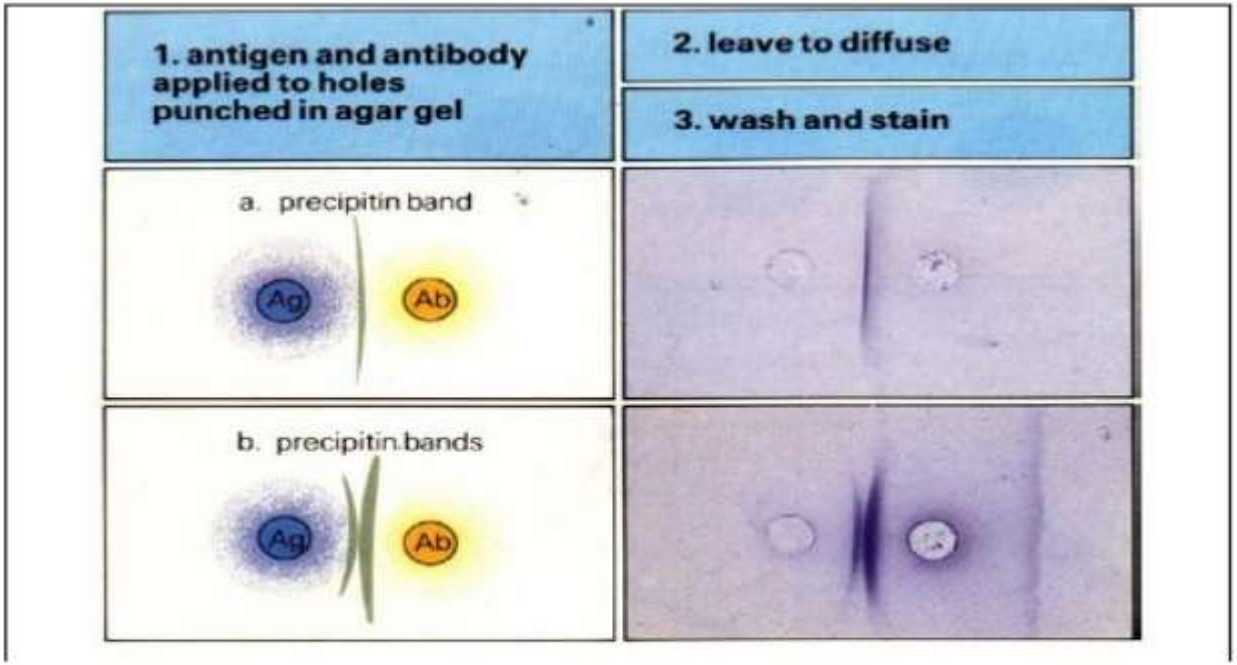
- Addition of a **specific antibody** (primary antibody) which will bind to the test molecule if it is present.
- **Washing** to remove unbound molecules.
- Addition of **secondary antibody** which will bind to the primary antibody.
- The secondary antibody usually has attached to it an **enzyme** e.g. **alkaline phosphatase**.
- **Wash** to remove unbound antibody.
- Addition of a **colourless substrate** which will react with the secondary antibody to give a **colour reaction** which indicates a positive result.

-> can be used for quasi High-throughput!!!


Step 1: Coat plate with capture antibody
Wash
Step 2: Block plate
Wash
vWF
Step 3: Add samples
Wash
B
vWF
Step 4: Add biotinylated antibody
Wash
B
vWF
Step 5: Add streptavidin-HRP
Wash
HRP
B
vWF
Step 6: Add TMB substrate
HRP
B
vWF
Step 7: Add stop solution and read plate

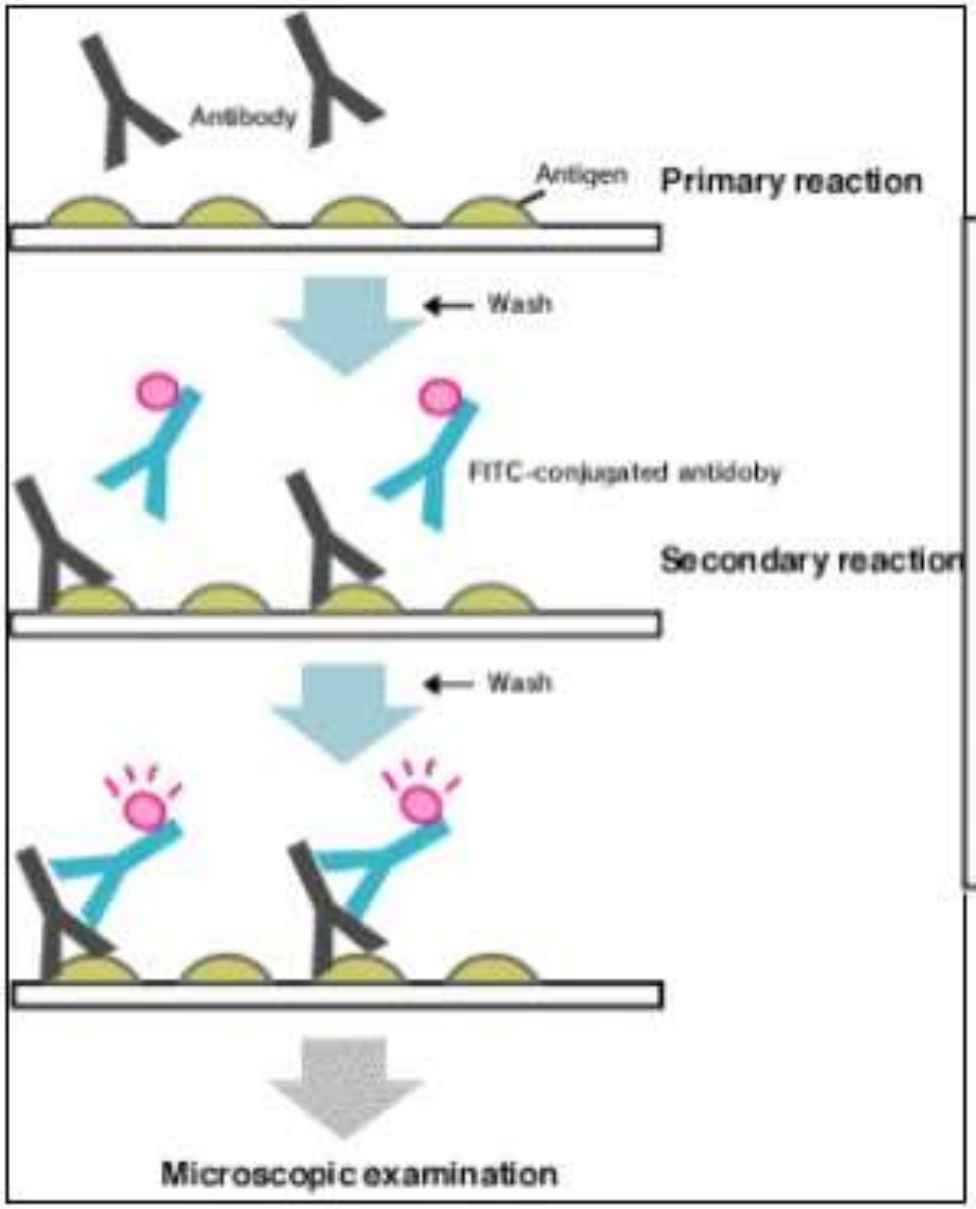
Ouchterlony Double Diffusion

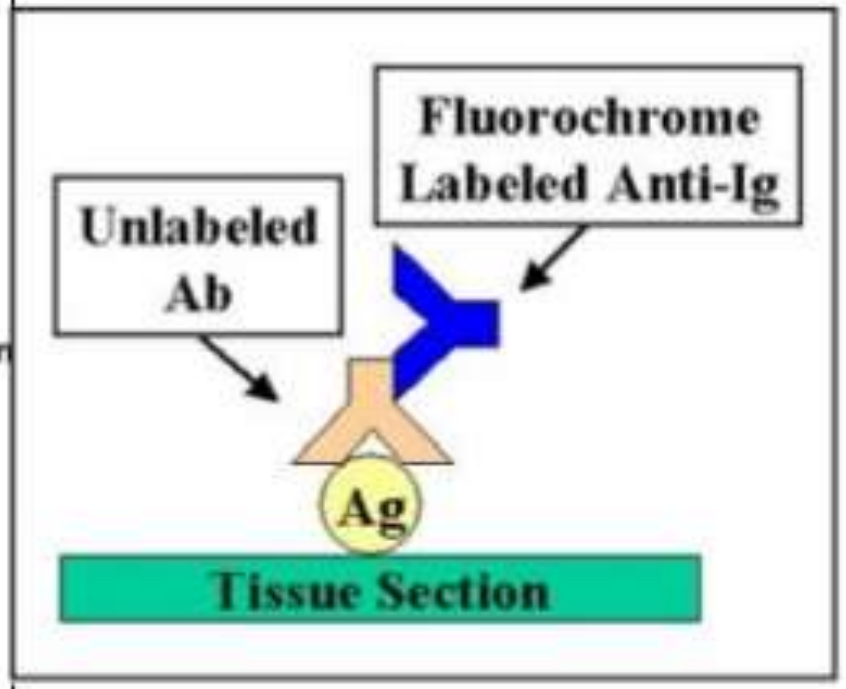
- Antigen and antibody diffuse independently through a semi solid medium (agar)

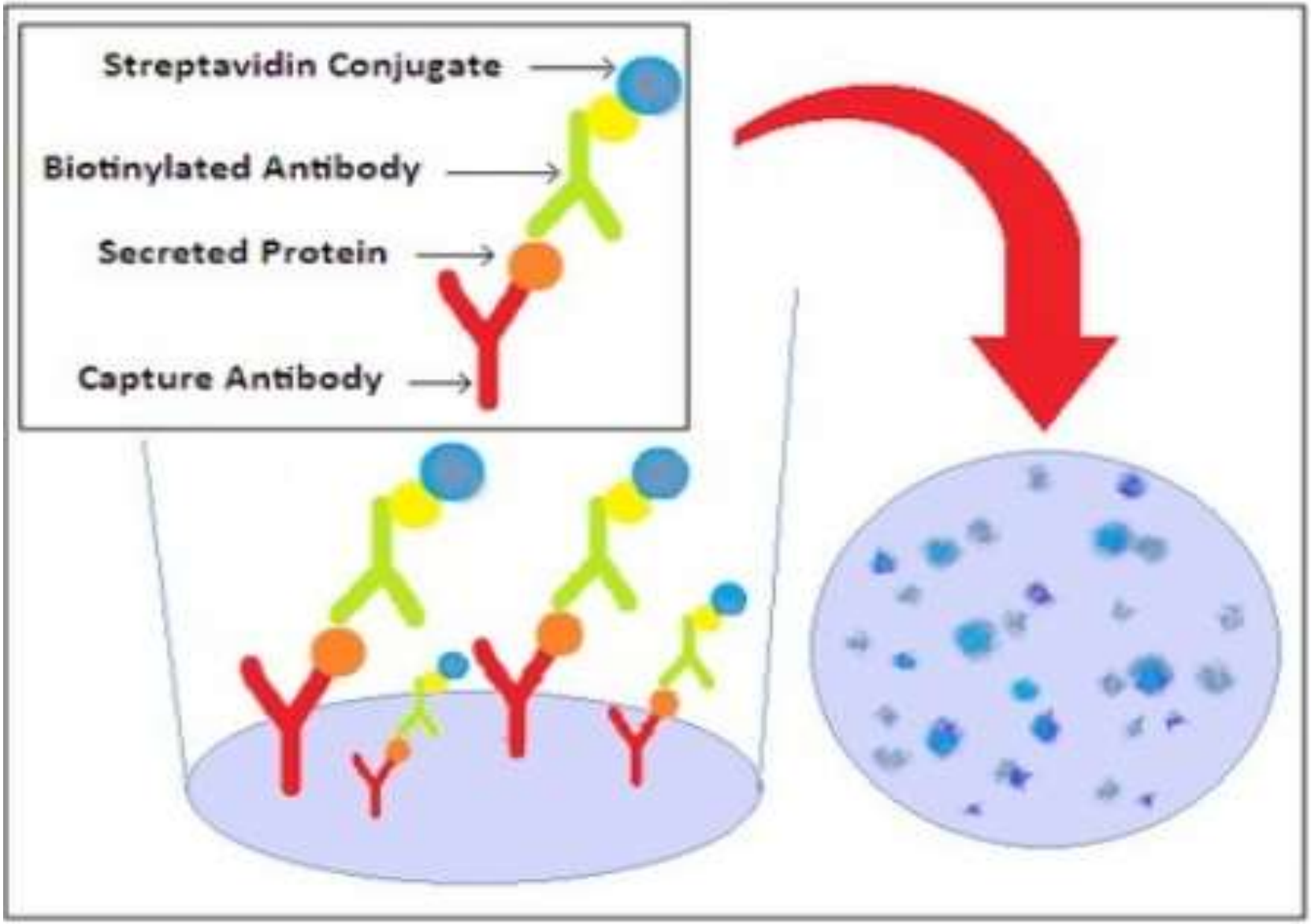


The density of the line reflects the amount of immune complexes formed

Fluorescent antibody technique (FAT)






Streptavidin Conjugate
Biotinylated Antibody
Secreted Protein
Capture Antibody

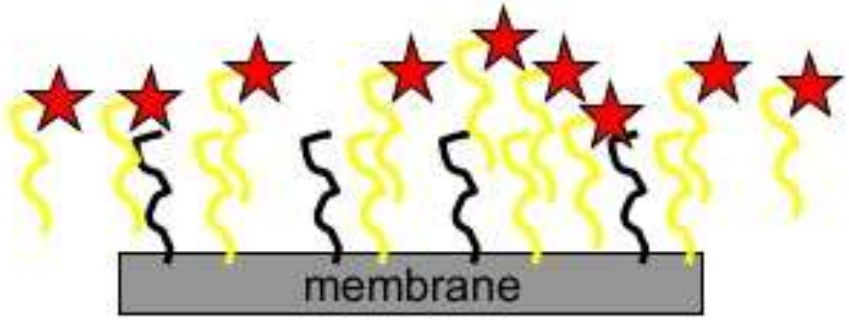
Flow cytometry

HIV INFECTION

- Determination of the numbers of CD4 +ve lymphocytes in the peripheral blood is used to monitor patients with HIV infections.
- The percentage of CD4 +ve cells can be obtained in a single tube by staining for CD45/CD3/CD4.
- A cytogram of SS versus CD45 is used to identify the lymphocytes and a cytogram of CD4 versus CD3 to enumerate the CD4+ve T cells.
- An extended panel is used to obtain a more complete picture of the peripheral blood lymphocytes.
- The absolute number of CD4 +ve cells is the clinically relevant parameter for a discussion on counting cells.

DNA diagnostic systems

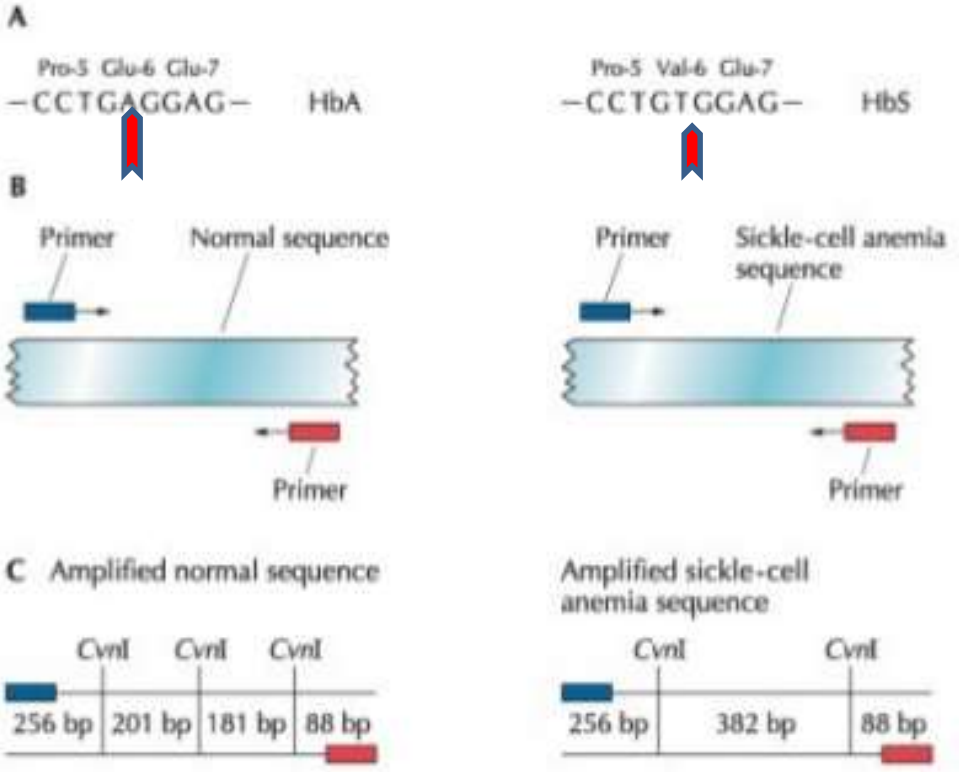
1. Bind ssDNA (target) to membrane
2. Hybridize to labeled ssDNA or RNA (probe)
3. Wash membrane to remove unbound probe
4. Detect hybrid sequences formed between the probe and target DNA (concern: false +s & -s)

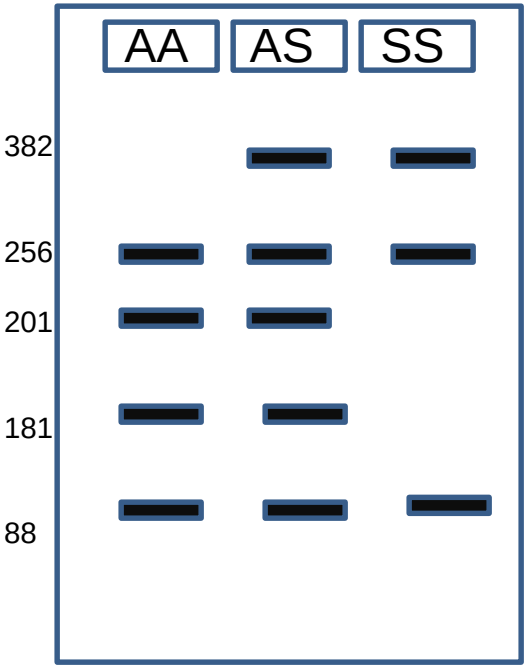


Molecular Diagnosis of Genetic Disease

- Cystic fibrosis

Sickle-cell anemia





What is cystic fibrosis (CF)?

- A multisystem disease
- Autosomal recessive inheritance
- Cause: mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene
  - chromosome 7
  - codes for a c-AMP regulated chloride channel

CFTR controls chloride ion movement in and out of the cell.

CFTR Sequence:

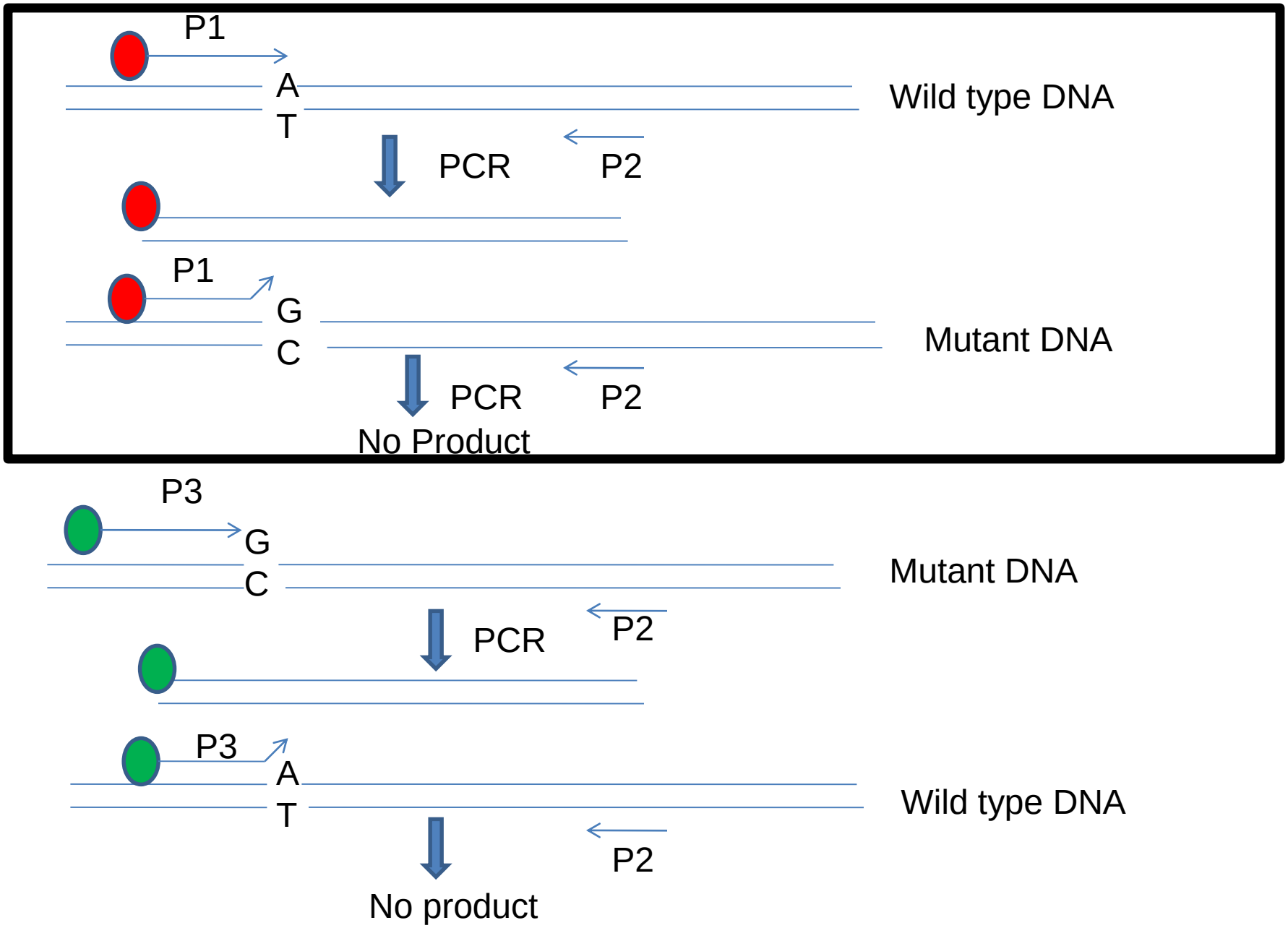
Nucleotide	A	T	C	A	T	C	T	T	T	G	G	T	G	T	T
Amino Acid	Ile			Ile			Phe			Gly			Val		
	506						508						510		

Deleted in ΔF508

ΔF508 CFTR Sequence:

Nucleotide	A	T	C	A	T	T	G	G	T	G	T	T
Amino Acid	Ile			Ile			Gly			Val		
	506											

Detection of single base mutation/ multiple



DNA based diagnosis of Malaria and *Typanosoma cruzi*

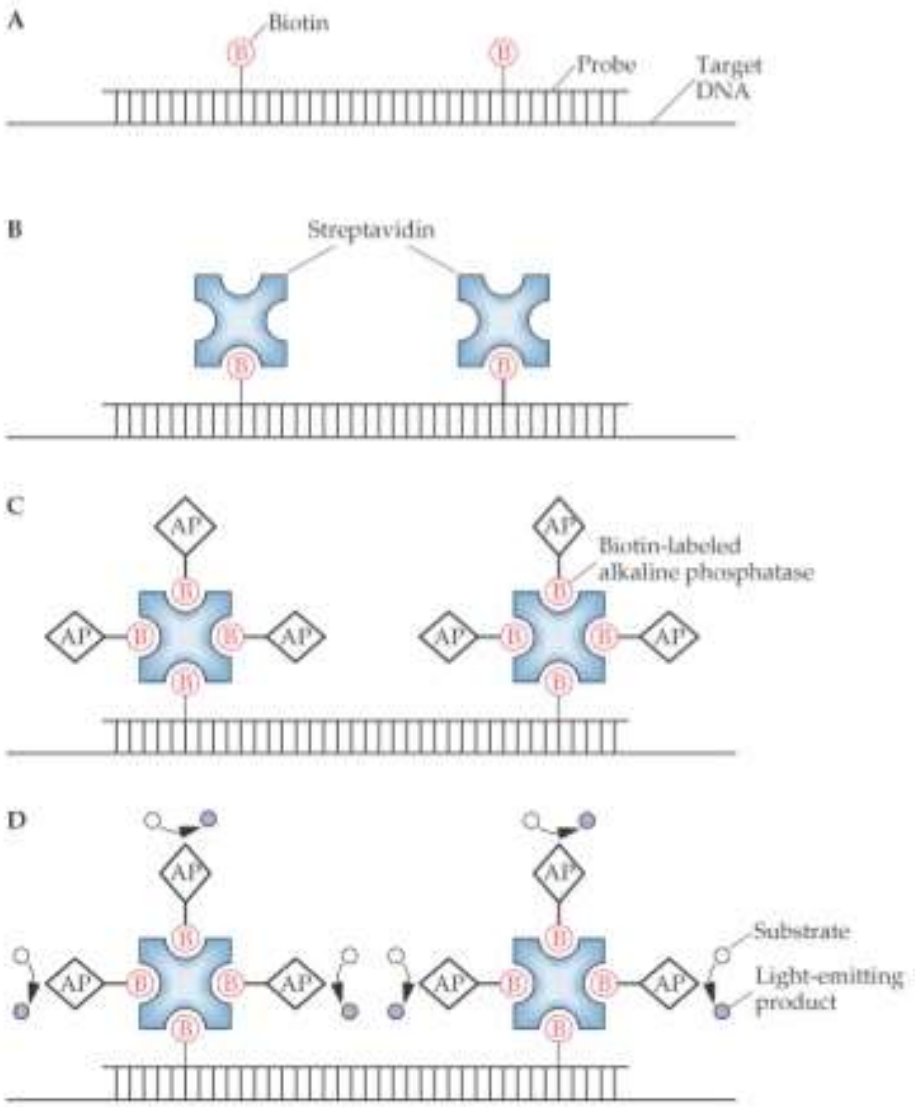
1. A DNA probe from a highly repeated DNA sequence of *Plasmodium falciparum*, the parasite that causes malaria, is used to screen blood samples via hybridization assays
2. DNA primers are made against the ends of a 188 bp repeated sequence contained in the protozoan parasite *Typanosoma cruzi*, the causative agent of Chagas disease and used in a PCR/polyacrylamide gel electrophoresis detection method

- Other examples of DNA-based detection: *Salmonella typhi* (food poisoning), certain *E. coli* (gastroenteritis), *Mycobacterium tuberculosis* (tuberculosis), etc.

Nonradioactive Hybridization Procedures

- Use of biotin-labeled nucleotides in DNA probes instead of ^{32}P, then add avidin (streptavidin) which binds to biotin, and then add biotin attached to an enzyme like alkaline phosphatase for detection

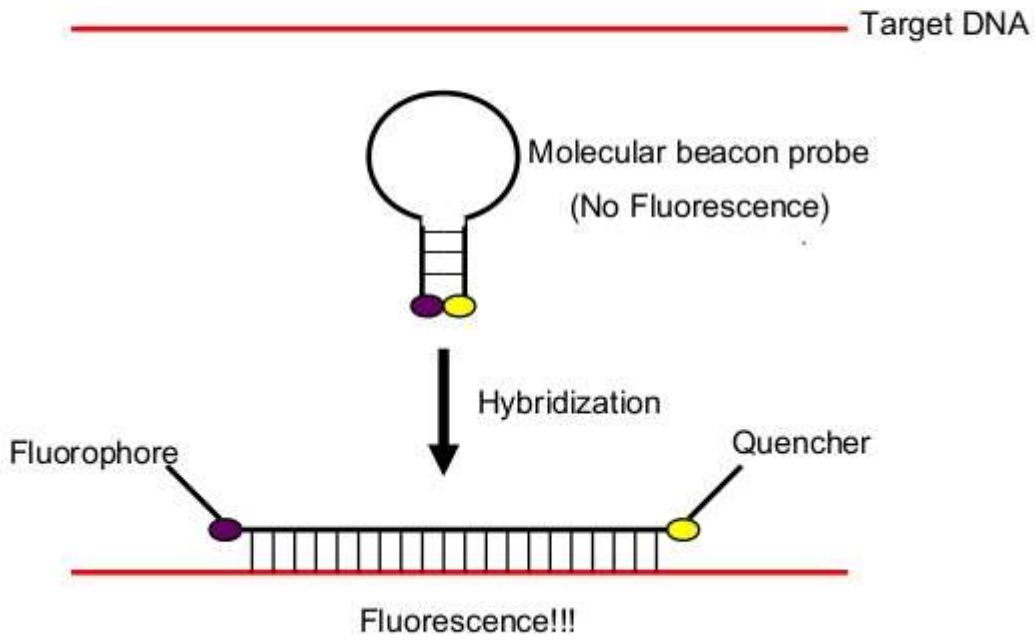
- Note that fluorescent dyes can also be attached to DNA primers for detecting amplified DNA products

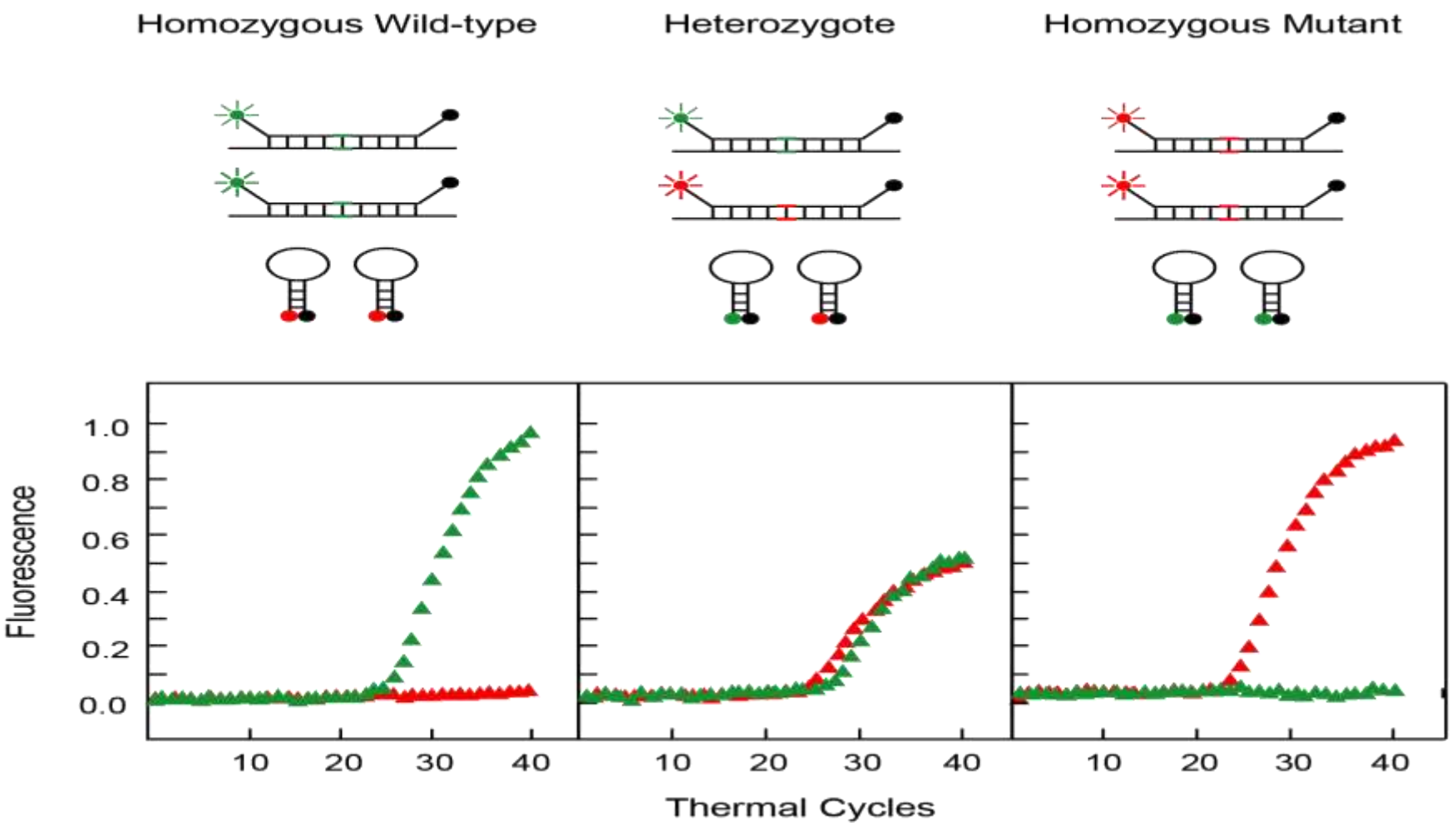


Nonradioactive Hybridization Procedures

In case of lack of
Hybridization probes
Are washed away hence
No signal

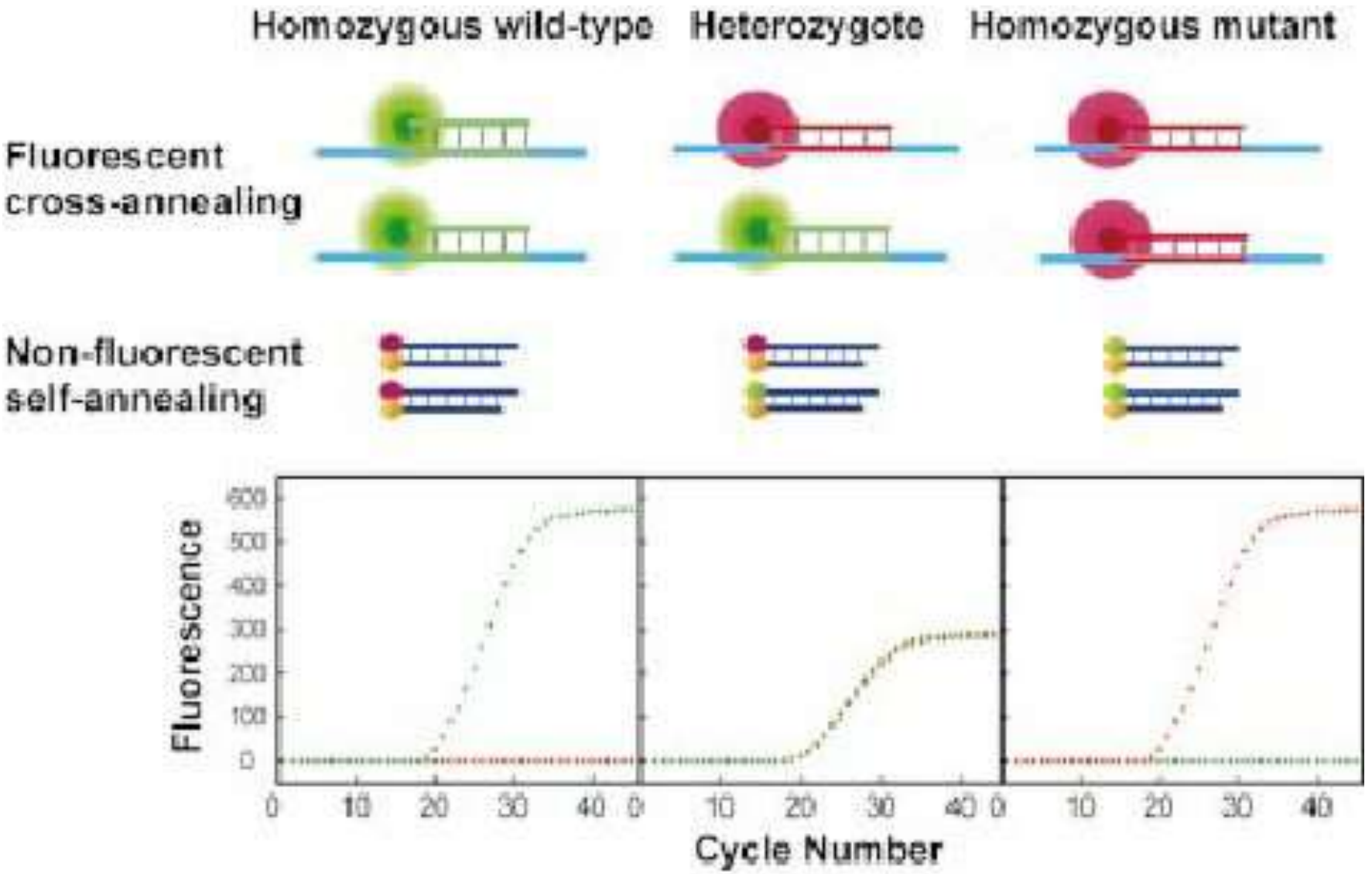
Fig. 9.13 Nonradioactive Hybridization Procedures: Molecular Beacons





to determine the genotype of an individual at a particular locus, the genetic region of interest is amplified in the presence of two different molecular beacons, one perfectly complementary to the wild-type allele and labeled with a fluorophore of a particular color, and the other perfectly complementary to the mutant allele and labeled with a differently colored fluorophore. If the assay results in the generation of only the first fluorescent color, then the individual is homozygous wild type at that locus. If the assay results in the generation of only the other fluorescent color, then the individual is homozygous mutant. And finally, if both fluorescent colors are produced, then the individual is heterozygous.

Principle of real-time PCR genotyping



DNA Fingerprinting & Forensics

- History
- Uses of DNA Profiling
- Hypervariable DNA sequences examined (RFLPs, VNTRs, STRs, SNPs, mitochondrial DNA, Y chromosomal DNA)
- Methods (Southerns & PCR)
- Statistical considerations
- Technical considerations
- Databases and Privacy

DNA Fingerprinting

- You're 99.9% identical
- But of course, you are unique--in a genome of three billion letters, even a 0.1 % difference translates into three million differences.
- These differences (or polymorphisms) reside in several places in the genome, often in microsatellites
- Examples of such polymorphisms include VNTRs, STRs, RFLPs and SNPs
  - Variable number tandem repeats
  - Short Tandem Repeats
  - Restriction fragment length polymorphism
  - Single Nucleotide Polymorphism

Preparation of a DNA fingerprint
Step 1

- Specimen collection
  - blood, semen, etc
  - Easy to contaminate a DNA sample with DNA from other sources (bacteria, DNA of person collecting sample)
  - DNA is not stable for very long-it degrades
    - sunlight
    - heat
    - moisture

Step 3

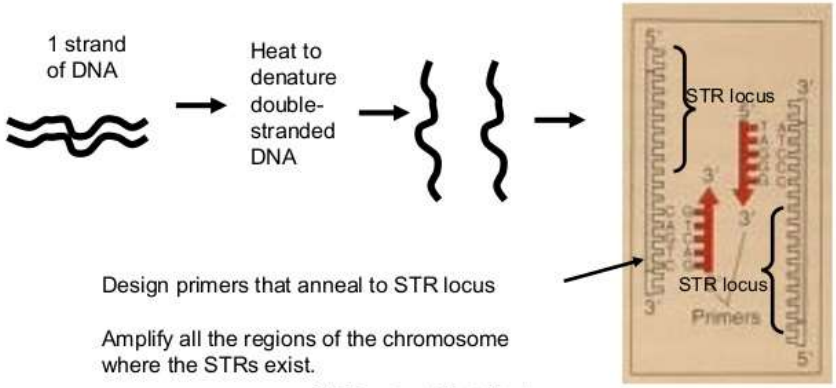
- PCR using primers targeting STRs at different loci
- PCR amplify STRs using target sites on chromosome

- DNA fingerprinting is a comparative process:
  - DNA from crime scene is compared with DNA of a suspect
  - So minimum of two samples must be prepared

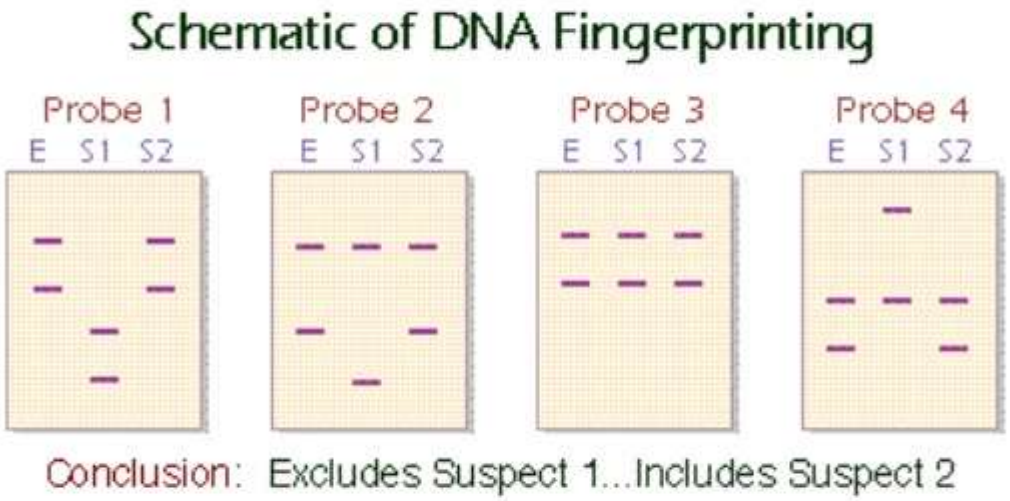
Step 2

- DNA extraction
  - standardized methods have been developed
  - need to separate DNA from other cell material and debris from crime scene.

Step 3
PCR amplification of DNA



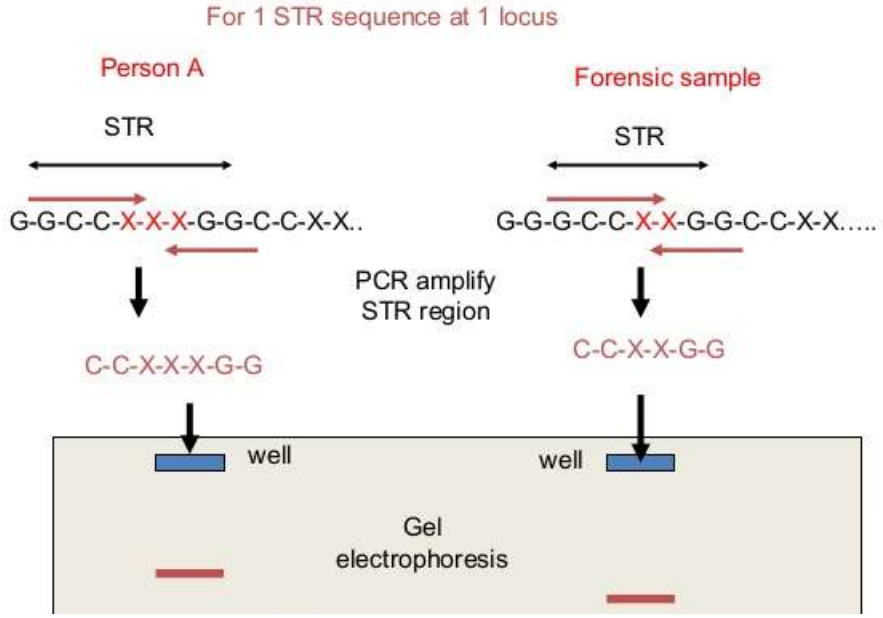
Example



E – reference sample, S1 – suspect 1 and S2 – suspect 2

Restriction Fragment Length Polymorphism

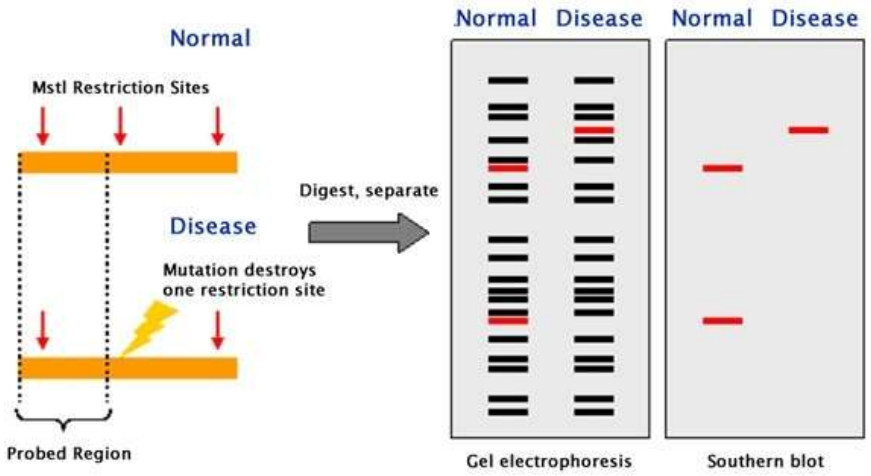
short tandemly repeated



Banding Patterns

- If you do this for 13 different repeat sequences at 13 different loci on the chromosome, each person produces a different band pattern when the fragments are separated by gel electrophoresis
- Banding patterns are identified using specific probes (see next slide)
- Since the patterns are unique to an individual, they are referred to as DNA finger prints

Restriction Fragment Length Polymorphism (RFLP



Applications of RFLP

RFLP has been used for several genetic analysis applications since its invention.

Some of these key applications of RFLP are listed below:

To determine the status of genetic diseases such as Cystic Fibrosis in an individual.

To determine or confirm the source of a DNA sample such as in paternity tests or criminal investigations.

In genetic mapping to determine recombination rates that show the genetic distance between the loci.

To identify a carrier of a disease-causing mutation in a family.

Disadvantages of RFLP

Since its invention, RFLP has been a widely used genome analysis techniques employed in forensic science, medicine, and genetic studies. However, it has become almost obsolete with the advent of relatively simple and less expensive DNA profiling technologies such as the polymerase chain reaction (PCR).

The RFLP procedure requires numerous steps and takes weeks to yield results, while techniques such as PCR can amplify target DNA sequences in a mere few hours.

Additionally, RFLP requires a large DNA sample, the isolation of which can be a laborious and time-consuming process. In contrast, PCR can amplify minute amounts of DNA in a matter of hours.

Due to numerous reasons such as these, the PCR technique has largely replaced RFLP in most applications requiring DNA sequencing such as paternity testing or forensic sample analysis.

Furthermore, the identification of single-nucleotide polymorphisms in the Human Genome Project has almost replaced the need for RFLP in disease status analysis.

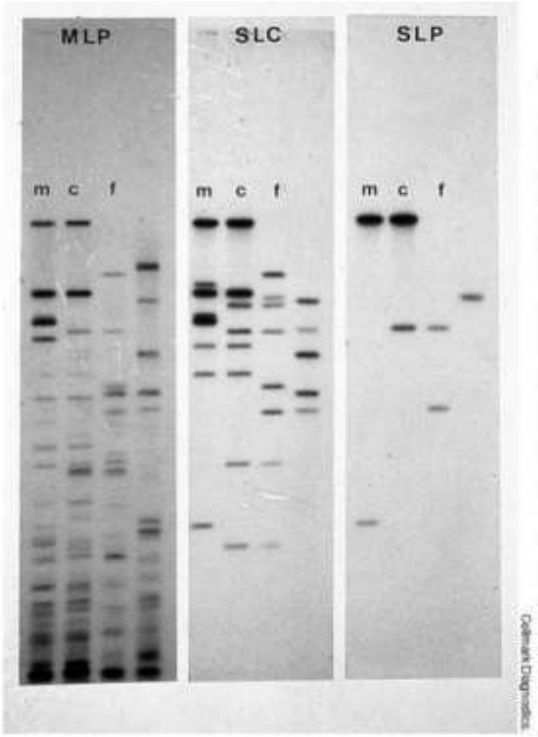
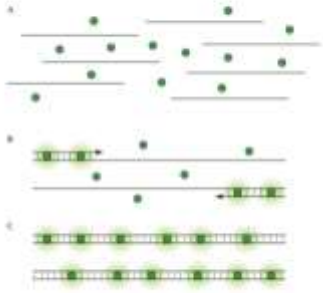


FIGURE 11.2

Two types of probes are used for identification testing: multilocus and single-locus. Shown here are three autoradiograms for a paternity test (m=mother's DNA, c=child's DNA, f=true father's DNA, unlabeled lane is excluded alleged father's DNA). Far left panel: Multilocus probes allow the detection of multiple repetitive DNA loci that are located on more than one chromosome. Far right panel: Single-locus probes allow the detection of a single repetitive DNA locus on one chromosome. Middle panel: Many single-locus probes are available; to increase the sensitivity of discrimination, several are used to examine different loci, as in this single-locus cocktail (SLC) comprised of four different single-locus probes.

- A way to quantitate DNA in a PCR
- Involves the use of SYBR green dye
- SYBR green only binds to and fluoresces with dsDNA (detect product)

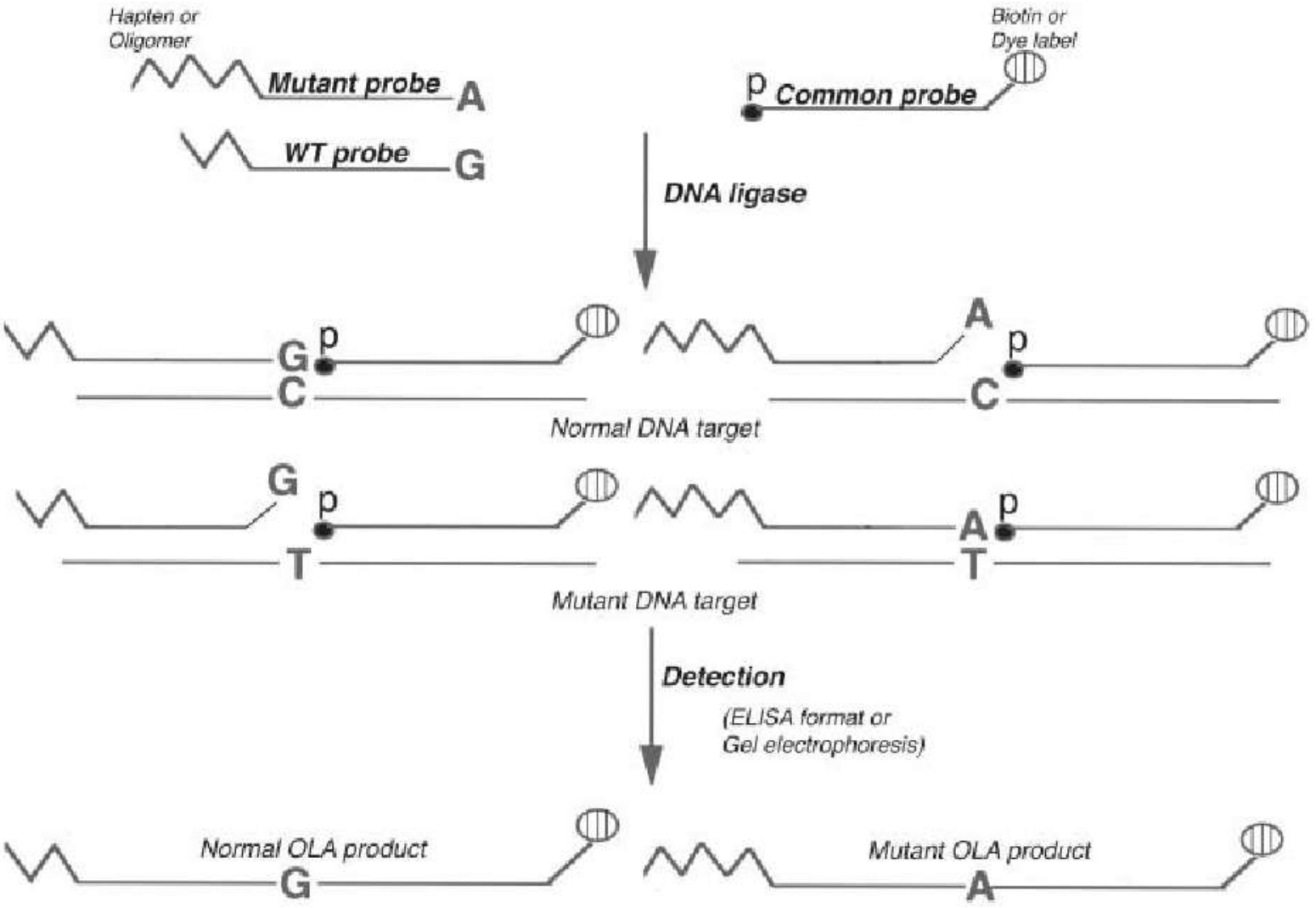


GKM/M.MEDPAEDS/LECT

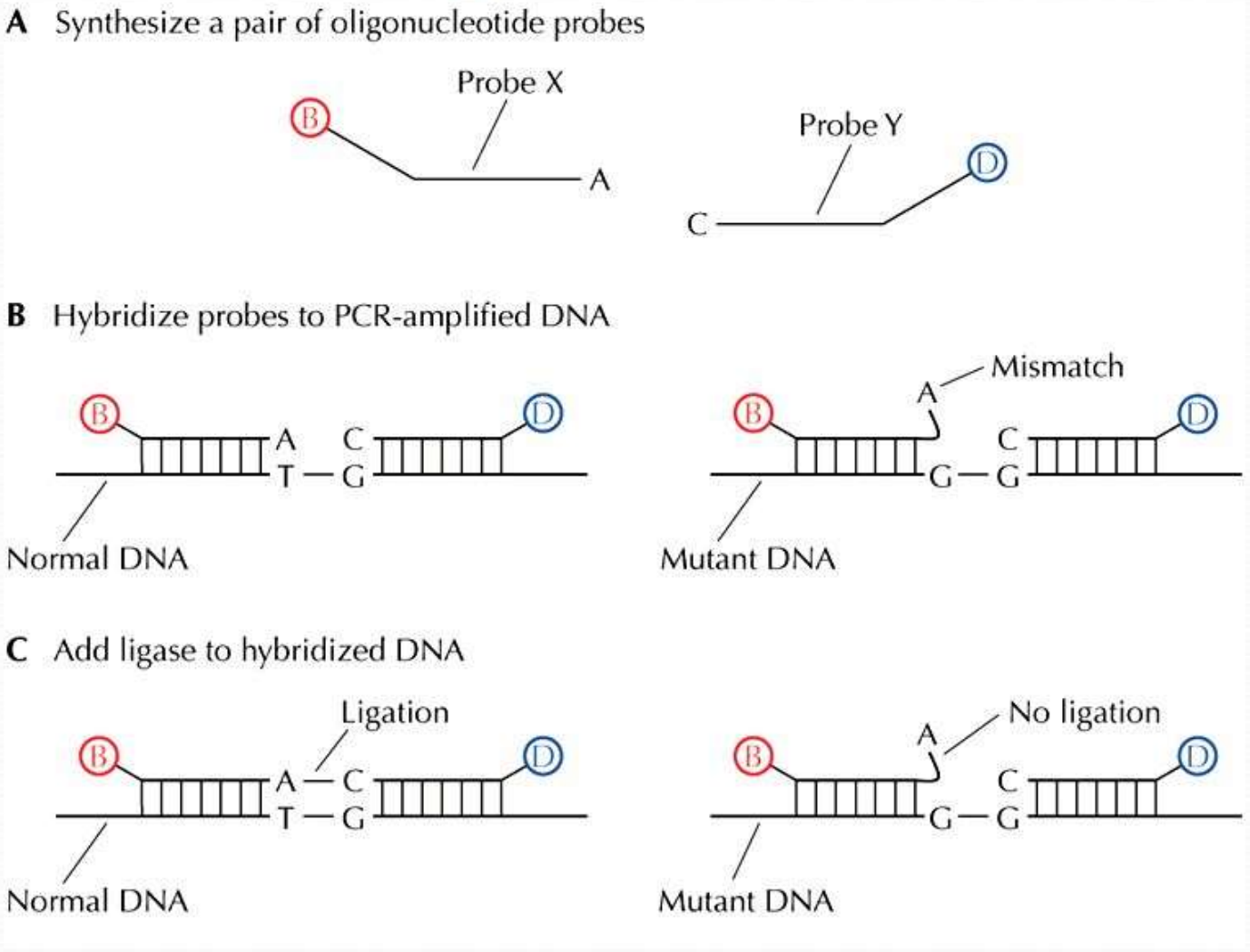
Technical Considerations

- Preserve the integrity of DNA sample
- Avoid DNA contamination & degradation
- Avoid incomplete digestions if REs are used
- Use standard hybridization conditions
- Use standard PCR primers and procedures
- Gel analysis is less reproducible than capillary electrophoresis of PCR products

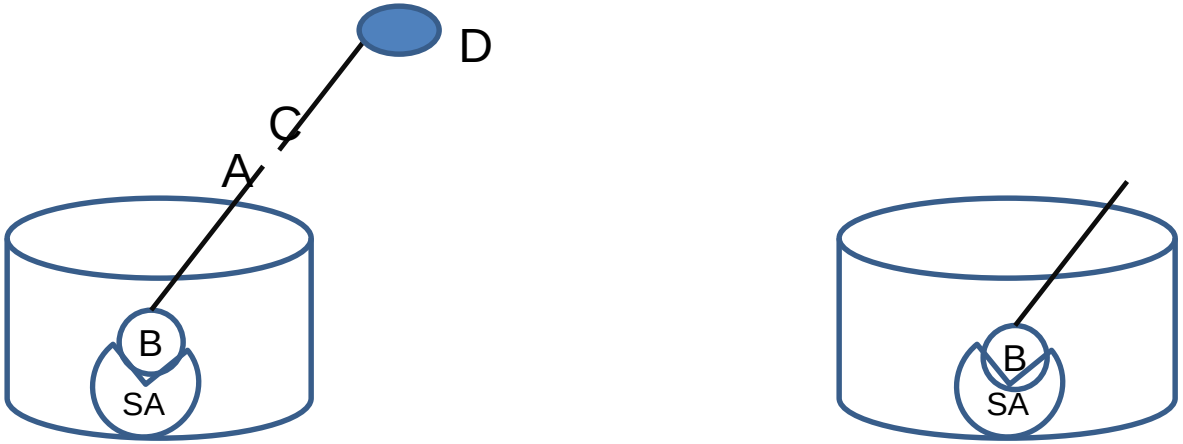
Oligonucleotide Ligation Assay



PCR/OLA


A Synthesize a pair of oligonucleotide probes
Probe X
B
A
Probe Y
D
C
B Hybridize probes to PCR-amplified DNA
B
D
A
C
T
G
Normal DNA
Mismatch
A
B
D
C
G
G
Mutant DNA
C Add ligase to hybridized DNA
Ligation
B
D
A
C
T
G
Normal DNA
No ligation
A
B
D
C
G
G
Mutant DNA

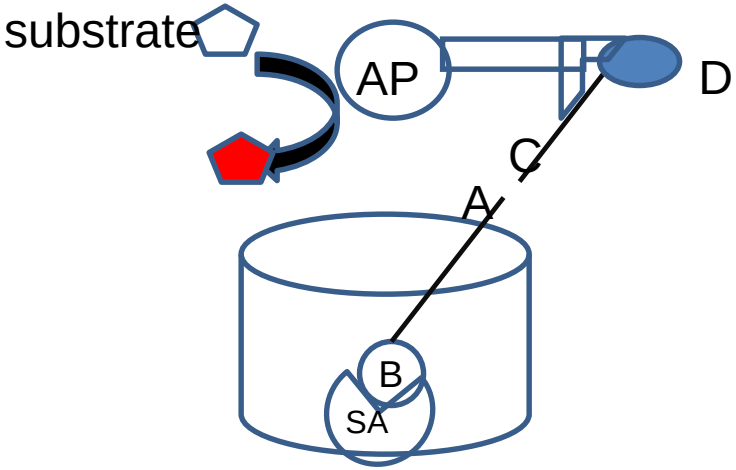
Bind probe to Streptavidin coated well

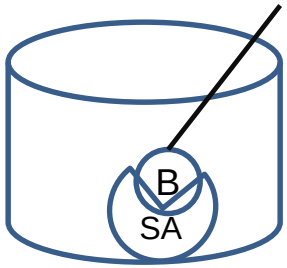


Normal

mutant

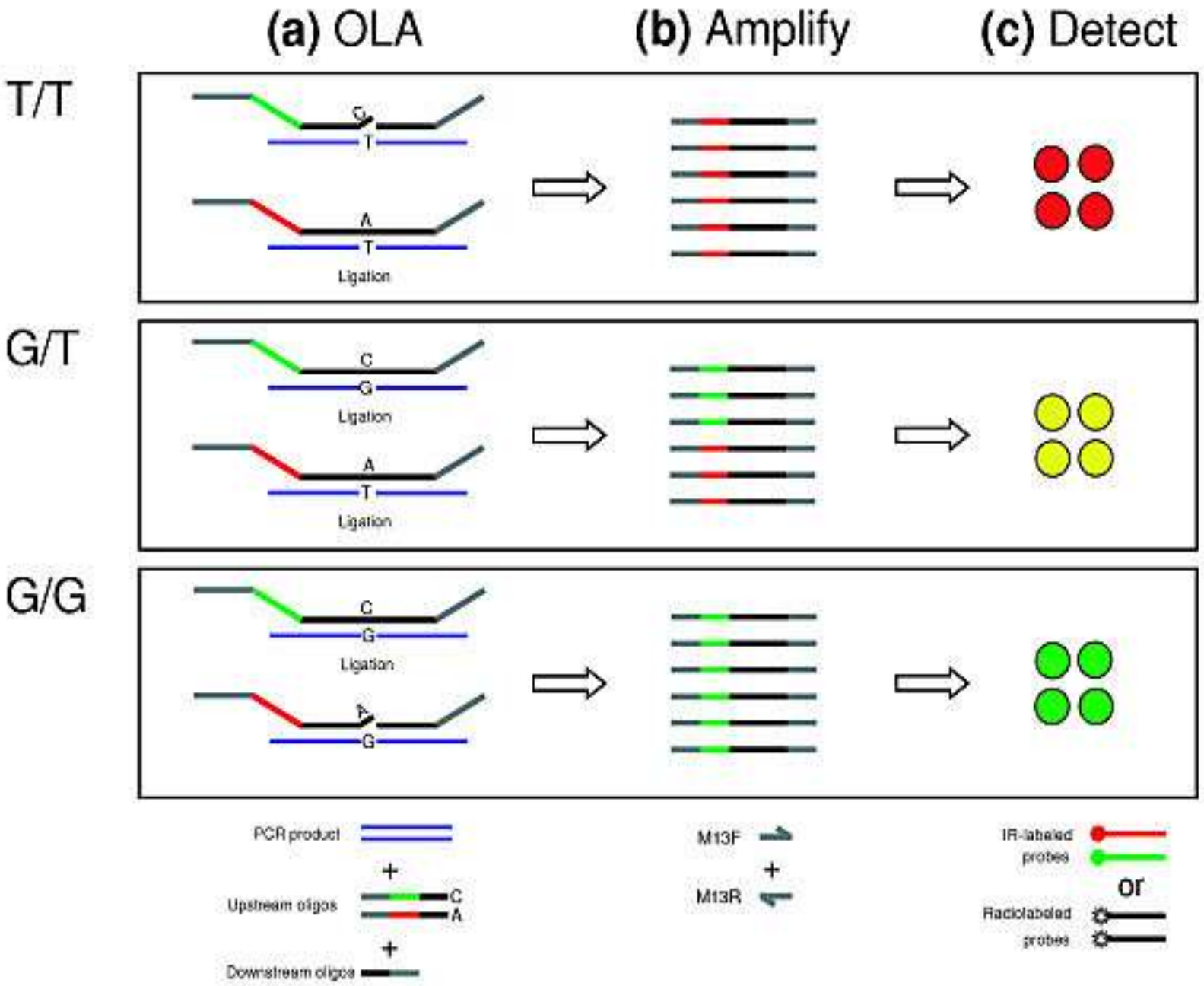
Add anti dig ab conjugated with AP and wash, add substrate





Color product

no color


(a) OLA
(b) Amplify
(c) Detect
T/T
C
T
A
T
Ligation
G/T
C
G
Ligation
A
T
Ligation
G/G
C
G
Ligation
A
G
PCR product
+
Upstream oligos
C
A
+
Downstream oligos
M13F
+
M13R
IR-labeled probes
or
Radiolabeled probes

Advantages and Limitations of Oligonucleotide Ligation

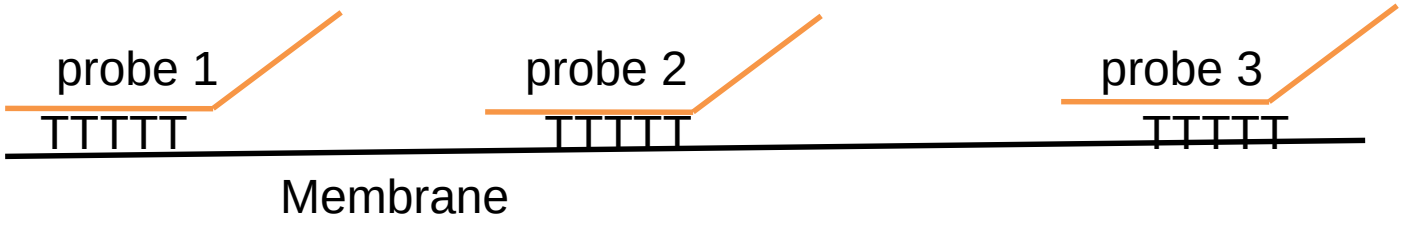
Advantages

- Accurate distinction of any base substitution or insertion/deletion mutation
- Specificity (chance of a false positive essentially zero)
- Sensitivity (detects rare sequence variants in background of normal DNA)
- Rapid, economical, and easy to perform (one-step reaction)
- Adapts well to multiplexing
- Minimal sample preparation required
- Results simple to interpret
- Tolerates some degree of sequence polymorphism
- Nonradioactive and nontoxic reagents
- Compatible with multiple detection formats
- Amenable to automation and computerized genotyping

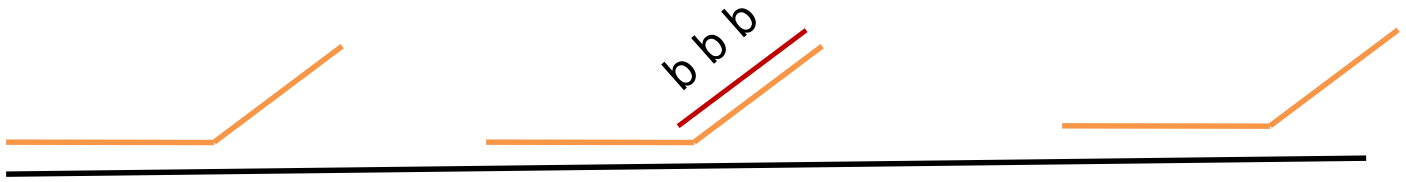
Limitation

- Detects only known mutations
- Special equipment might be required
- Sequence polymorphisms near ligation site might affect ligation
- G→T mismatches least well discriminated

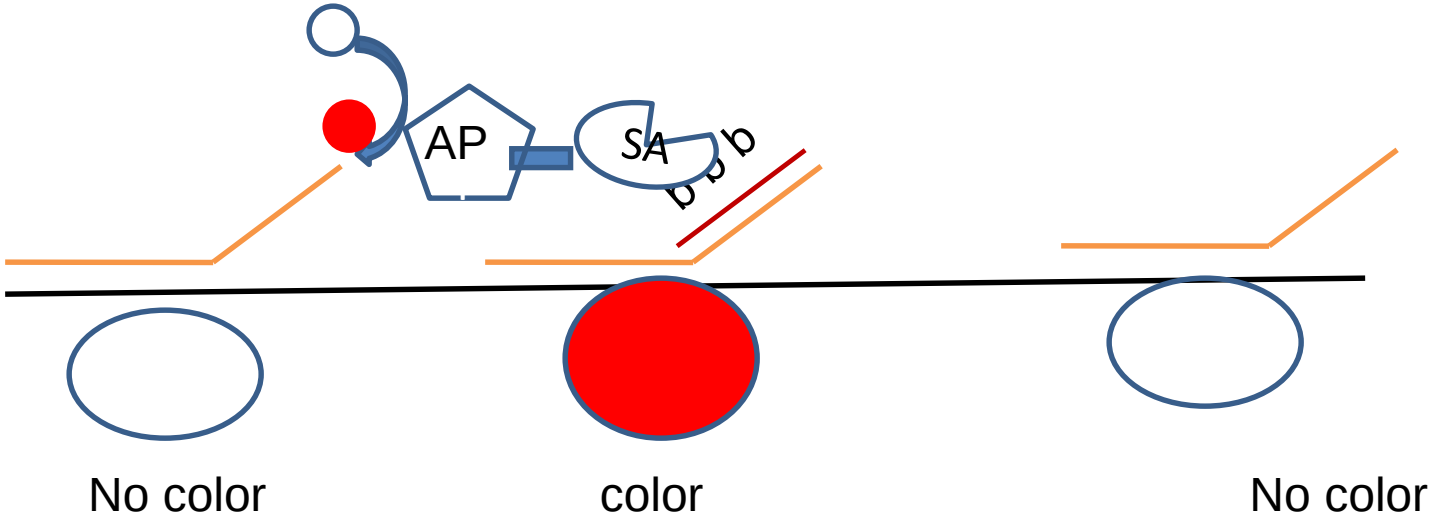
Mutation at different sites within one gene : Thalasemia



Hybridize with PCR amplified biotynaled fragment of target DNA

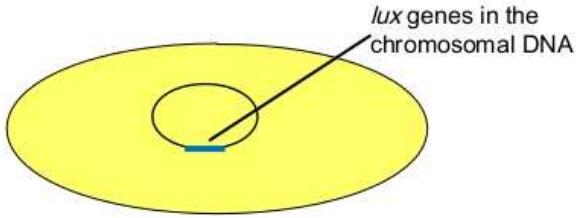


Add streptavidin-AP conjugated and substrate



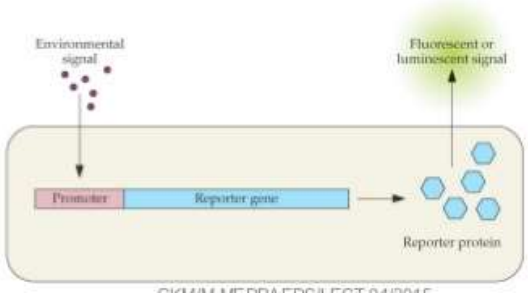
Bacterial biosensors

- One example involves using *Pseudomonas fluorescens* (genetically engineered for bioluminescence) to monitor pollutants
- If pollutants are present in a sample, then cell death occurs and "the light goes out"



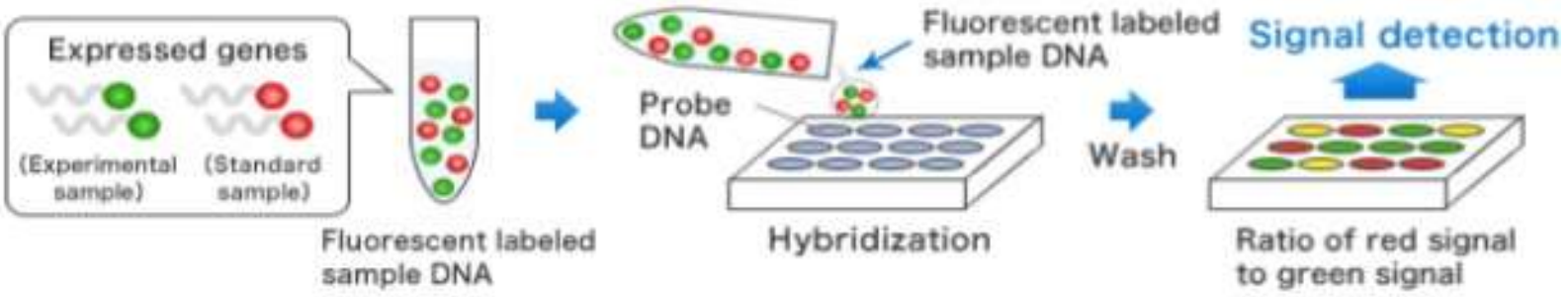
Bacterial biosensors (another example)

- Green fluorescent protein (GFP) can be used a reporter gene under the control of some inducible promoter (e.g., one that responds to some environmental signal such as a toxin)
- If the signal is present GFP will be produced

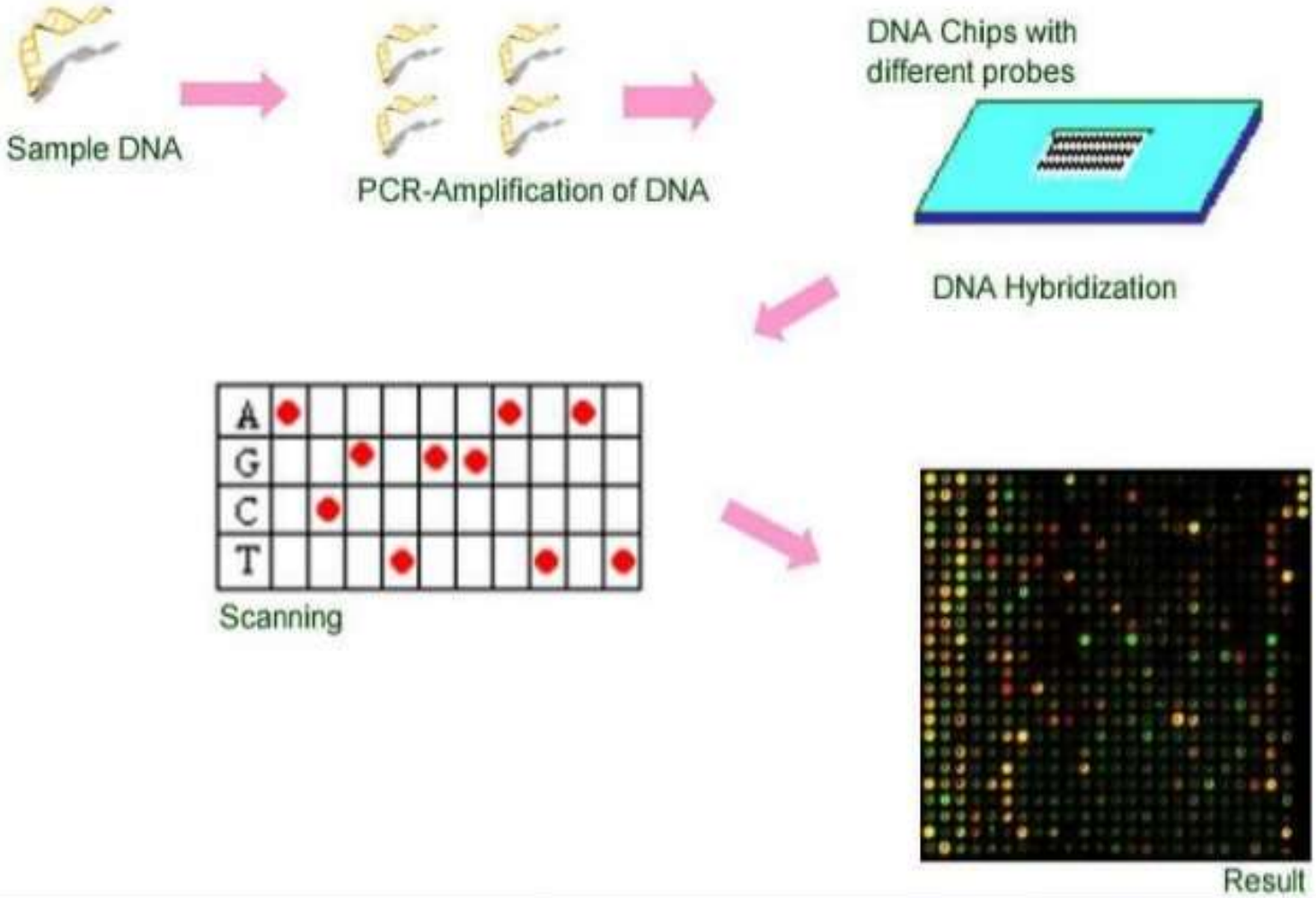


DNA CHIP MICRO ARRAY OF GENE PROBE

- The DNA chip are gene chip contains 1000's of DNA probes, arranged on a small glass slide of the postage stamp size.
- By this recent advanced approach, 1000's of target DNA molecules can be scanned simultaneously.
- Technique:
- Known DNA molecule cut into fragments by restriction endonuclease
- Fluorescent marker are attached to these DNA fragments.
- Allowed to react with probe of DNA chip.
- Target DNA fragments with complimentary sequences bind to DNA probes selectively.
- Wash remaining DNA fragments.
- Target DNA pieces can be identified by their fluorescence emission, by passing a laser beam.
- Computer recorded the pattern of fluorescence emission and DNA identification.



How Do DNA Chips work?

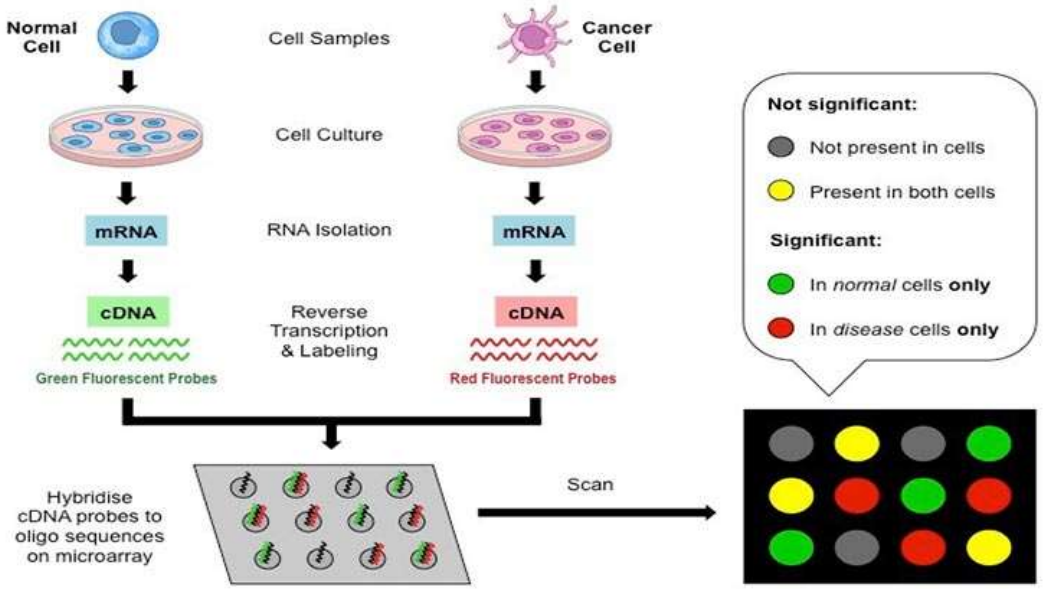


Application:

- The presence of mutation in a DNA can be conveniently identified.
- Gene chip probes used to find mutation in p53 and BRACI gene (involved in cancer).

Advantages:

- Very rapid.
- Sensitive and specific.
- Simultaneous analysis of many DNA is possible



Principle of DNA Microarray Technique

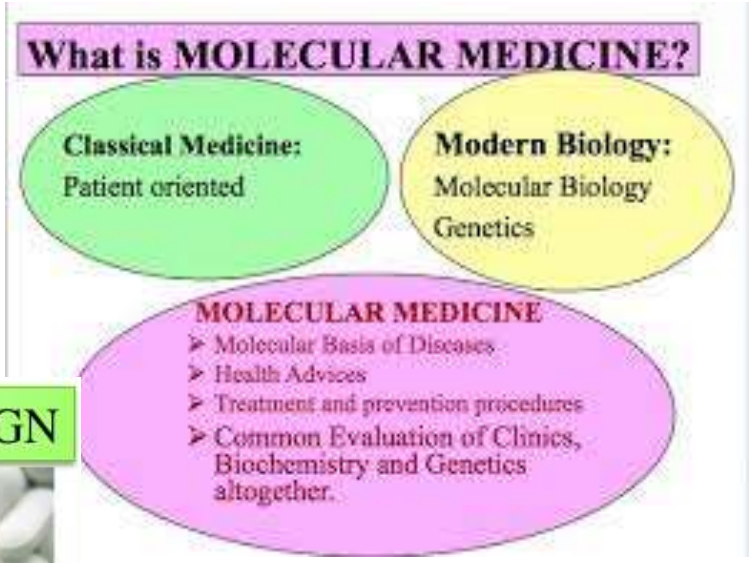
The principle of DNA microarrays lies on the hybridization between the **nucleic acid** strands.
The property of complementary nucleic acid sequences is to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs.
For this, samples are labeled using fluorescent dyes.
At least two samples are hybridized to chip.
Complementary nucleic acid sequences between the sample and the probe attached on the chip get paired via hydrogen bonds.
The non-specific bonding sequences while remain unattached and washed out during the washing step of the process.
Fluorescently labeled target sequences that bind to a probe sequence generate a signal.
The signal depends on the hybridization conditions (ex: temperature), washing after hybridization etc while the total strength of the signal, depends upon the amount of target sample present.
Using this technology the presence of one genomic or cDNA sequence in 1,00,000 or more sequences can be screened in a single hybridization

Molecular Medicine

(Graphics are collected from Internet)

Molecular Medicine

Molecular medicine is a broad field, where physical, chemical, biological, bioinformatics and **medical** techniques are used to describe **molecular** structures and mechanisms, identify fundamental
molecular and genetic errors of disease, and to develop **molecular** interventions to correct them



INTRODUCTION TO DRUG AND DRUG DESIGN

- The drug is most commonly an organic small molecule that activates or inhibits the function of a bio molecule such as a protein, which in turn results in a therapeutic benefit to the patient.
- Drug design, or rational drug design or simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target.
- Drug design involves the design of small molecules that are complementary in shape and charge to the bio molecular target with which they interact and therefore will bind to it.

2

Small Molecule and Large Molecule Drug Design

- "Small Molecule" Drugs
  - Low MW
  - Moderately hydrophobic/hydrophilic
  - Moderately chemical complexity
- "Biologicals"
  - Peptides/peptidomimetics
  - Engineered enzymes
  - Antisense/RNAi
  - Need help to enter cells!

Monoclonal antibodies and small molecule inhibitors are the two major types of targeted therapies. While monoclonal antibodies block the extracellular components of target proteins, small molecule inhibitors are highly cell permeable and can enter cells, thereby blocking the activities of intracellular target proteins and interfering with the downstream signaling pathways

How do small molecule therapies work?
A drug that can enter cells easily because it has a low molecular weight. Once inside the cells, it can affect other molecules, such as proteins, and may cause cancer cells to die

PROteolysis-TArgeting Chimeras (PROTACs) exploit the intracellular ubiquitin-proteasome system to selectively degrade target proteins.

Small molecule targeted agents... classification

ENZYME INHIBITORS

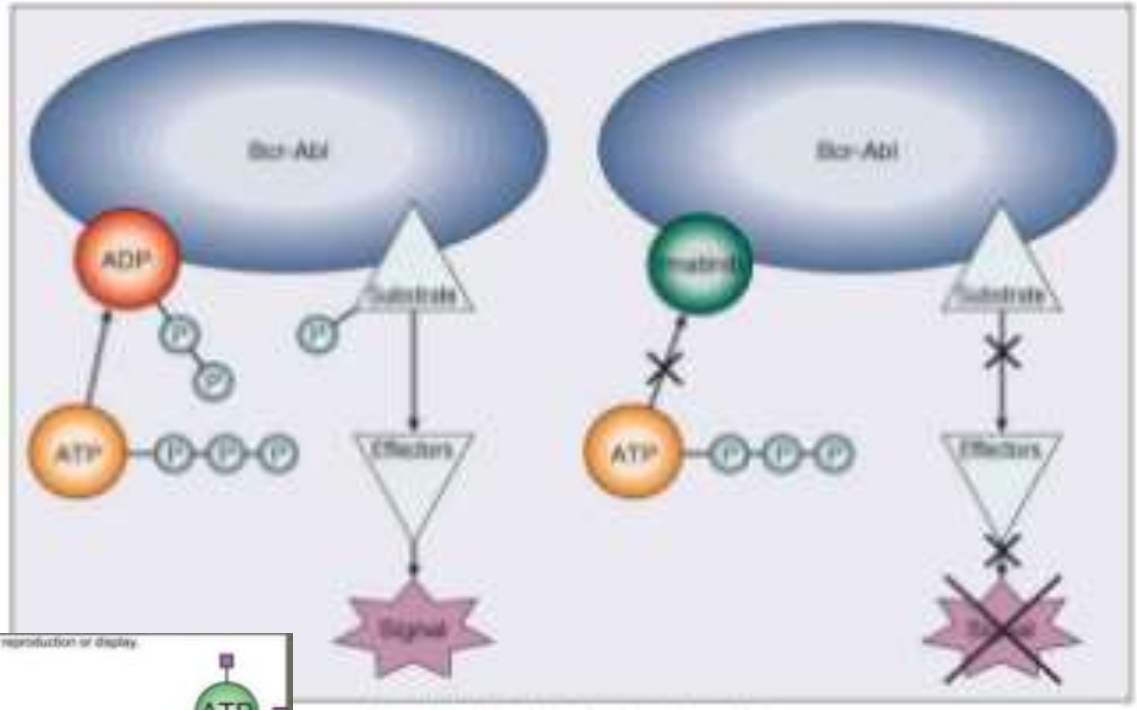
PARP Inhibitors	CDK Inhibitors	Proteasome Inhibitors
Fernesyl Transferase inhibitors	Histone Deacetylase Inhibitors	EMP Dehydrogenase inhibitors

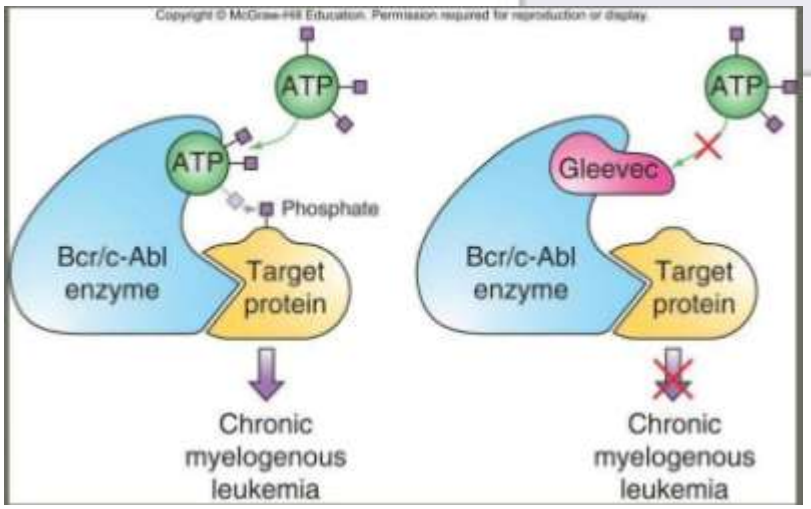
TYROSINE KINASE INHIBITORS

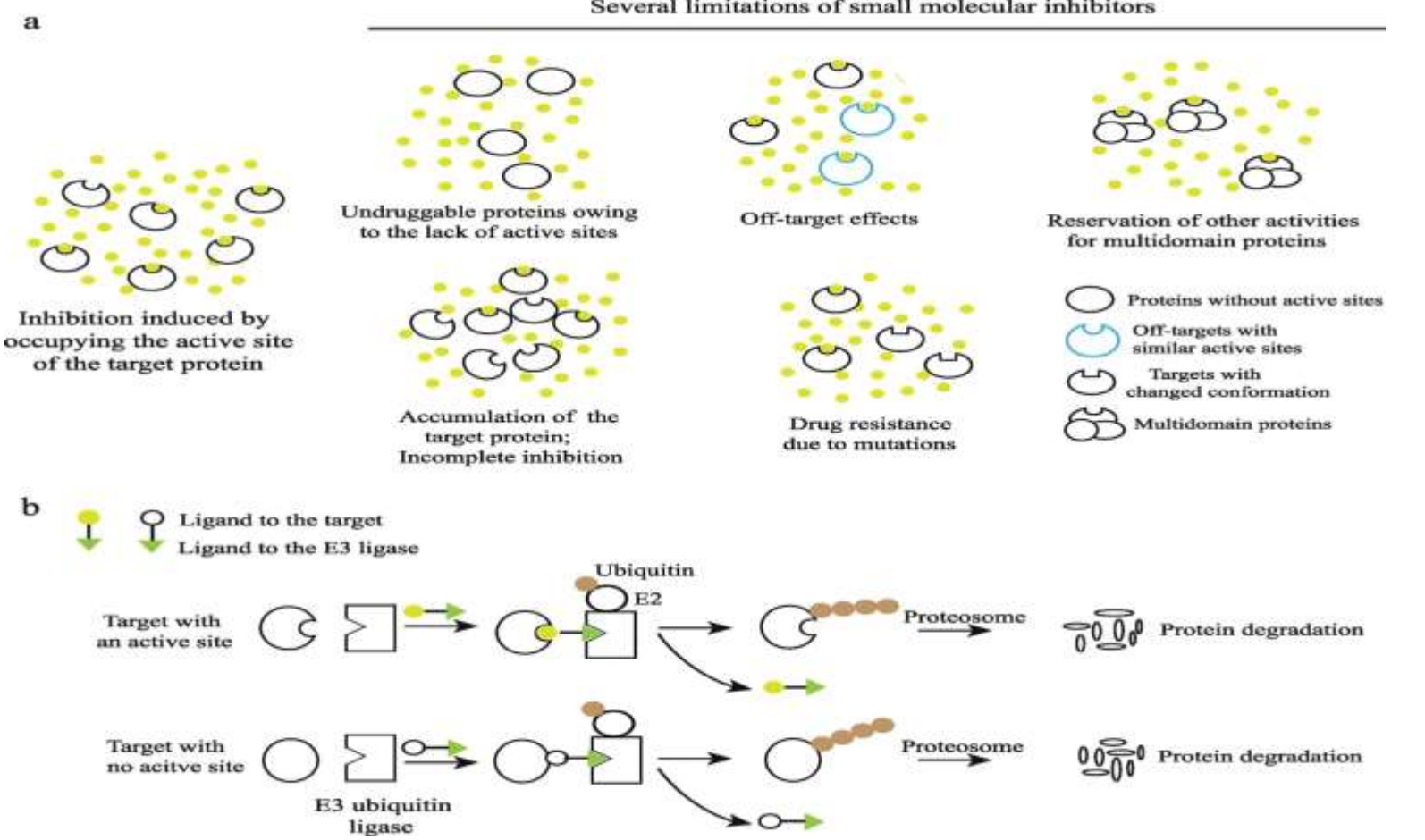
Single Receptor TKI	Multireceptor TKI	Non Receptor TKI
Erlotinib	Sunitinib	Imatinib

Example

Imatinib mesylate (Gleevec®) targets the BCR-ABL fusion protein, which is made from pieces of two genes that get joined together in some leukemia cells and promotes the growth of leukemic cells.







Overview of the mechanisms of small molecule inhibitors and PROTACs (a) In order to inhibit the activities of target proteins, small molecule inhibitors competitively bind to active sites on the target proteins. The limitations on developing and taking small molecule drugs are shown in this figure. (b) Heterobifunctional PROTAC molecules harness the ubiquitin proteasome system to selectively degrade target proteins. Currently, the generation of PROTACs relies on available small molecular inhibitors to be used as target binding ligands. Alternatively, PROTACs can bind to any crevice on the surface of the target proteins to induce their degradation.

Target specific

Human monoclonal **antibodies** are **produced** using transgenic mice or phage display libraries by transferring human immunoglobulin genes into the murine genome and vaccinating the transgenic mouse against the desired antigen, leading to the production of appropriate monoclonal **antibodies**

Recombinant **antibodies can** be cloned from any species of **antibody**-producing animal, **if** the appropriate oligonucleotide primers or hybridization probes are available. The ability to manipulate the **antibody** genes makes it possible to generate new **antibodies** and **antibody** fragments, such as Fab fragments and scFv in vitro.

mAb	Brand name	Company	Target	Format	Technology
Muromonab-CD3	Orthoclone OKT3	Centocor Ortho Biotech Products LP.	CD3	Murine IgG2a	Hybridoma/Janssen Biotech, Inc
Abciximab	Reopro	Centocor Inc./Eli Lilly/Janssen Biotech Inc.	GPIIb/IIIa	Chimeric IgG1 Fab	Hybridoma
Rituximab	MabThera, Rituxan	Biogen Inc./Roche, F. Hoffmann-La Roche Ltd./Genentech Inc.	CD20	Chimeric IgG1	Hybridoma
Palivizumab	Synagis	MedImmune/AbbVie Inc.	RSV	Humanized IgG1	Hybridoma
Infliximab	Remicade	Janssen Biotech Inc.	TNFα	Chimeric IgG1	Hybridoma
Trastuzumab	Herceptin	Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc.	HER2	Humanized IgG1	Hybridoma
Alemtuzumab	Campath, Lemtrada	Berlex Inc./Genzyme Corp./Millennium Pharmaceuticals Inc.	CD52	Humanized IgG1	Hybridoma
Adalimumab	Humira	AbbVie Inc.	TNFα	Human IgG1	Phage display
Ibritumomab tiuxetan	Zevalin	Biogen Inc./Schering AG/Spectrum Pharmaceuticals Inc.	CD20	Murine IgG1	Hybridoma
Omalizumab	Xolair	Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc./Novartis Pharmaceuticals Corp./Tanox Inc.	IgE	Humanized IgG1	Hybridoma
Cetuximab	Erbitux	Bristol-Myers Squibb/Merck & Co. Inc./Eli Lilly/ImClone Systems Inc.	EGFR	Chimeric IgG1	Hybridoma
Bevacizumab	Avastin	Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc.	VEGF-A	Humanized IgG1	Hybridoma
Natalizumab	Tysabri	Biogen Inc./Elan Pharmaceuticals International, Ltd.	ITGA4	Humanized IgG4	Hybridoma
Panitumumab	Vectibix	Amgen	EGFR	Human IgG2	Transgenic mice

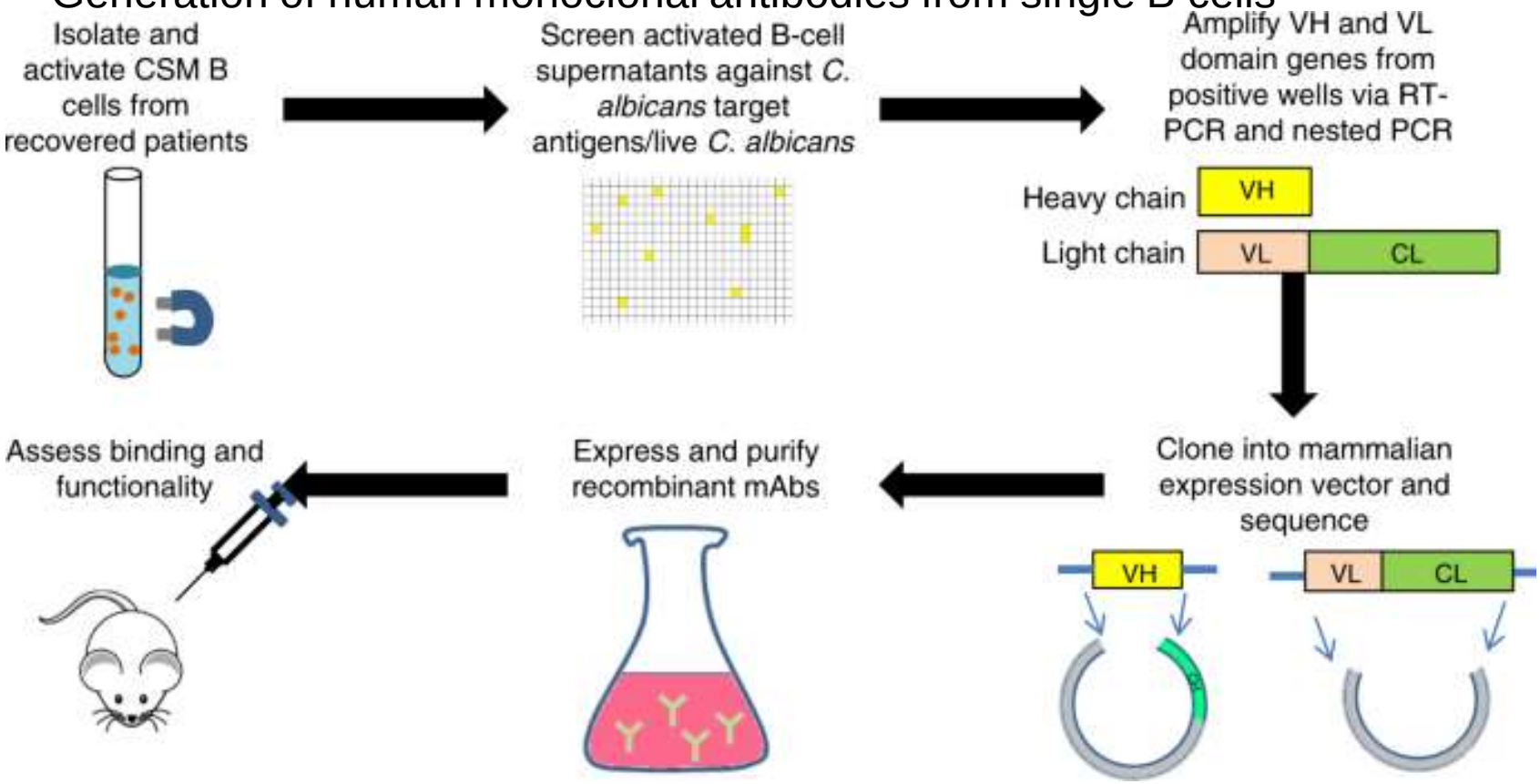
Ranibizumab	Lucentis	Roche, F. Hoffmann-La Roche Ltd./Genentech Inc./Novartis Pharmaceuticals Corp.	VEGF-A	Humanized IgG1 Fab	Hybridoma
Eculizumab	Soliris	Alexion Pharmaceuticals Inc.	C5	Humanized IgG2/4	Hybridoma
Certolizumab pegol	Cimzia	Celltech, UCB.	TNFα	Humanized Fab, pegylated	Hybridoma
Ustekinumab	Stelara	Medarex/Centocor Ortho Biotech Inc./Janssen Biotech Inc.	IL-12/23	Human IgG1	Transgenic mice
Canakinumab	Ilaris	Novartis Pharmaceuticals Corp.	IL-1β	Human IgG1	Transgenic mice
Golimumab	Simponi	Centocor Ortho Biotech Inc./Janssen Biotech Inc.	TNFα	Human IgG1	Transgenic mice
Ofatumumab	Arzerra	Genmab A/S /GlaxoSmithKline /Novartis.	CD20	Human IgG1	Transgenic mice
Tocilizumab	RoActemra, Actemra	Chugai Pharmaceutical Co., Ltd./Roche, F. Hoffmann-La Roche. Ltd./Genentech Inc.	IL-6R	Humanized IgG1	Hybridoma
Denosumab	Xgeva, Prolia	Amgen	RANKL	Human IgG2	Transgenic mice
Belimumab	Benlysta	GlaxoSmithKline /Human Genome Sciences Inc.	BLyS	Human IgG1	Phage display
Ipilimumab	Yervoy	Bristol-Myers Squibb/Medarex	CTLA-4	Human IgG1	Transgenic mice
Brentuximab vedotin	Adcetris	Seattle genetics Inc./Takeda Pharmaceutical Co., Ltd.	CD30	Chimeric IgG1; ADC	Hybridoma
Pertuzumab	Perjeta	Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc.	HER2	Humanized IgG1	Hybridoma
Trastuzumab emtansine	Kadcyla	Roche, F. Hoffmann-La Roche Ltd./Genentech Inc./ImmunoGen Inc.	HER2	Humanized IgG1; ADC	Hybridoma

Raxibacumab	Abthrax	GlaxoSmithKline/Human Genome Sciences Inc. (HGSI)	*B. anthracis* PA	Human IgG1	Transgenic mice
Obinutuzumab	Gazyva, Gazyvaro	Biogen Inc./Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc.	CD20	Humanized IgG1 Glycoengineered	Hybridoma
Siltuximab	Sylvant	Centocor Inc./Janssen Biotech Inc./Janssen-Cilag International NV	IL-6	Chimeric IgG1	Hybridoma
Ramucirumab	Cyramza	Eli Lilly/ImClone Systems Inc.	VEGFR2	Human IgG1	Phage display
Vedolizumab	Entyvio	Genentech Inc./Millennium Pharmaceuticals Inc./Takeda Pharmaceuticals U.S.A. Inc.	α4β7 integrin	Humanized IgG1	Hybridoma
Blinatumomab	Blincyto	Amgen	CD19, CD3	Murine bispecific tandem scFv	Hybridoma
Nivolumab	Opdivo	Bristol-Myers Squibb/Ono Pharmaceutical Co., Ltd.	PD-1	Human IgG4	Transgenic mice
Pembrolizumab	Keytruda	Merck & Co. Inc.	PD-1	Humanized IgG4	Hybridoma
Idarucizumab	Praxbind	Boehringer Ingelheim Pharmaceuticals	Dabigatran	Humanized Fab	Hybridoma
Necitumumab	Portrazza	Eli Lilly/ImClone Systems Inc.	EGFR	Human IgG1	Phage display
Dinutuximab	Unituxin	United Therapeutics Corporation	GD2	Chimeric IgG1	Hybridoma
Secukinumab	Cosentyx	Novartis Pharmaceuticals Corp.	IL-17α	Human IgG1	Transgenic mice
Mepolizumab	Nucala	Centocor Inc./GlaxoSmithKline	IL-5	Humanized IgG1	Hybridoma
Alirocumab	Praluent	Regeneron Pharmaceuticals Inc./Sanofi.	PCSK9	Human IgG1	Transgenic mice
Evolocumab	Repatha	Amgen/Amgen Astellas BioPharma K.K.	PCSK9	Human IgG2	Transgenic mice
Daratumumab	Darzalex	Genmab A/S/Janssen Biotech Inc.	CD38	Human IgG1	Transgenic mice

Elotuzumab	Empliciti	Bristol-Myers Squibb/AbbVie Inc.	SLAMF7	Humanized IgG1	Hybridoma
Ixekizumab	Taltz	Eli Lilly	IL-17α	Humanized IgG4	Hybridoma
Reslizumab	Cinqaero, Cinqair	Celltech, UCB/Schering-Plough/Teva Pharmaceutical Industries, Ltd.	IL-5	Humanized IgG4	Hybridoma
Olaratumab	Lartruvo	Eli Lilly/ImClone Systems Inc.	PDGFRα	Human IgG1	Transgenic mice
Bezlotoxumab	Zinplava	Merck & Co. Inc.	*Clostridium difficile* enterotoxin B	Human IgG1	Transgenic mice
Atezolizumab	Tecentriq	Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc.	PD-L1	Humanized IgG1	Hybridoma
Obiltoxaximab	Anthim	Elusys Therapeutics Inc.	*B. anthrasis* PA	Chimeric IgG1	Hybridoma
Inotuzumab ozogamicin	Besponsa	Wyeth Pharmaceuticals/Pfizer.	CD22	Humanized IgG4	Hybridoma
Brodalumab	Siliq, Lumicef	MedImmune/Amgen/Kyowa Hakko Kirin /AstraZeneca/Valeant Pharmaceuticals International Inc.	IL-17R	Human IgG2	Transgenic mice
Guselkumab	Tremfya	MorphoSys/Janssen Biotech Inc.	IL-23 p19	Human IgG1	Phage display
Dupilumab	Dupixent	Regeneron Pharmaceuticals Inc./Sanofi	IL-4Rα	Human IgG4	Transgenic mice
Sarilumab	Kevzara	Regeneron Pharmaceuticals Inc./Sanofi	IL-6R	Human IgG1	Transgenic mice
Avelumab	Bavencio	Merck Serono International S.A./Pfizer	PD-L1	Human IgG1	Phage display
Ocrelizumab	Ocrevus	Biogen Inc./Roche, F. Hoffmann-La Roche, Ltd./Genentech Inc./SIGMA-TAU Industrie Farmaceutiche Riunite S.p.A.	CD20	Humanized IgG1	Hybridoma

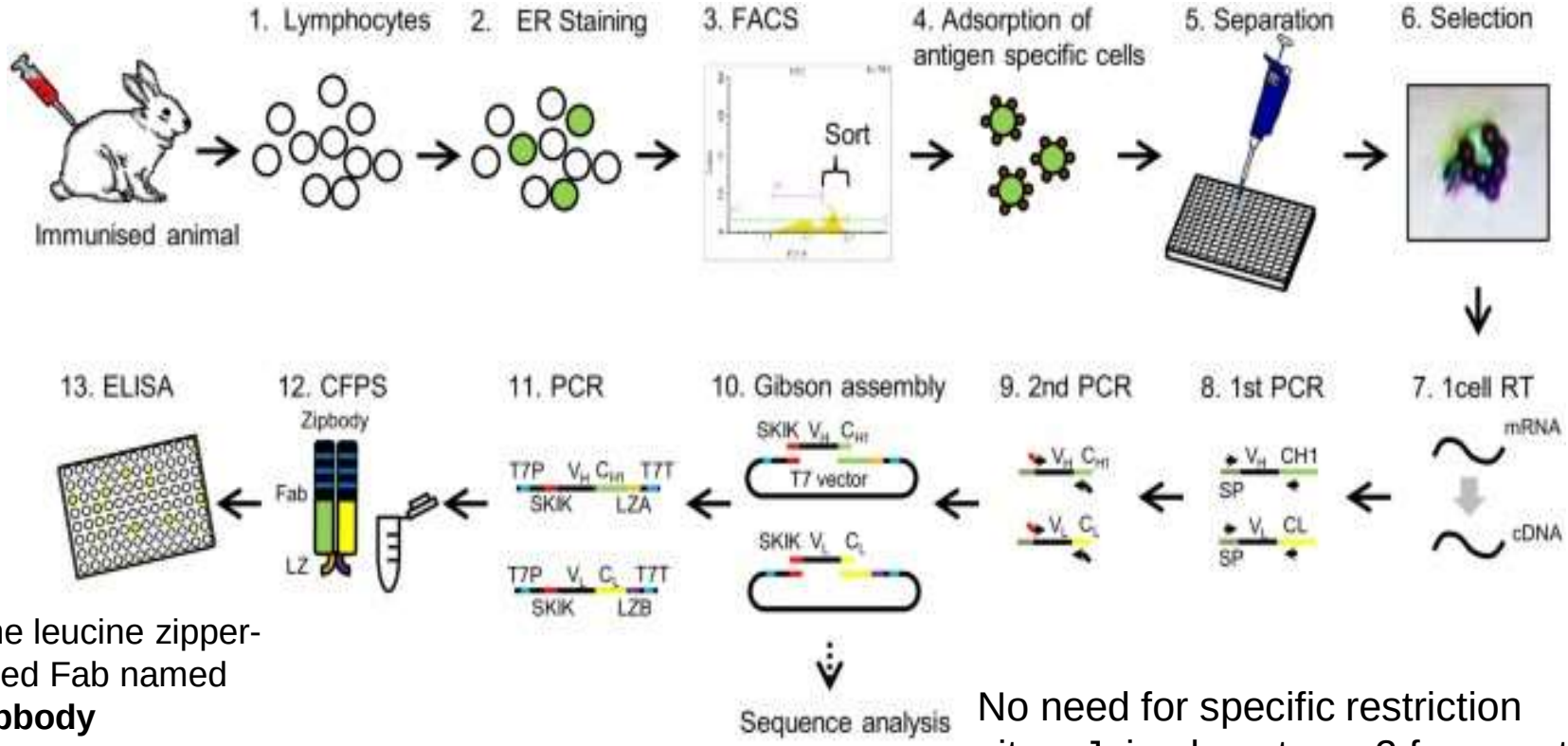
Approaches for the development of therapeutic antibodies.
a The traditional mouse hybridoma technique starts by immunization of mice with desired antigens to trigger an immune response. Harvested splenocytes are fused with myeloma cells to produce hybridoma cells that persistently secrete antibodies. After the screening, selected leads are used to generate chimeric or humanized antibodies.
b Phage display. A human phage-displayed human antibody library is used to select antigens of interest. After 3–5 rounds of biopanning, immuno-positive phage clones are screened by ELISA; then DNA sequences are analyzed to construct and express human IgGs.
c Transgenic mouse. Similar to the mouse hybridoma technique or single B cell methods.
d The single B cell technique. From infected or vaccinated donors, PBMCs are prepared for isolation of suitable B cells by flow cytometry. Following the RT-PCR, V_H and V_L information of each B cell informs the generation of human mAbs

Generation of human monoclonal antibodies from single B cells

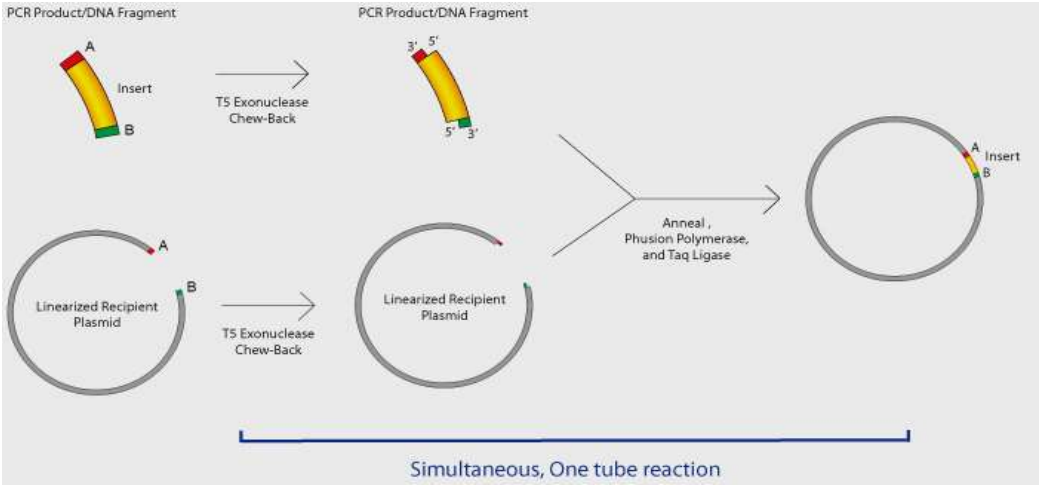


Class switched memory (CSM) B cells were isolated from individuals and microcultured in activating media to induce differentiation into plasmblast/plasma cells to promote IgG secretion for screening against target antigens. VH and VL genes from B-cell cultures positive for the target were recovered by RT-PCR and cloned into a mammalian expression vector for expression as full-length human IgG1 followed by standard IgG purification via fast protein liquid chromatography. Following QC, recombinant mAbs were assessed for functional activity in vitro and in vivo. V_H: heavy chain variable domain, V_L: light chain variable domain, C_H: heavy chain constant domain, C_L: light chain constant domain

Single B cell methods



The leucine zipper-fused Fab named '**Zipbody**



No need for specific restriction sites. Join almost any 2 fragments regardless of sequence.
No scar between joined fragments.
Fewer steps. One tube reaction.
Can combine many DNA fragments at once

Expression of **Zipbody** with SKIK tag by cell-free protein synthesis (**CFPS**)

(1) Collection of lymphocytes by density gradient centrifugation from a few millilitres of animal blood.
(2) Fluorescent staining of plasma cells with endoplasmic reticulum (ER)-tracker.
(3) Sorting of stained cells by fluorescence-activated cell sorting (FACS).
(4) Adsorption of cells binding to the antigen using antigen-coated magnetic beads.
(5) Separation of one cell per 10 μL into 384-well plate.
(6) Selection and confirmation of cell-bead complexes under an inverted phase-contrast microscope.
(7) Reverse transcription using gene specific primers and SuperScript IV reverse transcriptase.
(8) First PCR, using primers annealing to the signal peptide (SP) sequence and constant region of antibody genes.
(9) Second PCR, using primers with tails required for the subsequent Gibson assembly step.
(10) Gibson assembly to combine T7 promoter and terminator with antibody genes.
(11) PCR to amplify DNA fragments for **cell-free protein synthesis**. HA and His tags are present downstream of leucine zipper A (LZA) and B (LZB), respectively.
(12) Expression of Zipbody with SKIK tag (Ser-Lys-Ile-Lys) by cell-free protein synthesis (CFPS) with DsbC and oxidised glutathione (GSSG).
(13) Enzyme-linked immunosorbent assay (ELISA) evaluation.

Oxidative protein folding in the periplasm of Escherichia coli is catalyzed by the thiol-disulfide oxidoreductases DsbA and DsbC

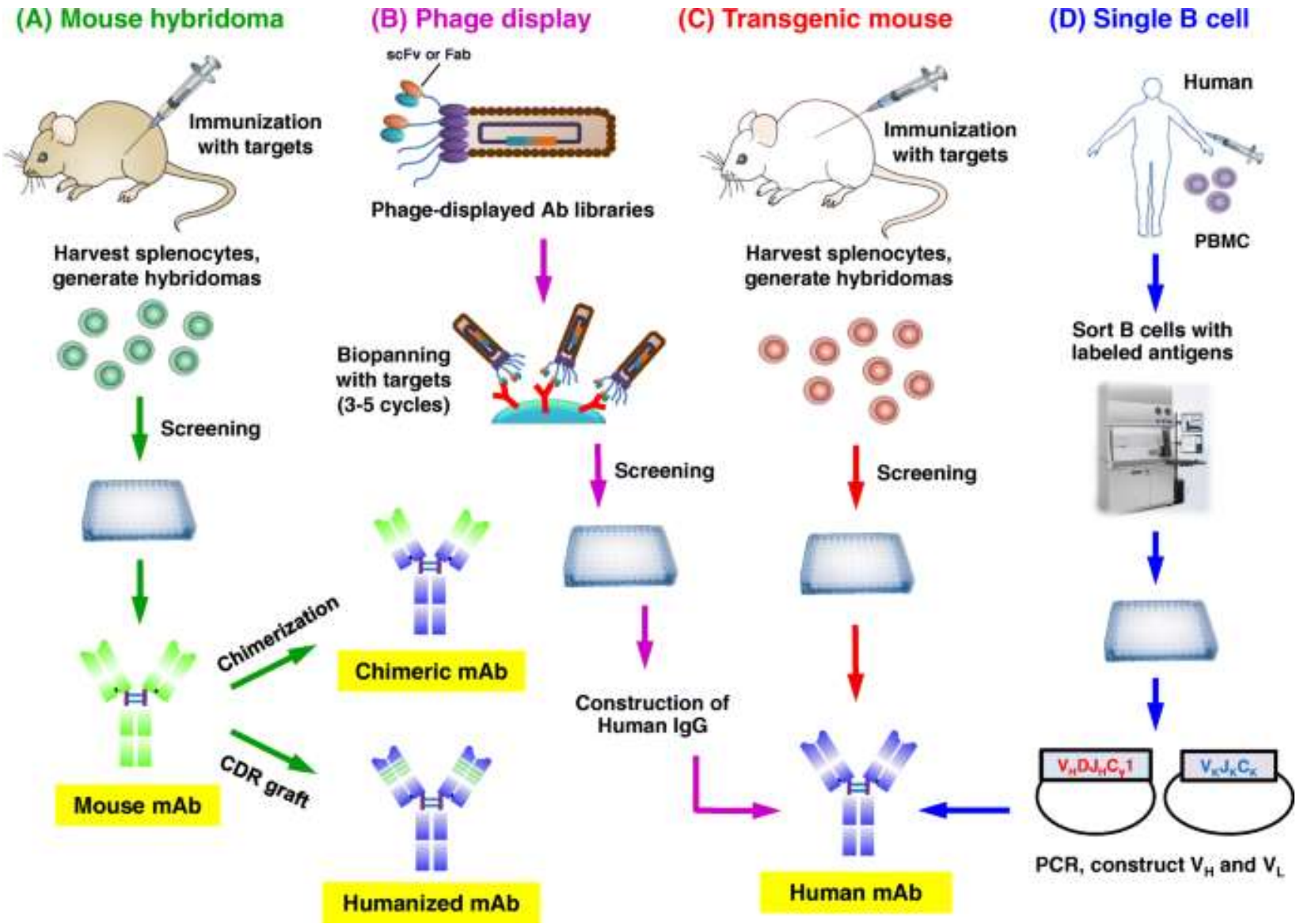
insertion of a short peptide tag with the sequence Ser-Lys-Ile-Lys (SKIK) at the N-terminus of the protein. The insertion of SKIK tag was effective to increase the production of the difficult-to-express proteins in *Saccharomyces cerevisiae* and an *E. coli* cell-free protein synthesis system

Zipbody or leucine zipper (LZ) to enhance the association of the light chain and the heavy chain of Fab. The zipbody format significantly increased the production of active Fab in an *Escherichia coli* cell-free protein synthesis system and *in vivo E. coli* expression systems

Cell-**free protein** (CFP) expression, also termed in vitro translation, facilitates the production of a given target **protein** by performing the translational machinery in **cell** lysates rather than within cultured **cells**

DsbC is a soluble protein of the bacterial periplasm that was identified genetically as being involved in protein disulfide formation

Reactions with molecular oxygen or small disulfide molecules, such as oxidized glutathione (GSSG), are required for the chemical oxidation of thiol groups to disulfide bonds


(A) Mouse hybridoma
Immunization with targets
Harvest splenocytes, generate hybridomas
Screening
Mouse mAb
Chimerization
Chimeric mAb
CDR graft
Humanized mAb
(B) Phage display
scFv or Fab
Phage-displayed Ab libraries
Biopanning with targets (3-5 cycles)
Screening
Construction of Human IgG
(C) Transgenic mouse
Immunization with targets
Harvest splenocytes, generate hybridomas
Screening
Human mAb
(D) Single B cell
Human
PBMC
Sort B cells with labeled antigens
$V_HDJ_HC_\gamma1$
$V_KJ_KC_K$
PCR, construct V_H and V_L

Bispecific antibody

The recent development of bispecific antibodies offers attractive new opportunities for the design of novel protein therapeutics. A bispecific antibody can be generated by utilizing protein engineering techniques to link two antigen binding domains (such as Fabs or scFvs), allowing a single antibody to simultaneously bind different antigens.

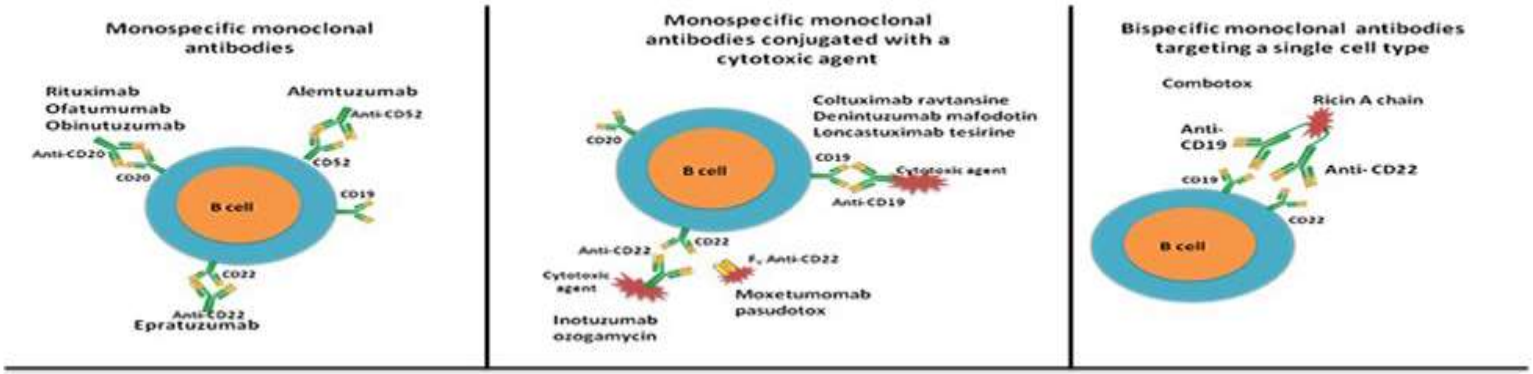
Thus, bispecific antibodies may be engineered to exhibit novel functions, which do not exist in mixtures of the two parental antibodies. Most bispecific antibodies are designed to recruit cytotoxic effector cells of the immune system to target pathogenic cells .

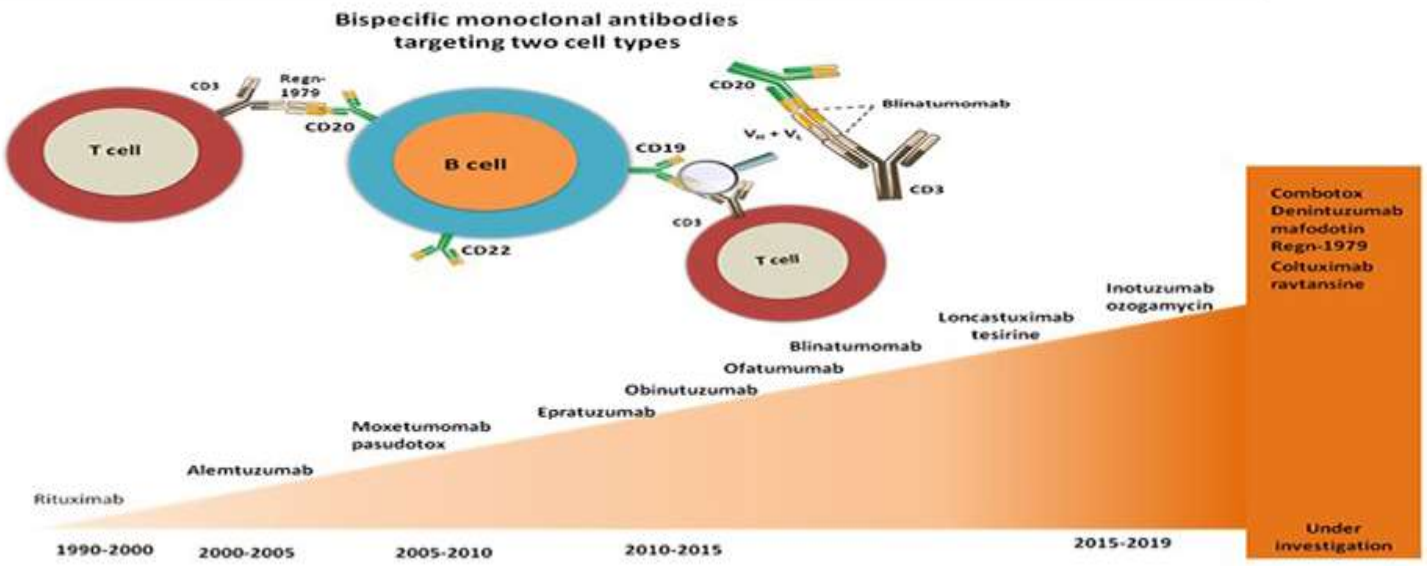
The first approved bispecific antibody was catumaxomab in Europe in 2009 . **Catumaxomab targets CD3 and EpCAM to treat solid tumors** in patients with malignant ascites. However, this drug was withdrawn from the market in 2017 for commercial reasons.

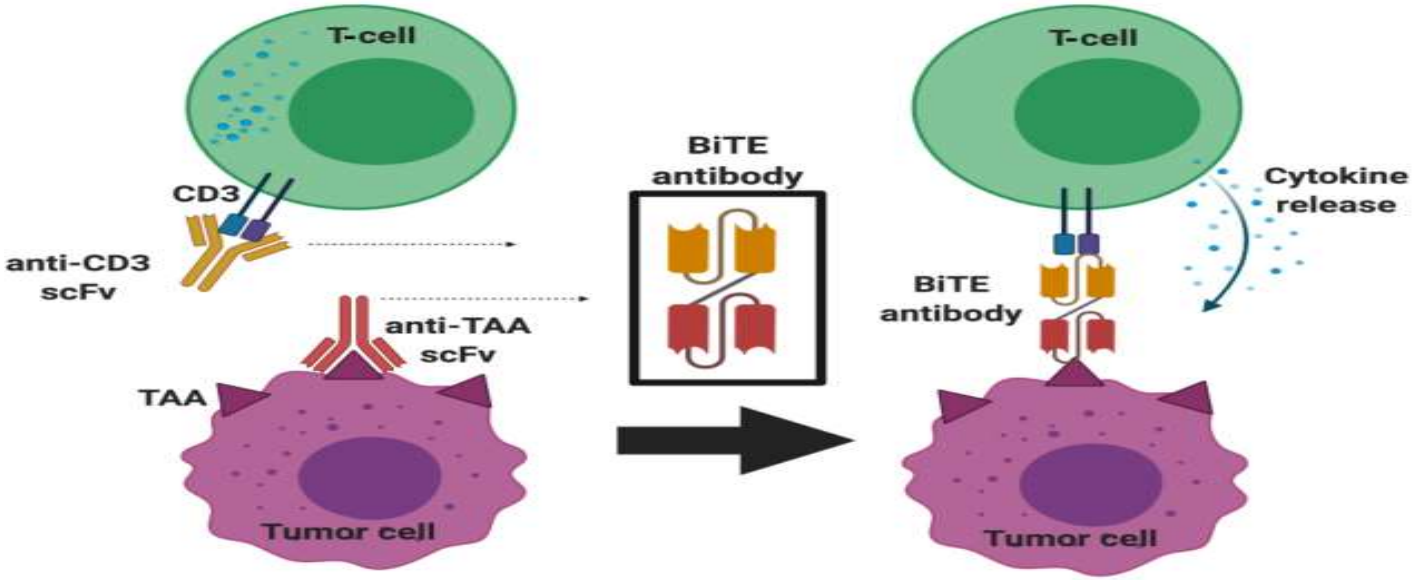
Currently, two bispecific antibodies have obtained US FDA approval and are on the market. First, **blinatumomab is a bispecific T-cell engager (BiTE) that targets CD3 and CD19 for treatment of B-cell precursor acute lymphoblastic leukemia** (ALL) . Second, **emicizumab is a full-size bispecific IgG with natural architecture, which binds to activated coagulation factors IX and X for the treatment of haemophilia A .**

To date, there are more than 85 bispecific antibodies in clinical trials, about 86% of which are under evaluation as cancer therapies . The concepts and platforms driving the development of bispecific antibodies continue to advance rapidly, creating many new opportunities to make major therapeutic breakthroughs

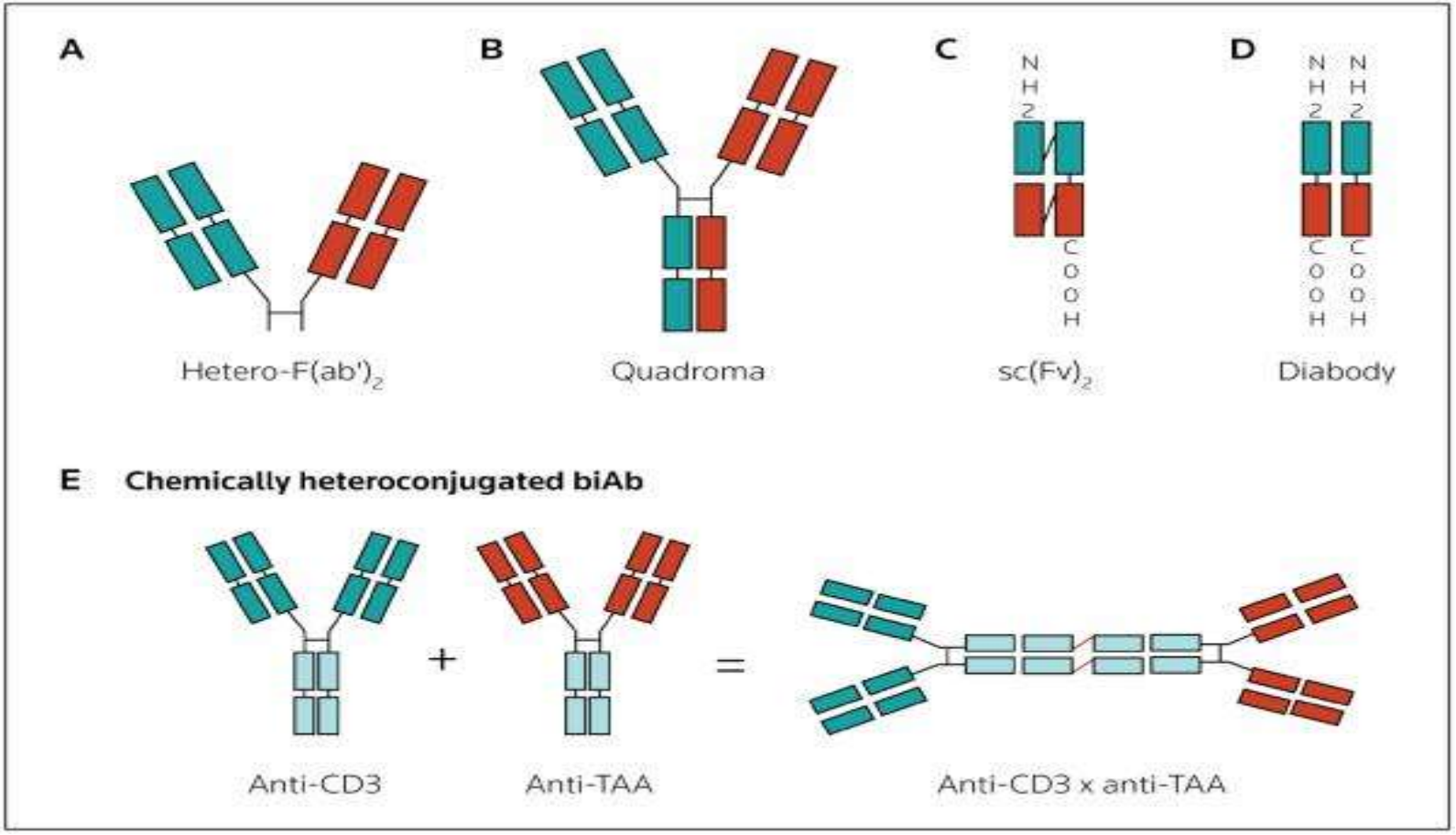
Blinatumomab is composed of two single-chain variable antibody fragments (scFv) connected by a flexible linker. One scFv binds to the CD19 antigen, which is expressed on more than 90% of B-cell cancer lineages, and the other scFv binds to the T-cell receptor/CD3 complex. Thus, blinatumomab brings B-cells and T-cells in contact, activating cytotoxic T-cells to release cytolytic proteins that induce apoptosis



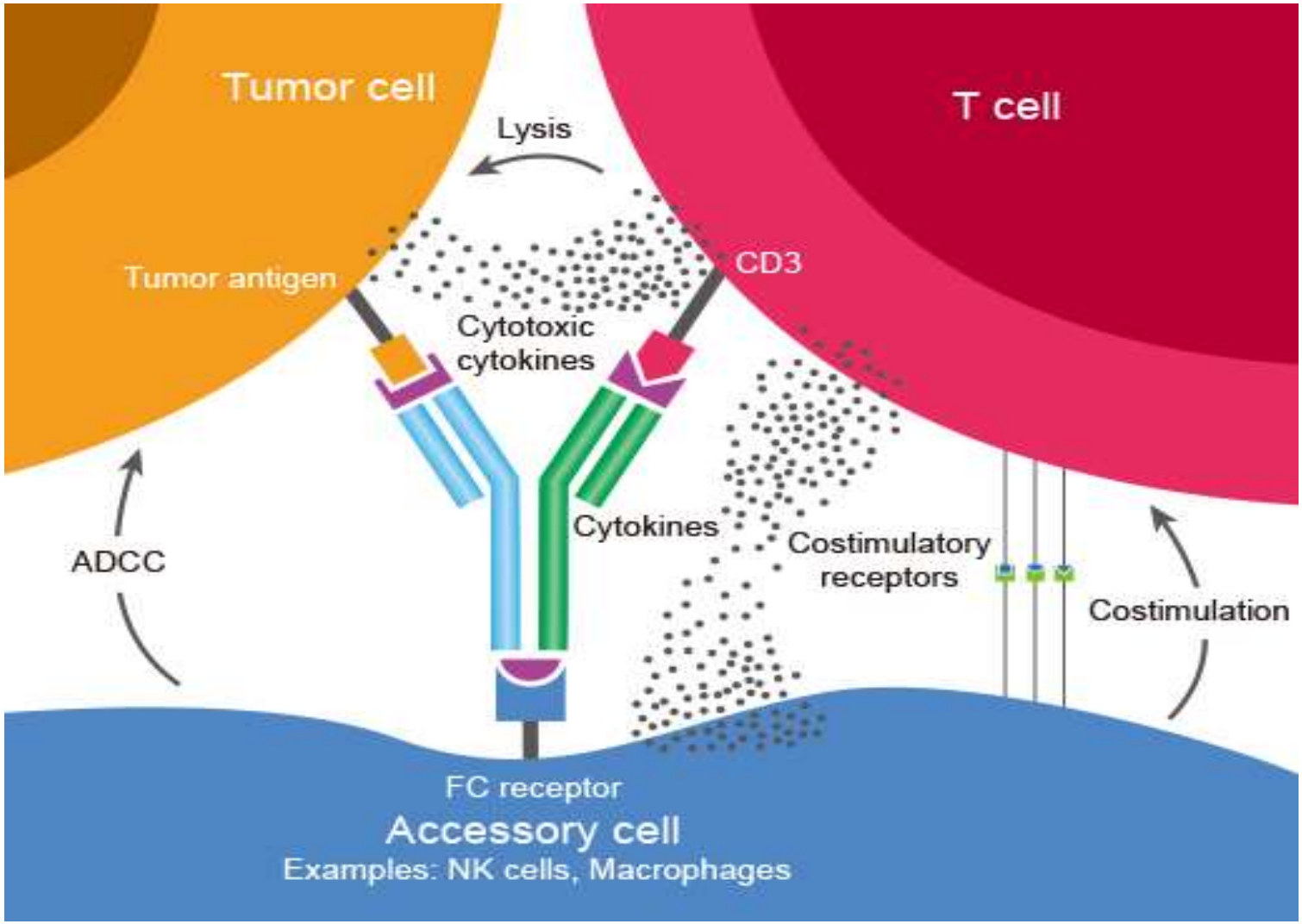


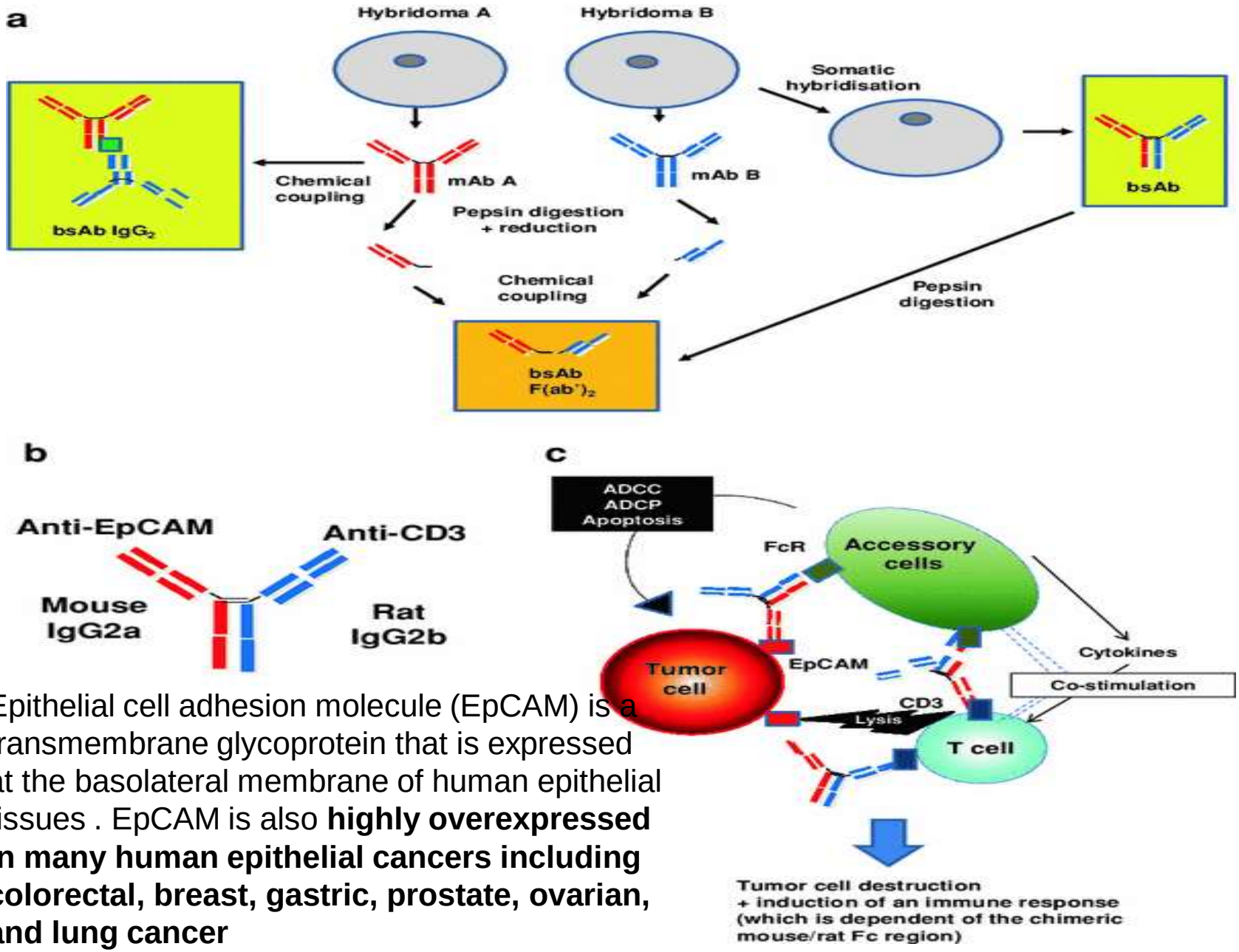


Bispecific T-cell Engager (BiTE) structure and mechanism of action. An anti-tumour-associated antigen (TAA) single-chain variable fragment (scFv) is linked via a small linker molecule to an anti-CD3 scFv . Binding of anti-TAA to tumour cell surface antigens redirects T-cells to target the tumour cell via anti-CD3 binding causing the formation of a lytic immune synapse with cytokine release.



Bispecific antibody formats Genetically engineered antibody fragments or the heteroconjugation of intact antibodies to generate bispecific antibodies (**biAb**s) in various formats are shown: (**A**) **fragment antigen-binding (Fab) format**, (**B**) **quadroma (IgG) formats constructed by fusing two hybridomas secreting antibodies of different specificities**, (**C**) **single-chain antibody (scFv [single-chain variable fragment])-based formats,** (**D**) **diabody formats** or (**E**) **chemical heteroconjugation** of two IgG molecules [$(IgG)_2$] of different antigen specificities. **Hetero-F(ab)$_2$** Heterogeneous fragment antigen-binding, **TAA** tumor-associated antigen


Tumor cell
T cell
Lysis
Tumor antigen
CD3
Cytotoxic
cytokines
Cytokines
Costimulatory
receptors
ADCC
Costimulation
FC receptor
Accessory cell
Examples: NK cells, Macrophages



Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein that is expressed at the basolateral membrane of human epithelial tissues . EpCAM is also **highly overexpressed in many human epithelial cancers including colorectal, breast, gastric, prostate, ovarian, and lung cancer**

Even though bispecific antibodies have similar characteristics as monoclonal antibodies, they remain an artificial molecule that can not be produced by normal B-cells.

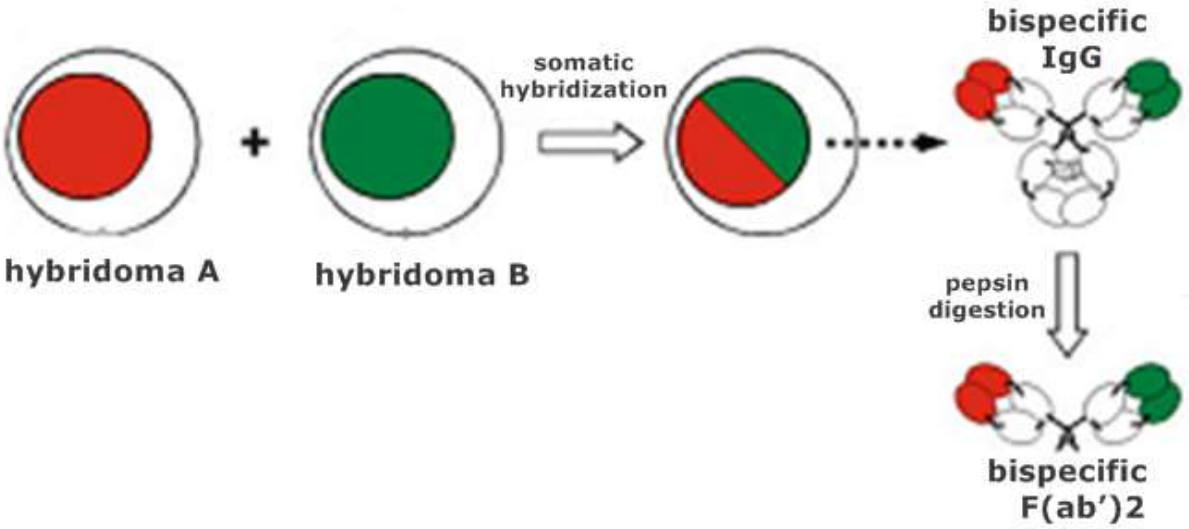
Bispecific antibodies can be obtained by different biochemical methods such **as chemical conjugation of two antibodies, fusion of two antibody-producing cell lines, or genetic approaches resulting in recombinant bispecific antibody molecules.**

Currently, **two major classes of bispecific antibodies have been described: Immunoglobuline-G (IgG)-like bsAbs and small bsAbs.**

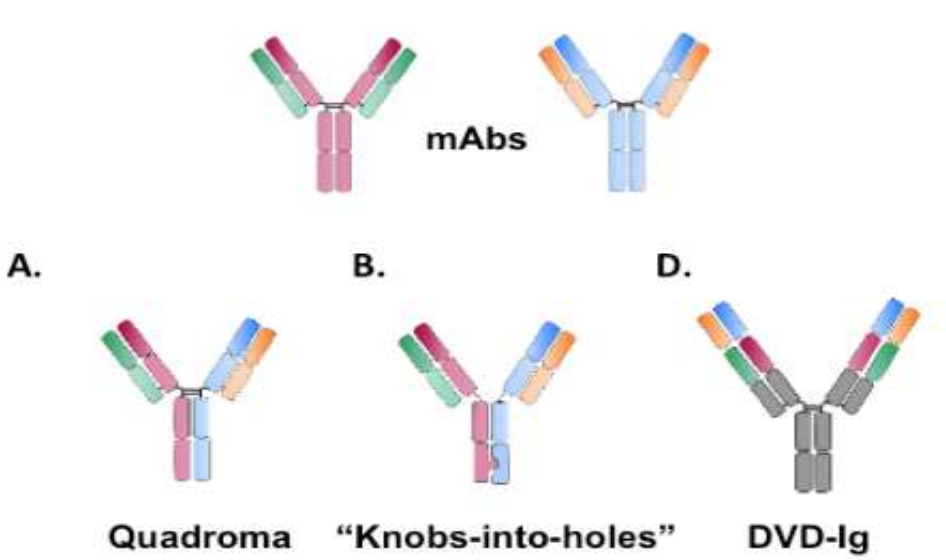
The IgG-like bispecific antibodies have a conserved immunoglobulin constant domain, thus they can **exhibit Fc-mediated activities**. Although these antibodies share the same properties as conventional IgG antibodies, they differ in size and geometry. Therefore, a significant effort has been made to produce bsAbs with an IgG design that is as natural as possible.

On the other hand, small bsAbs are genetically engineered recombinant antibodies **lacking a constant domain.** They have been primarily designed as effector cell recruiters, and consisting of only the Fab regions, and various types of bivalent and trivalent single-chain variable fragments (scFvs). There are also fusion proteins mimicking the variable domains of two antibodies. The furthest developed of these newer formats are the bi-specific T-cell engagers (BiTEs).

The **quadroma or hybrid hybridomas** was the first approach used to create bispecific antibodies, when the idea for bispecific antibodies arose in the 1980s. The quadroma technology consists in constructing and producing bispecific antibodies **by somatic fusion of two hybridoma cells** (obtained by a somatic fusion of an antibody producing lymphocyte and a myeloma cell). Both hybridoma cells express monoclonal antibodies with different specificities, the ones desired for the bispecific antibody. The quadromas cell line from the two fused hybridomas secretes the two antibodies including the bispecific antibody with two distinct arms . However, this method has not been adequate to produce bispecific antibodies in a quality and quantity required for therapeutic use, because of random chain association. The random pairing of the light and heavy Ig chains, resulted in less then 1/10 of functional bsAbs, hence, the bsAbs purification has been complicated



To overcome this chain association issue **a chimeric quadroma** was created from a murine and a rat hybridoma cell line•. The chimeric quadroma expressing mouse IgG2a and rat IgG2b chimeric antibodies had three major technical improvements: (I) enrichment of functional bsAbs because of preferential intra-species heavy/light chain association; (II) efficient heterologous inter-species heavy chain association; (III) simple purification of the desired bsAbs with a specific affinity for a protein A and ion exchange chromatography. This method allows a better purification, and allows a large scale supply of bispecific antibodies .



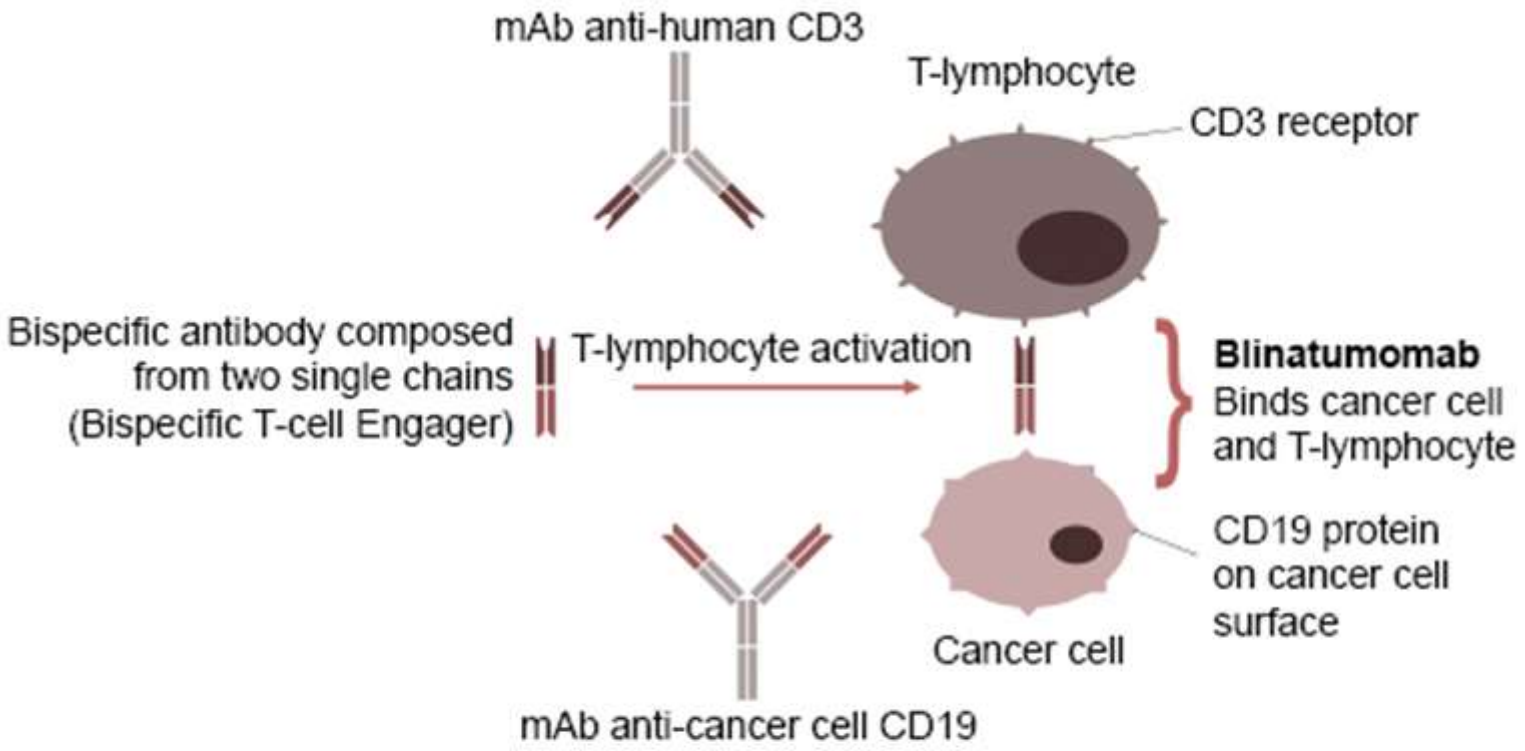
dual variable domain immunoglobulin

Recently, an alternative redox method has been described as an improvement of the traditional quadroma method, in order to produce rapidly bispecific antibodies using the mild reducing agent 2-mercaptoethanesulfonic acid sodium salt (MESNA). During the oxidative reaction, a mixture of antibodies is produced, including bispecific antibodies and parental antibodies. The bsAbs are highly purified over two sequential affinity columns. The redox method can be used to produce bsAbs from mAbs of a different or the same subclass and the same species .

The bispecific antibodies obtained by the these methods have maintained the structure of conventional IgG antibodies. Despite being mouse/rat chimeric, the Fc part has retained the effector function, and therefore these antibodies have also been described as **trifunctional or Triomabs**®

"KNOBS INTO HOLES" APPROACH

The progress in genetic engineering and in DNA recombinant technology has led to a new strategy to facilitate the heavy chain association – the "knobs-into-holes" (KiH) approach. The "knobs-into-holes" approach is based on a **transfection of modified human genes coding for the antibodies, in mammalian cells**. The advantage of this method is that the resulting bispecific antibodies are of human instead of murine/rat nature, and consequently they are less immunogenic. The production of bispecific antibodies with the "knobs-into-holes" strategy is based on **a single amino acid substitution in the opposite CH3 domains, that promotes heavy chain heterodimerization**. In one of the heavy chains referred as a "knob" variant, **a small amino acid has been replaced with a larger one (T366Y) in the CH3 domain**. Subsequently, in the **other heavy chain, a large amino acid has been replaced with a smaller one (Y407T)** .A sort of a hole is formed, permitting the interaction with the "knobs" variant miming a key-lock concept . Furthermore, to optimize the heavy chains association as well as the stability of bispecific antibodies, **six mutations were introduced: two in the "knob" heavy chain (S354A, T166W) and four in the "hole" heavy chain (Y349C, T366S, L368A, Y407V).** Moreover, the heterodimeric Fc part **can be further stabilized by an additional disulfide bridge created artificially**. The co-expression of the two variants of the heavy chains is sufficient to allow efficient production of bsAbs, with more than 90% correct chain association.



Mechanism of blinatumomab therapeutic action: recruitment of T cells to tumors through binding of tumor-cell-surface antigens to immune cells.

Abbreviation: mAb, monoclonal antibody.

Targeted Intracellular Delivery of Antibodies

Although it is generally believed that antibodies do not penetrate the plasma membrane, there is a considerable data pool that illustrates the presence of various antibodies within cells as a result of antigen-driven or antibody-driven cell entry of the free antibody or antibody–antigen complex.

The property of most antibodies that limits their applications to targeting extracellular proteins is their inability to cross cellular membranes to reach the cytosol and compartments such as the nucleus. **One exception is the small subset of naturally occurring mouse and human autoantibodies that target host self-antigens during autoimmune diseases, such as multiple sclerosis**

Various technologies have been developed and tested to accomplish this task; some success has occurred. Those strategies can be grouped into several major classes: **direct physical delivery, direct intracellular expression, fusion with the part of internalizing autoantibodies responsible for their intrinsic ability to enter the cells, the use of protein-transduction domains or their mimics, and the use of various nanoparticle carriers** (including inorganic nanoparticles, liposomes, polymersomes, and viral envelopes).

All these physical delivery methods offer the advantage of direct antibody delivery inside the cytosol with the ability to target other compartments (e.g., the nucleus) upon the attachment of the specific localization signal, as shown **for the nuclear delivery of anti-PCNA antibody conjugates with NLS delivered into the cells via electroporation** (Freund et al., 2013). Possible limitations of these methods include a lack of cell-specificity, in addition to rather difficult *in vivo*, and difficulties in clinical

Delivery approach	Antibody type	Targeted antigen	Reference
Microinjection	monoclonal antibody (mAb) (IgG)	α-tubulin	Lessman et al., 1997
Microinjection	polyclonal antibody labeled with Alexa Fluor 488	inosine-5′-monophosphate dehydrogenase 2	Keppeke et al., 2015
Microinjection	mAbs	N-terminal transactivation region of p53	Gire and Wynford-Thomas, 1998
Microinjection	four different mAbs	α-p21	Ma et al., 1999
Microinjection	polyclonal antibody	fos	Riabowol et al., 1988
Microinjection	polyclonal antibody (IgG)	actin	Scheer et al., 1984
Microinjection into either the nuclei or cytoplasm	NLS-conjugated polyclonal antibody (IgG)	lamin A/C histone-binding site	Dixon et al., 2017
Electroporation	two different mAbs	bovine asparagine synthetase	Chakrabarti et al., 1989
Electroporation	mAbs	proliferating cell nuclear antigen (PCNA) DNA polymerase α, HPV16 E6 oncogene	Freund et al., 2013
Electroporation	mAb and Fab	PCNA DNA polymerase alpha	Desplancq et al., 2016
In situ electroporation	mAbs	TF-1 apoptosis-related gene 19 (TFAR19), or Programmed Cell Death 5(PDCD5)	Rui et al., 2002
Electroporation	scFv-Fc	myosin, tubulin	Marschall et al., 2014
Electroporation	polyclonal antibody	pp60c-src	Marrero et al., 1995
Electroporation	mAb	cyclin D1	Lukas et al., 1994
Electroporation	mAbs and Fabs labeled with Alexa Fluor 488	γH2AX, α-tubulin, heptapeptide repeats of nonphosphorylated C-terminal domain of the largest subunit of RNA Pol II, TATA binding protein (TBP), TBP-associated factor 10	Conic et al., 2018
Microfluidics	mAb labeled with Alexa Fluor 488	tubulin	Sharei et al., 2013
Sonoporation	mAb	E6 HPV 16 oncoprotein	Togtema et al., 2012
Laser-induced cavitation bubbles	mAb labeled with Alexa Fluor 488	α-tubulin	Wu et al., 2015

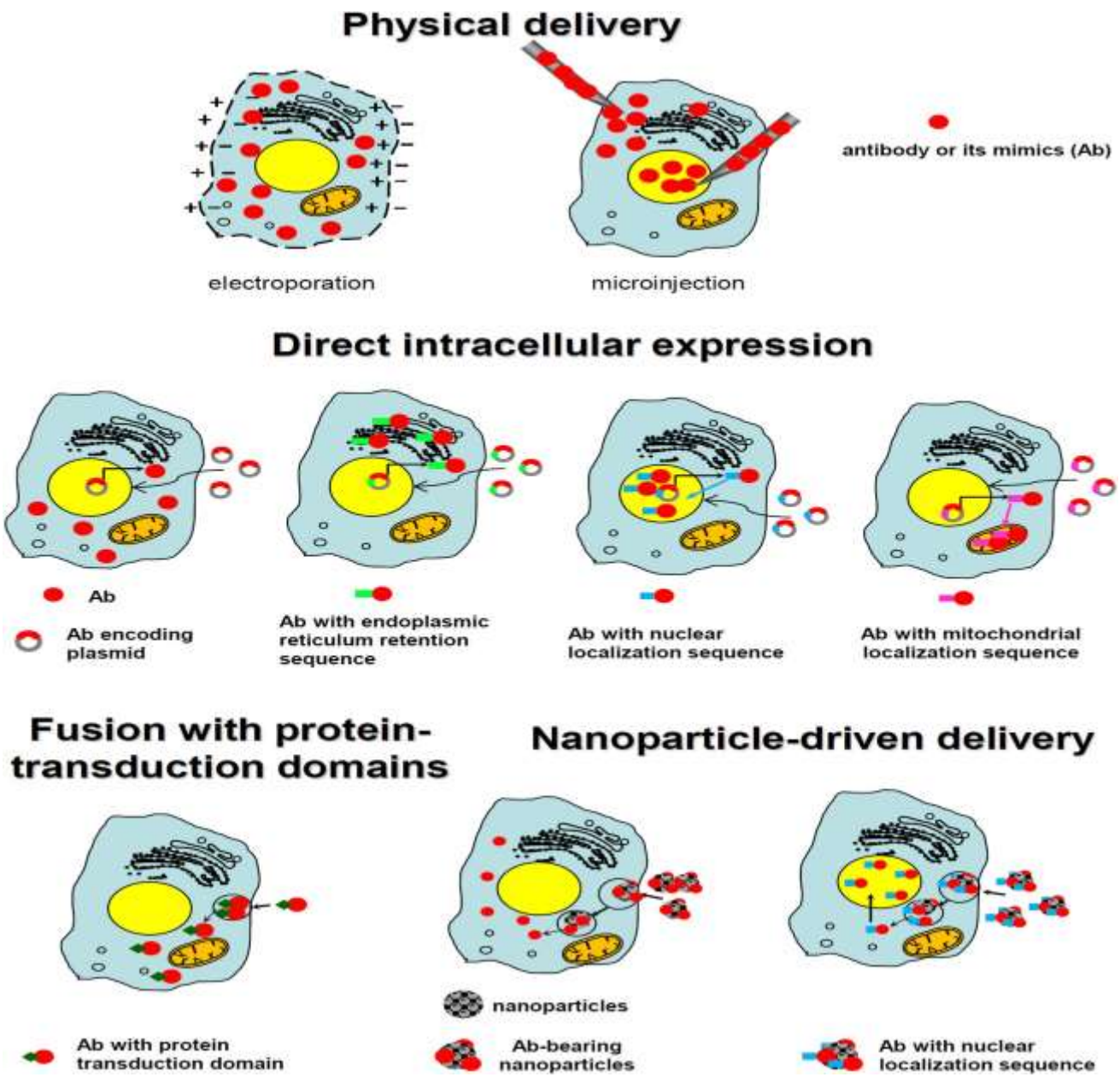
a well-established straightforward method for intracellular antibody delivery is the **so-called intrabody technique**. An intrabody is an antibody or its derivative produced within the same cell where the antigen is located. Intrabody expression can be **achieved by cell transfection with a plasmid or virus carrying the gene encoding the antibody or its fragment**. This technology has become more attractive since one of the pioneering proof-of-principle works demonstrating the inhibition of **alcohol dehydrogenase I in *Saccharomyces* transfected with cDNA-coding antibody** was published in 1988 (Carlson, 1988). The advantages of this approach are the direct expression within the cell and relatively easy direction of intrabody to the desired cell compartment where the specific antigen should be bound (e.g., membrane or secreted protein in the ER or nuclear protein in the nucleus). The latter is usually achieved by the attachment of a targeting sequence. The targeting of intrabodies to be retained in the ER appears to be the most straightforward approach, enabling their maturation and folding in the native environment.

Since the discovery of the first and prototypical HIV 1 trans-activating (TAT) protein, capable of **cell membrane penetration** (Green and Loewenstein, 1988; Frankel and Pabo, 1988) in 1988, a wide variety of **CPP**s and some of their mimics was derived and recruited for the intracellular delivery of various agents for research studies or potential therapeutic applications

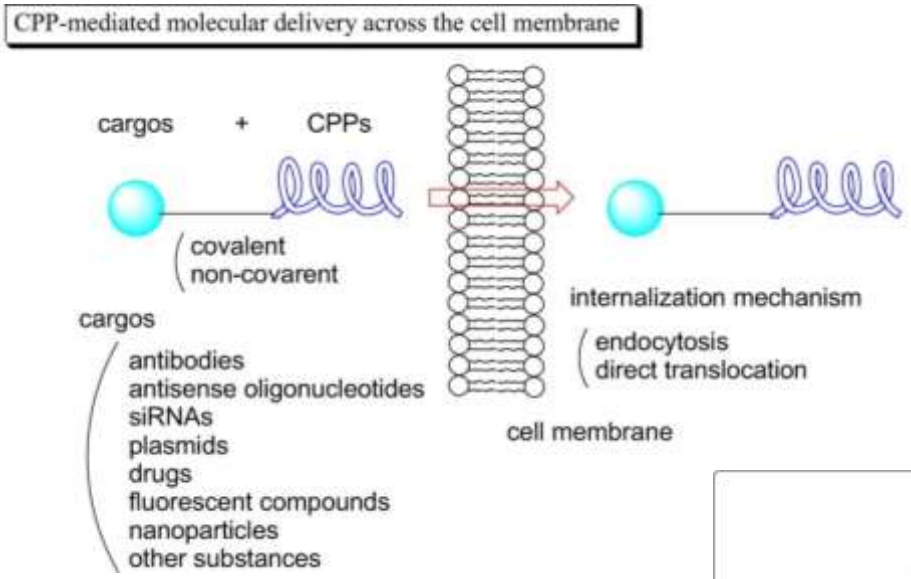
Approach relying on fusion to protein transduction domains, or CPPs, is a widely utilized technique to shuttle various cargos (proteins, plasmid DNA, RNA, oligonucleotides, liposomes, imaging agents, and anti-cancer drugs) into living cells

Enhanced cell retention and internalization of Fab fragments decorated with a TAT protein-derived peptide, was followed more than a decade later by a set of articles demonstrating that antibody-based fusion proteins containing a short membrane transport-facilitating peptide were transported into the cells and bound to intracellular structures (Zhao et al., 2001) and **that anti-caspase-3 antibody based TransMabs were able to inhibit apoptosis**

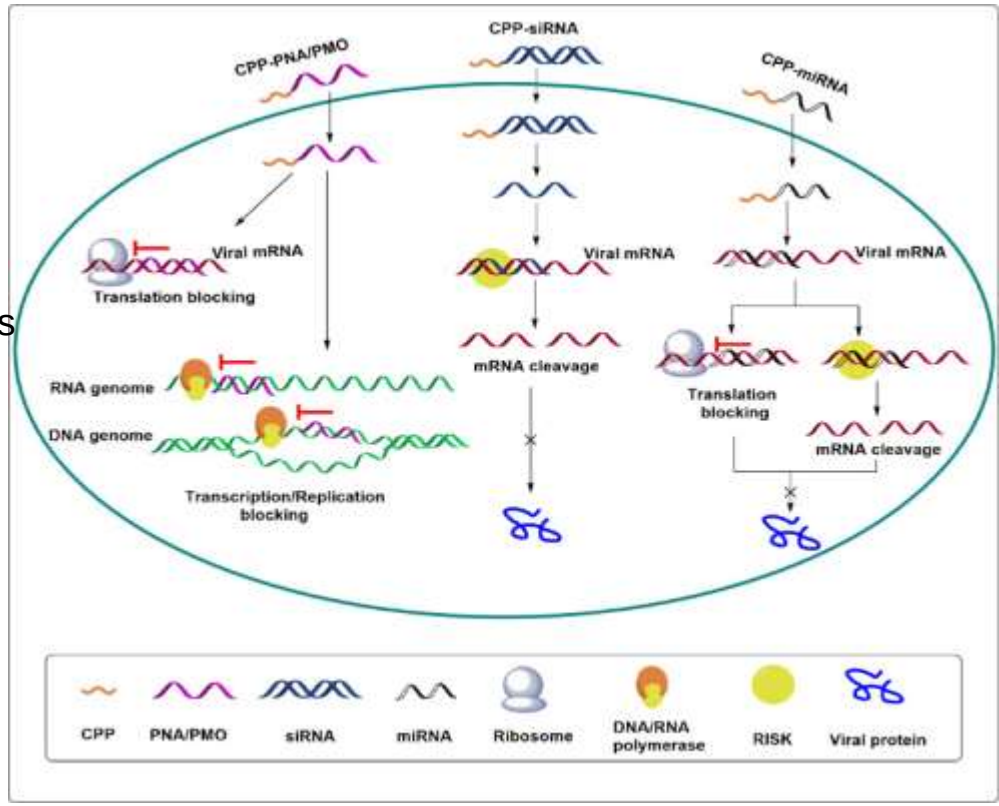
Nanocarriers can be easily designed to provide a controlled payload release in response to thermal (body, increased tumor temperature, or localized heating), specific enzymatic (e.g., tumor microenvironment or intracellular enzymes) and specific pH microenvironments (e.g., acidic tumor microenvironment or acidifying endocytic vesicles) as well as to external light exposure or any combination of these.



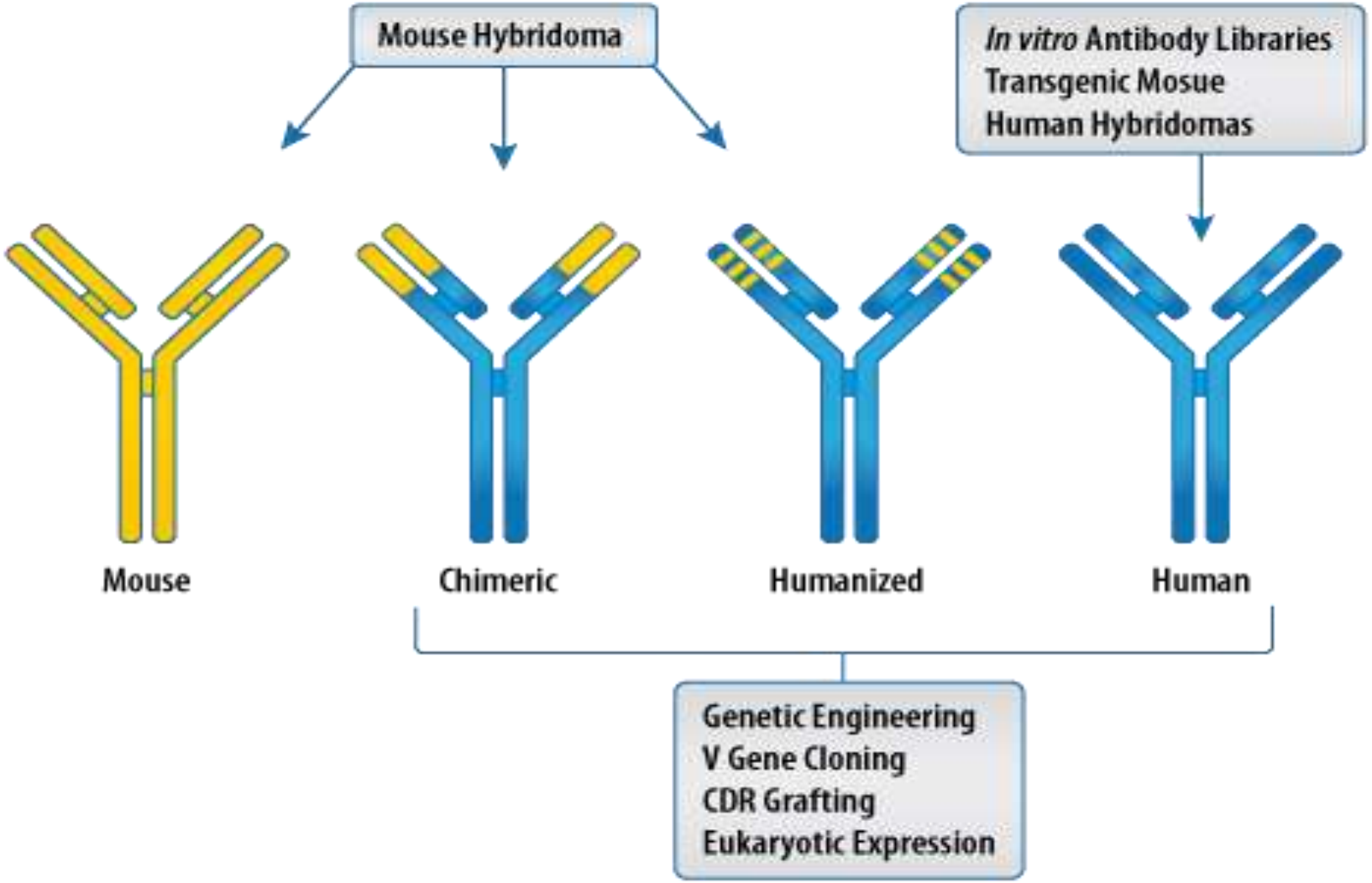
Schematic presentation of the main approaches utilized for the intracellular targeting of antibodies.

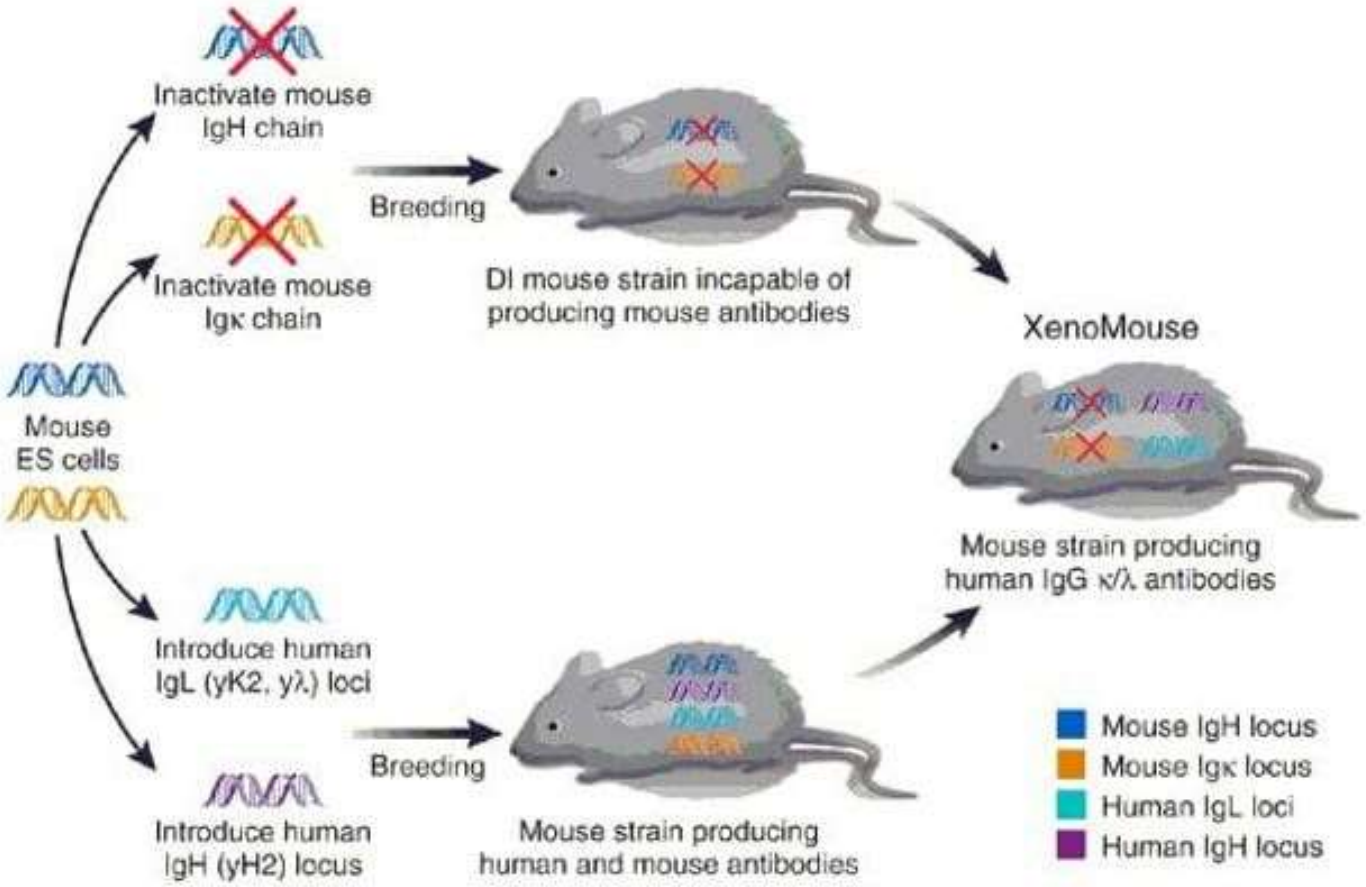


CPPs provide a novel and efficient approach for oligonucleotide delivery , which were used either as covalently linked conjugates or non-covalent complexes Successful delivery and therapeutic efficiency of CPP-siRNA conjugates against various infectious viruses particularly those with no currently available efficient therapy were shown. CPP-siRNA targeting the virus essential genes or the sequences of a vast range of viruses, such as HIV ,HCV ,HBV, influenza , exhibited significant inhibitory effects on virus replication *in vitro* and *in vivo*

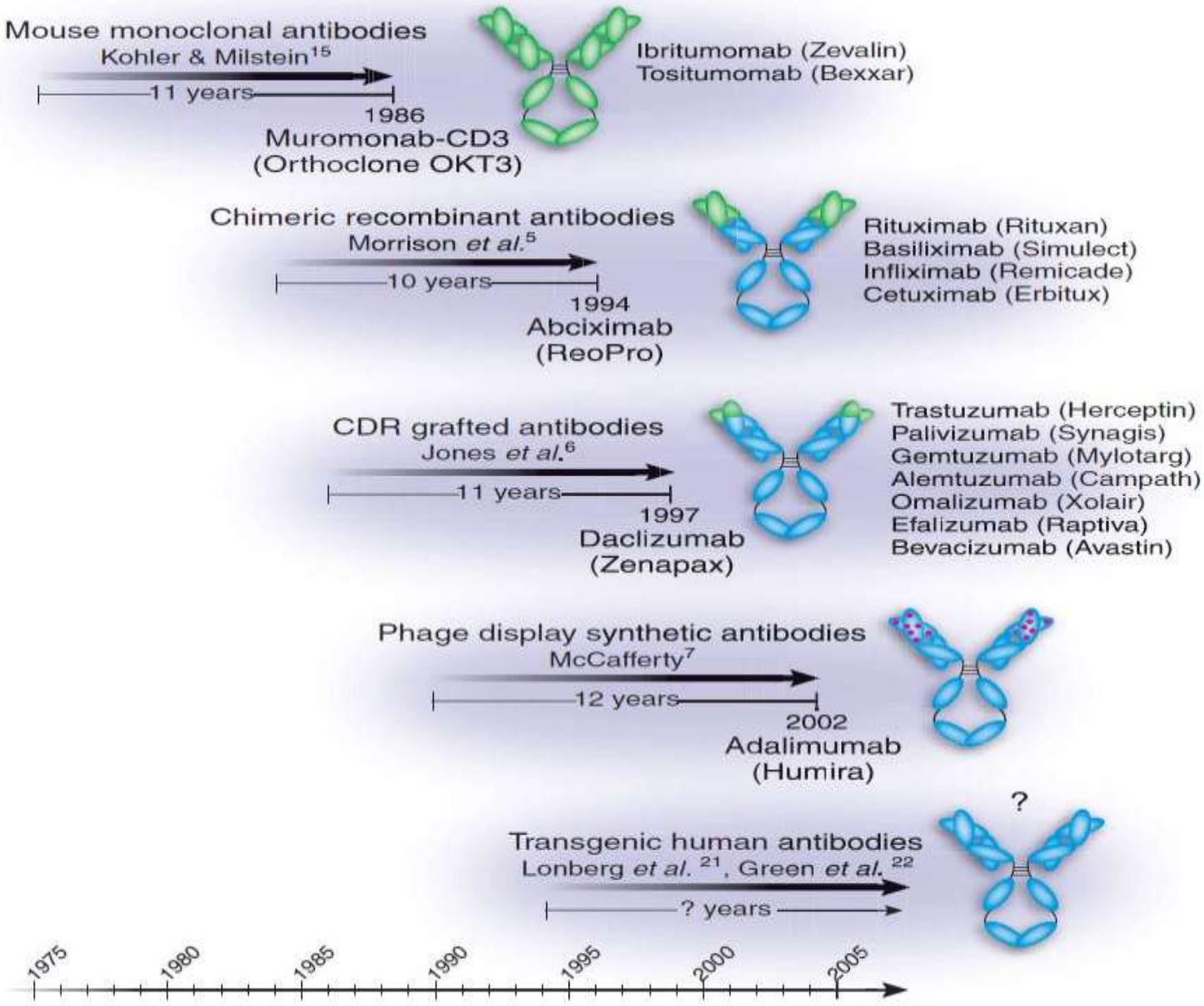


Humanization can be necessary when the process of developing a specific **antibody** involves generation in a non-**human** immune system (such as that in mice). ... The latter also **have** their protein sequences made more similar to **human antibodies**, but carry a larger stretch of non-**human** protein.

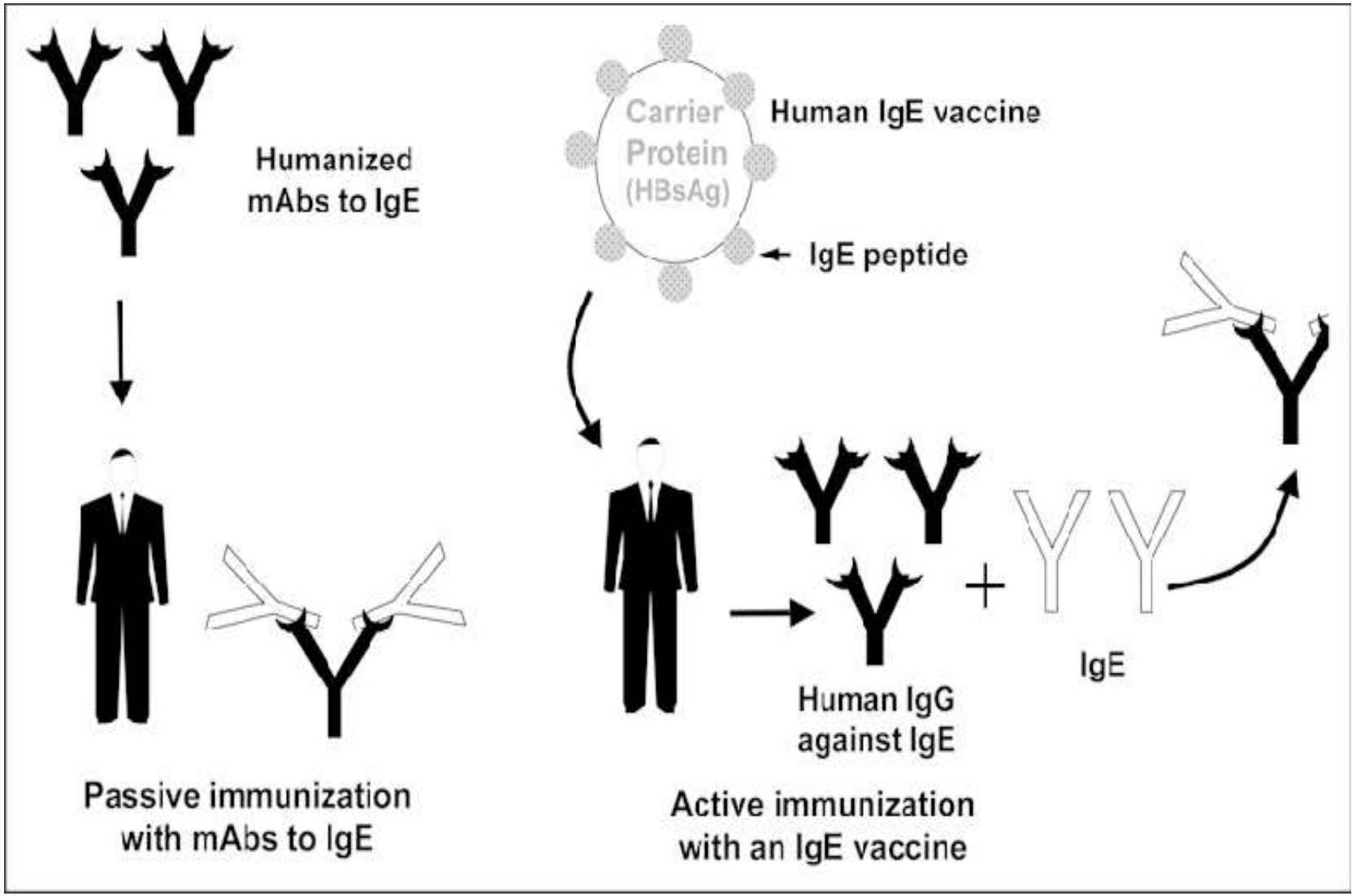




Jakobovits A; Amado RG, *et al.* (2007) "From XenoMouse technology to panitumumab, the first fully human antibody product from transgenic mice." *Nat Biotechnol.* 25(10):1134-43.

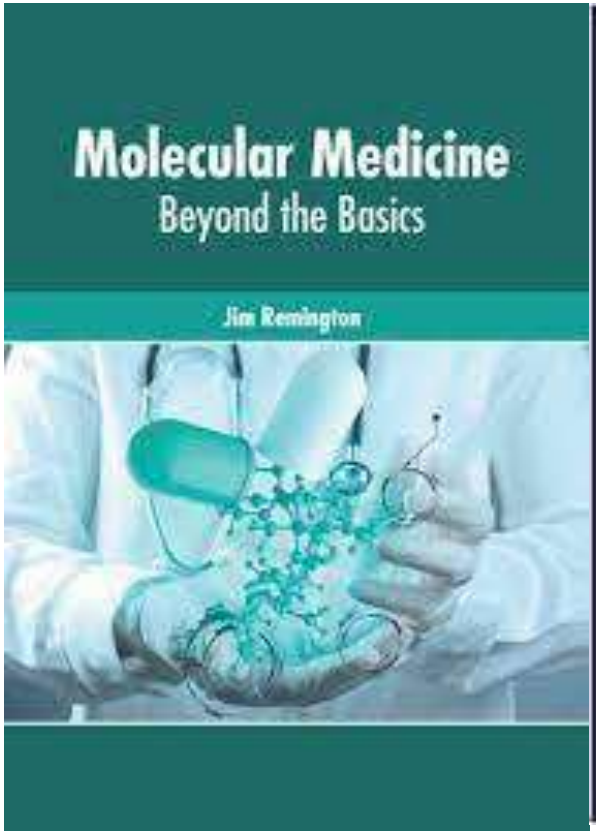

Mouse monoclonal antibodies
Kohler & Milstein[15]
11 years
1986
Muromonab-CD3
(Orthoclone OKT3)
Ibritumomab (Zevalin)
Tositumomab (Bexxar)
Chimeric recombinant antibodies
Morrison et al.[5]
10 years
1994
Abciximab
(ReoPro)
Rituximab (Rituxan)
Basiliximab (Simulect)
Infliximab (Remicade)
Cetuximab (Erbitux)
CDR grafted antibodies
Jones et al.[6]
11 years
1997
Daclizumab
(Zenapax)
Trastuzumab (Herceptin)
Palivizumab (Synagis)
Gemtuzumab (Mylotarg)
Alemtuzumab (Campath)
Omalizumab (Xolair)
Efalizumab (Raptiva)
Bevacizumab (Avastin)
Phage display synthetic antibodies
McCafferty[7]
12 years
2002
Adalimumab
(Humira)
Transgenic human antibodies
Lonberg et al. [21], Green et al. [22]
? years
?
1975
1980
1985
1990
1995
2000
2005

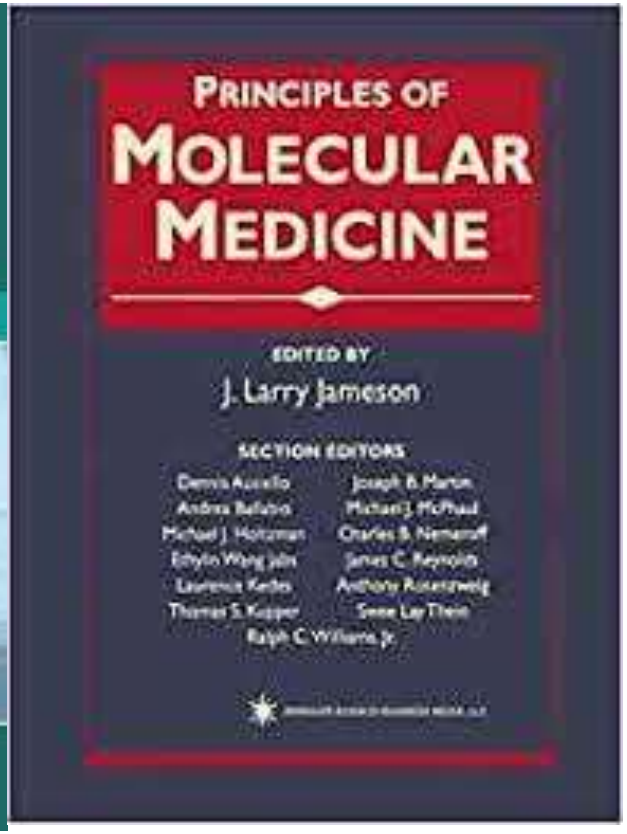
Passive immunization with monoclonal antibodies to IgE and active immunization with an IgE vaccine.



Name	Source	Target	Status
Omalizumab	Chimerical mAb	IgE	FDA-approved
Lumiliximab	Chimerical mAb	FcγRII/CD23	Phase II
Soluble IL-4 R	Fusion protein	IL-4	Phase II
Keliximab	Humanized mAb	IL-5	Phase II
Lumiliximab	Chimerical mAb	CD4	Phase II
Mepolizumab	Humanized mAb	IL-5	Phase II
Daclizumab	Humanized mAb	IL-2Rα	Phase II
CAT-354	Human mAb	IL-13	Animal model
IL-13Rα2-IgFc	Fusion protein	IL-13	Animal model
Infliximab	Chimerical mAb	TNFα	Phase II in asthma*
Etanercept	Fusion protein	TNFα	Phase II in asthma^
CAT-192,-152	Human mAb	TGFβ	Animal model#

*FDA-approved in Crohn disease and rheumatoid arthritis. ^FDA-approved in rheumatoid arthritis, ankylosing spondylitis, etc. #Phase II in systemic sclerosis.


Molecular Medicine
Beyond the Basics
Jim Remington


PRINCIPLES OF
MOLECULAR
MEDICINE
EDITED BY
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SECTION EDITORS
Dennis Ausiello
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James C. Reynolds
Anthony Rosenzweig
Steve Lee Thein
Ralph C. Williams, Jr.

Transgenic animals (from inetrnet)

Selective mating: Improve live stock milk ,wool , egg lying frequency etc
But combination and selection : time consuming and costly
: But exceptionally sucessful
: difficult to introduce new gene by this method

Advance of rDNA technology along with sucessful gene transfer technology made it possible to create animals that carry genes from other species

Animal carry new gene -----transgenic animal
New gene --------- transgene

Strategy in Brief:

- **A cloned gene is injected into the nucleus of fertilized egg**
- **The egg is then implanted into receiptive female**
- **Some offsprig will carry the cloned gene in all their cells**
- **Breeding to generate new genetic lines**

Some animals are produced for specific requirement or economic traits like Cattle for milk, pig for meat etc

Transgenic cattle was created to produce milk containing particular human protein drugs or pharmaceuticals

Why mammary gland:

- **Milk are renewable**
- **Secreted body fluid**
- **Produce sufficient amount**
- **Collected frequently without harm of the animals**
- **No side effect of normal physiological process**
- **Posttranslational modulation**
- **Purification , if needed is simple**

Other transgenic animals are produced as diseases model --- animals genetically Manipulated to exhibit disease symptom, so that effective treatment can be established

Ex;
Harvard mouse -- Oncogen carrying that promote development of various cancer
--tumor development, immunological specificity, molecular genetics of development

Many transgenic animals produce human therapeutic agents

Examples of the expression of human recombinant proteins (RPs) in the milk of transgenic animals (TAs)

RP (construct)	Regulatory elements	TA/method of production
Albumin (native gene)	β-casein promoter (goat) + insulator	Cow/NT
α-fetoprotein (native gene)	β-casein promoter (goat) + insulator	Goat/NT
Butyrylcholinesterase (cDNA)	–"–	Goat/NT
Granulocyte colony-stimulating factor (native gene)	–"–	Goat/NT
Growth hormone (native gene)	β-casein promoter (goat)	Goat/NT
Antithrombin (cDNA)	β-casein promoter (goat)	Goat/MI
Coagulation Factor IX (mini-gene)	–"–	Mouse/MI
Tissue plasminogen activator (cDNA)	–"–	Goat/MI
Coagulation Factor IX (cDNA)	β-casein promoter (cow)	Goat/MI
Growth hormone (native gene)	β-casein promoter (cow)	Cow/NT
Granulocyte colony-stimulating factor (native gene)	α-S1- casein promoter (goat)	Mouse/MI

Production of transgenic animals

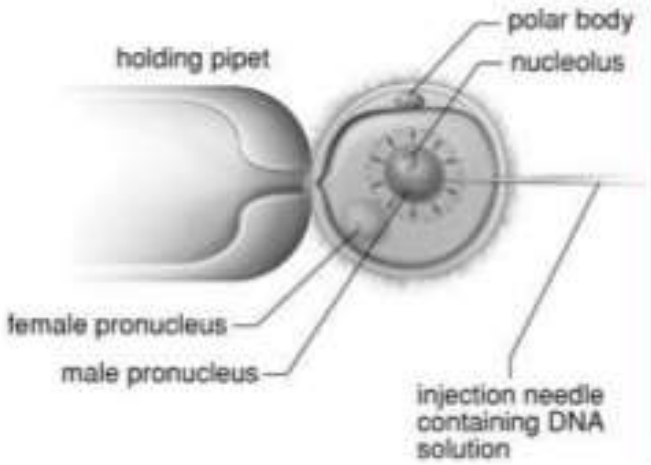
The methodology

- **Step 1 – Construction of a transgene**
  - Transgene made of 3 parts:
    - Pomoter
    - Gene to be expressed
    - Termination sequence

- **Step 2 – Introduction of foreign gene into the animal**
  - Pronuclear microinjection method
  - Embryonic stem cell method.

MICROINJECTION METHOD

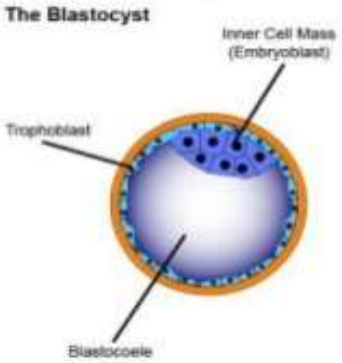
- A female animal is superovulated and eggs are collected.
- The eggs are fertilized in vitro.
- The transgene containing solution is injected into the male pronucleus using a micropipette.



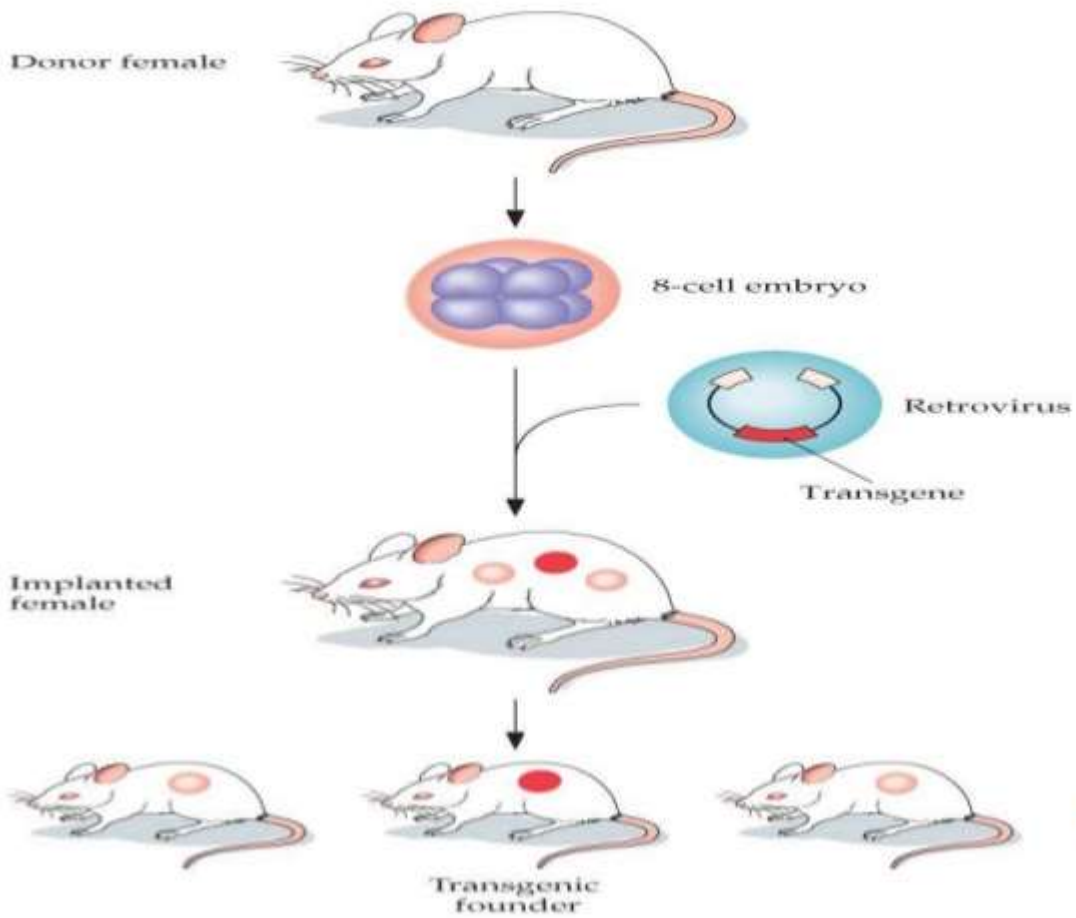
- Eggs with the transgenes are kept overnight in an incubator to develop to a 2 cell stage.
- The eggs are then implanted into the uterus of a pseudo - pregnant female (female which has been mated with a vasectimized male the previous night)

EMBRYONIC STEM CELL METHOD

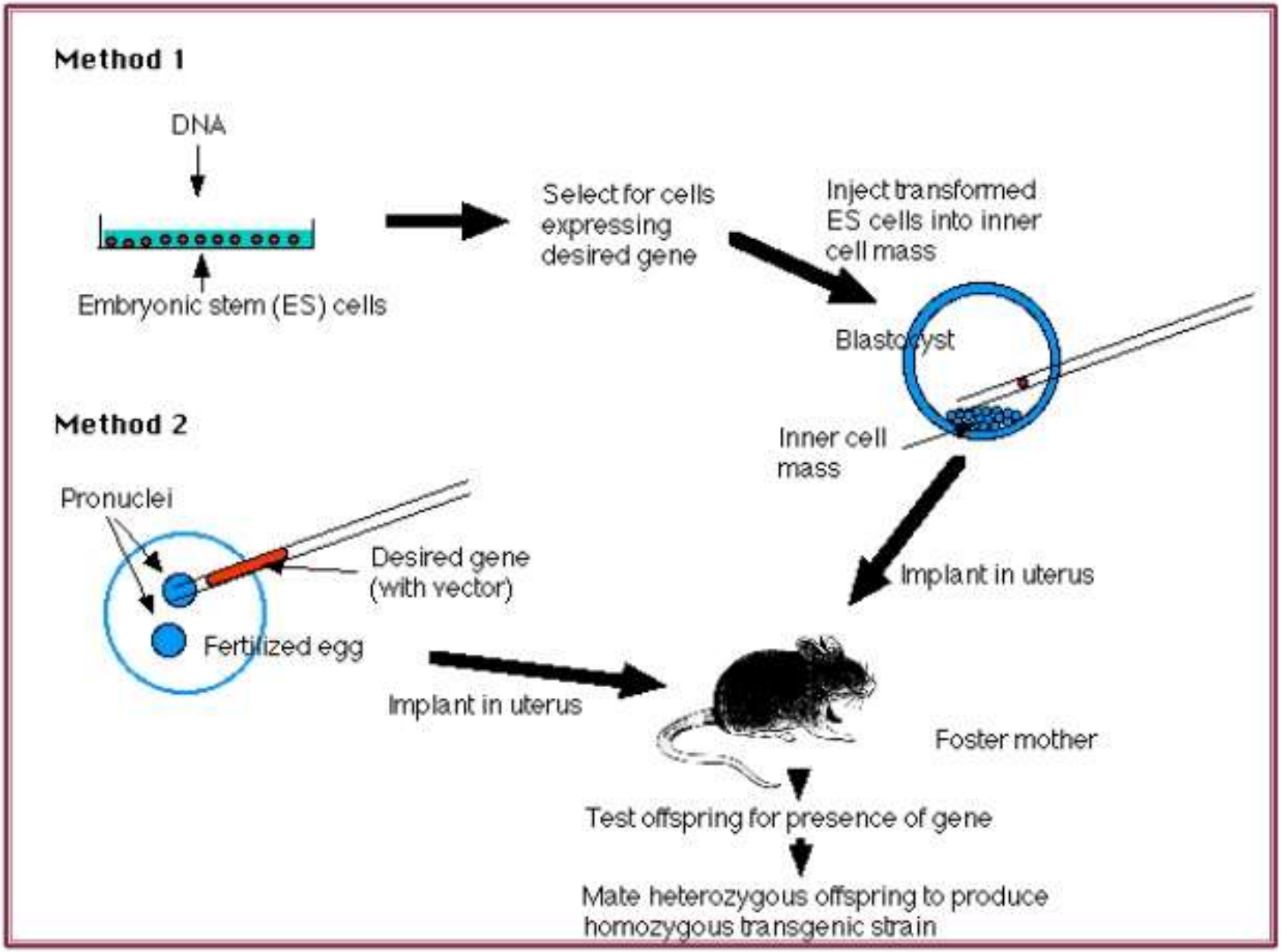
- Transgenic animals can be created by manipulating embryonic stem cells.
- ES cells are obtained from the inner cell mass of a blastocyst.
- Transgene is incorporated into the ES cell by
  - Microinjection
  - By a retro virus
  - By electroporation
- Transgenic stem cells are grown in vitro.
- Then they are inserted into a blastocyst and implanted into a host's uterus to grow normally.



BLASTOCYST MICROINJECTION


Donor female
8-cell embryo
Retrovirus
Transgene
Implanted female
Transgenic founder

- **Step 3: Screening for transgenic positives**
  - Transgenic progenies are screened by PCR to examine the site of incorporation of the gene
  - Some transgenes may not be expressed if integrated into a transcriptionally inactive site.
- **Step 4: Further animal breeding is done to obtain maximal expression.**
  - Heterozygous offsprings are mated to form homozygous strains.


Method 1
DNA
Embryonic stem (ES) cells
Select for cells expressing desired gene
Inject transformed ES cells into inner cell mass
Blastocyst
Inner cell mass
Method 2
Pronuclei
Desired gene (with vector)
Fertilized egg
Implant in uterus
Implant in uterus
Foster mother
Test offspring for presence of gene
Mate heterozygous offspring to produce homozygous transgenic strain

PROBLEMS

- Multiple insertion – too much proteins
- Insertion into an essential gene – lethality
- Insertion into a gene leading to gene silencing
- Insertion into a different area can affect the gene regulation

IDENTIFICATION OF TRANSGENIC CELLS

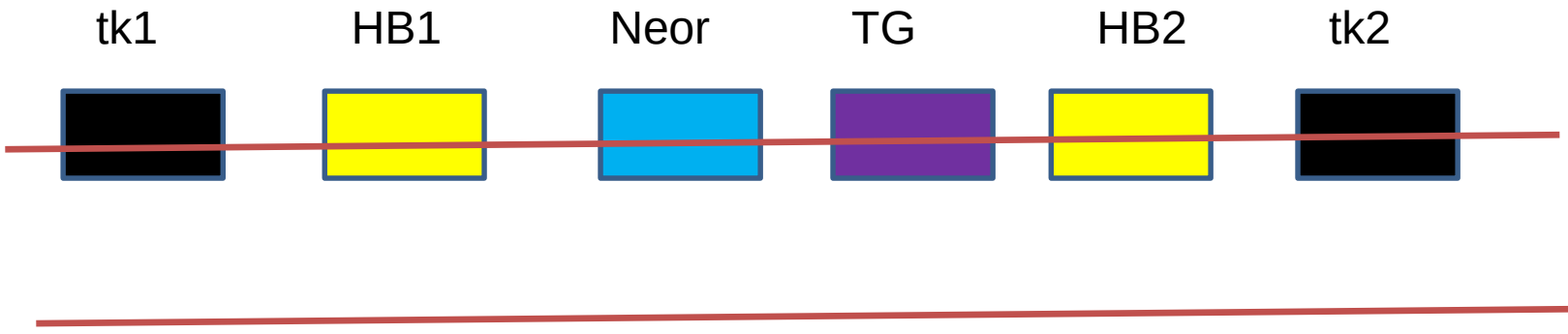
- Since only a relatively small fraction of cells take up DNA, a selective technique must be available to identify the transgenic cells
- In most cases the exogenous DNA includes two additional genes
- The small fraction of cells in which homologous recombination takes place can be identified by a combination of positive and negative selection

POSITIVE AND NEGATIVE SELECTION

- **Positive Selection-** One of the additional genes (*neo^R*) confers neomycin resistance; it permits positive selection of cells in which either homologous (specific) or non-homologous (random) recombination has occurred.

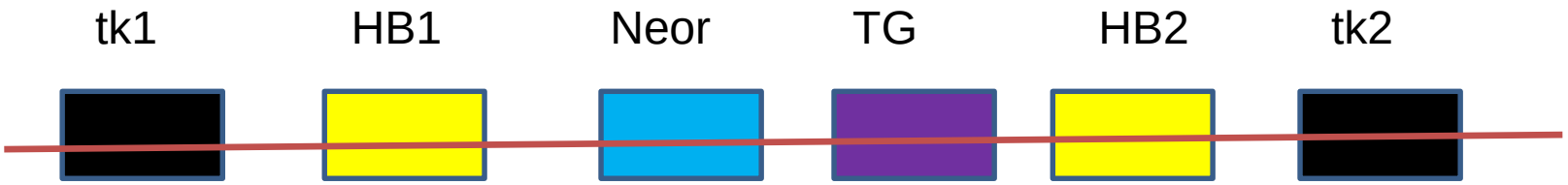
- **Negative selection-** The second gene, thymidine kinase gene from Herpes Simplex Virus (*tk^{HSV}*) confers sensitivity to gancyclovir (a cytotoxic nucleotide analog). This gene permits negative selection of ES cells in which non-homologous recombination has occurred.

- ***Only ES cells that undergo homologous recombination (i.e. gene-targeted specific insertion of the DNA construct) can survive this selection scheme.***

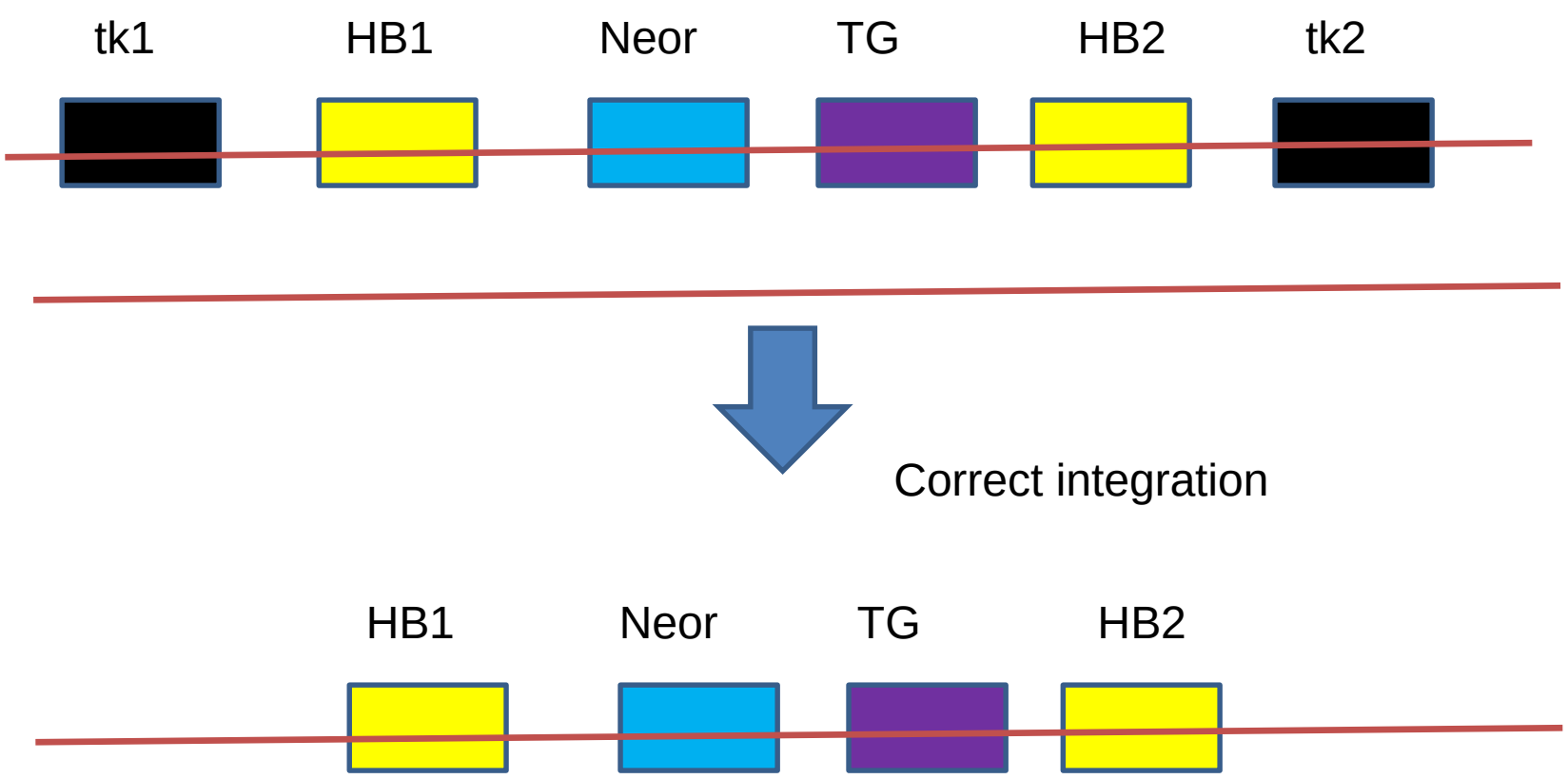


Chromoso

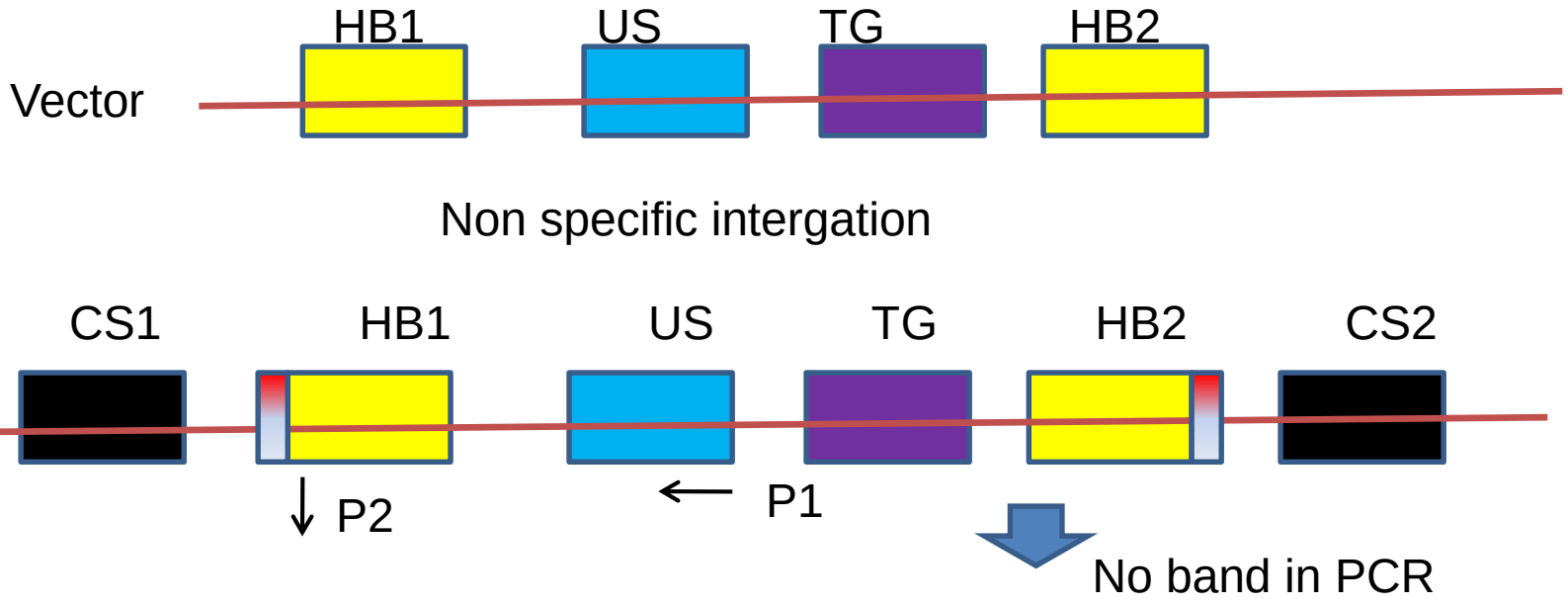
Non specific integration



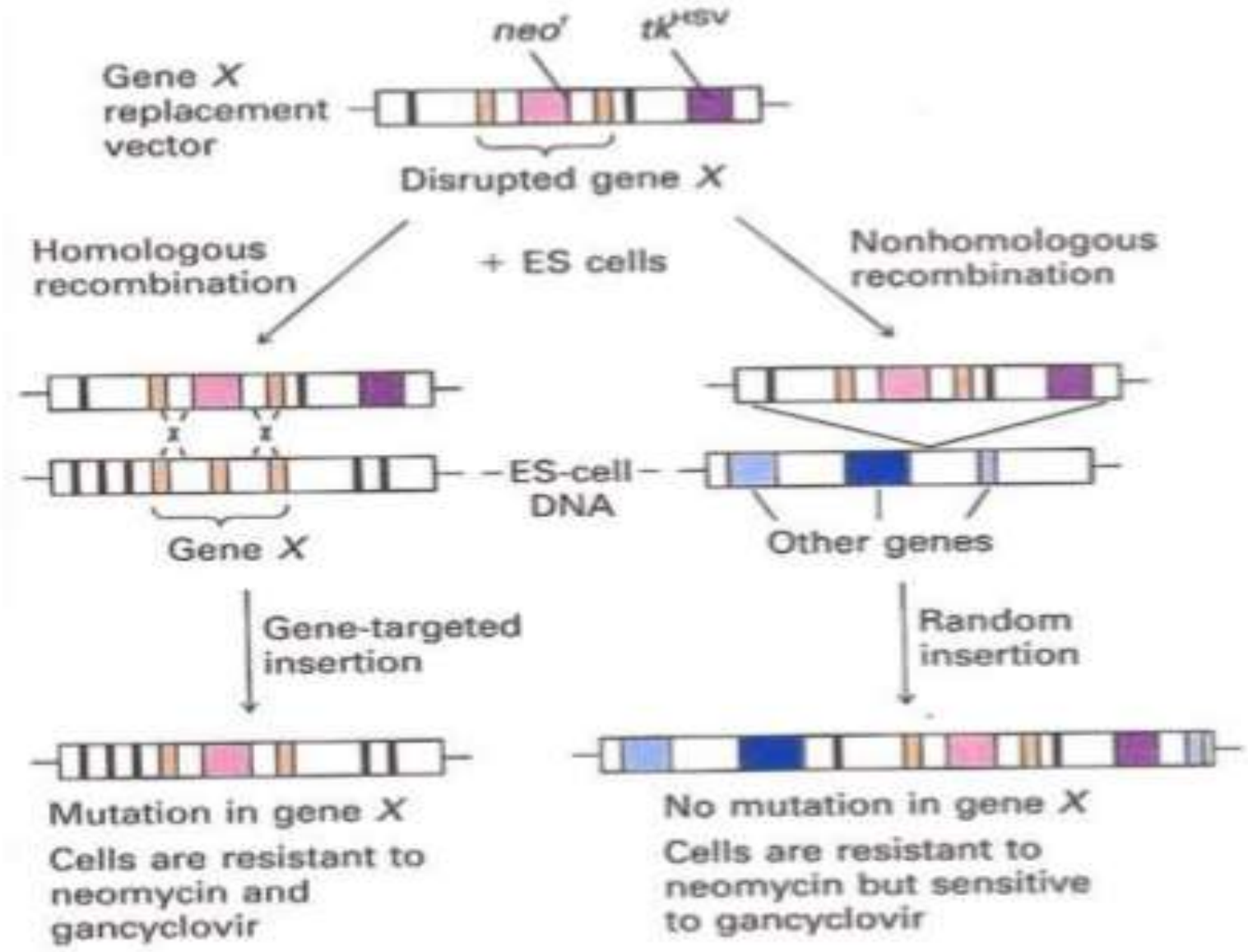
Will not survive when treated with G418+ Gancliclover as tk convert Gancliclover to toxic chemicals


tk1
HB1
Neor
TG
HB2
tk2
Correct integration
HB1
Neor
TG
HB2

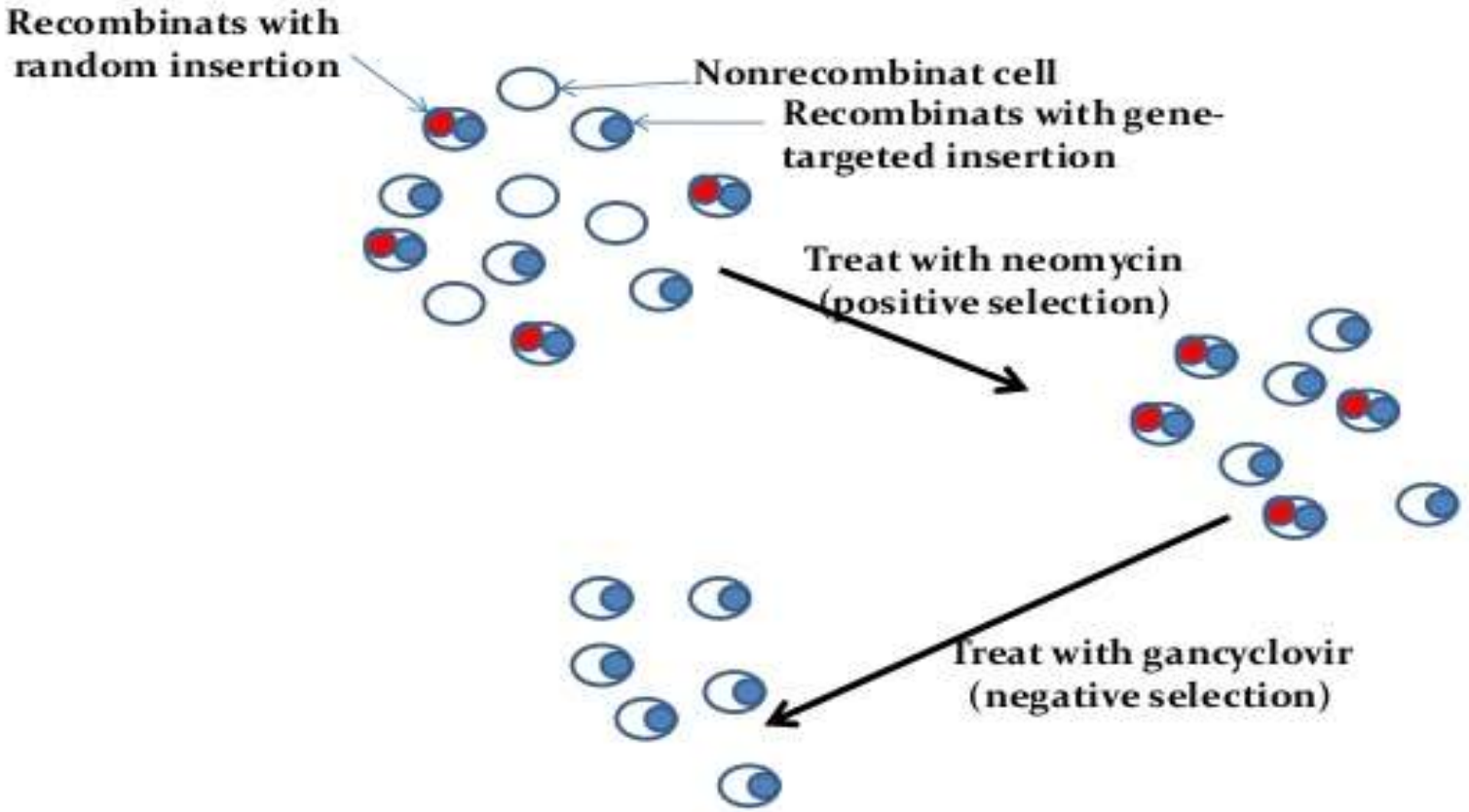
PCR based method



a) Formation of ES cells carrying a knockout mutation



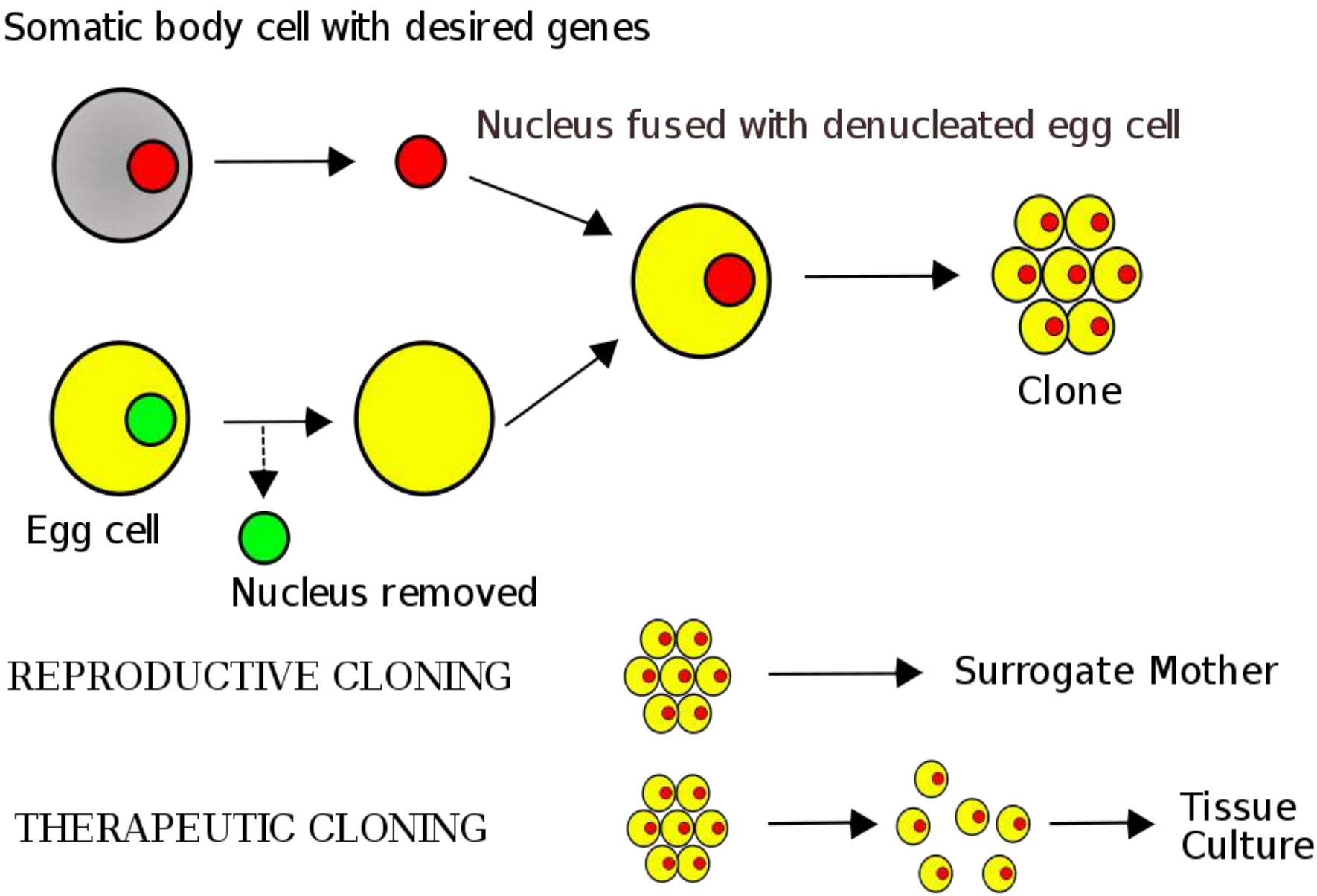
Positive and Negative selection of recombinant ES cells

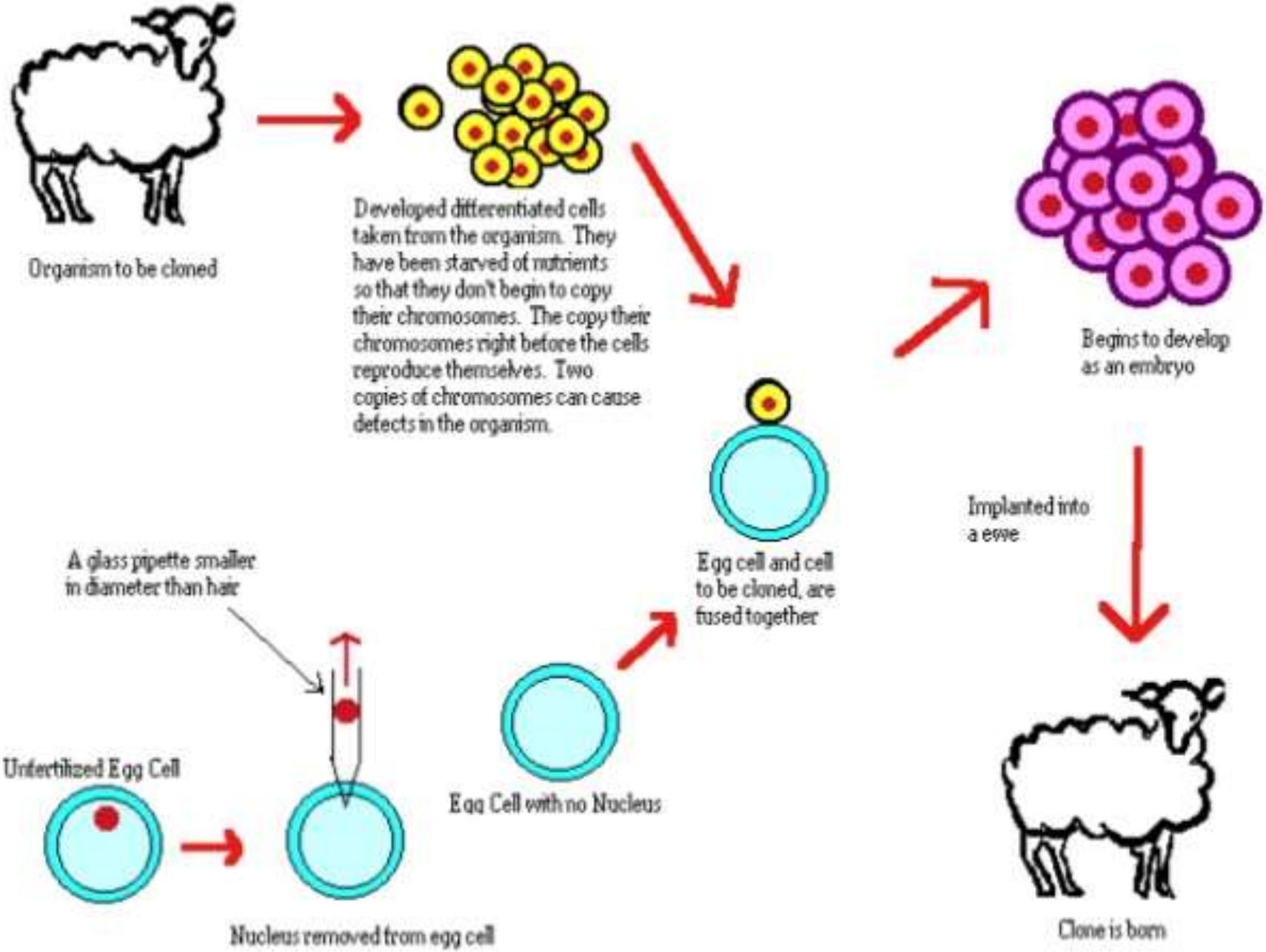


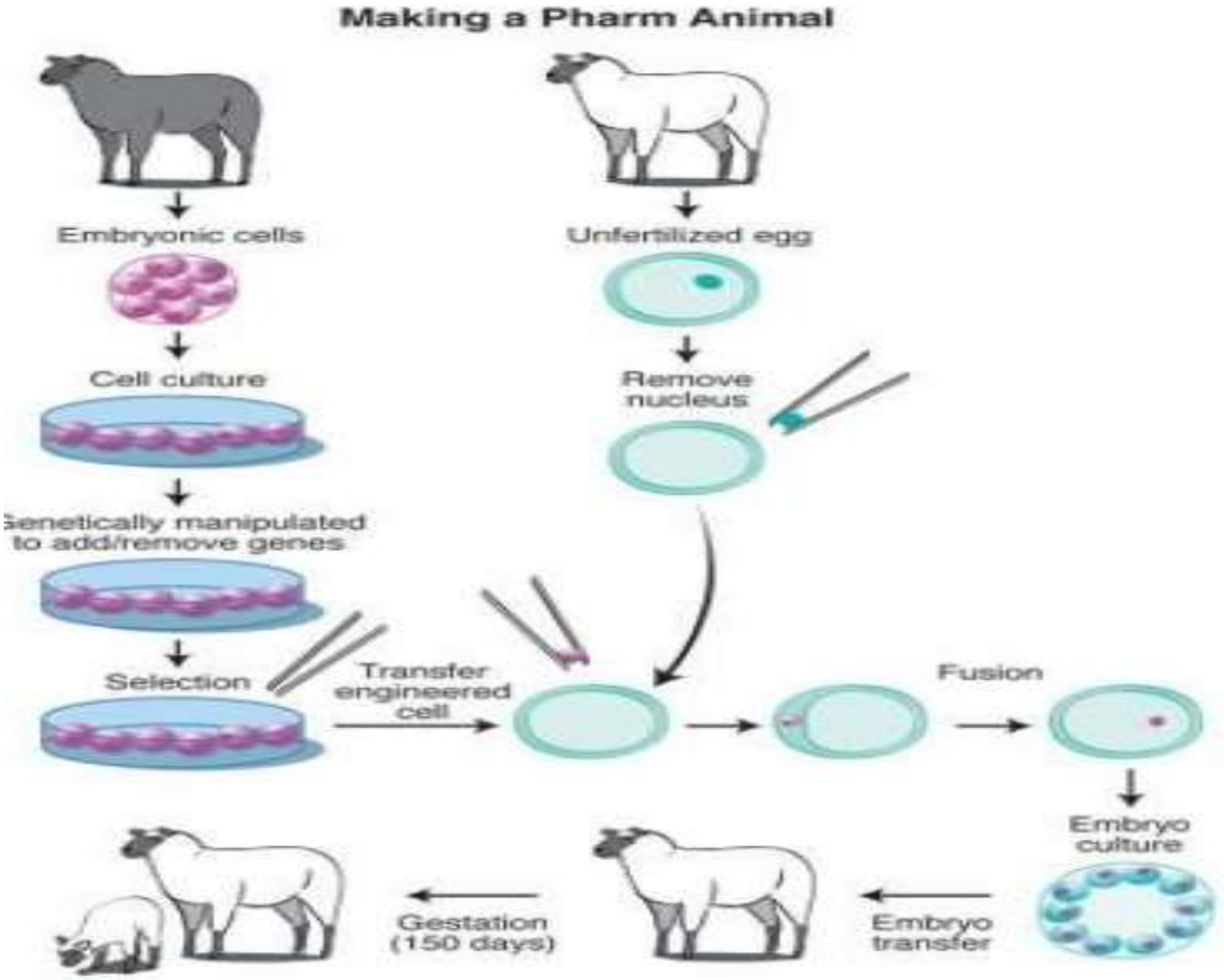
Transgenic using nuclear transfer

Nuclear transfer is a technique that can be used to create a genetically identical copy, or a clone, of an animal. ... The ability to **use** cells that can be cultured increased the number of cells available to clone **with** as well as facilitated the ability to make **transgenic** cattle

The steps involve removing the DNA from an oocyte (unfertilised egg), and injecting the nucleus which contains the DNA to be cloned. In rare instances, the newly constructed cell will divide normally, replicating the new DNA while remaining in a pluripotent state. If the cloned cells are placed in the uterus of a female mammal, a cloned organism develops to term in rare instances. **This is how Dolly the Sheep and many other species were cloned.** Cows are commonly cloned to select those that have the best milk production. On 24 January 2018, two monkey clones were reported to have been created with the technique for the first time


Somatic body cell with desired genes
Nucleus fused with denucleated egg cell
Clone
Egg cell
Nucleus removed
REPRODUCTIVE CLONING
Surrogate Mother
THERAPEUTIC CLONING
Tissue
Culture


Organism to be cloned
Developed differentiated cells taken from the organism. They have been starved of nutrients so that they don't begin to copy their chromosomes. The copy their chromosomes right before the cells reproduce themselves. Two copies of chromosomes can cause defects in the organism.
Begins to develop as an embryo
Implanted into a ewe
Egg cell and cell to be cloned, are fused together
A glass pipette smaller in diameter than hair
Unfertilized Egg Cell
Egg Cell with no Nucleus
Nucleus removed from egg cell
Clone is born


Making a Pharm Animal
Embryonic cells
Unfertilized egg
Cell culture
Remove nucleus
Genetically manipulated to add/remove genes
Selection
Transfer engineered cell
Fusion
Embryo culture
Gestation (150 days)
Embryo transfer

Other methods:

1. Chemical or

2. Viral delivery into ES cells, or homologous recombination with ES cells.

Virus mediated gene transfer

- Earliest method for successful gene transfer in mammals

- Virus has transfection property

- Killed virus is replication defective

- The virus gene is replaced with transgene gene

- The transgene is delivered to the host cell by transfection (gene therapy)

- Can be used to transfect a wide range of cells, e.g., ES cells

Virus mediated gene transfer

- Direct transfection of embryos has resulted nongermline transgenics

- ES cells transfection has resulted in germline transgenics

- Has succeeded in chickens and fish

- Transfecting oocytes resulted in 100% transgenics

- Only small transgene construct is usable (8 kb or less)
- More research is needed on the safety of the method

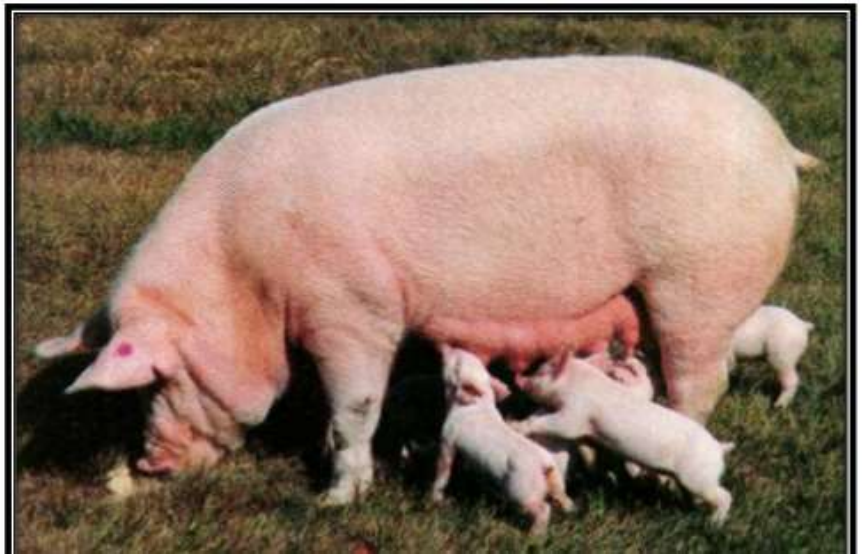
Applications of transgenic farm animals

- Agricultural applications
- Bioreactors
- Organ/cell/tissue donors
- Basic research/disease model

Human Protein C

- Blood protein.
- Functions to control blood clotting.
- Some individuals have inborn deficiency require exogenous Protein C.

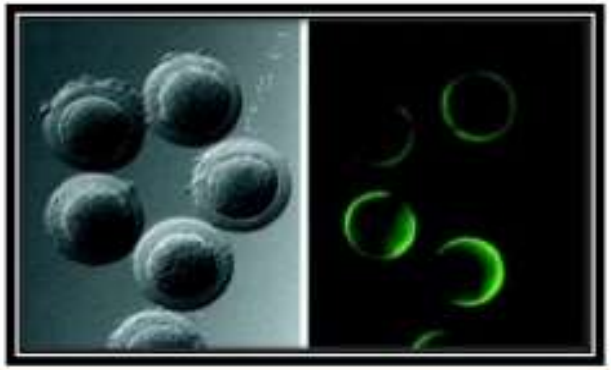
"Genie" The first genetically engineered animal to produce a human protein drug (human protein C) in her milk.



Some examples of therapeutic protein production using transgenic animals

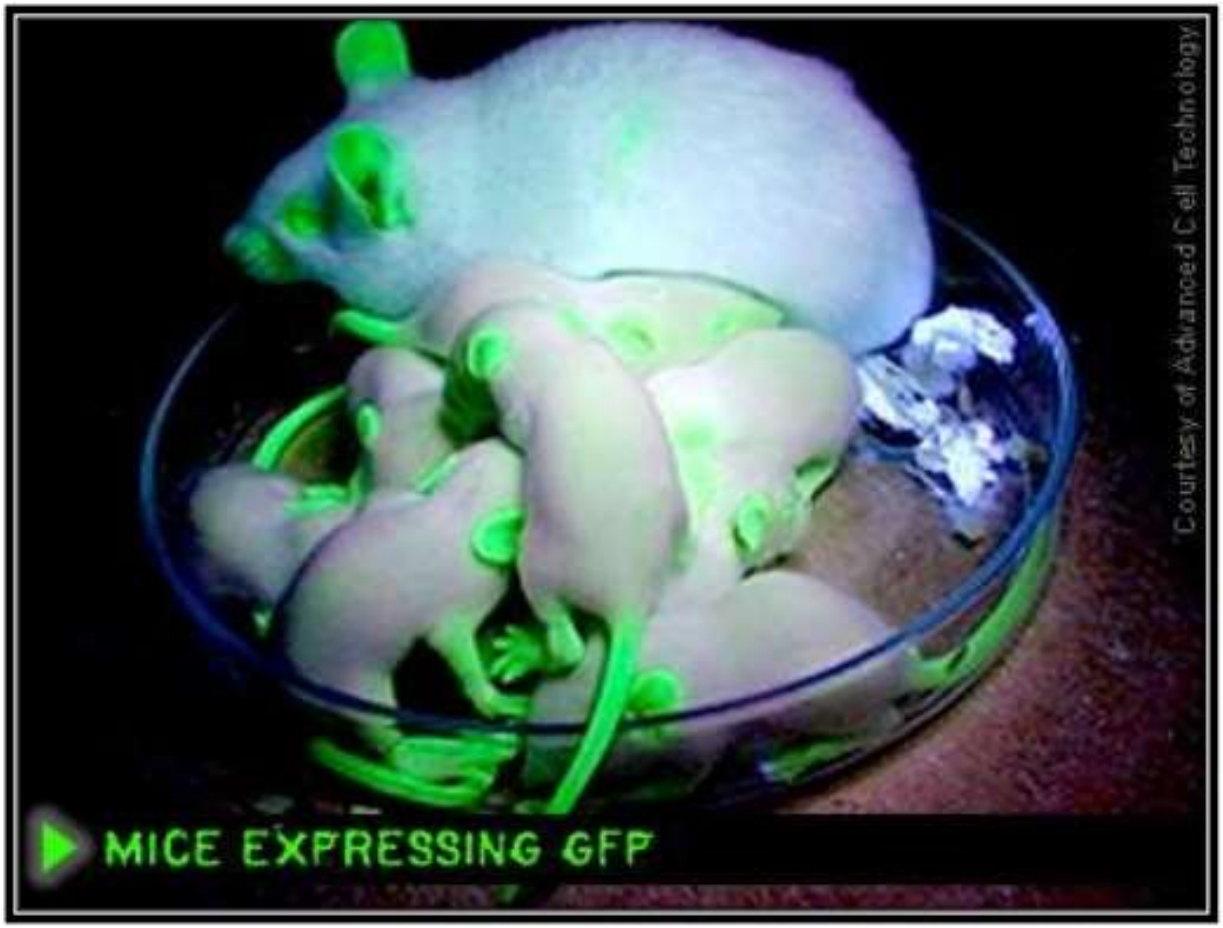
1. Growth hormone (gigantism / dwarfism) -- goat
2. Human fertility hormones – cow
3. Fibrinogen – for burn patients -- sheep

TRANSGENIC PRIMATE



- ANDi, the first transgenic primate born in January, 2000
- "ANDi" stands for "inserted DNA" spelled backwards.
- 224 unfertilized rhesus eggs were infected with a GFP virus
- ~Half of the fertilized eggs grew and divided
- 40 were implanted into twenty surrogate mothers
- five males were born, two were stillborn.
- ANDi was the only live monkey carrying the GFP gene

TRANSGENIC MICE



Why Use Transgenic Mouse Models?

- Model the development of human diseases in a controlled environment
- Test possible new drug treatments and get faster results

- Target specific genes to study
- Replicate specific characteristics, symptoms, or pathology of the disease

TRANSGENIC MOUSE

ALZHEIMER'S MOUSE

- In the brain of Alzheimer's patients, dead nerve cells are entangled in a protein called **amyloid.**
- Mouse made by introducing amyloid precursor gene into fertilized egg of mice.

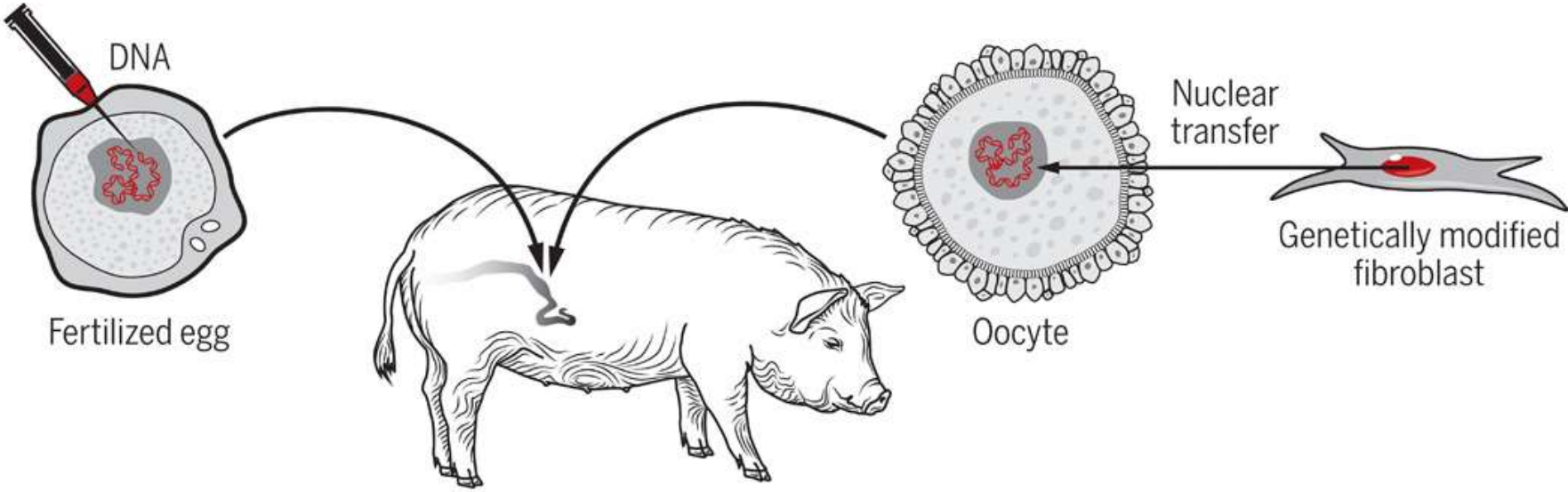
ONCOMOUSE

- Mouse model to study cancer
- Made by inserting activated **oncogenes.**

SMART MOUSE

- Biological model engineered to overexpress **NR2B receptor** in the synaptic pathway.
- This makes the mice **learn faster** like juveniles throughout their lives.

Glutamate [NMDA] receptor subunit epsilon-2, also known as ***N*-methyl D-aspartate receptor subtype 2B** (**NMDAR2B** or **NR2B**), is a protein that in humans is encoded by the *GRIN2B* gene. It is activated when **glutamate** and glycine (or D-serine) bind to it, and when activated it allows positively charged ions to flow through the cell membrane. The NMDA receptor is very important for controlling synaptic plasticity and **memory function**. The NMDAR is a specific type of ionotropic **glutamate** receptor



Transgenics		Knockouts
Complement inhibition	Immunosuppressive molecules	α1,3-galactosyltransferase
hDAF	Anti-CD2	CMAH
hCD46	CTLA4Ig	B4GalNT2
hCD59	hCD47	vWF
		PERVs
Coagulation inhibition	PERV siRNA	
hCD39	MHC genes	
Human thrombomodulin	Class I (NK inhibition)	
Anti-inflammatory genes		
HO-1		
A20		

Transgenic Animals Model Of Human Disease

1. **Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS):**

- **Tg26 HIVAN Mouse Model was the first transgenic model developed in 1991 for HIV.**
- **These transgenic animals can express HIV-1 proteins develop symptoms and immune deficiencies similar to the manifestations of AIDS in humans.**
- **Other models are AIDS Mouse and Smart Mouse.**

HIV-associated nephropathy (HIVAN) is a renal disease that affects HIV+ people of African ancestry with a high viral load and a genetic predisposition to develop this disease. From the clinical point of view, HIVAN is characterized by heavy proteinuria, nephrotic syndrome, and rapid progression to end-stage kidney disease (ESKD)

Transgenic Animals Model Of Human Disease

2. Alzheimer's disease:

- **Mouse models like PDAPP mice, Tg2576 mice, TAU transgenic mouse models like ALZ7 mice, 7TauTg mice.**
- **Immunization of Amyloid precursor protein A42 in transgenic mice showed that vaccination against Alzheimer's disease could have potential as a therapeutic approach.**

Transgenic Animals Model Of Human Disease

3. Cardiovascular disease:

- Transgenic models of heart failure and hypertrophy like Gene overexpression of Calmodulin, Gene mutation of alpha cardiac myosin heavy chain and Knockout gene model of transforming growth factor are developed.
- Mutation of the ApoE gene that is critical for uptake of chylomicrons and very low density lipoprotein particles, results in a model that develops atherosclerotic lesions histologically similar to those found in humans.

large triglyceride-rich lipoproteins produced in enterocytes

Transgenic Animals Model Of Human Disease

4. Diabetes Mellitus:

- **A transgenic mouse model that expressed Insulin Dependent Diabetes Mellitus by inserting a viral gene in the animal egg stage is also developed.**
- **There are other models like beta receptor knockout mouse, uncoupling protein (UCP1) knockout mouse , acute and chronic models for the evaluation of anti-diabetic agents.**

Transgenic Animals Model Of Human Disease

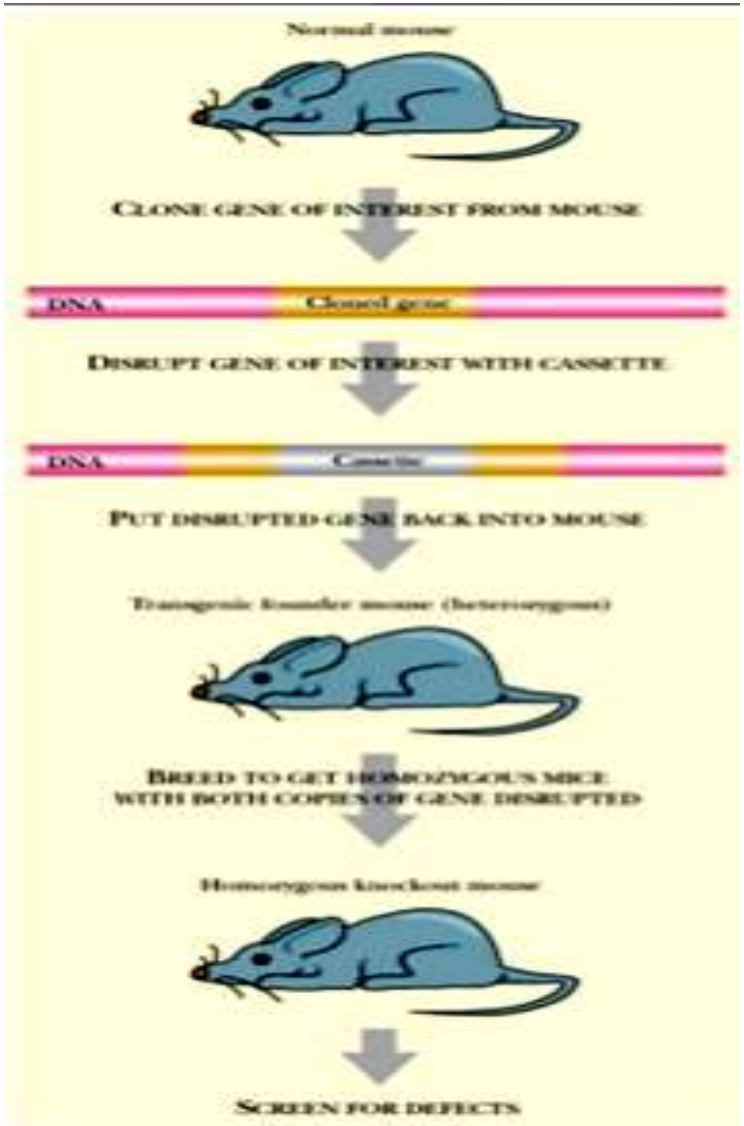
5. Angiogenesis:-

- **Mouse models of angiogenesis, arterial stenosis, atherosclerosis, thrombosis, thrombolysis and bleeding addresses techniques to evaluate vascular development.**
- **With the help of angiogenesis transgenic animal models inhibitors are identified which act on specific mechanisms of angiogenesis.**

Transgenic Animals Model Of Human Disease

6. Cancer diseases:

- **Oncomouse was first transgenic animal to be patented.**
- **Mechanisms for tumor progression and metastasis via E-cadherin, and other adhesion molecules is possible by various transgenic knockout models.**

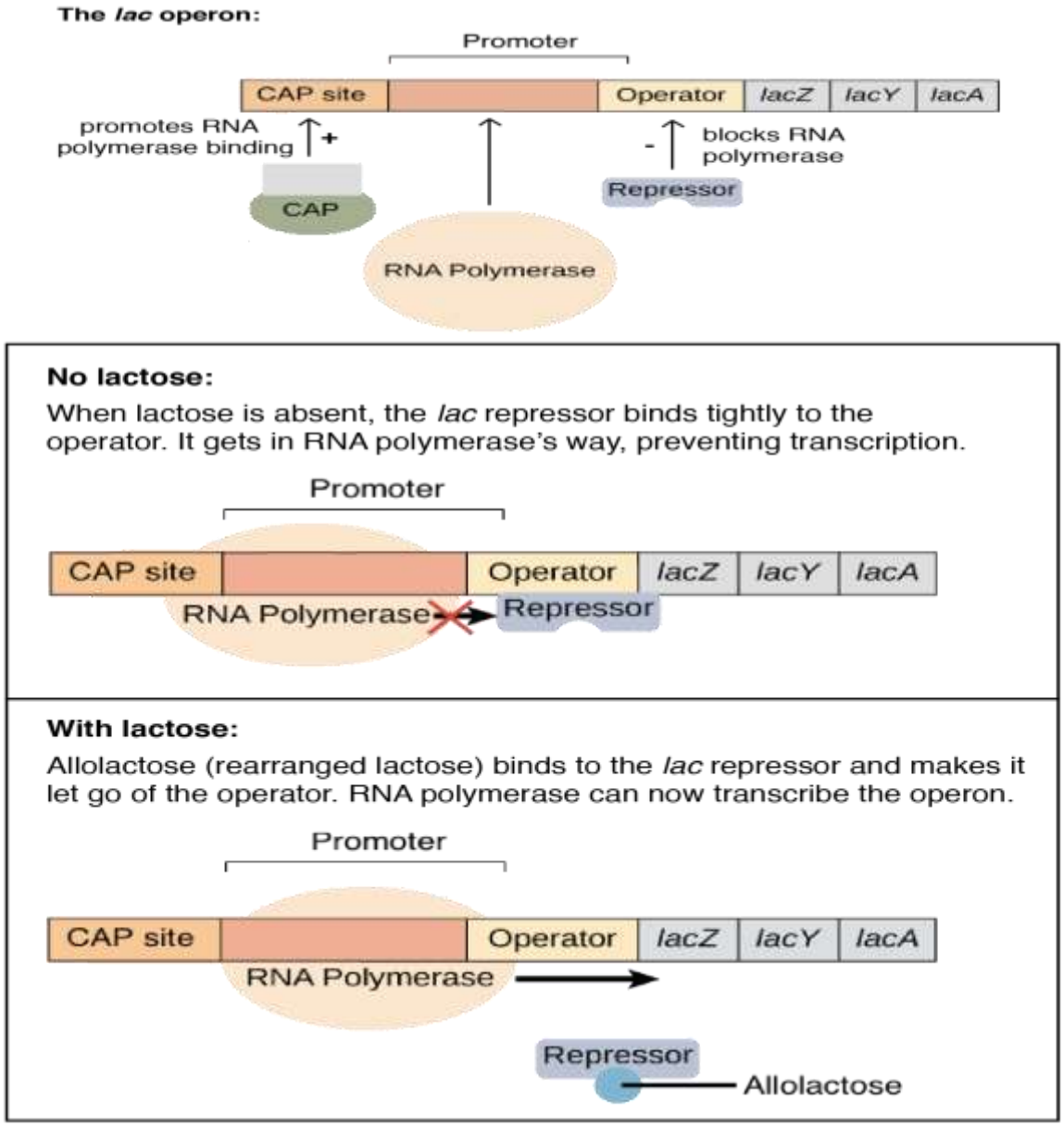


Knockout Mice

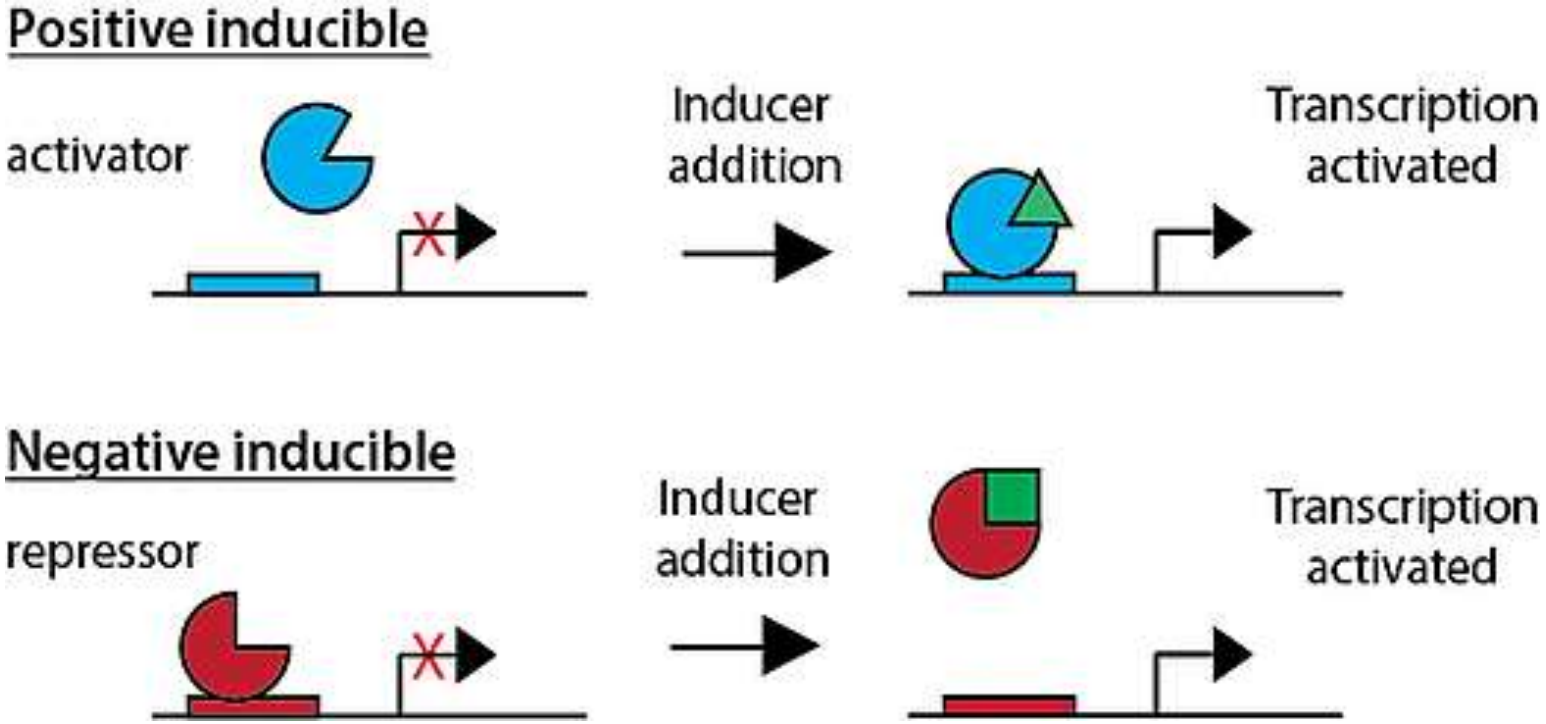
Like traditional transgenic mice, knockout mice are generated in vitro. The target gene is cloned and disrupted by inserting a DNA cassette. This work is usually done in bacteria. Once the construct is made, it is put back into a mouse by injection into the male pronucleus during fertilization

After the transgenic offspring are born, two heterozygotes are crossed to create a homozygous knockout mouse. These are then screened for defects due to inactivation of the target gene.

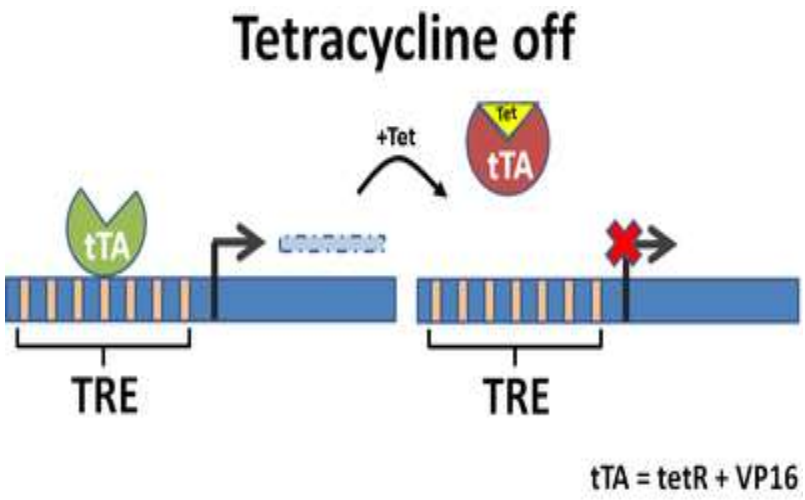
inducible operon



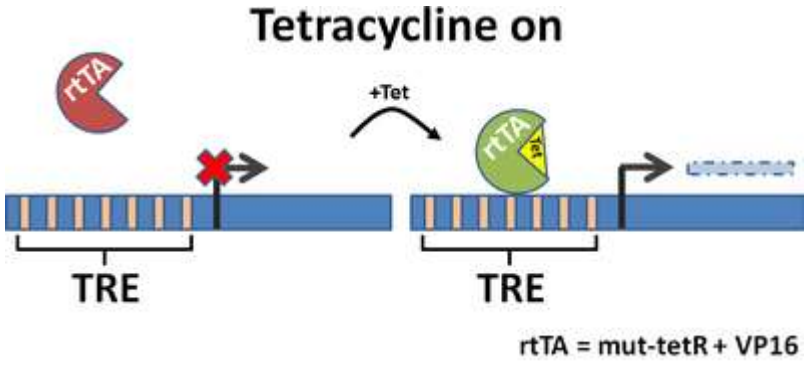
Inducible transgenic mouse models allow for the activation of genes in specific cells and tissues at specific times. ... The most commonly used methods to control gene **expression** in mouse models are based on the tet-operon/repressor bi-**transgenic** system and the estrogen receptor (ER) ligand-binding domain.



o use tetracycline as a regulator of gene expression, a tetracycline-controlled transactivator (tTA) was developed. tTA was created by fusing tetR with the C-terminal domain of VP16 (virion protein 16), an essential transcriptional activation domain from HSV (herpes simplex virus).



In the absence of tetracycline, the tetR portion of tTA will bind these tetO sequences and the activation domain promotes expression. In the presence of tetracycline, tetracycline binds to tetR. This precludes tTA binding to the tetO sequences and subsequent increase in expression by the activation domain, resulting in reduced gene expression



Tet repressor, or rTetR, which reversed the phenotype and created a reliance on the presence of tetracycline for induction, rather than repression. The new transactivator rtTA (**r**everse **t**etracycline-controlled **t**rans**a**ctivator) was created by fusing rTetR with VP16. The tetracycline on system is also known as the rtTA-dependent system.

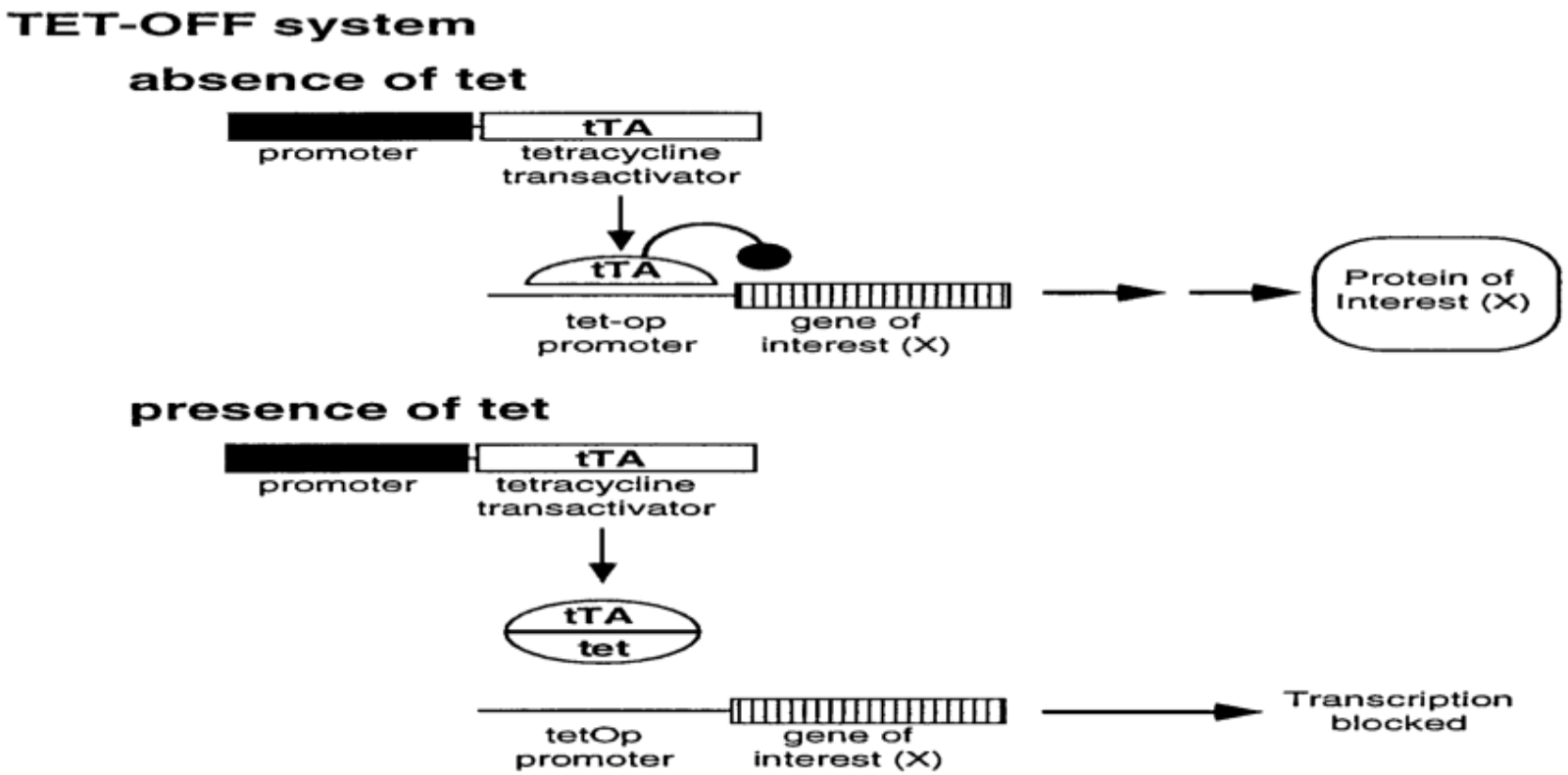
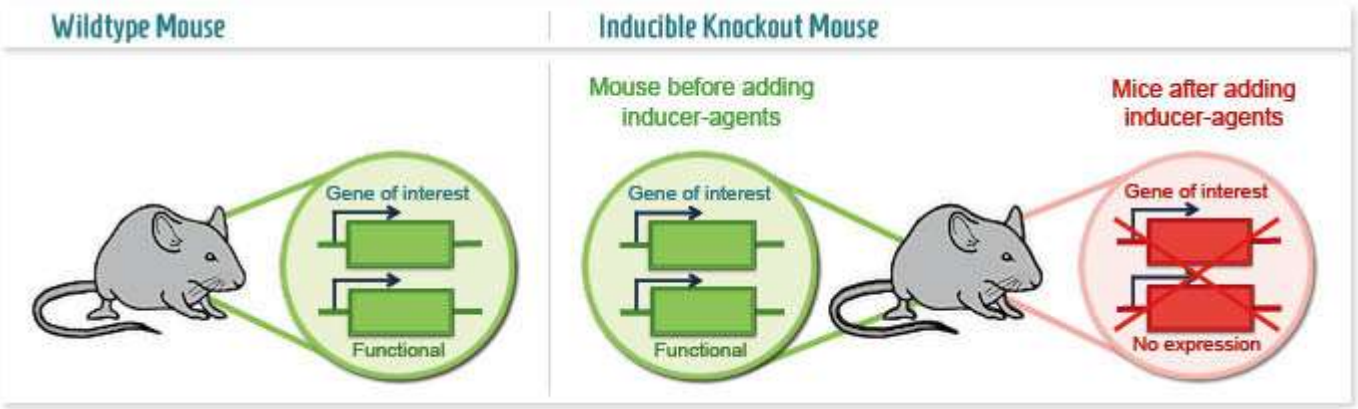
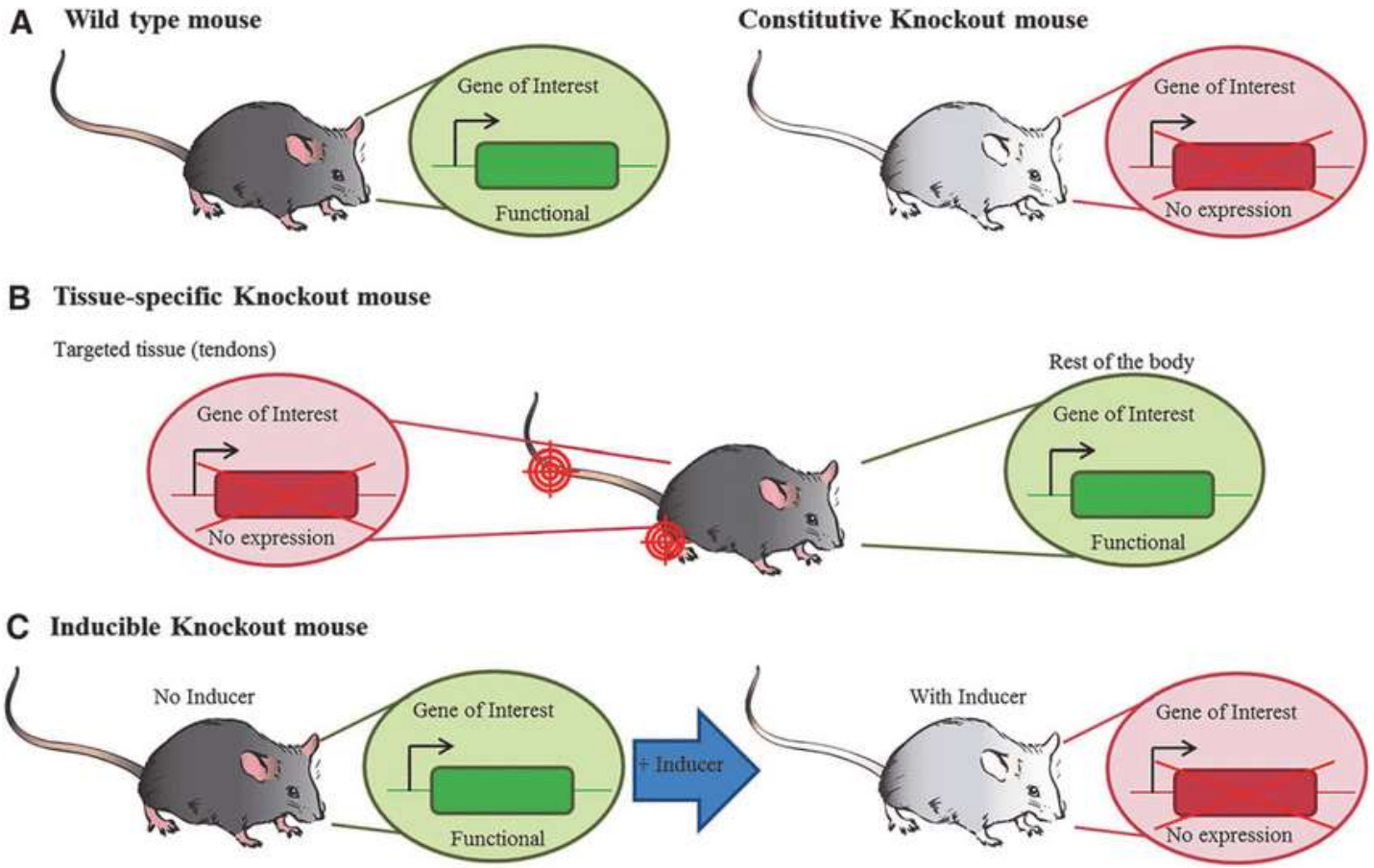
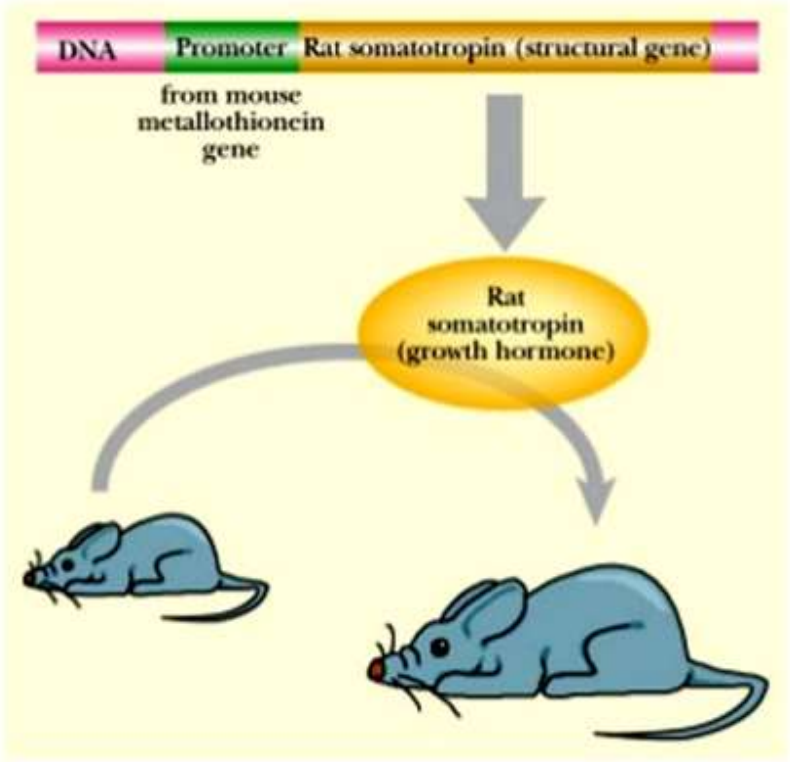


Diagram of the tetracycline-inducible expression system. The mechanisms of action of the *tet* system are shown. The tetracycline-controlled transactivator (tTA) is a fusion protein containing the *tet* repressor of *E. coli* and the carboxyl-terminal domain of the VP16 transcription factor from herpes simplex virus. tTA binds to an array of seven cognate operator sequences (*tetO*) and activates transcription of a minimal human cytomegalovirus promoter. In the presence of tetracycline or its derivatives [such as doxycycline (Dox)], tTA cannot bind its target and the system is inactive (*tet*-OFF system). Depending of the promoter used in the tTA fusion gene, tTA is expressed ubiquitously or with a specific-tissue expression pattern.


Wildtype Mouse
Inducible Knockout Mouse
Mouse before adding inducer-agents
Mice after adding inducer-agents
Gene of interest
Functional
Gene of interest
Functional
Gene of interest
No expression


A Wild type mouse
Gene of Interest
Functional
Constitutive Knockout mouse
Gene of Interest
No expression
B Tissue-specific Knockout mouse
Targeted tissue (tendons)
Gene of Interest
No expression
Rest of the body
Gene of Interest
Functional
C Inducible Knockout mouse
No Inducer
Gene of Interest
Functional
+ Inducer
With Inducer
Gene of Interest
No expression

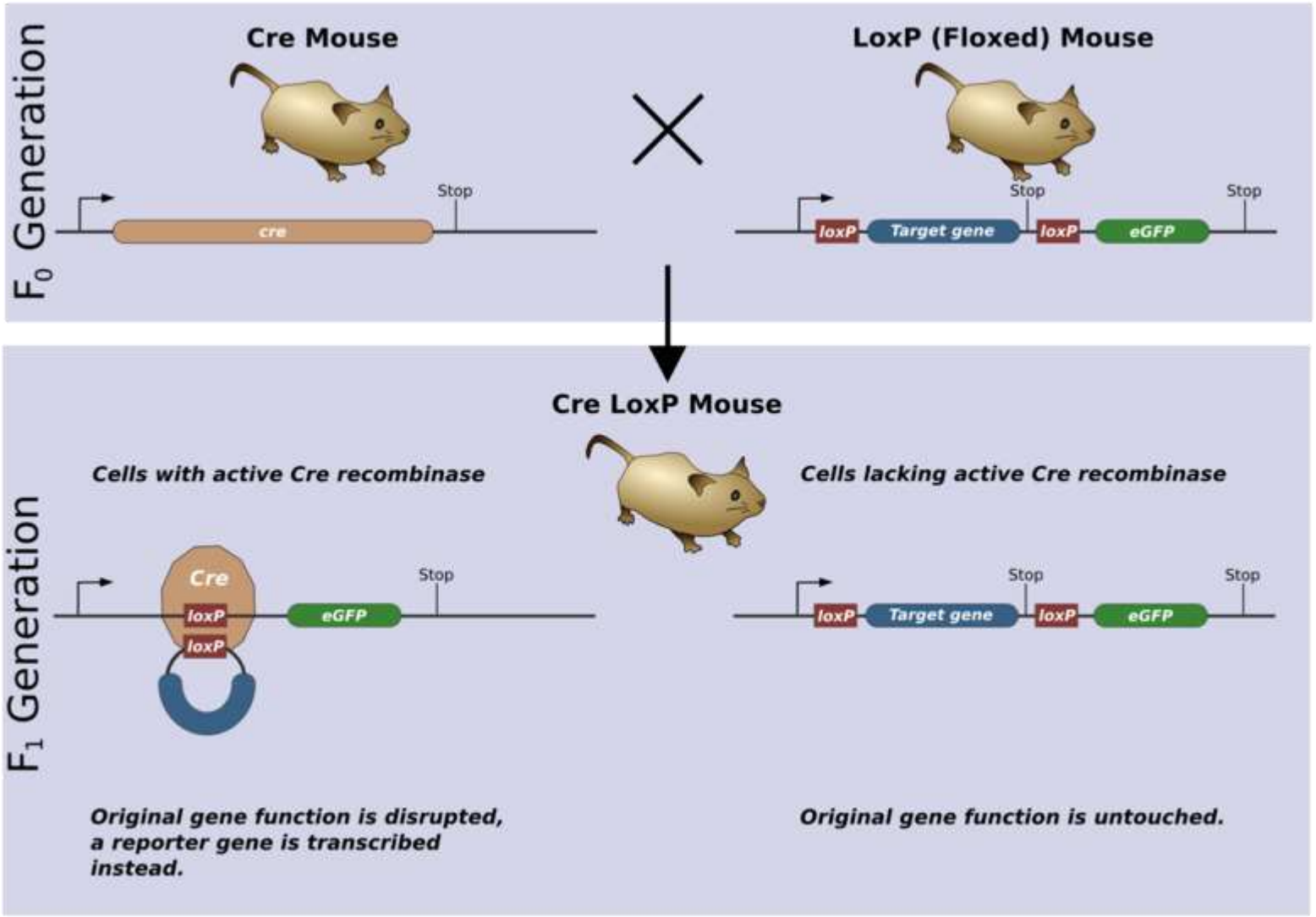


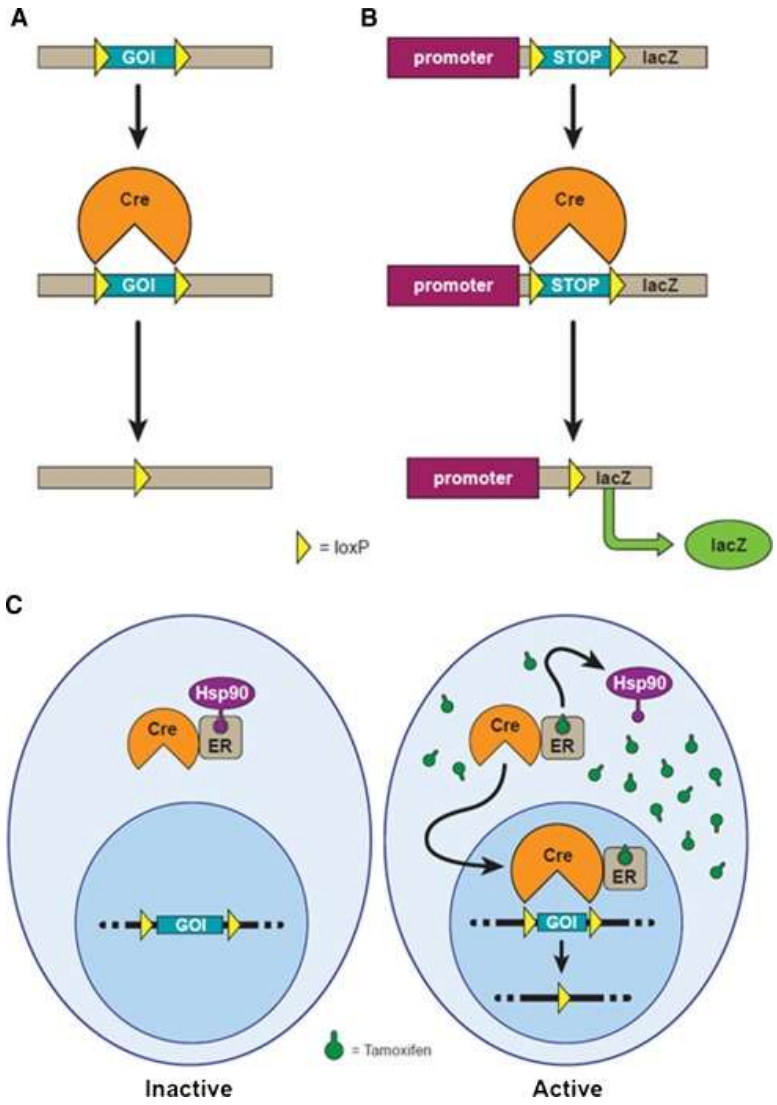
Large Transgenic Mice
A DNA construct containing the rat somatotropin gene under the control of the mouse metallothionein promoter was used to make a transgenic mouse. The transgene cause the mouse to grow to twice its normal body size.

Such endogeneous supply may
High background level of expression
Low level of induction
Differential rate of uptake and elimination of ligand by animal

Cre*lox* System

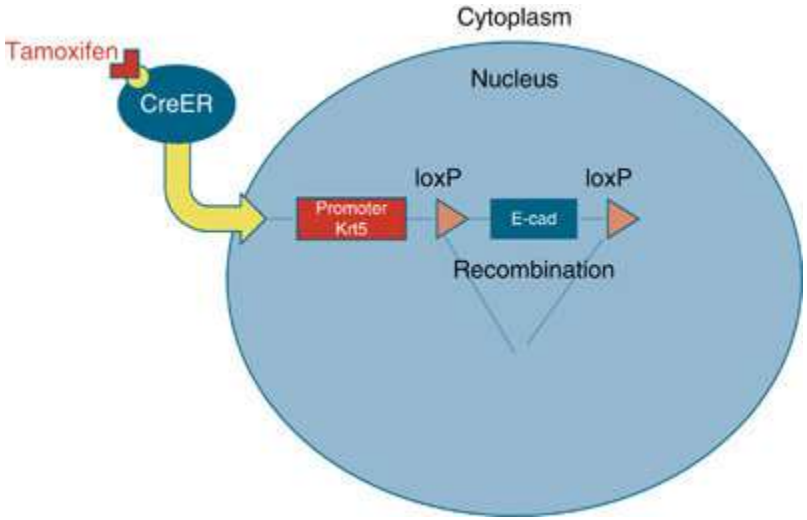
The Cre*lox* system uses the **Cre recombinase isolated from the P1 bacteriophage**. The Cre recombinase catalyzes site-specific recombination by crossover between two distant Cre recognition sequences, *i.e., loxP* sites . The *loxP* sites include two 13-bp inverted repeats separated by an 8-bp spacer sequence. Any DNA sequence introduced between the two 34-bp *loxP* sequences (termed “floxed” DNA) is excised because of Cre-mediated recombination. Therefore, control of Cre expression in a transgenic animal, using either spatial control (with a tissue- or cell-specific promoter) or temporal control (with an inducible system), results in the spatial or temporal control of DNA excision between the two *loxP* sites. In addition to conditional gene inactivation (conditional knockout), this approach can be applied to protein over-expression. In that case, a floxed stop codon is inserted between the promoter sequence and the cDNA of interest . Transgenic animals do not express the transgene until Cre is expressed, leading to excision of the floxed stop codon. This strategy has been successfully used to develop reporter mice that express *LacZ* after Cre-mediated recombination . This system has been applied to tissue-specific oncogenesis and controlled antigene receptor expression in B lymphocytes .


F0 Generation
Cre Mouse
LoxP (Floxed) Mouse
Stop
cre
Stop
Stop
loxP
Target gene
loxP
eGFP
Cre LoxP Mouse
F1 Generation
Cells with active Cre recombinase
Cells lacking active Cre recombinase
Cre
loxP
loxP
Stop
eGFP
Stop
Stop
loxP
Target gene
loxP
eGFP
Original gene function is disrupted, a reporter gene is transcribed instead.
Original gene function is untouched.

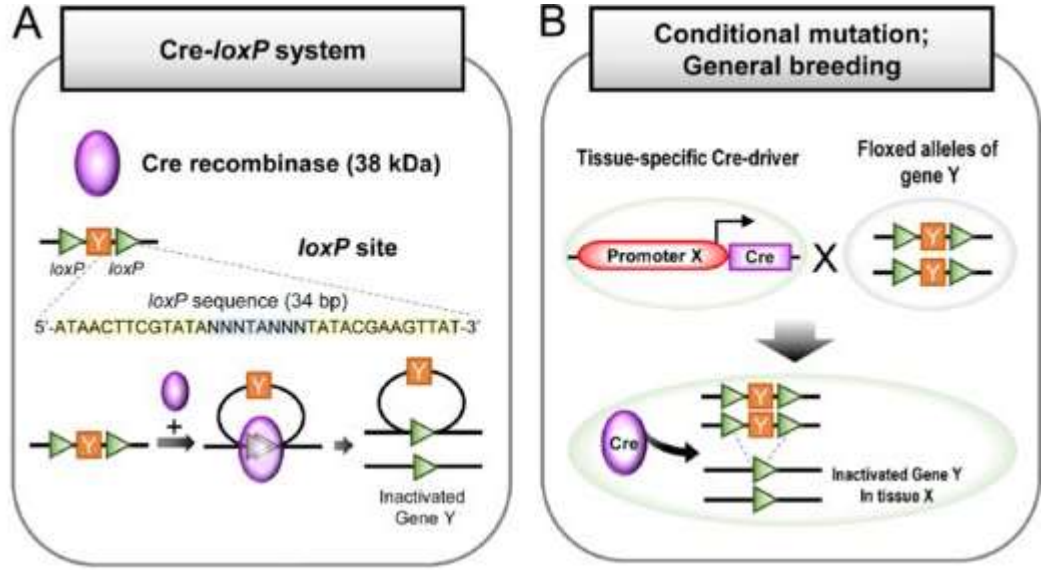


To allow for cell type-specific control of gene deletions, mouse models have been created where Cre expression is controlled by a cell type-specific promoter. These lines can then be crossed with mouse lines containing a relevant part of a GOI that is surrounded by *loxP* sites in the genome to generate conditional gene deletion .The overexpression or ectopic expression of a gene can also be induced using the Cre-*loxP* system. In this case, a construct containing a promoter and a floxed “stop” sequence upstream of a GOI (i.e., promoter-*loxP*-stop-*loxP*-GOI) is inserted into the genome; thus, only Cre^+ cells are able to remove the “stop” sequence and overexpress or ectopically express the GOI This strategy is also used for reporter alleles so that lacZ, green fluorescent protein (GFP), or other fluorescent molecules are expressed in Cre^+ cells only when the floxed “stop” sequence is removed.

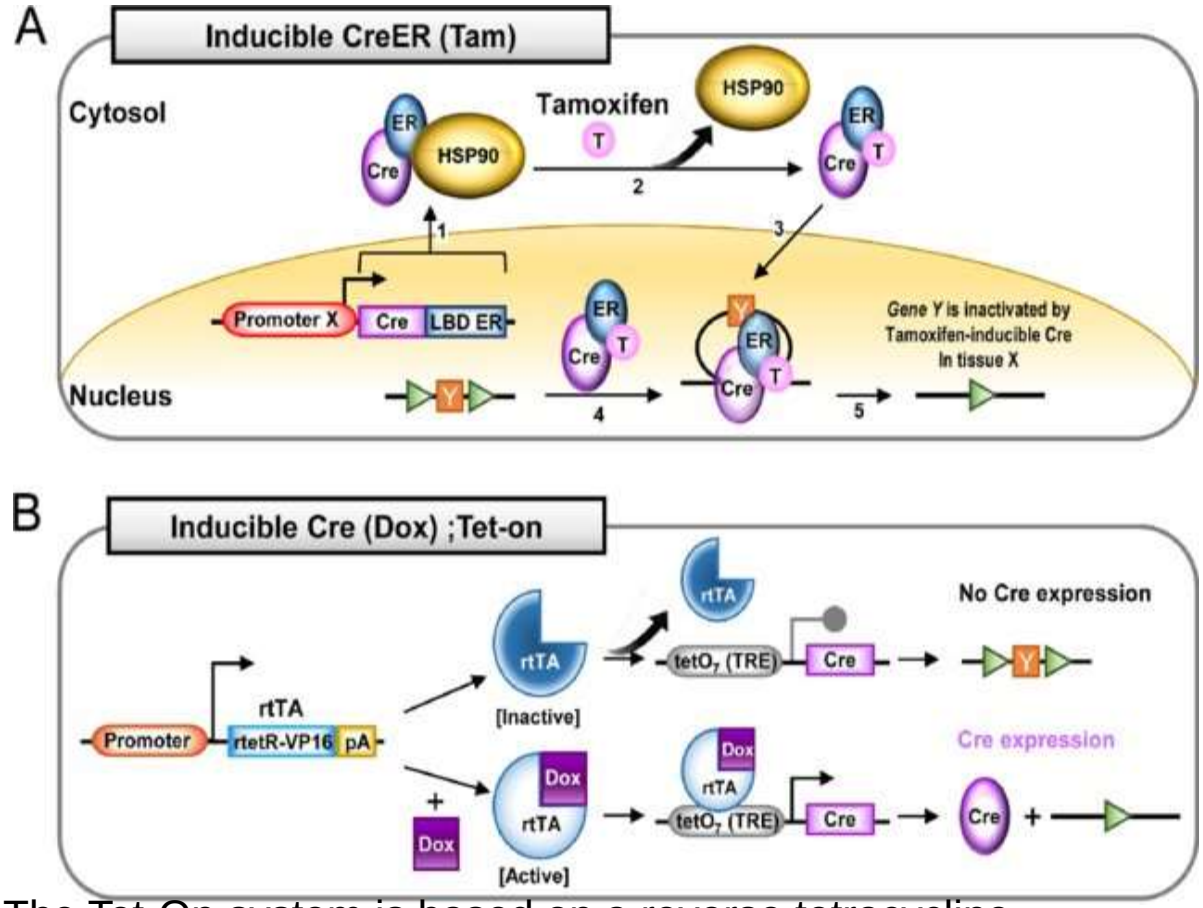
Cre-mediated excision and the mechanism of inducible CreER. **A** Diagram of Cre-mediated deletion of a GOI flanked by *loxP* sites. **B** Diagram of a reporter allele where Cre-mediated deletion of a floxed “stop” sequence results in the expression of lacZ. **C** Diagram of inducible CreER-mediated excision. In the absence of tamoxifen, CreER is sequestered in the cytoplasm by Hsp90. In the presence of tamoxifen, CreER is translocated to the nucleus where it recognizes *loxP* sequences and cleaves the DNA



The CreER/LoxP system. CreER is a tamoxifen-inducible Cre recombinase fused to the estrogen receptor (ER). In the current studies, expression of CreER fusion gene is driven by an esophageal basal cell-specific promoter, Krt5. When tamoxifen binds to CreER protein, CreER translocates into the nucleus, and then mediates site-specific recombination (deletion of e-cadherin).

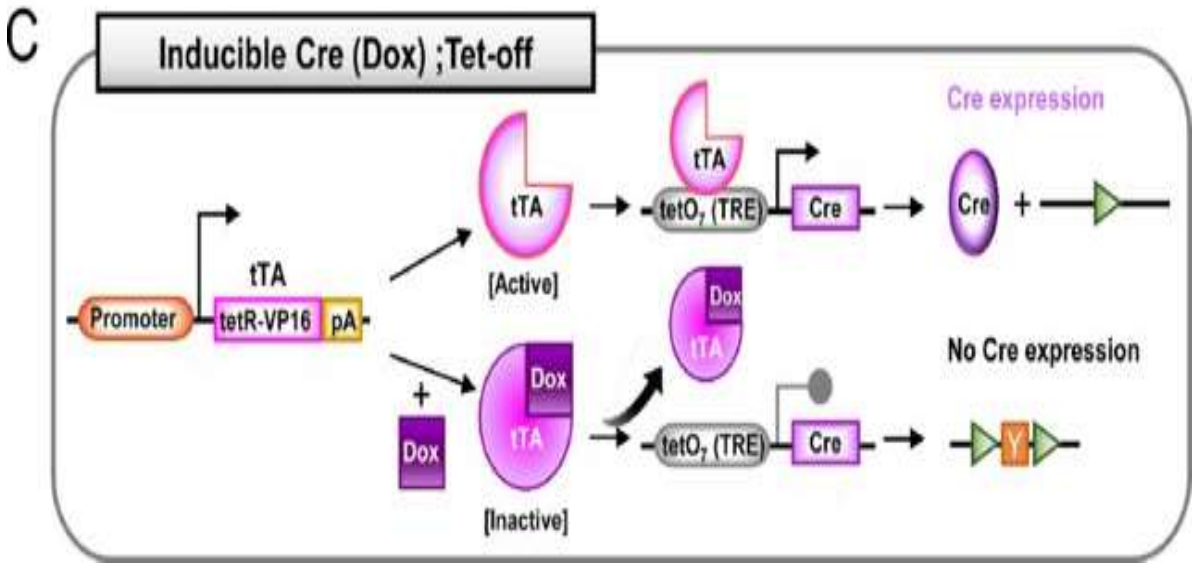


Mechanism of Cre-loxP system. (A) An overview of Cre-loxP system. 38 kDa Cre recombinase recognizes the loxP sites of specific 34 bp DNA sequences. (B) General breeding strategy for conditional mutation using loxP and Cre driving mouse line. In principle, one mouse must have a tissue-specific driven cre gene and another mouse have loxP flanked (floxed) alleles of interest gene Y. Expression of Cre recombinase excises floxed loci and inactivates the gene Y.



Principles of Inducible Cre-loxP mutation system. (A) Tamoxifen (Tam)-inducible System of estrogen receptor fused to Cre (CreER). In the absence of tamoxifen, expressed fusion protein, CreER, interacts with heat shock protein 90 (HSP90) and exists in cytoplasm (1). Administration of Tam disrupts the interaction of HSP90 with CreER (2). Interaction of ER with Tam induces nuclear translocation of Cre (3). In the nucleus, the CreER recognizes the loxP sites (4) and inactivates the gene Y in tissue X (5). (B and C) Doxycycline (Dox)-induced Tetracyclin (Tet)-on and-off systems. (B) In Tet-on system, ubiquitous or tissue-specific promoter driven rtTA is expressed. In the absence of Dox, inactivated rtTA is unable to bind tetO 7 (7 repeats of a 19 nuclotide tetO minimal promoter, also referred to as TRE) sequence of cre gene. Cre is not expressed. Following Dox administration, Dox interacts with the rtTA and allows to activate. Activated rtTA binds to tetO 7 promoter of cre and induces the Cre expression..

The Tet-On system is based on a reverse tetracycline-controlled transactivator, *rtTA*. Like *tTA*, *rtTA* is a fusion protein comprised of the TetR repressor and the VP16 transactivation domain; however, a four amino acid change in the tetR DNA binding moiety alters *rtTA's* binding characteristics such that it can only recognize the tetO sequences in the TRE of the target transgene in the presence of the Dox effector. Thus, in the Tet-On system, transcription of the TRE-regulated target gene is stimulated by *rtTA* only in the presence of Dox.



(C) In Tet-off system, in the absence of Dox, activated tTA is able to bind tetO 7 (TRE) sequence of cre and induces the Cre expression. Upon Dox administration, tTA interacted with Dox is inactivated. Inactivated rTA is not able to bind to tetO 7 promoter and therefore Cre expression is inhibited

The two vector design of the Tet-Off and Tet On systems allows tissue-specific promoters to drive *tTA* or *rtTA* expression, resulting in tissue-specific expression of the TRE-regulated target transgene. Further, the ability to strictly regulate the level of *rtTA* and *tTA* activity allows the investigator to regulate activation of the target gene both quantitatively and temporall

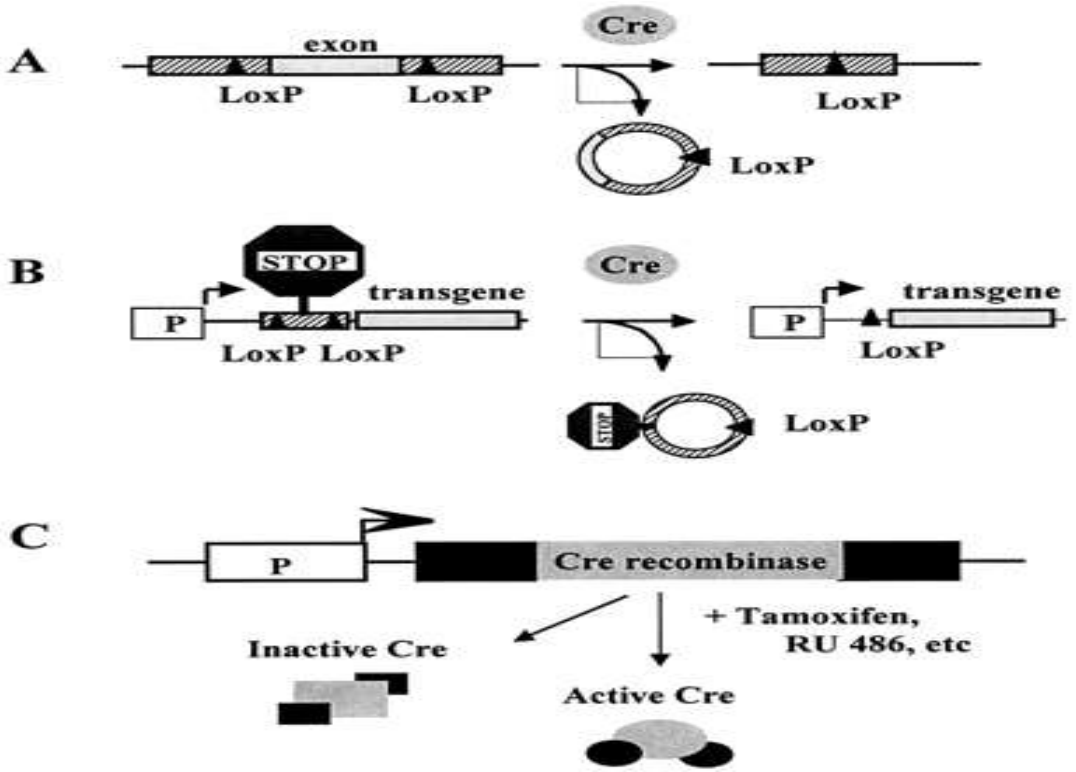


Diagram of the Cre/*lox* system and its use as an inducible expression system. (A) The Cre recombinase has been isolated from the P1 phage of *E. coli*. Cre induces recombination between two 34-bp, site-specific recognition sequences (*loxP*), allowing excision of the DNA flanked by these *loxP* sites (floxed DNA). In transgenic mice, expression of Cre recombinase is under the control of ubiquitous or tissue-specific promoters (P) or follows transient infection with a Cre adenovirus. This allows conditional gene targeting when two *loxP* sites have been introduced into the genome by homologous recombination. (B) Conditional gene expression can be achieved using Cre recombinase. In this case, a floxed stop sequence is placed between the promoter and the cDNA to be expressed. In double-transgenic mice, the cDNA cannot be expressed in tissues or cells in which Cre recombinase is not expressed. However, Cre recombinase expression results in excision of the floxed stop sequence and allows the cDNA to be expressed. (C) Temporal control of Cre recombinase activity can be added to the spatial control described in B. Chimeric recombinases with the ligand-binding domain of several steroid hormone receptors have been produced. The activity of the chimeric fusion protein is dependent on ligand binding to the ligand-binding domain. Mutated ligand-binding domains have been used to avoid Cre recombinase activation by endogenous steroids rather than exogenous ligands such as tamoxifen (a synthetic glucocorticoid agonist) or RU486 (a synthetic antiprogesterone).

Genetically engineered animal models that reproduce human diseases are very important for the pathological study of various conditions. The development of the clustered regularly interspaced short palindromic repeats (CRISPR) system has enabled a faster and cheaper production of animal models compared with traditional gene-targeting methods using embryonic stem cells. Genome editing tools based on the CRISPR-Cas9 system are a breakthrough technology that allows the precise introduction of mutations at the target DNA sequences. In particular, this accelerated the creation of animal models, and greatly contributed to the research that utilized them

Gene editing system *Streptococcus pyogenes* Cas9 (SpCas9) can efficiently introduce mutations at desired target positions in the genome in a guide RNA (gRNA)-dependent manner (Cong et al. 2013). The target recognition sequence of SpCas9, called the protospacer-adjacent motif (PAM) sequence, is 5′–NGG–3′ (N = A or T or G or C). To form a functional Cas9/gRNA complex, SpCas9 requires a gRNA consisting essentially of a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). The Cas9 protein, which can target specific genes for editing and correction, generates DNA double-strand breaks at 20 base pair of target sequence positions that are complementary to the short gRNAs. The cleaved DNA is repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR) endogenous repair mechanisms, to produce insertion or deletion (indel) mutations

Natural CRISPR Pathway

1. DNA Invasion
Foreign DNA from a virus or plasmid invades the cell.

2. Invading DNA is Incorporated Into CRISPR Array
DNA fragments from the invading DNA are incorporated into the CRISPR locus as spacers. The exact mechanism of incorporation remains unkonwn.

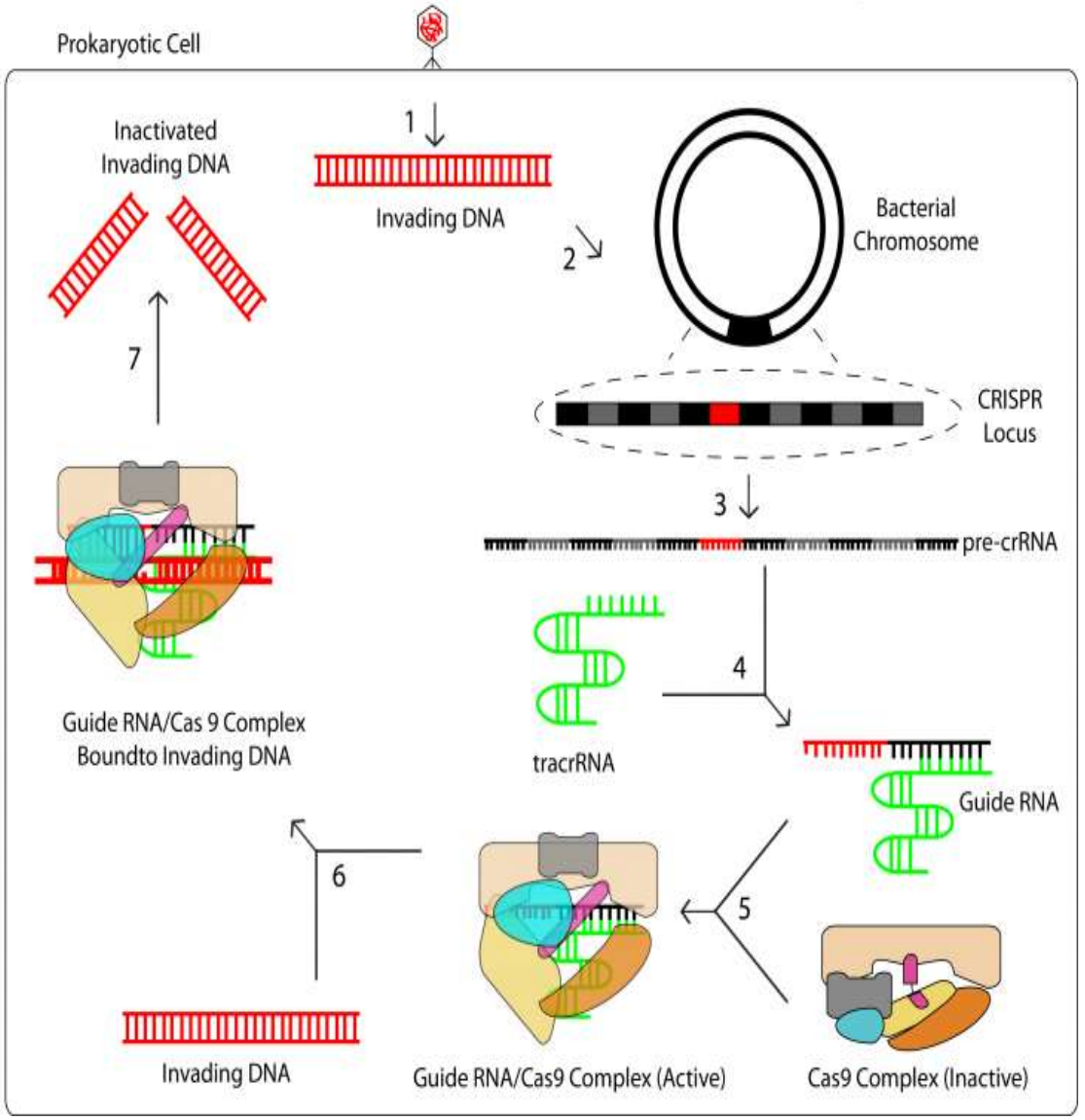
3. Pre-crRNA Transcription
The cell constitutively transcribes a repeat/spacer group into pre-crRNA. Black boxes represent repeats. Grey boxes represent spacers. The red box represents the spacer corresponding to the invading DNA.

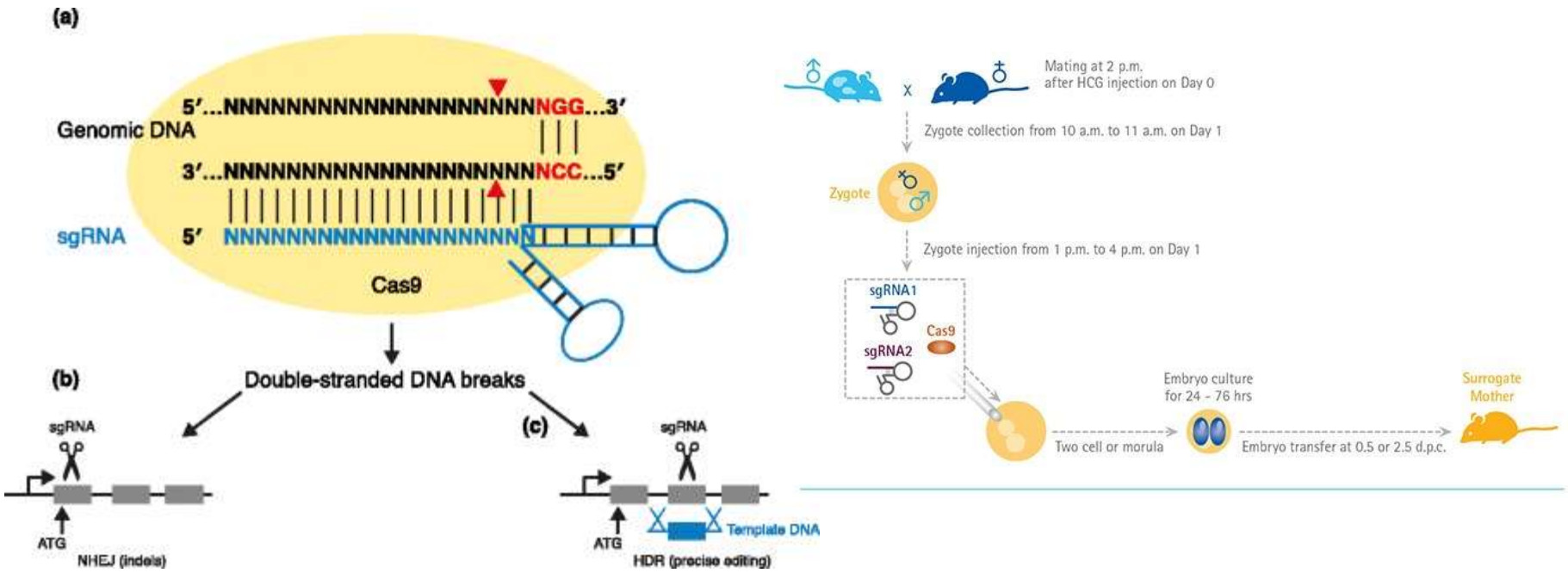
4. Guide RNA Formation
Constitutively expressed transactivating RNA (tracrRNA) base pairs with the CRISPR repeat sequences on the pre-crRNA. RNase III, Csn 1, and other unidentified CRISPR-associated pro-tiens modify the pre-crRNA/tracrRNA duplex to form a guide RNA. (Deltcheva et al. 2011)

5. Cas9 Activation
Inactive Cas9 protien binds to the guide RNA and becomes activated.

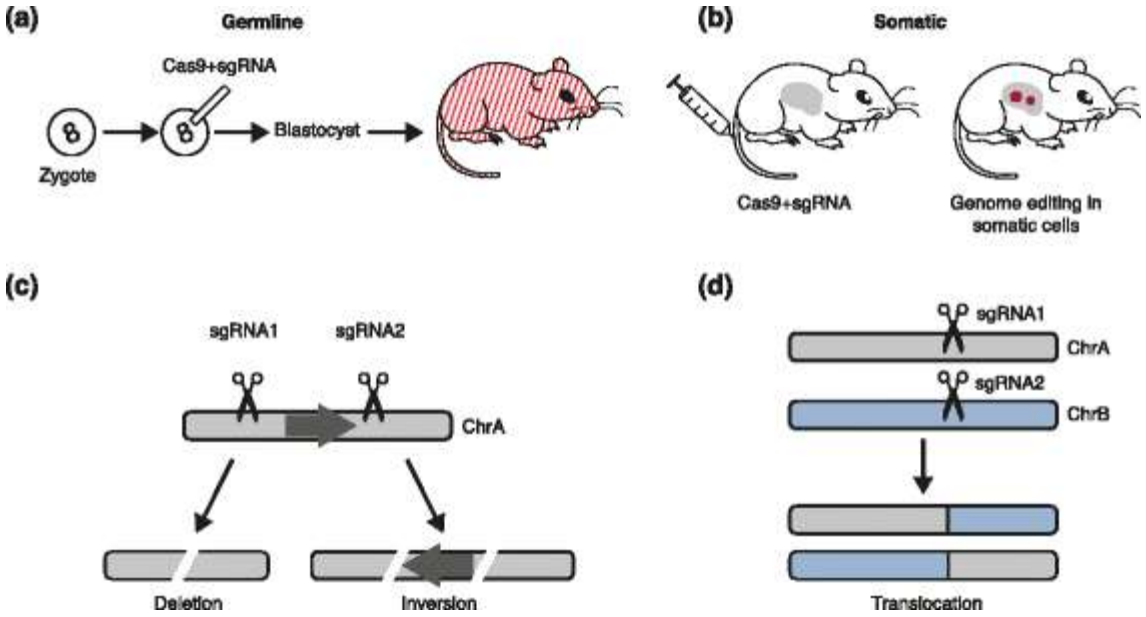
6. Target Binding
The activated guide RNA/Cas9 complex binds with the target DNA. The localization occurs stochastically (Sternberg et al. 2014).

7. Target Cleavage
The Cas9 protein cleaves the invading DNA and inactivates it.

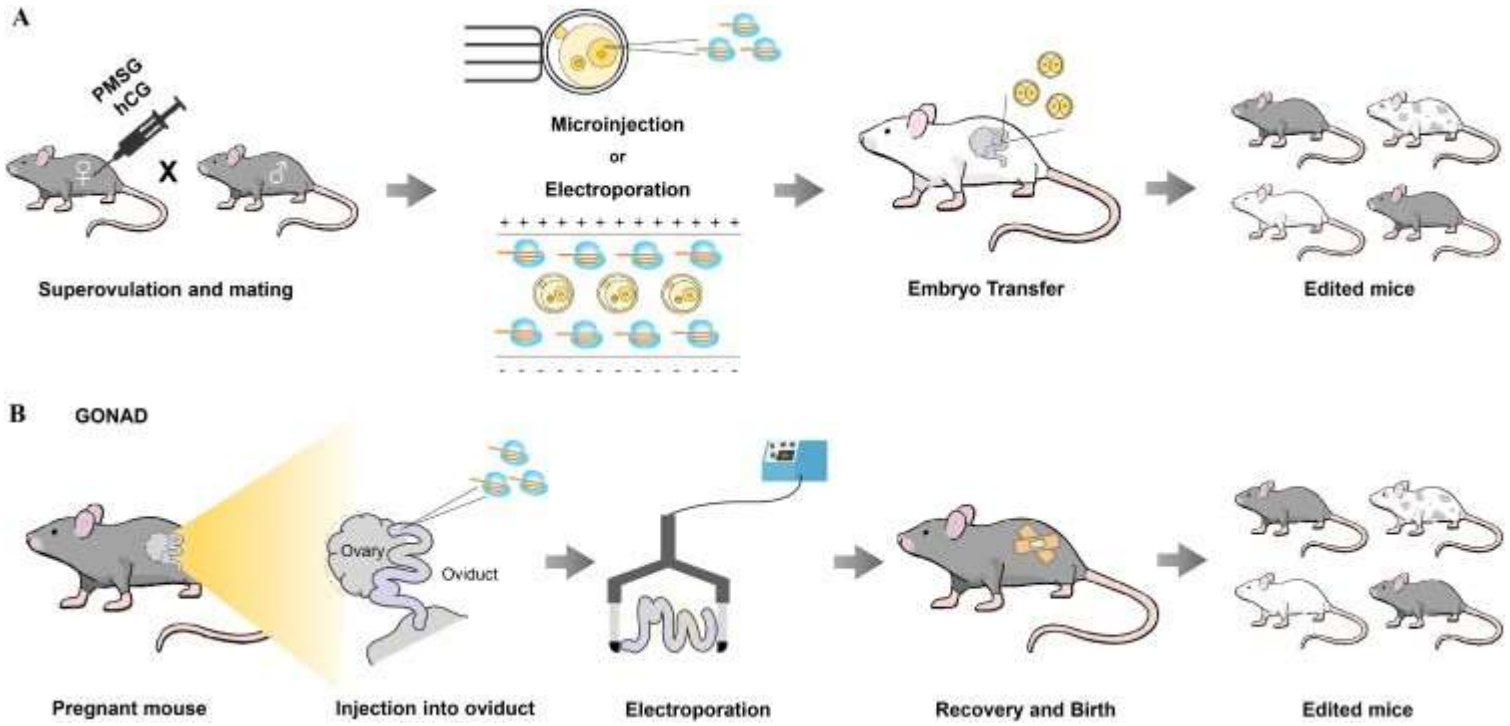




CRISPR-Cas9 mechanism. **a** A single guide RNA (sgRNA) is a fusion between crRNA (CRISPR RNA) and tracrRNA (trans-activating CRISPR RNA). This complex recognizes the protospacer-adjacent motif (PAM) sequence and the complementary 20-nucleotide upstream genomic sequence. Cas9 cuts approximately three nucleotides upstream of the PAM to induce DNA double-strand breaks. Then, the cellular DNA repair system, either non-homologous end joining (NHEJ) or homology-directed repair (HDR), results in indels or precise editing, respectively. Cas9 proteins from different bacteria recognize different PAM sequences;



Rapid generation of cancer models in mouse through genome editing. **a** Germline CRISPR mouse models. Cas9 and single guide RNA (sgRNA) can be microinjected into mouse zygotes. The resulting mouse will carry cells harboring CRISPR-mediated indels or homology-directed repair. This method can generate mosaic mice. **b** Somatic CRISPR mouse models. Cas9 and sgRNA can be delivered to mouse tissue in vivo, for example through hydrodynamic injection to the liver or viral vehicles to various tissue. **c** Two sgRNAs targeting one chromosome can lead to deletion or inversion between sgRNA cutting sites. **d** Two sgRNAs targeting two chromosomes can lead to chromosomal translocation, allowing rapid modeling of cancer-associated chromosomal rearrangement.



A, CRISPR delivery to zygote embryos using microinjection (pronucleus/cytoplasm) or electroporation. Edited 2-cell-stage embryos are transplanted into a surrogate mouse and the edited offspring are obtained.

B**, Genome editing via oviductal nucleic acid delivery** (GONAD) is a new method of introduction of the Cas9/gRNA complex into embryos. Direct injection of the Cas9/gRNA complex for genome editing into oviduct of pregnant mouse, followed by an electrical impulse.

VACCINE (from internet)

What is Vaccine?

A substance used to stimulate the production of antibodies and provide immunity against one or several diseases, prepared from the causative agent of a disease, its products, or a synthetic substitute, treated to act as an antigen without inducing the disease.

What is the role of a vaccine?

Vaccines are like a training course for the immune system. They prepare the body to fight disease without exposing it to disease symptoms. When foreign invaders such as bacteria or viruses enter the body, immune cells called lymphocytes respond by producing antibodies, which are protein molecules.

What is the difference between vaccines and immunizations?

Vaccination is the term used for getting a **vaccine** – that is, actually getting the injection or taking an oral **vaccine** dose. Immunisation refers to the process of both getting the **vaccine** and becoming immune to the disease following **vaccination**.

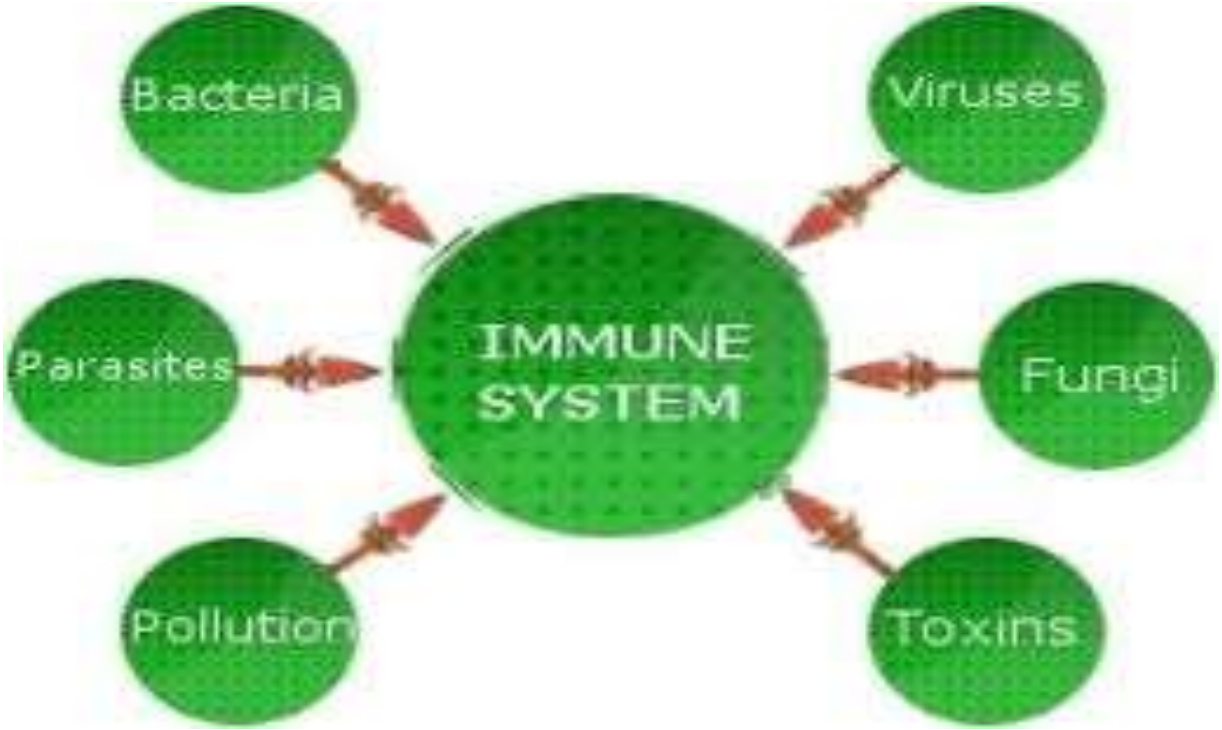
How do vaccines work?

A **vaccine works** by training the immune system to recognize and combat pathogens, either viruses or bacteria. To **do** this, certain molecules from the pathogen must be introduced into the body to trigger an immune response. These molecules are called antigens, and they are present on all viruses and bacteria.

How a vaccine is prepared?

Vaccines are **made** by taking viruses or bacteria and weakening them so that they can't reproduce (or replicate) themselves very well or so that they can't replicate at all.

Different antigens

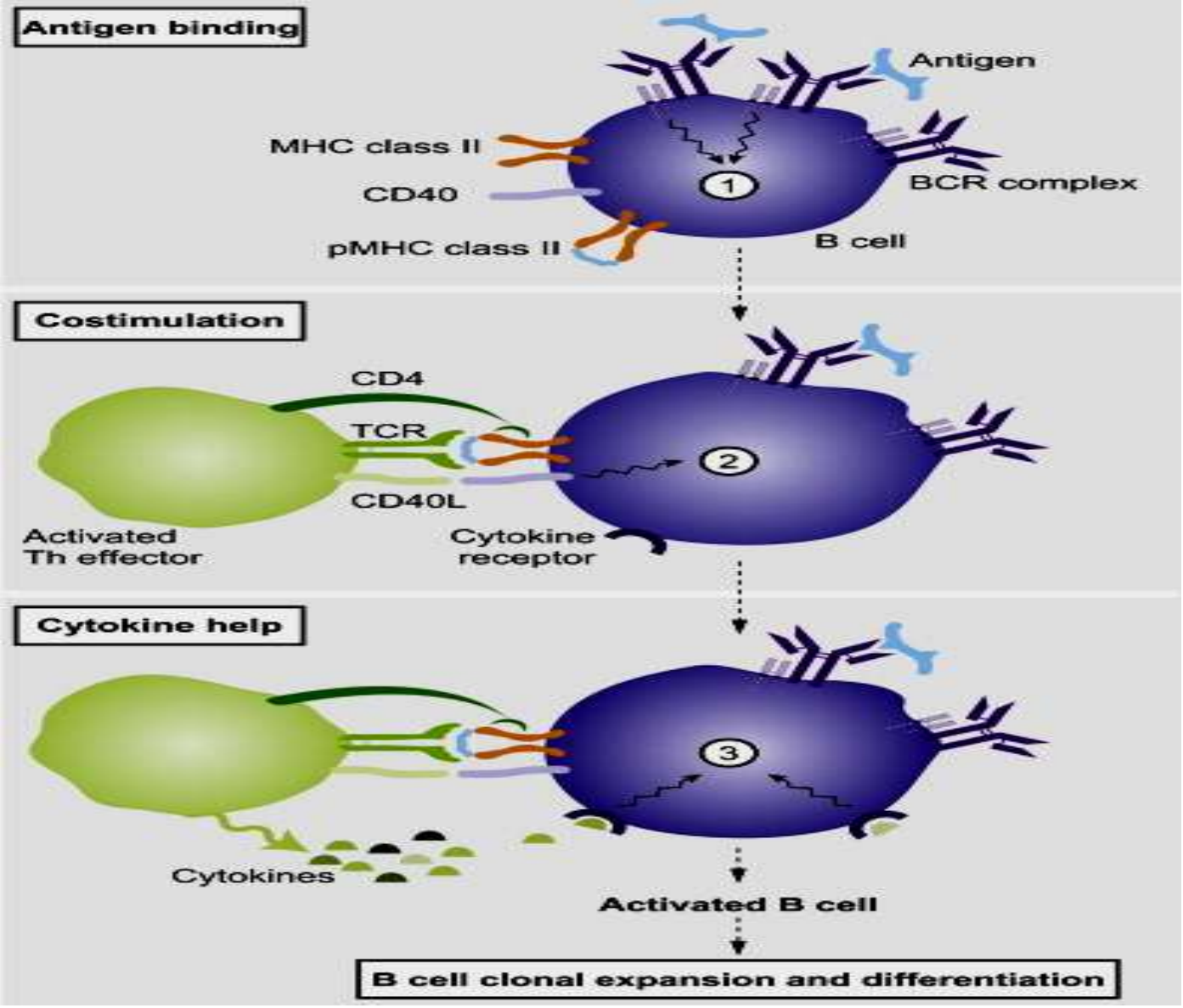


Basic immune system

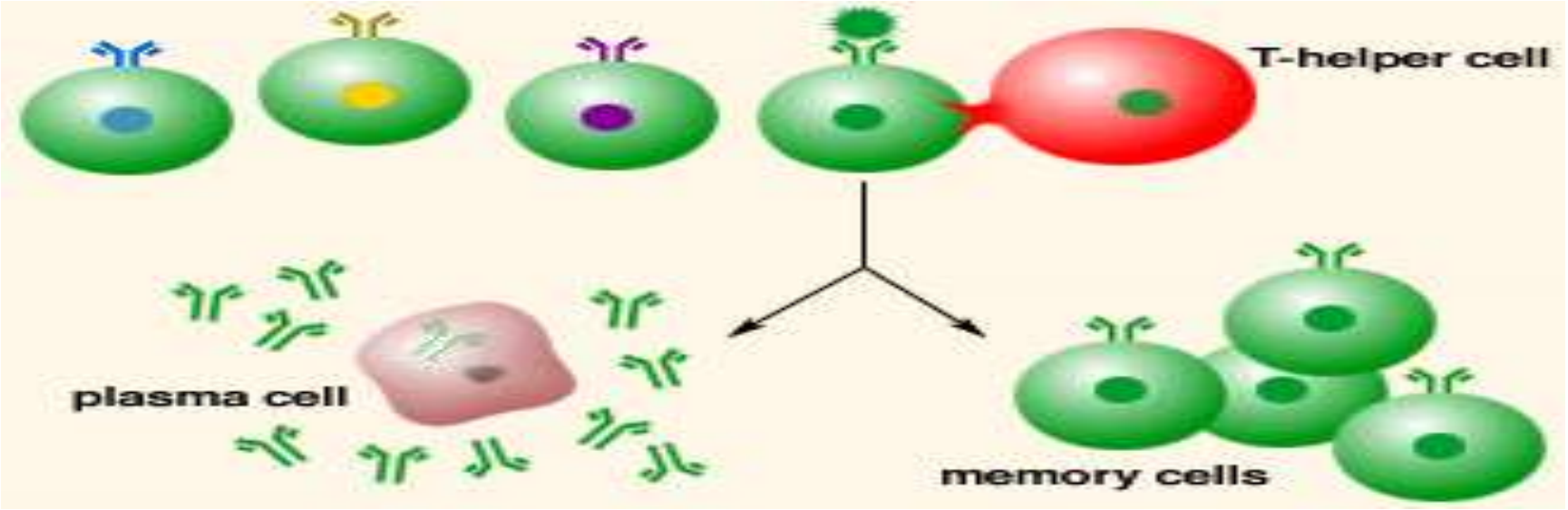
Immune response

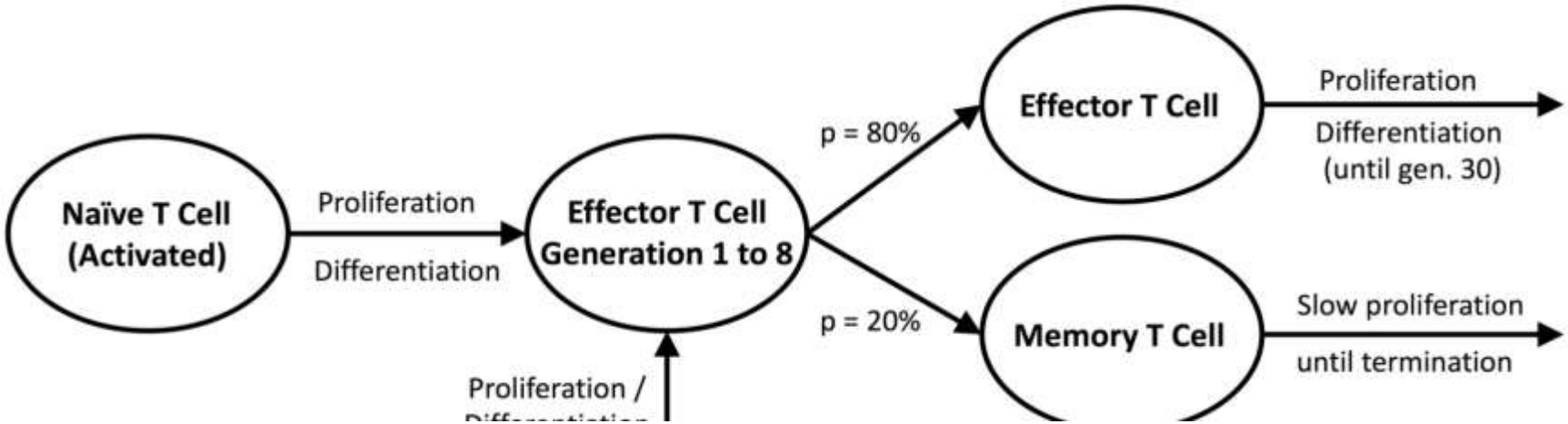
- **Innate (non specific)**
- **Adaptive (specific)**
  - **Humoral (B cells)**
  - **Cell mediated (T cells)**

Adaptive immune response: antibody production

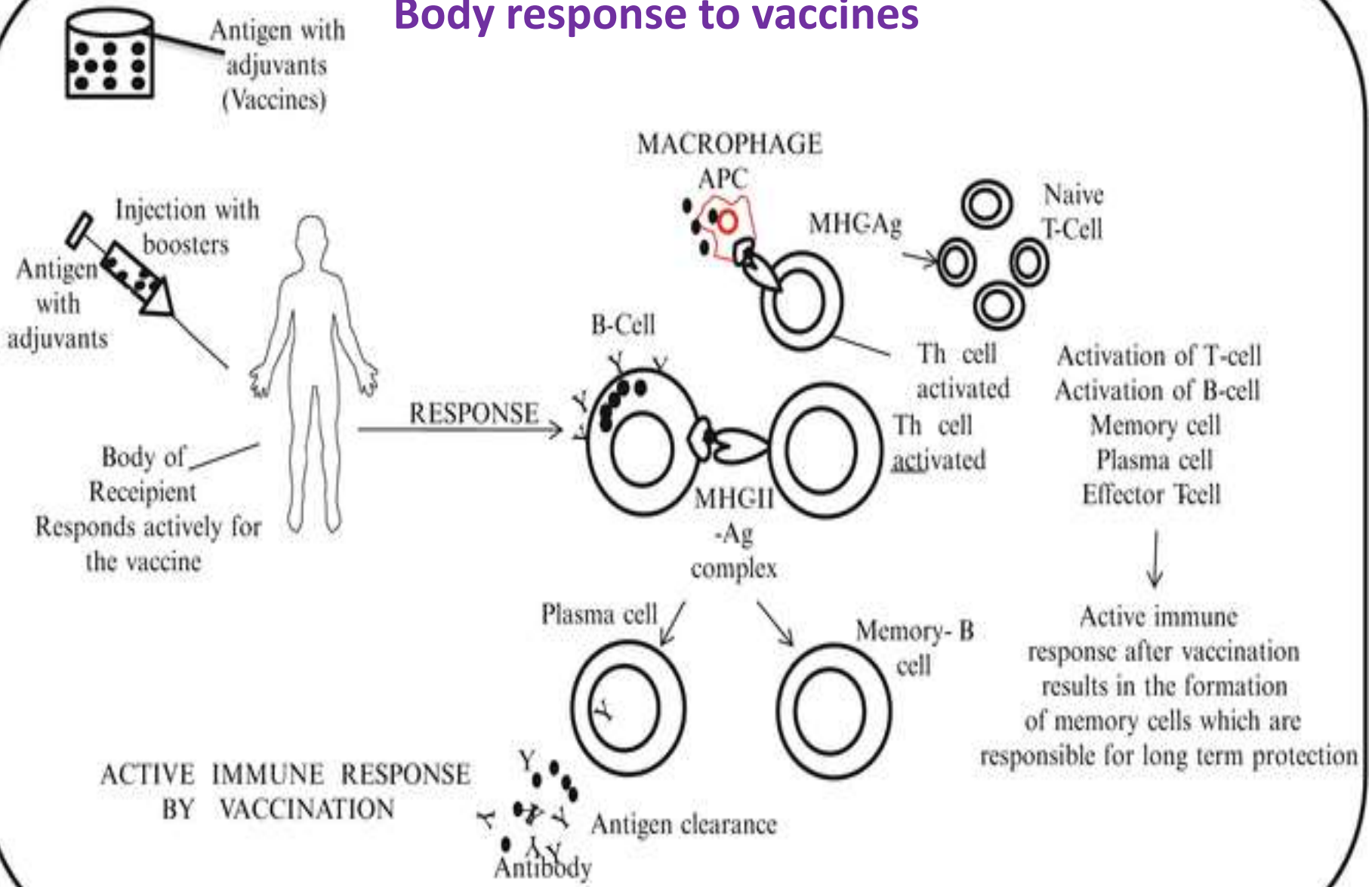


Development of Memory cells: key to the vaccination





Body response to vaccines

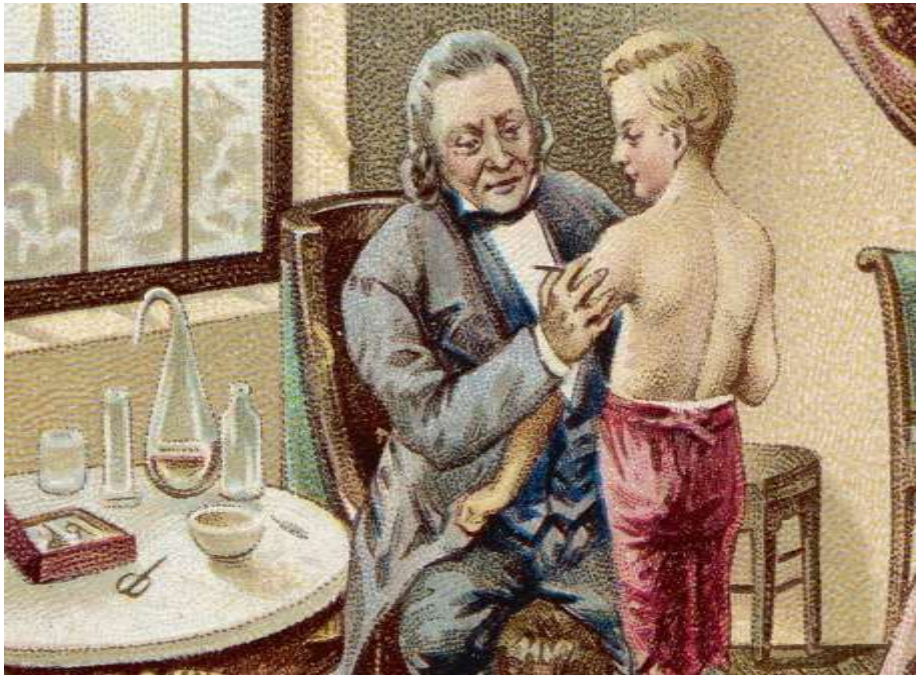


How do vaccines work?

- When inactivated or weakened disease-causing microorganisms enter the body, they initiate an immune response.
- This response mimics the body's natural response to infection.
- But unlike disease-causing organisms, vaccines are made of components that have limited ability, or are completely unable, to cause disease The components of the disease-causing organisms or the vaccine components that trigger the immune response are known as "antigens".
- These antigens trigger the production of "antibodies" by the immune system.
- Antibodies bind to corresponding antigens and induce their destruction by other immune cells

First vaccination

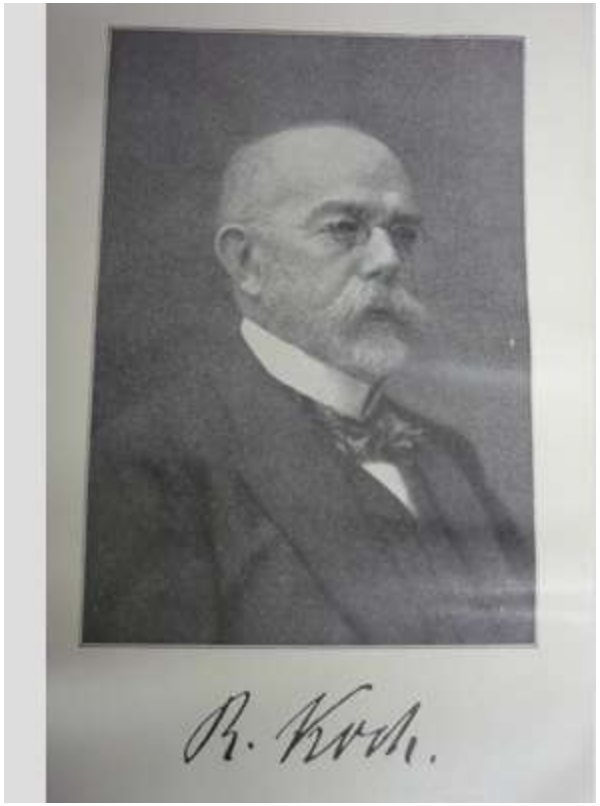
On May 14, 1796, **Jenner** took fluid from a cowpox blister and scratched it into the skin of James Phipps, an eight-year-old boy. A single blister rose up on the spot, but James soon recovered. On July 1, **Jenner** inoculated the boy again, this time with smallpox matter, and no disease developed. The **vaccine** was a success



Louis Pasteur

Almost 90 years after Jenner initiated immunization against smallpox, Pasteur developed another vaccine—the first vaccine against rabies.

After accidentally exposing chickens to the attenuated form of a culture, he demonstrated that they became resistant to the actual virus. Pasteur developed vaccinations for diseases such as anthrax, cholera, TB and smallpox.



German physician and scientist Robert Koch (1843-1910) is considered a founder of microbiology. He made important discoveries in identifying many bacteria, such as anthrax, tuberculosis, and cholera, and establishing their relation to disease. He also contributed important work in methods of growing, staining, identifying, and controlling bacteria in the lab. Koch's postulates are a landmark set of rules for linking a disease to the pathogen that causes it,

IMMUNIZATION

Passive : antibody from human or animals

Active: is achieved by inoculation with microbial pathogen that induce immunity but donot cause diseases

Passive immunization

Disease	Agent
Black widow spider bite	Horse antivenin
Botulism	Horse antitoxin
Diphtheria	Horse antitoxin
Hepatitis A and B	Pooled human immune gamma globulin
Measles	Pooled human immune gamma globulin
Rabies	Pooled human immune gamma globulin
Snake bite	Horse antivenin
Tetanus	Pooled human immune gamma globulin or horse antitoxin

Types of vaccines...

*Vaccines are **dead** or **inactivated organisms** or **purified products** derived from them. The different types of vaccines are:*

a) ***Traditional vaccines***

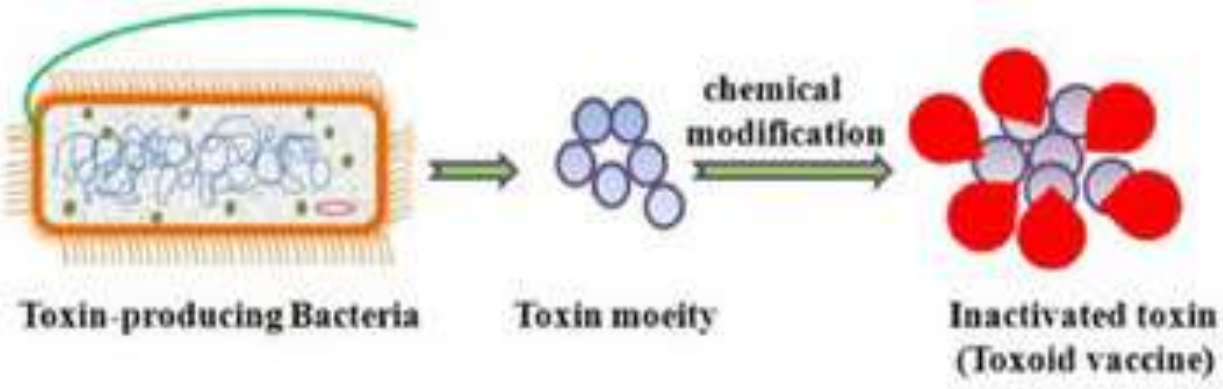
b) ***Innovative vaccines***

Traditional vaccine

Killed : Killed whole organism either chemically or heat

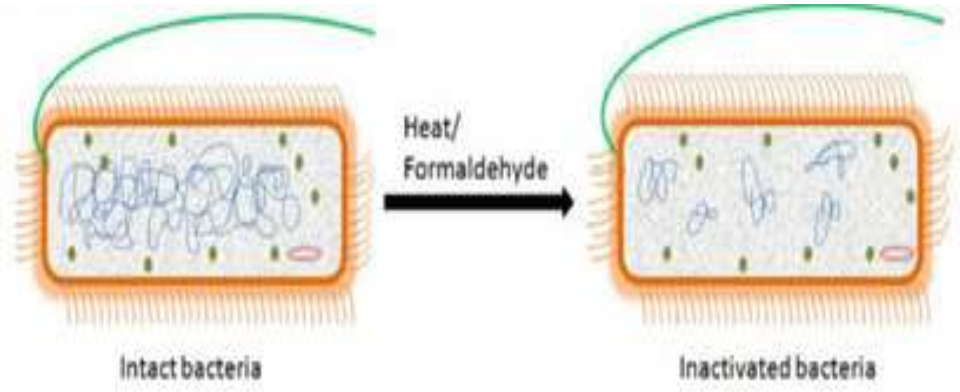
Live attenuated : A disease-causing **virus** or bacterium that is weakened in a laboratory so it cannot cause disease. **Live attenuated viruses** are often used as vaccines because, although weakened, they can stimulate a strong immune response

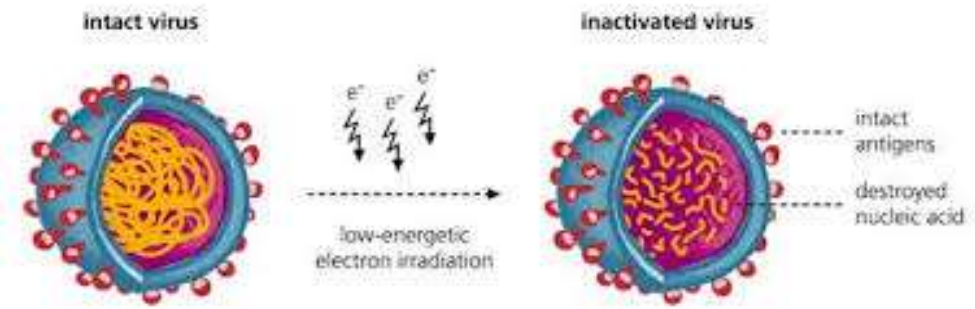
Toxiod: Modification of toxin to toxiod



Killed or inactivated vaccines

Pathogens for **inactivated vaccines** are grown under controlled conditions and are killed as a means to reduce infectivity (virulence) and thus prevent infection from the **vaccine**. The virus is killed using a method such as heat or formaldehyde.





Advantages

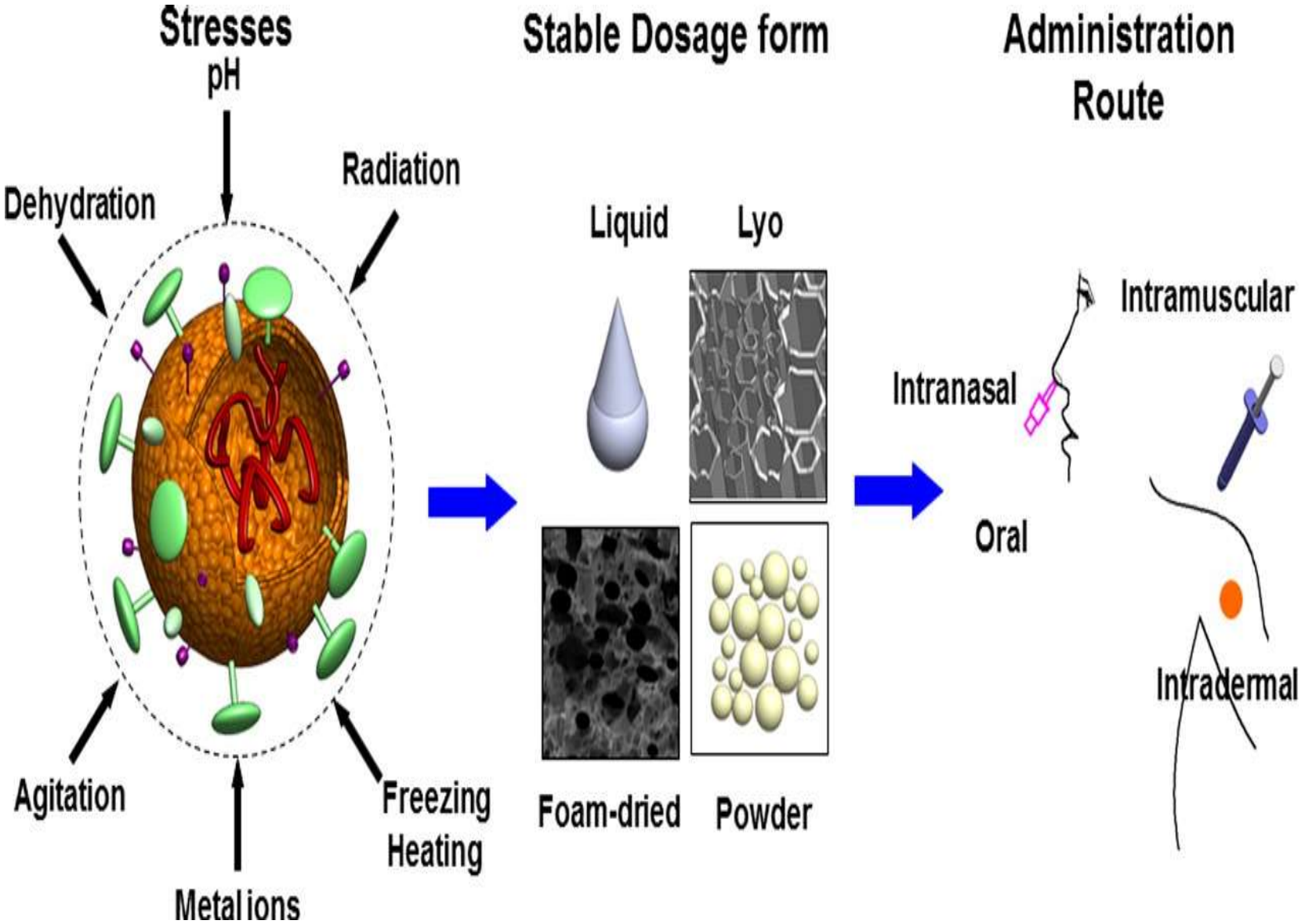
- Safe to use and can be given to immuno-deficient and pregnant individuals.
- Cheaper than live attenuated vaccine
- Storage not as critical as live vaccine

Disadvantages

- microorganisms cannot multiply so periodic boosters must be given to maintain immunity.
- Only humoral immunity can be induced.
- Most killed vaccines have to be injected.
- Some vaccines such as *Bordetella pertussis induce ill effects like post-vaccinial encephalomyelitis.*
- Inactivation, such as by formaldehyde in the case of the Salk vaccine, may alter antigenicity.

LIVE ATTENUATED VACCINE

- These vaccines are composed of live, attenuated microorganisms that cause a limited infection in their hosts sufficient to induce an immune response, but insufficient to cause disease.
- To make an attenuated vaccine, the pathogen is grown in foreign host such as animals, embryonated eggs or tissue culture, under conditions that make it less virulent.
- The strains are altered to a non-pathogenic form; for example, its tropism has been altered so that it no longer grows at a site that can cause disease. Some mutants will be selected that have a better ability to grow in the foreign host.
- These tend to be less virulent for the original host. These vaccines may be given by injection or by the oral route.
- A major advantage of live virus vaccines is that because they cause infection, the vaccine very closely reproduces the natural stimulus to the immune system.


Stresses
pH
Radiation
Dehydration
Agitation
Metal ions
Freezing
Heating
Stable Dosage form
Liquid
Lyo
Foam-dried
Powder
Administration
Route
Intramuscular
Intranasal
Oral
Intradermal

Live

Advantages

- stimulate generation of cellular as well as humoral immune responses.
- Since these can multiply in the host, single administration of vaccine produce long-lived immunity.
- Multiple booster doses may not be required
- Oral preparations are less expensive than giving injections.
- elimination of wild type virus from the community

Disadvantages

- May very rarely convert to its virulent form and cause disease.
- Live vaccines cannot be given to immuno-suppressed individuals,can cause serious illness or death in the vaccine recipient.
- Since they are live and because their activity depends on their viability, proper storage is critical.

Not all infectious agents are culturable
Production of animal and human virus need cell culture
Animal and human virus yeild is often very low
Extensive precaution is needed to produce the virus
Chances of revert
Batch to batch variation

Actually what our immune system recognize is the specific surface proteins or Polysaccharides of the infectious agents

So we donot need to use whole pathogen only the proteins is sufficient to induce Immune response and keep the system alert for future invasion

Thus the idea of subunit vaccine developed

Advantages

- can safely be given to immuno-suppressed people
- less likely to induce side effects.

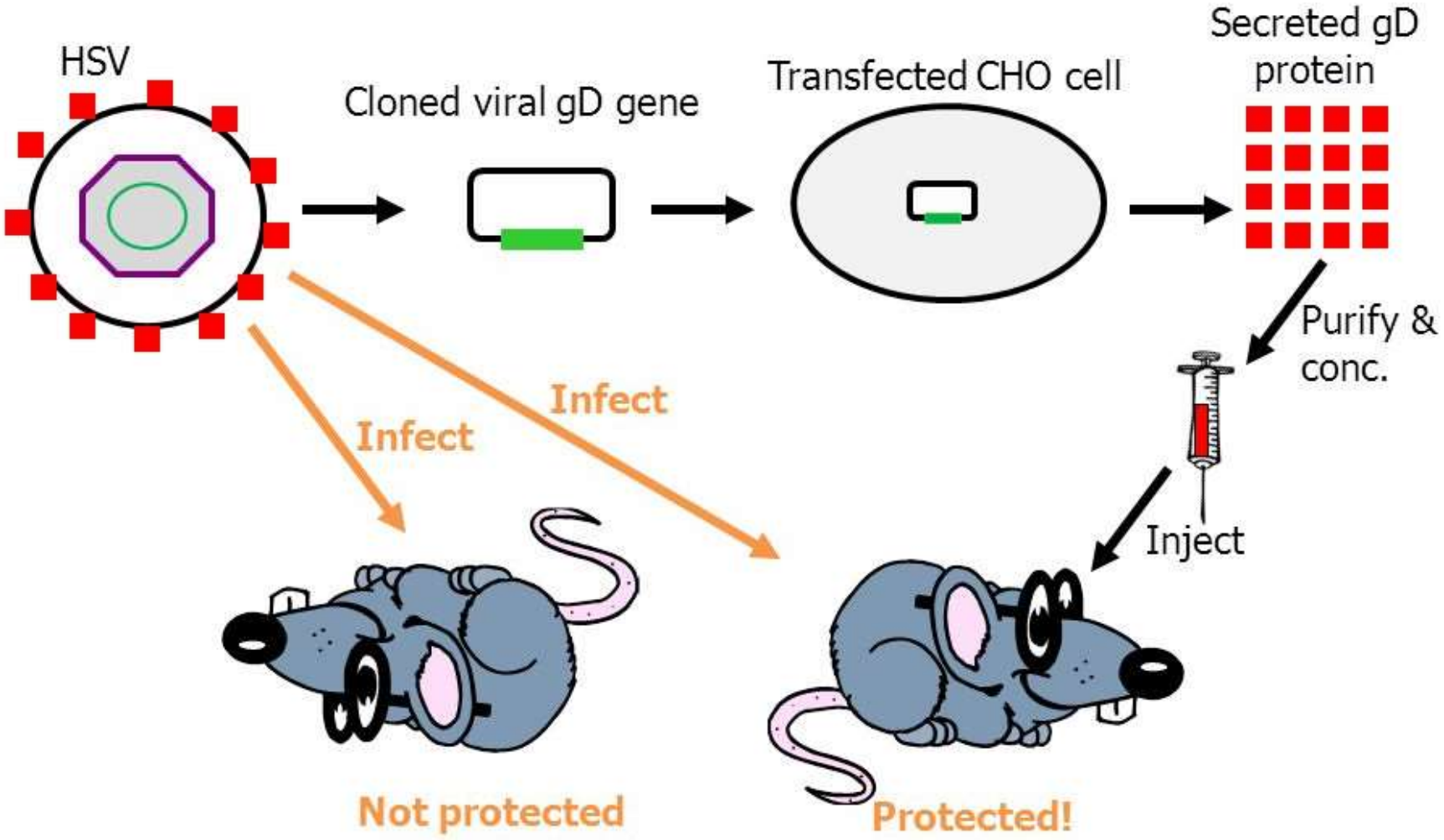
Disadvantages

- Antigens may not retain their native conformation, so that antibodies produced against the subunit may not recognize the same protein on the pathogen surface.
- Isolated protein does not stimulate the immune system as well as a whole organism vaccine.

Subunit vaccine

- **Protein subunit** (toxoid)- diphteria, tetanus
- **Bacterial polysaccharide**- ***S. pneumoniae, Nisseria meningitidis***
- **Viral glycoproteins**- Herpes simplex virus glycoprotein D

Subunit against HSV



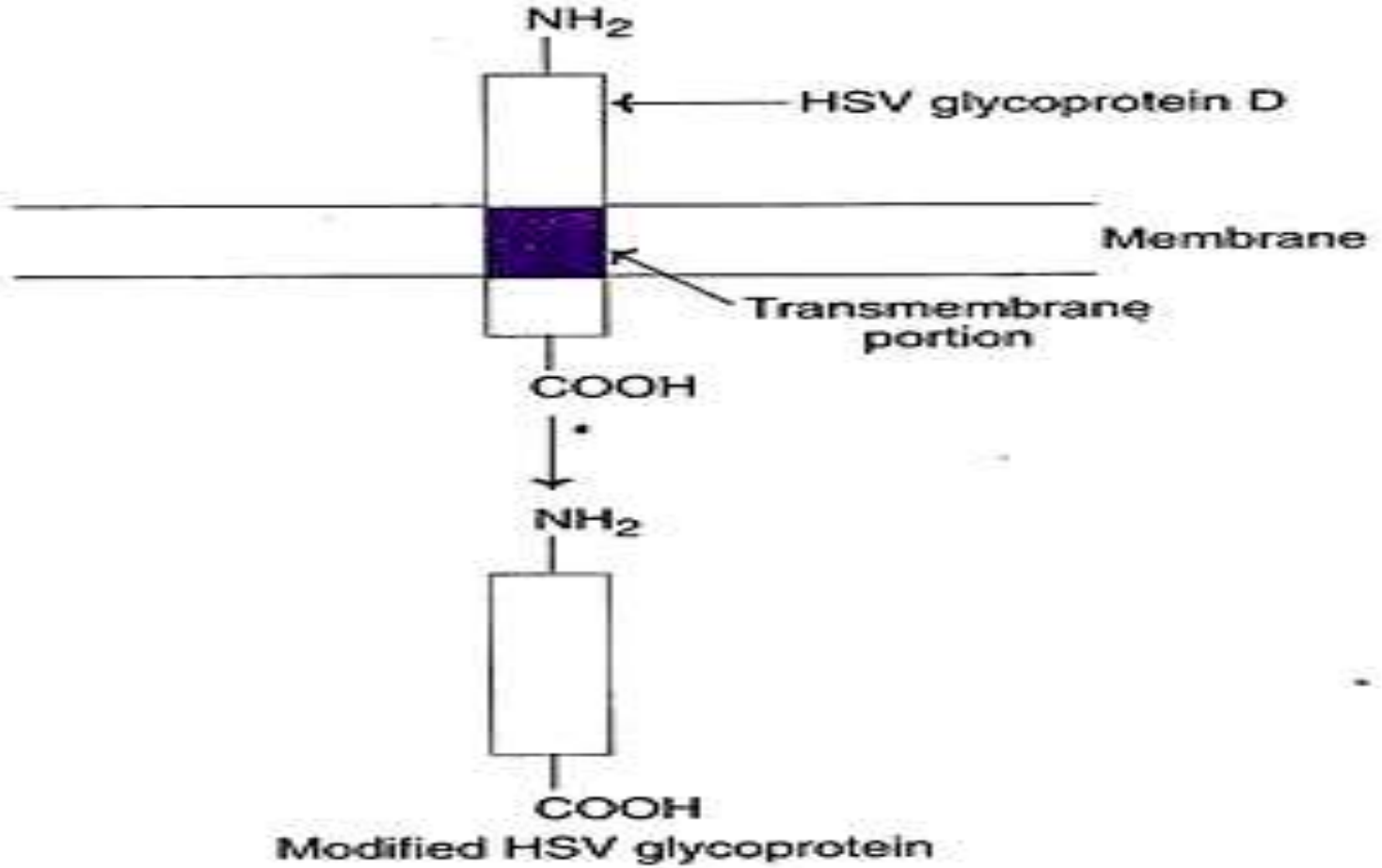
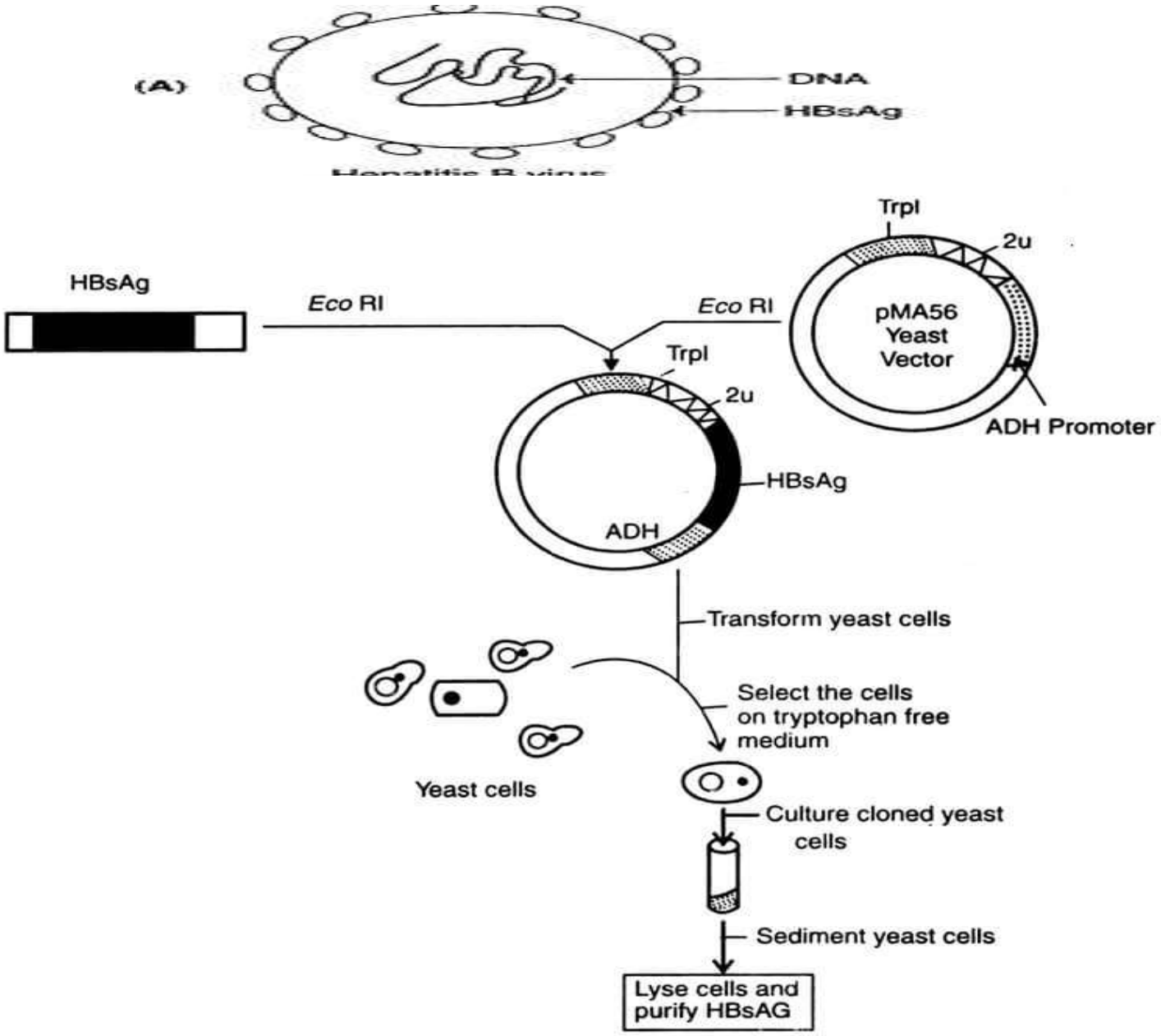


Fig. 16.3 : Modification of HSV glycoprotein D by deleting transmembrane portion. (HSV–Herpes simplex virus)

Production of hepatitis B surface antigen protein for subunit vaccine



Examples of some subunit vaccines that are in use which employ antigens either purified from microorganisms or produced by recombinant DNA technology

	organism	remarks
virus	hepatitis B virus	surface antigen can be purified from blood of carriers or produced in yeast by recombinant DNA technology
bacteria	*Neisseria meningitidis*	capsular polysaccharides or conjugates of groups A, C, γ and W-135 are effective B is non-immunogenic
	Streptococcus pneumoniae	84 serotypes; capsular polysaccharide vaccines contain 23 serotypes; conjugates with five or seven bacterial serotypes now available
	Haemophilus influenzae B	good conjugate vaccines now in use

Conjugate vaccines

Principle

Immunization with capsular polysaccharide antigen of one pathogen (weak immunogen in children) conjugated to protein antigen of another pathogen (strong immunogen)

Example

Vaccines against pneumococcus, menigococcus and *H. influenzae* type B (capsular polysaccharide bound to diptheria toxoid)

Advantages

Same as for subunit vaccines

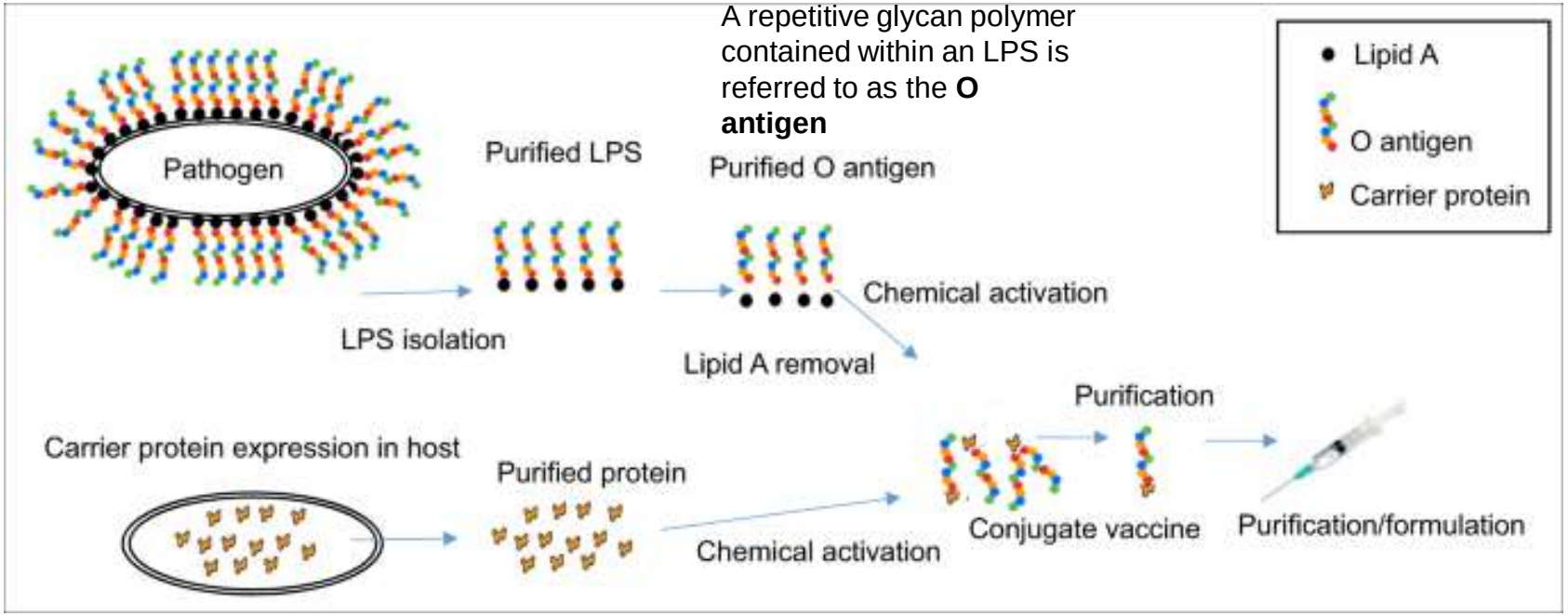
Good immune response to capsular antigens

Efficient in children in the first two years of life and asplenic persons

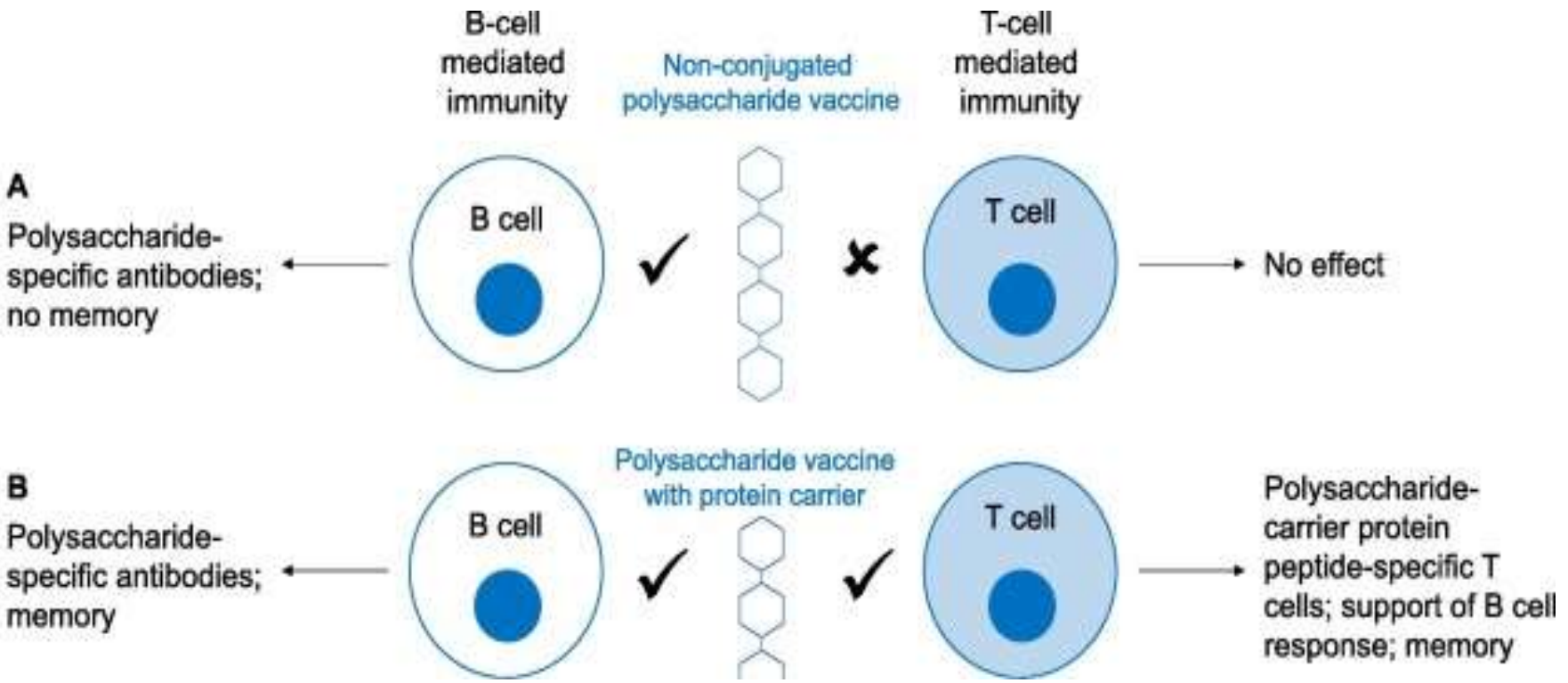
Limitations

Same as for subunit vaccines

Relatively high cost



Traditional chemical conjugation method for the production of glycoconjugate vaccines.


B-cell mediated immunity
Non-conjugated polysaccharide vaccine
T-cell mediated immunity
A
Polysaccharide-specific antibodies; no memory
B cell
T cell
No effect
Polysaccharide vaccine with protein carrier
B
Polysaccharide-specific antibodies; memory
B cell
T cell
Polysaccharide-carrier protein peptide-specific T cells; support of B cell response; memory

Peptide vaccine

Theoretically, it is expected that only small portions of a given protein (i.e., domains) are immunogenic and bind to antibodies. Logically, it is possible to use short peptides that are immunogenic as vaccines. These are referred to as peptide vaccines.

- **Synthetic peptides produced from active epitopes of antigen are used as vaccines.**
- **Synthetic peptide vaccines for HIV, influenza, diphtheria toxin, hepatitis B virus and malaria parasite are currently being evaluated for their efficacy**
- **Not very successful as peptides are not as immunogenic as proteins**

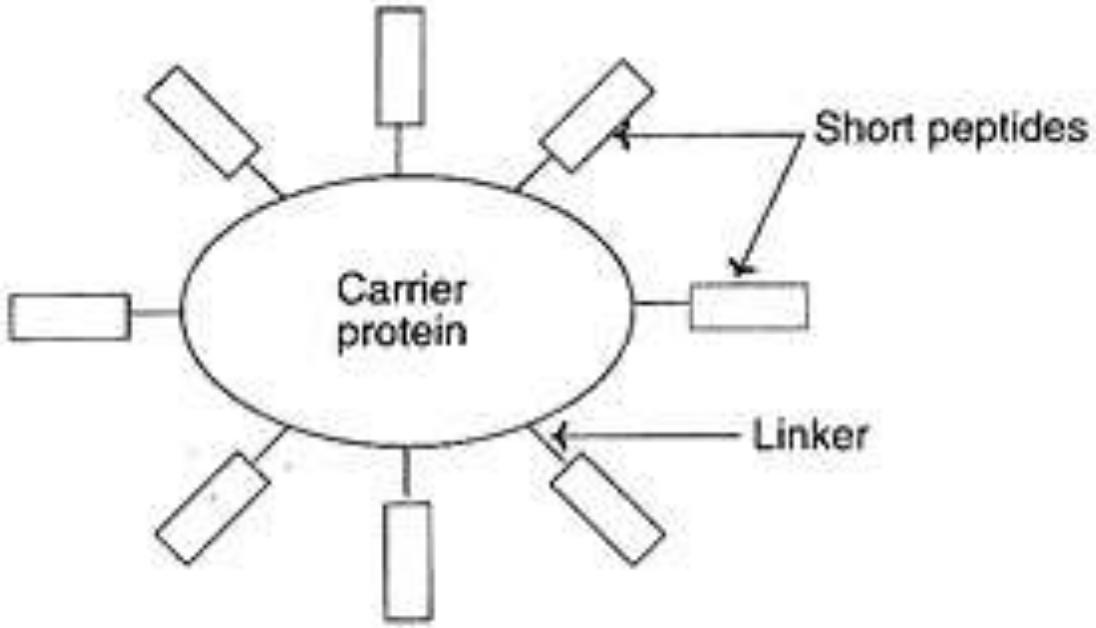


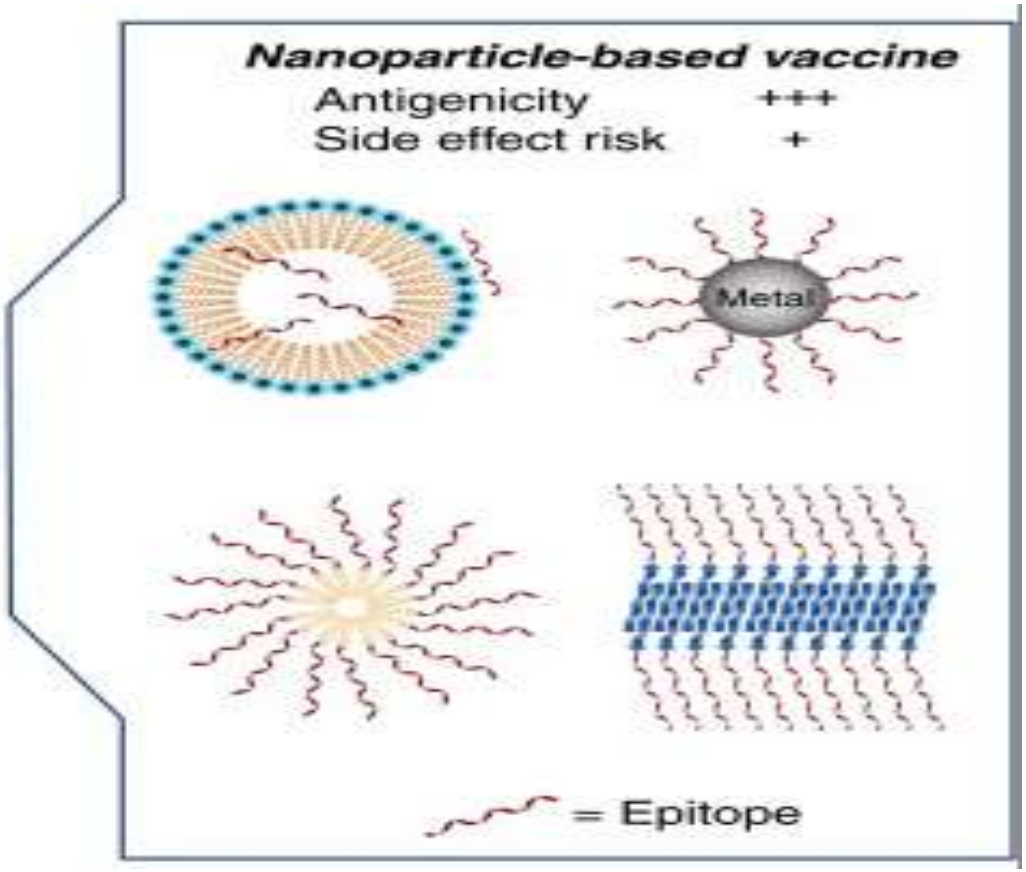
Fig. 16.2 : A general structure of a synthetic peptide vaccine.

PEPTIDE VACCINE

- The vaccine in which peptide of the original pathogen is used to immunize an organism.
- The best known example is foot and mouth disease, where protection was achieved by immunizing animals with a linear sequence of 20 amino acids.
- Synthetic peptide vaccines would have many advantages. Their antigens are precisely defined and free from unnecessary components which may be associated with side effects.
- They are stable and relatively cheap to manufacture.
- Furthermore, less quality assurance is required. Changes due to natural variation of the virus can be readily accommodated, which would be a great advantage for unstable viruses such as influenza.

- **Advantages**
- • If the peptide that induces protective immunity is identified, it can be synthesized easily on a large scale.
- • It is safe and can be administered to immunodeficient and pregnant individuals.
- **Disadvantage**
- • Poor antigenicity. Peptide fragments do not stimulate the immune system as well as a whole organism vaccine.

NANO Vaccines



Nanocarrier-based delivery systems offer an opportunity to enhance the humoral and cellular immune responses. This advantage is attributable to the nanoscale particle size, which facilitates uptake by phagocytic cells, the gut-associated lymphoid tissue, and the mucosa-associated lymphoid tissue, leading to efficient antigen recognition and presentation. Modifying the surfaces of nanocarriers with a variety of targeting moieties permits the delivery of antigens to specific cell surface receptors, thereby stimulating specific and selective immune responses.

Nano Particle as Carrier for peptide vaccine

Colloidal gold nanoparticles (GNPs) are ideal for this purpose primarily because of their biocompatibility and lack of immunogenicity. They can be easily prepared from gold salt [$H(AuCl_4)$] in water, and their particle sizes are controllable within the nano range. The peptides containing Cys can be readily conjugated to the surface of GNPs via their thiol groups, resulting in the presentation of multiple peptides on GNPs surface.

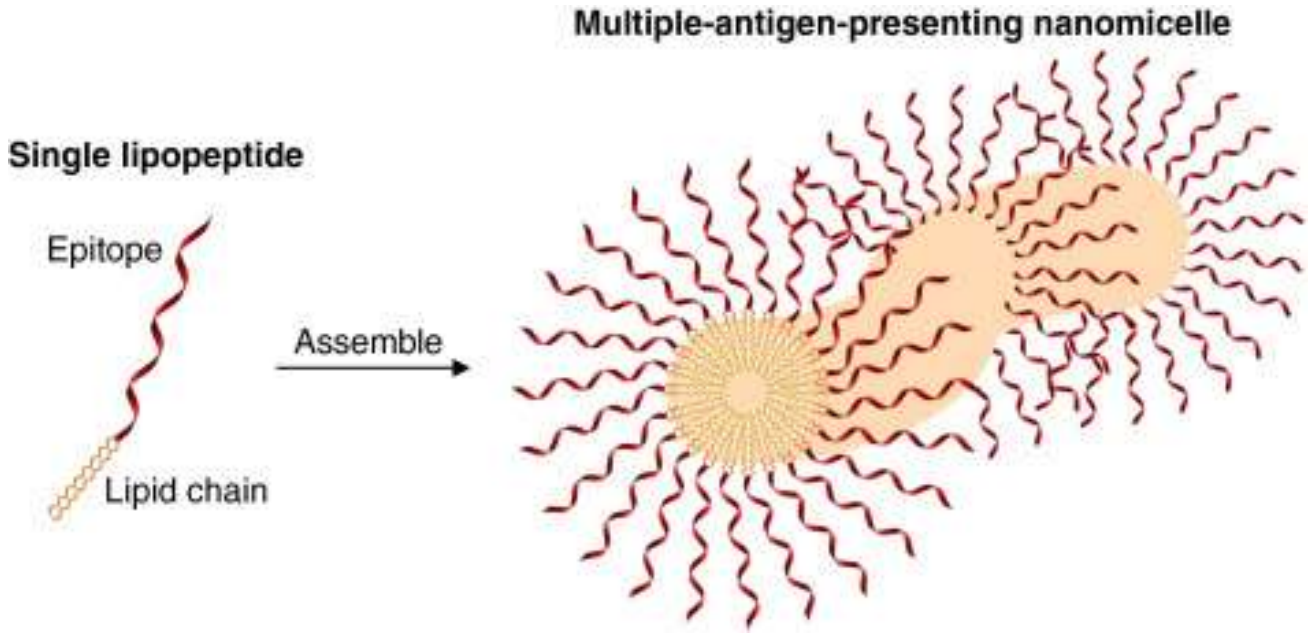
GNP-epitope conjugates containing epitopes derived from the VP1 protein of FMDV with an extra Cys residue, which provided thiol functionality for the conjugation. The conjugates using midsized nanoparticles (8 and 12 nm) showed a stronger antibody production compared with other sizes of particles or carrier protein conjugate.

The lyophilized nanovaccines are stable

The conjugation of peptides onto gold nanoparticles leads to increased stability and reproducibility of the conjugates and has the potential advantage of storage in a solid form following lyophilization.

Furthermore, addition of CpG (TLR9 ligand) as an adjuvant to the conjugate did not disturb the stability of the colloidal suspension.

Lipopeptide-Based Nanovaccines



Self-assembly of amphiphilic lipopeptides leads to the formation of micelles with multiple peptides at high density on the surface-lipopeptides induce humoral and cellular immune responses against the attached peptide antigen without addition of adjuvant or carrier proteins

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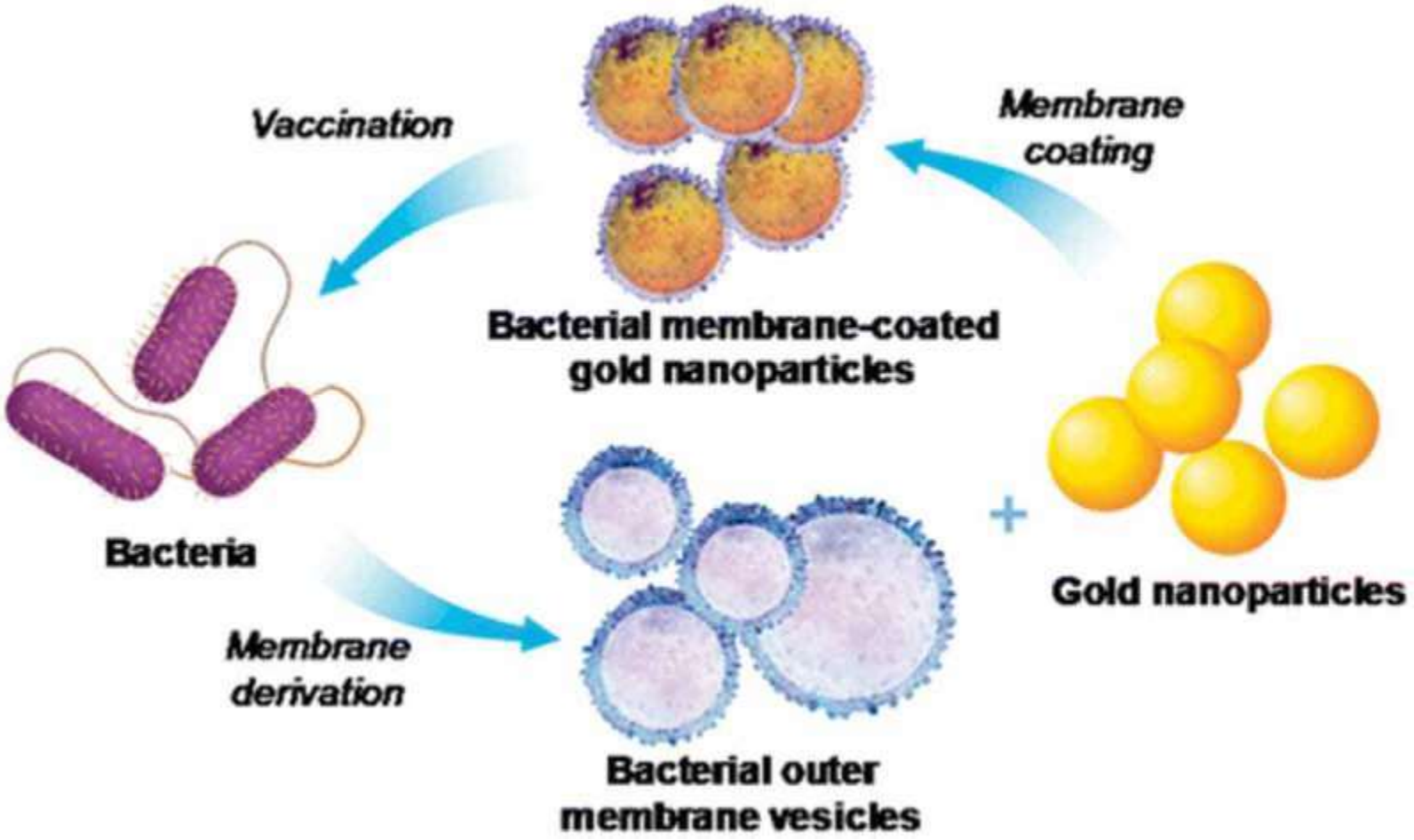


Schematic illustration of modulation of antibacterial immunity with bacterial-membrane-coated nanoparticles

ADVANTAGES OF NANOVACCINE

- Nanovaccine have potential to deliver safe and more effective vaccine.
- Nanobead covalently coupled with antigen offer distinct advantages – a low dose of antigen is required, efficient processing by antigen-presenting cells and stability during storage.
- Encapsulated nanoparticles easily deliver antigen, protects the antigen from degradation and is found to be effective with a single dose due to slow release of the antigen.

DISADVANTAGES OF NANOVACCINE

- Cost of production.
- Nanomaterials can change size, shape but not composition, which may change their toxicity.
- Small nanoparticles are cleared quickly from the body, large counterparts may accumulate in vital organs causing toxic problems.
- Reproducibility of formulation during manufacturing is one of the major hurdles in the use of nanoparticles as vaccines.

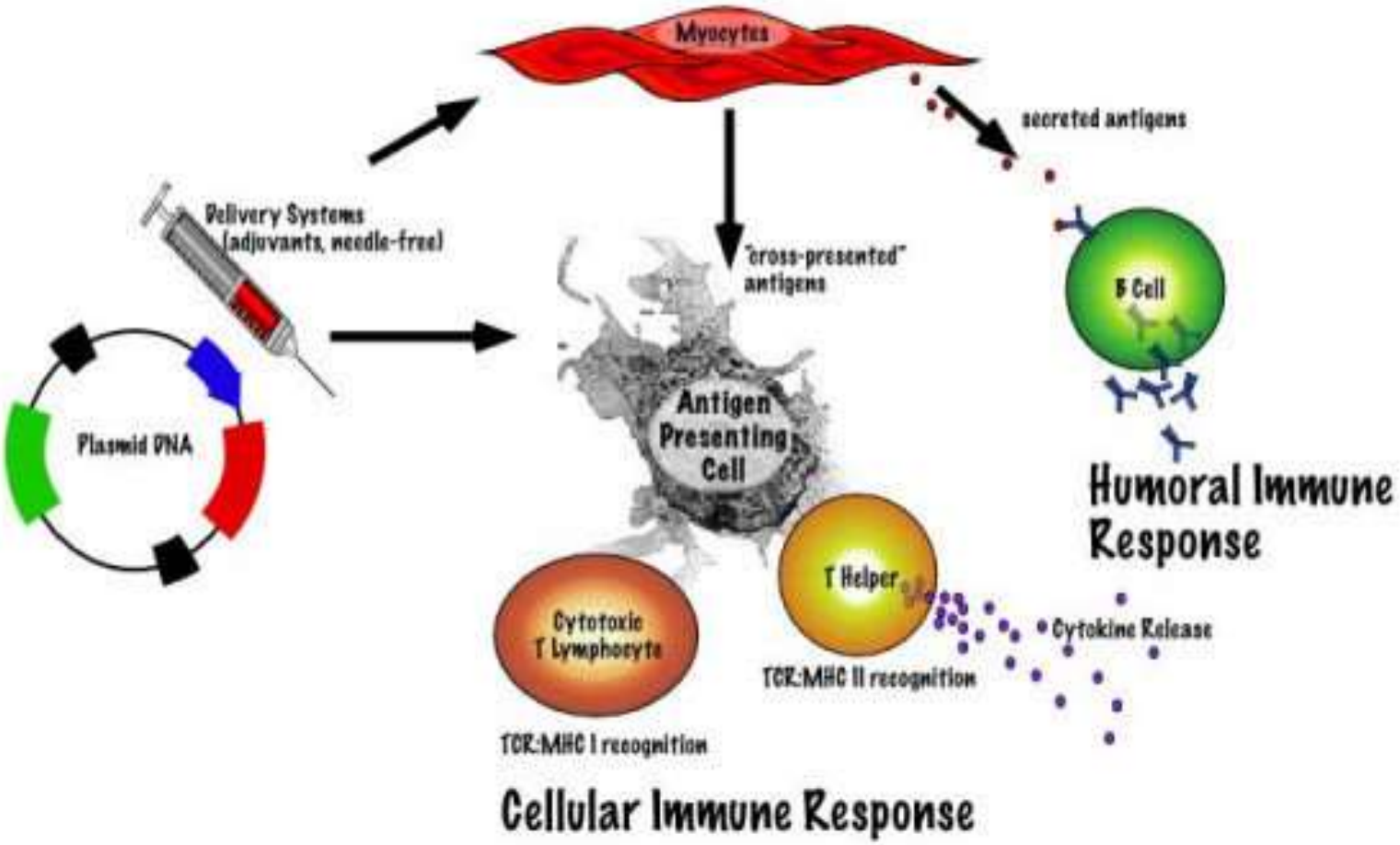
Genetic immunization or DNA vaccines

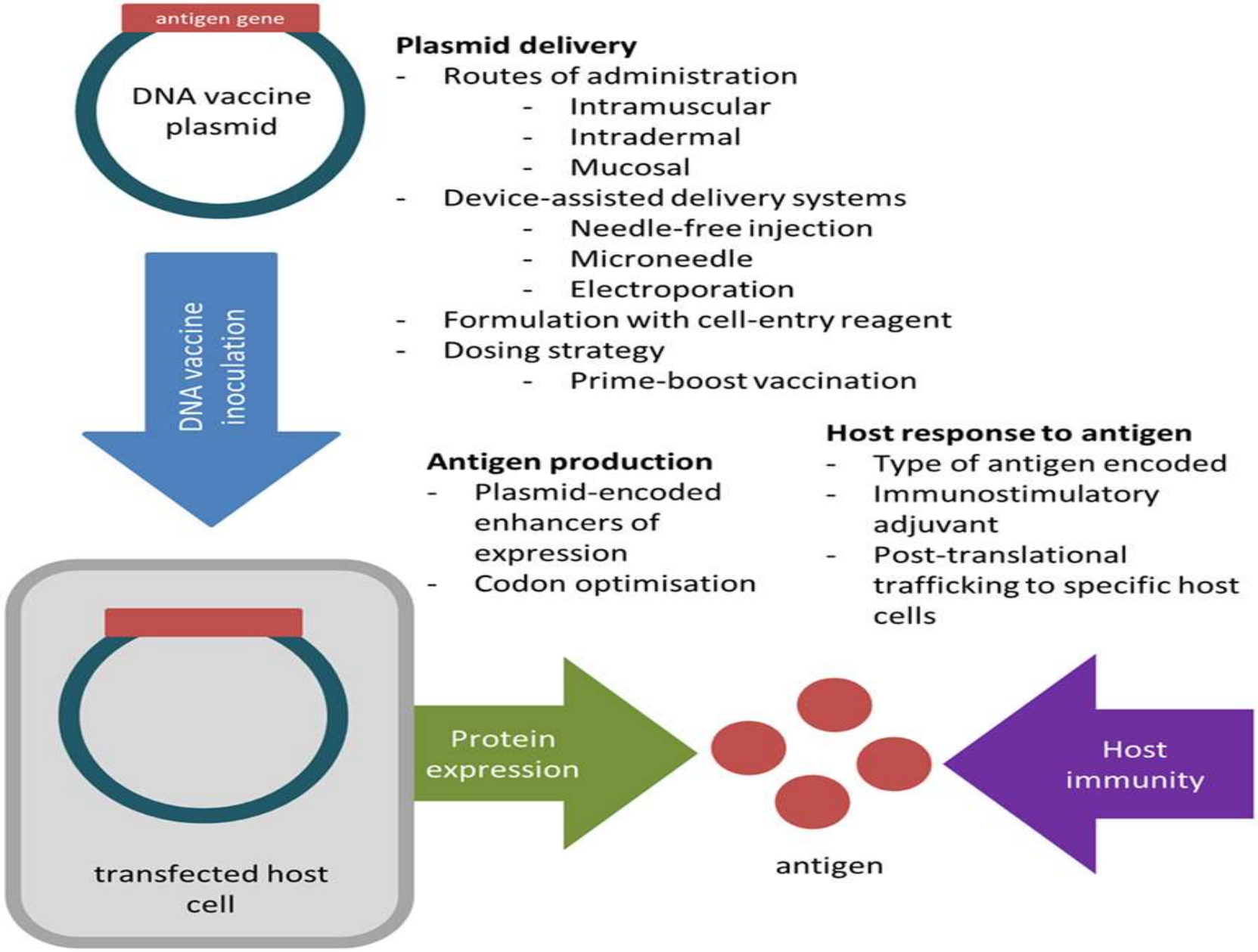
Peptide are naturally (within cells) coming from DNA. Thus instead of using peptide antigenic DNA can be introduced into the cells for vaccination

DNA vaccines are third generation vaccines. They contain DNA that codes for specific proteins (antigens) from a pathogen. The DNA is injected into the body and taken up by cells, whose normal metabolic processes synthesize proteins based on the genetic code in the plasmid that they have taken up.

Several DNA vaccines are available for veterinary use. Currently no DNA vaccines have been approved for human use. Research is investigating the approach for viral, bacterial and parasitic diseases in humans, as well as for several cancers.

Mechanisms of Action of DNA Vaccines





Plasmid delivery

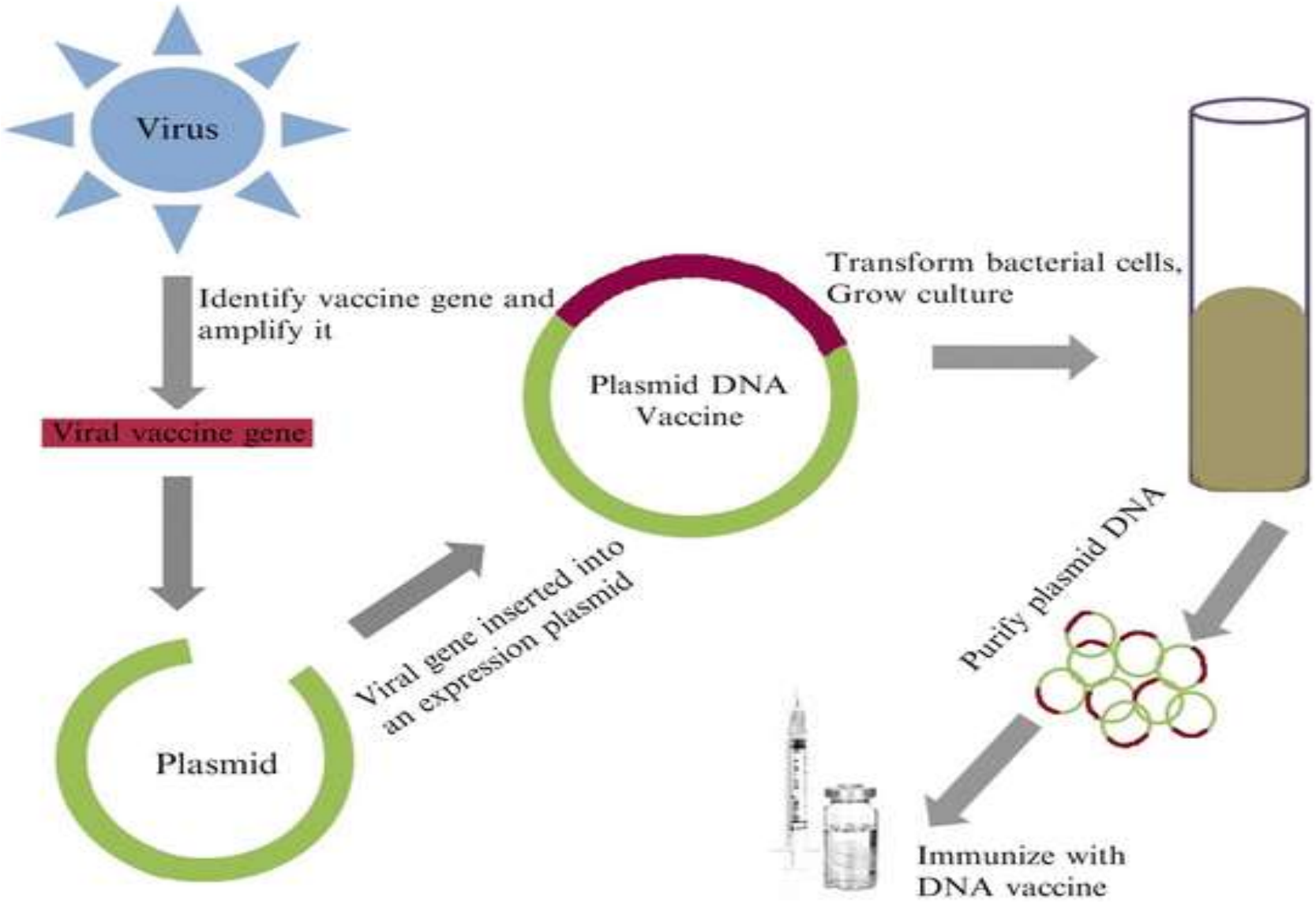
- Routes of administration
  - Intramuscular
  - Intradermal
  - Mucosal
- Device-assisted delivery systems
  - Needle-free injection
  - Microneedle
  - Electroporation
- Formulation with cell-entry reagent
- Dosing strategy
  - Prime-boost vaccination

Antigen production

- Plasmid-encoded enhancers of expression
- Codon optimisation

Host response to antigen

- Type of antigen encoded
- Immunostimulatory adjuvant
- Post-translational trafficking to specific host cells


Virus
Identify vaccine gene and amplify it
Viral vaccine gene
Plasmid
Viral gene inserted into an expression plasmid
Plasmid DNA Vaccine
Transform bacterial cells, Grow culture
Purify plasmid DNA
Immunize with DNA vaccine

Advantages

No risk for infection

Antigen presentation by both MHC I and II molecules

Immune response focused on antigen of interest

Ease of development and production

Stability for storage and shipping

Cost-effectiveness

Obviates need for peptide synthesis, expression and purification of recombinant proteins and use of toxic adjuvants

Long-term persistence of immunogen

***In vivo* expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications[**

Adverse effects

Limited to protein immunogens (not useful for non-protein based antigens such as bacterial polysaccharides)

Risk of affecting genes controlling cell growth

Possibility of inducing antibody production against DNA

Possibility of tolerance to the antigen (protein) produced

Plasmid-based DNA vaccines are **a novel, economic, and effective strategy which induces antigen-specific immunity capable of conferring effective protection against various infectious diseases and tumor cells**

At present, human trials are under way with several DNA vaccines, including those for **malaria, AIDS, influenza, Ebola and herpesvirus**.

Attenuated Shigella as a DNA-mediated immunization

Direct inoculation of DNA, in the form of purified bacterial plasmids that are unable to replicate in mammalian cells but are able to direct cell synthesis of foreign proteins, is being explored as an approach to vaccine development. Highly attenuated Shigella vector invaded mammalian cells and delivered such plasmids into the cytoplasm of cells, and subsequent production of functional foreign protein. Because this Shigella vector was designed to deliver DNA to colonic mucosa, the method is a potential basis for oral and other mucosal DNA immunization and gene therapy strategies.

Why Can *Shigella* Be Used as Vaccine-vectors?

Several features of the attenuated *Shigella* make it an attractive vaccine vector: i) it effectively escapes from the endosome and directly enters the cytoplasm host cell, possibly mimicking the vaccine carrier characteristics of *Listeria monocytogenes*; ii) it naturally targets lymphoid tissue in the colonic mucosa and therefore may elicit mucosal and systemic immune responses against passenger antigens. iii) attenuated *Shigella* might enter host cells efficiently at sites of induction for mucosal immunity following administration by the oral or intranasal route, without causing significant pathology or disease.

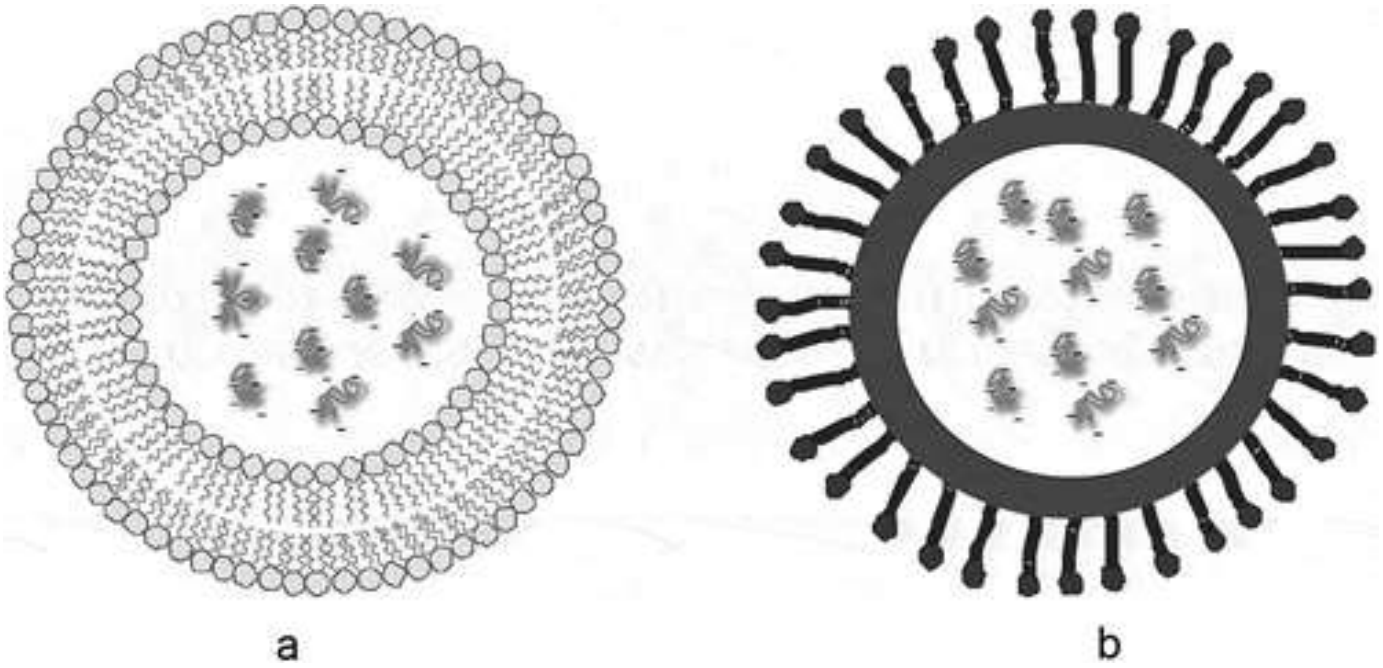
The DNA vaccines have been emerged as best remedy for problematic diseases being capable of producing humoral and cellular immune responses as well as the safest vaccines so far.

There are a large number of infectious diseases against which traditional vaccines failed to respond effectively. Especially, viral diseases and cancer where DNA vaccines seem to be the better option. However, the magnitude of immune responses produced by them in primates is not sufficient to be used in human beings.

There is an evidence that these immune responses can be augmented by using **properly structured nano-sized particles** that may avoid DNA degradation and facilitate targeted and controlled delivery to antigen presenting cells.

Adsorption, formulation or encapsulation with particles has been found to stabilize DNA formulations. The use of nanoparticles for vaccine delivery is a platform technology and has been applied for delivery of a variety of existing and potential vaccines successfully.

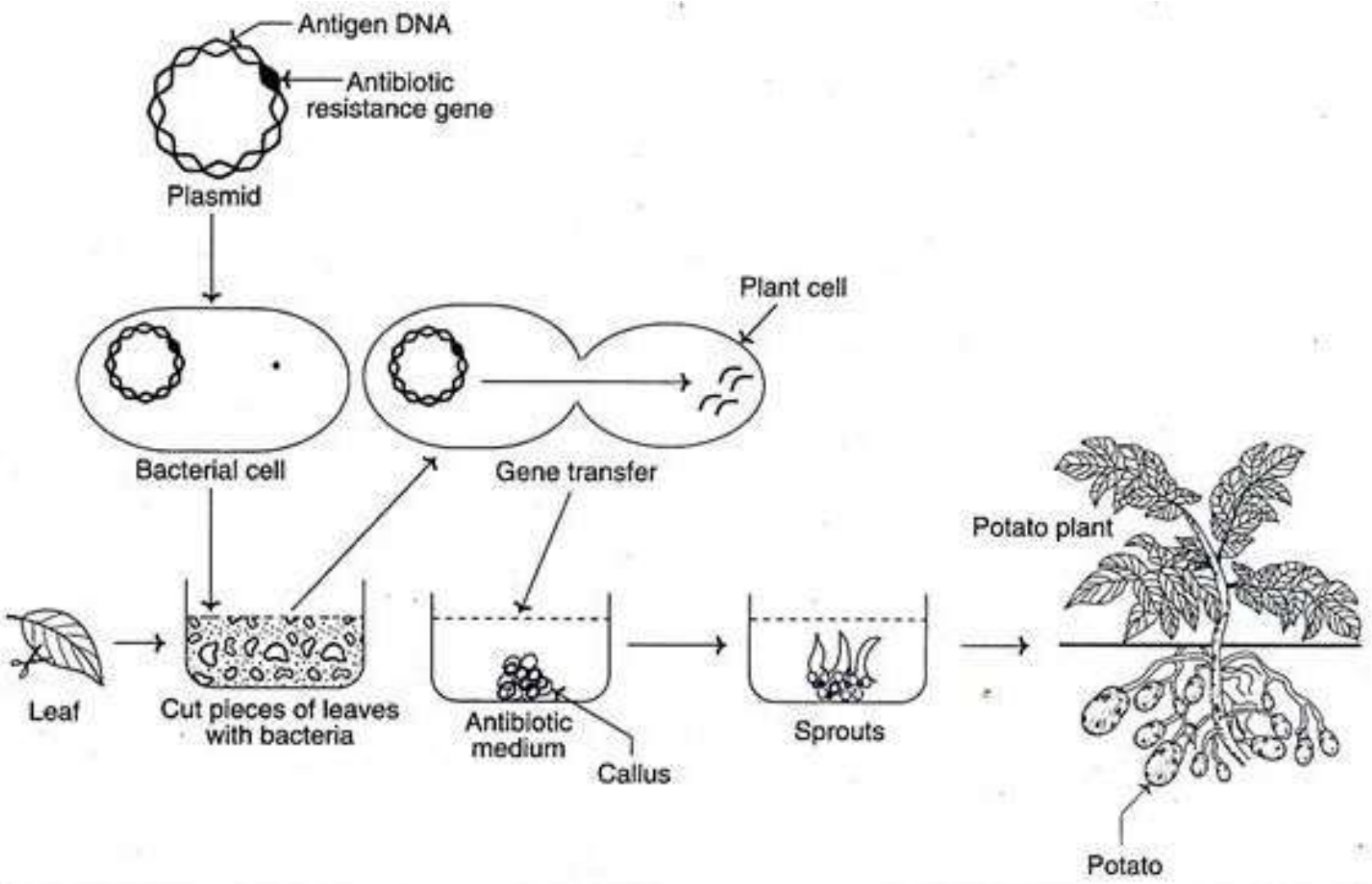
Nanoparticle mediated DNA vaccines



Nanoparticles of cationic lipids and cell-surface active polymers.

An important advantage of nanosystems in vaccine delivery is the possibility to co-encapsulate additional immunostimulatory molecules, such as small molecules, targeted at specific receptors present on immune cells (pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), that have the ability to trigger stronger immune responses

Production of edible vaccines in plant



Selected list of plant edible subunit vaccines

Antigen	*Host plant*
Rabies glycoprotein	Tomato
Foot and mouth virus (VPI)	*Arabidopsis*
Herpes virus B surface antigen	Tobacco
Cholera toxin B subunit	Potato
Human cytomegalovirus glycoprotein B	Tobacco

Attenuated Recombinant Vaccines

In the early years of vaccine research, attenuated strains of some pathogenic organisms were prepared by prolonged cultivation — weeks, months or even years. Although the reasons are not known, the infectious organism would lose its ability to cause disease but retains its capability to act as an immunizing agent. This type of approach is almost outdated now

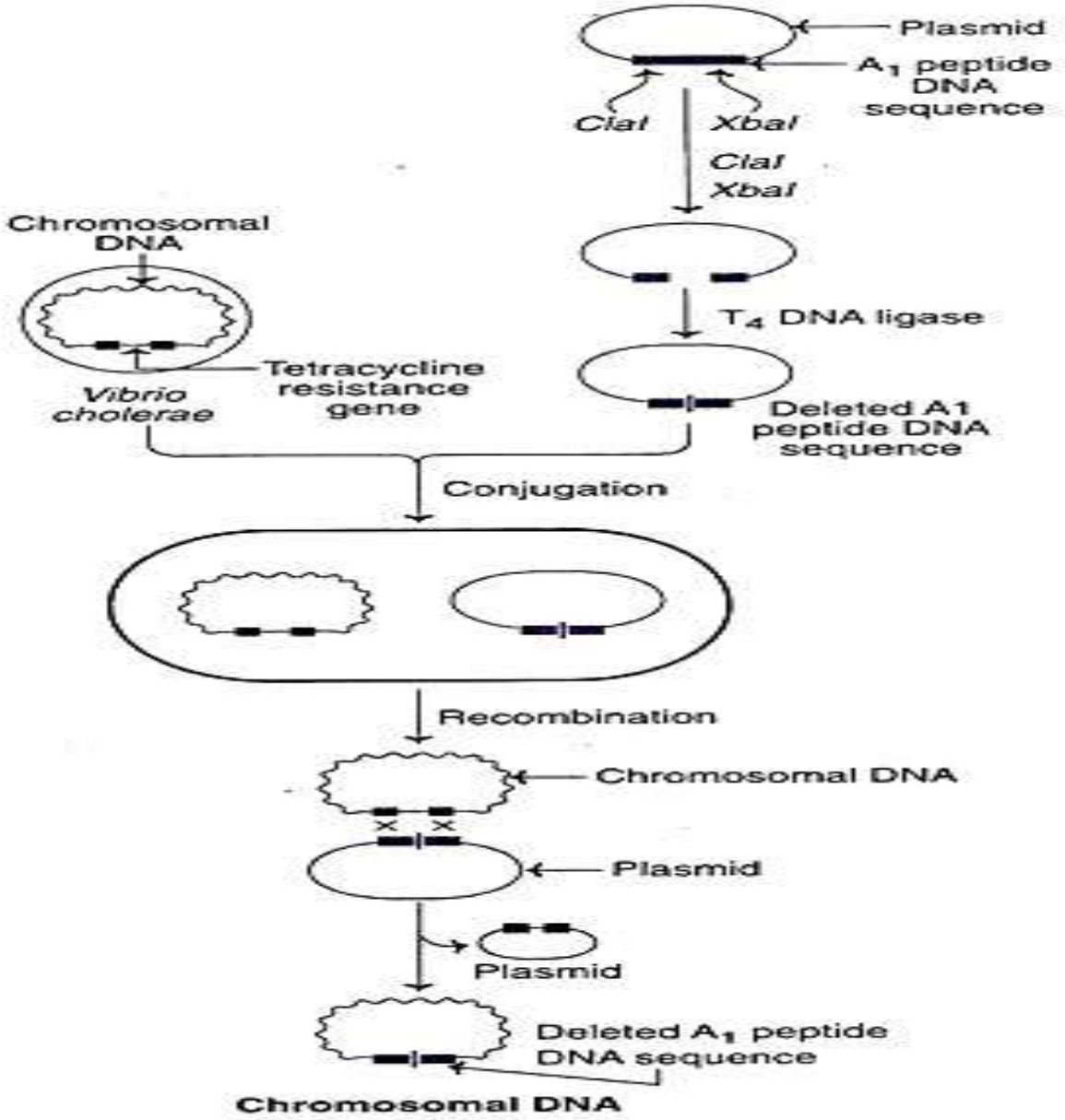
It is now possible to genetically engineer the organisms (bacteria or viruses) and use them as live vaccines, and such vaccines are referred to as attenuated recombinant vaccines. The genetic manipulations for the production of these vaccines are broadly of two types:

1. Deletion or modification of virulence genes of pathogenic organisms.

2. Genetic manipulation of non-pathogenic organisms to carry and express antigen determinants from pathogenic organisms.

The advantage with attenuated vaccines is that the native conformation of the immunogenic determinants is preserved; hence the immune response is substantially high. This is in contrast to purified antigens which often elicit poor immunological response.

Development of new strain of V. Cholerae as attenuated vaccine



A group workers have developed a gene altered potato containing attenuated cholera vaccine. These potatoes when fed to mice induced immunity against cholera.

Salmonella Species:

The different strains of Salmonella genus are responsible for causing typhoid, enteric fever, food poisoning and infant death. Immunoprotection against Salmonella pathogens is really required.

Some workers have been successful in deleting **aro genes and pur genes** in Salmonella.

Aro genes encode for the enzymes responsible for the biosynthesis of aromatic compounds, while pur genes encode for enzymes of purine metabolism.

The new strains of Salmonella can be grown in vitro on a complete medium. The doubly deleted strains have a very restricted growth in vivo, while they can stimulate immunological response. The genetically engineered attenuated vaccines of Salmonella have been shown to be effective as oral vaccines in experimental animals (mice, cattle, sheep, and chickens). Some workers claim that the new strain of Salmonella offers immunoprotection in humans also.

Leishmania Species:

Leishmania species are flagellated protozoan parasites and are responsible for the disease leishmaniasis. This disease is characterized by cutaneous, visceral and mucosal leisons. Leishmaniasis is transmitted by sand flies.

An attenuated strain of leishmania has been created and successfully used in mice to offer immunoprotection against leishmaniasis. In Leishmania major, the genes encoding **dihydrofolate reductase-thymidylate synthase** can be replaced by the genes encoding resistance to antibiotics G-418 and hygromycin.

This new strain of L. major invariably requires thymidine in the medium for its growth and multiplication. The attenuated strain of L. major can survive only a few days when administered to mice. This short period is enough to induce immunity in mice against the lesions of leishmania. However, more experiments on animals have to be carried out before the leishmania attenuated vaccine goes for human trials.

Vaccines against Viruses- Vaccinia Virus:

Vaccinia viruses is basically the vaccine that was originally used by Jenner for the eradication of smallpox. The molecular biology of this virus has been clearly worked out. Vaccinia virus contains a double-stranded DNA (187 kb) that encodes about 200 different proteins. The genome of this virus can accommodate stretches of foreign DNA which can be expressed along with the viral genes.

The vaccinia virus can replicate in the host cell cytoplasm (of the infected cells) rather than the nucleus. This is possible since the vaccinia virus possesses the machinery for DNA replication, transcription-DNA polymerase, RNA polymerase etc. The foreign genes inserted into the vaccinia virus can also be expressed along with the viral genome. Thus, the foreign DNA is under the control of the virus, and is expressed independently from the host cell genome.

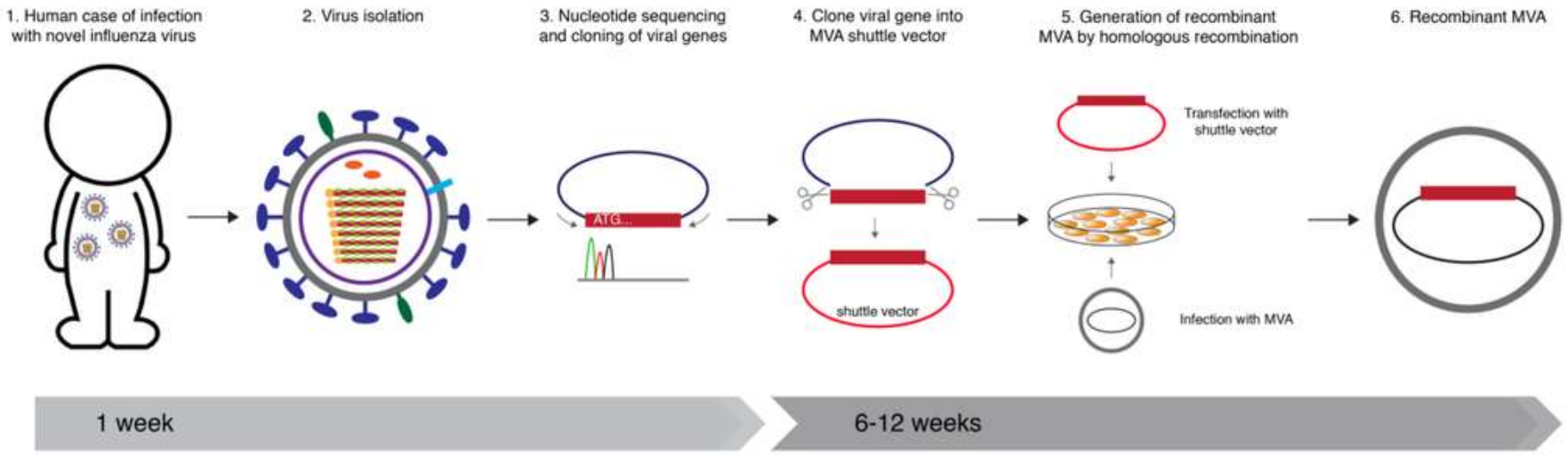
The vaccinia viruses are generally harmless, relatively easy to cultivate and stable for years after lyophilization (freeze-drying). All these features make the vaccinia virus strong candidates for vector vaccine. The cloned foreign genes (from a pathogenic organism) can be inserted into vaccinia virus genome for encoding antigens which in turn produces antibodies against the specific disease- causing agent.

The advantage with vector vaccine is that it stimulates **B-lymphocytes (to produce antibodies) and T-lymphocytes (to kill virus infected cells)**. This is in contrast to a subunit vaccine which can stimulate only B-lymphocytes. Thus, vaccinia virus can provide a high level of immunoprotection against pathogenic organisms.

Another advantage of vaccinia virus is the possibility of vaccinating individuals against **different diseases** simultaneously. This can be done by a recombinant vaccinia viruses which carries genes encoding different antigens.

Antigen genes for certain diseases have been successfully incorporated into vaccinia virus genome and expressed. Thus, vector vaccines have been developed against hepatitis, influenza, herpes simplex virus, rabies and malaria. **However, none of these vaccines has been licensed for human use due to fear of safety. It is argued that recombinant vaccinia virus might create life threatening complications in humans.**

Modified Vaccinia Virus Ankara (MVA) is a replication-deficient viral vector that holds great promise as a vaccine platform.



Ideal timeline for construction of an MVA-based vaccine after a human case of infection with a novel respiratory virus. Influenza virus is used as an example.

(1) After the emergence of a novel respiratory virus with the ability of infecting humans,

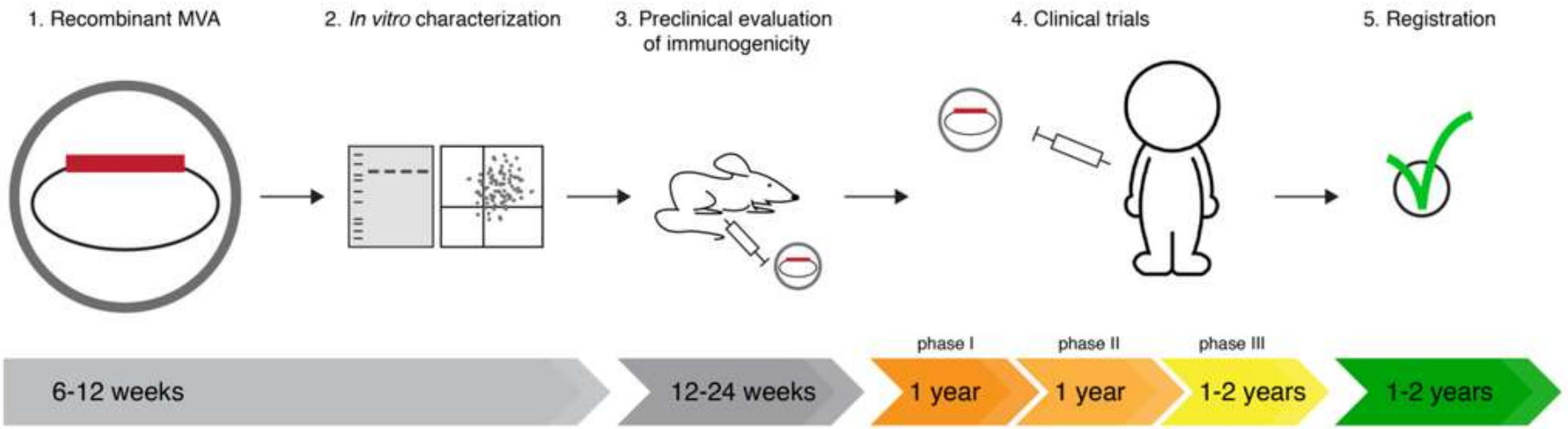
(2) the virus is isolated

(3) and the sequence of a target gene of interest is obtained within a week

(4) Subsequently, the gene of interest is cloned or simply synthesized and subcloned into an MVA shuttle vector plasmid.

(5) This shuttle vector is then transfected in cells infected with MVA. Through homologous recombination the gene of interest is inserted into the MVA genome.

(6) By serial plaque passages on CEF, a good laboratory practice (GLP) compliant rMVA is clonally isolated . The process from cloning to obtaining the rMVA takes about 6–12 weeks.



Ideal timeline for evaluation of a novel MVA-based vaccine.

(1) A newly developed rMVA vaccine

(2) is tested in vitro to assess correct gene insertion and protein expression in rMVA infected cells, e.g., by Western Blot or flow cytometry.

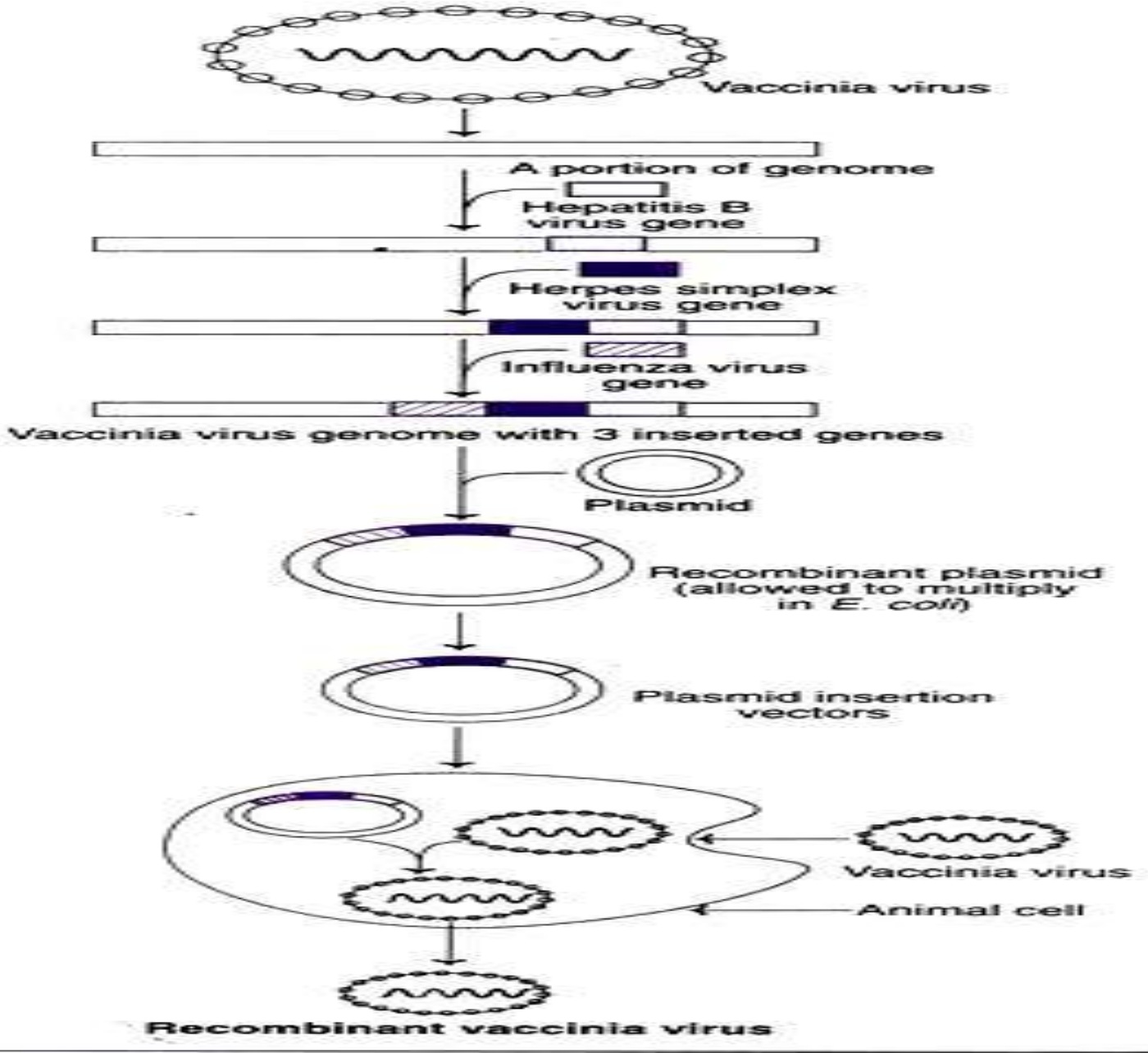
(3) Subsequently, the vaccine immunogenicity and efficacy is tested in mice, ferrets and/or macaques.

(4) If the MVA-based vaccine is successful in the pre-clinical tests, the vaccine is tested in phase I, II and III clinical trails.

(5) Finally, when the vaccine has proven safe and effective, it can be filed for market authorization.

Production of recombinant vaccinia viruses:

The development of recombinant vaccinia virus is carried out by a two-step procedure



Advantages:

1. Authenticated antigens that closely resemble natural antigens can be produced.
2. The virus can replicate in the host cells. This enables the amplification of the antigens for their action on B-lymphocytes and T-lymphocytes.
3. There is a possibility of vaccinating several diseases with one recombinant vaccinia virus.

Disadvantages:

1. The most important limitation is the yet unknown risks of using these vaccines in humans.
2. There may be serious complications of using recombinant viral vaccines in immunosuppressed individuals such as AIDS patients.

Other viral recombinant vaccines:

Most of the work on the development of live viral vaccines has been carried out on vaccinia virus. Other viruses such as adenovirus, poliovirus and varicella-zoster virus are also being tried as recombinant vaccines. Scientists are attracted to develop a recombinant poliovirus as it can be orally administered. It might take many more years for the recombinant viral vaccines to become a reality for human use.

Immuno compromised individual like AIDS , vaccinia virus can lead to serious infection

To avoid this Il2 gene is inserted along with antigenic gene, presence of IL2 activated T cells to stop virus infection

But if the proliferation of virus has serious effects, it would be better to inhibit the virus After vaccinization
Wt type vaccinia virus is resistance to interferon, thus interferon sensitive virus may be used

Interferon acivate PI kinase which phosphorylate eIF-2α which inhibits protein synthesis
Vaccinia virus code for a protein K3L, which compete with phosphorylation of eIF-2α
Thus interferon has little effect on vaccinia virus

So K3l deleted Vaccinia virus is used, suitable for oral vaccine

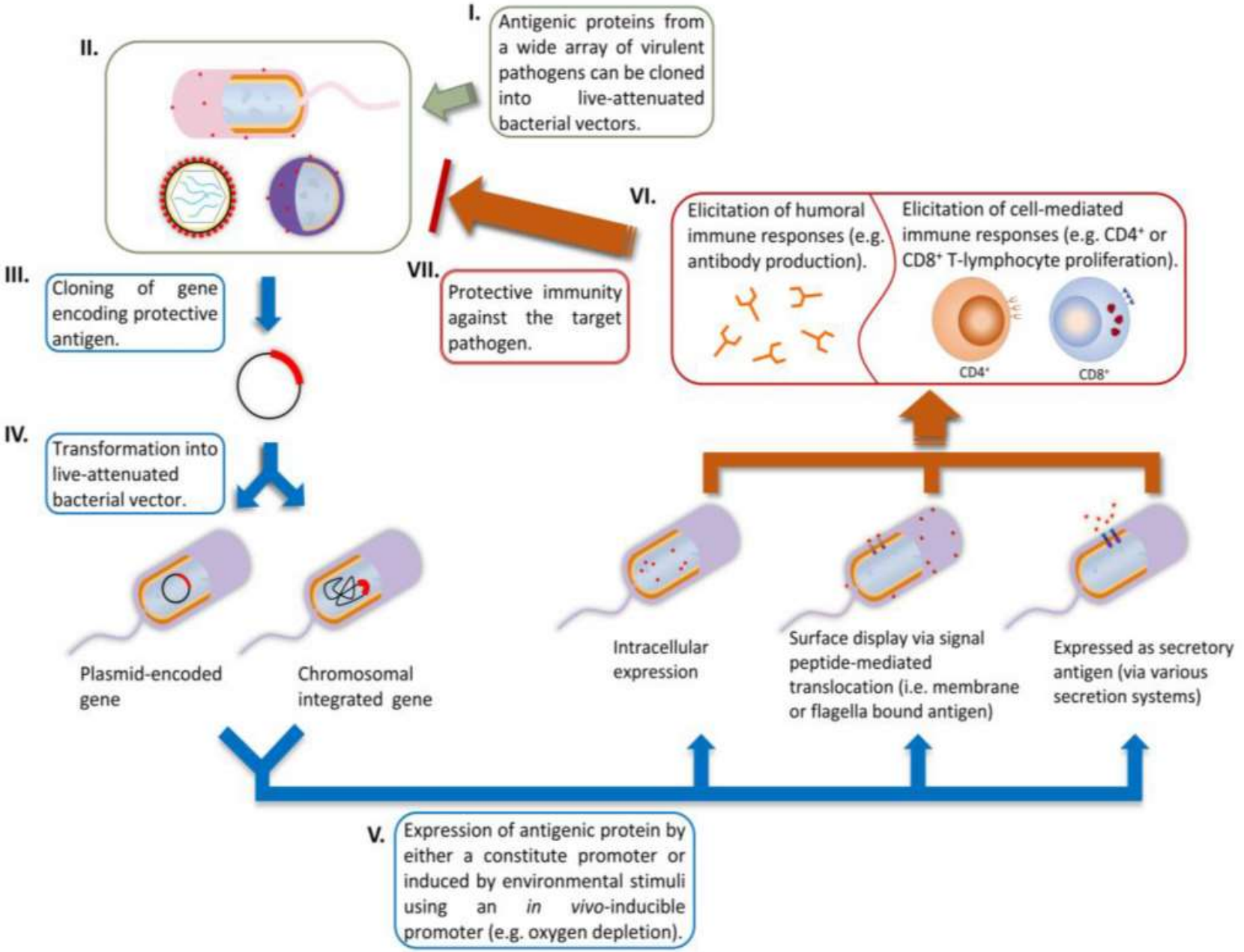
Interferon resistance of vaccinia virus is mediated by specific inhibition of phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2 alpha) by the double-stranded-RNA-activated (DAI) protein kinase. Vaccinia virus encodes a homolog of eIF-2 alpha, K3L, the deletion of which renders the virus sensitive to interferon treatment

Delivery of Antigens by Bacteria:

It is known that the antigens located on the surface of a bacterial cell are more immunogenic than the antigens in the cytoplasm. Based on this observation, scientists have developed strategies to coat the surfaces of non-pathogenic organisms with antigens of pathogenic bacteria.

Flagellin is a protein present in the fragella (thread like filaments) of Salmonella. A synthetic oligonucleotide encoding the epitope of cholera toxin B subunit was inserted into Salmonella flagellin gene. This epitope was in fact found on the flagellum surface. These flagella-engineered bacteria, when administered to mice, raised antibodies against the cholera toxin B subunit peptide. It may be possible in future to incorporate multiple epitopes (2 or 3) into the flagellin gene to create multivalent bacterial vaccines.

Lactic acid bacterium is one of the most promising **bacteria** used as a live **bacterial** vector. However, some other attenuated pathogenic **bacteria**, such as Salmonella spp. and Shigella spp., have been used as DNA **vaccine** carriers


I.
Antigenic proteins from a wide array of virulent pathogens can be cloned into live-attenuated bacterial vectors.
II.
III.
Cloning of gene encoding protective antigen.
IV.
Transformation into live-attenuated bacterial vector.
V.
Expression of antigenic protein by either a constitute promoter or induced by environmental stimuli using an *in vivo*-inducible promoter (e.g. oxygen depletion).
VI.
Elicitation of humoral immune responses (e.g. antibody production).
Elicitation of cell-mediated immune responses (e.g. CD4⁺ or CD8⁺ T-lymphocyte proliferation).
CD4⁺
CD8⁺
VII.
Protective immunity against the target pathogen.
Plasmid-encoded gene
Chromosomal integrated gene
Intracellular expression
Surface display via signal peptide-mediated translocation (i.e. membrane or flagella bound antigen)
Expressed as secretory antigen (via various secretion systems)

Probiotics & Vaccines

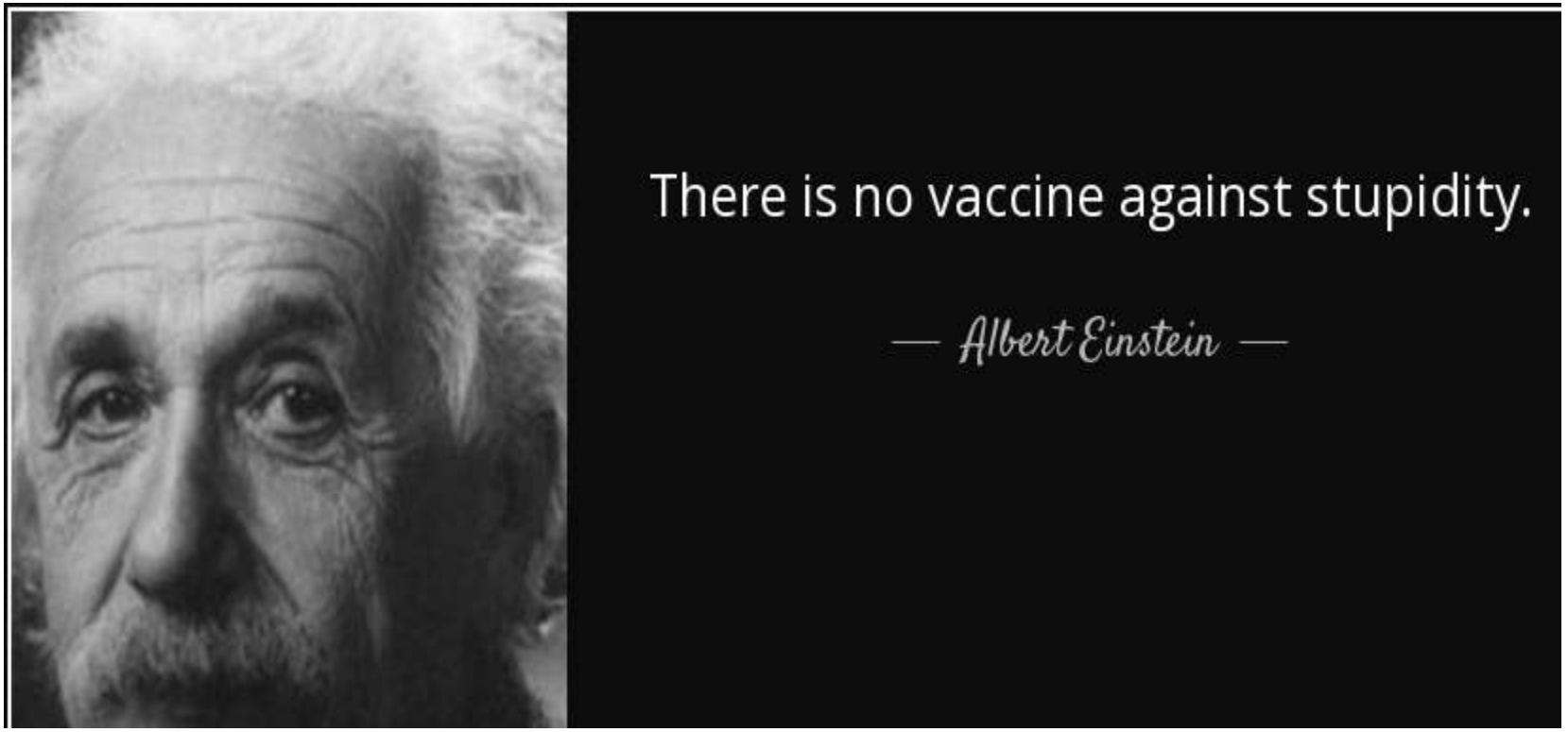
The oral or intranasal administration of **Lactobacillus**-based **vaccines** is a promising method to control mucosal infection because these **vaccines** could induce strong humoral and cellular immune responses both in the blood and at mucosal sites.

- Lactobacillus genus, are **able to colonise cavities** such as the mouth, the urogenital or the gastrointestinal tracts, where they play a critical role in maintaining a balanced normal micro flora
- Preventing or lowering the incidence or recurrent urinary or digestive tract infections
- LAB are **quite acid resistant** and certain strains are able to effectively **survive passage through the stomach**
- Absence of **LPS in their cell wall** virtually eliminates the risk of endotoxic shock

Vaccines against COVID-19

In April, CEPI (Coalition for Epidemic Preparedness Innovations) scientists reported that 10 different technology platforms were under research and development during early 2020 to create an effective vaccine against COVID-19. Major platform targets advanced into Phase I safety studies include:
nucleic acid (DNA and RNA) (Phase I developer and vaccine candidate: Moderna, mRNA-1273)
viral vector (Phase I developer and vaccine candidate: CanSin Biologics, adenovirus type 5 vector)
virus-like particle involved in DNA replication (Phase I developer and vaccine candidate: Shenzhen Geno-Immune Medical Institute, LV-SMENP)

According to CEPI, the platforms based on DNA or messenger RNA offer considerable promise to alter COVID-19 antigen functions for strong immune responses, and can be rapidly assessed, refined for long-term stability, and prepared for large-scale production capacity.Other platforms being developed in 2020 focus on peptides, recombinant proteins, live attenuated viruses, and inactivated viruses.


There is no vaccine against stupidity.
— Albert Einstein —