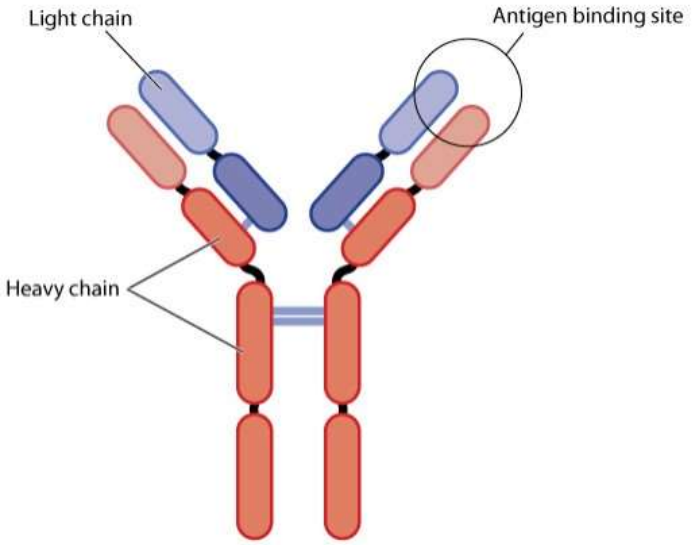
Cell Culture Techniques

Course 8

Monoclonal Antibodies & Hybridoma Technology

What is an Antibody?

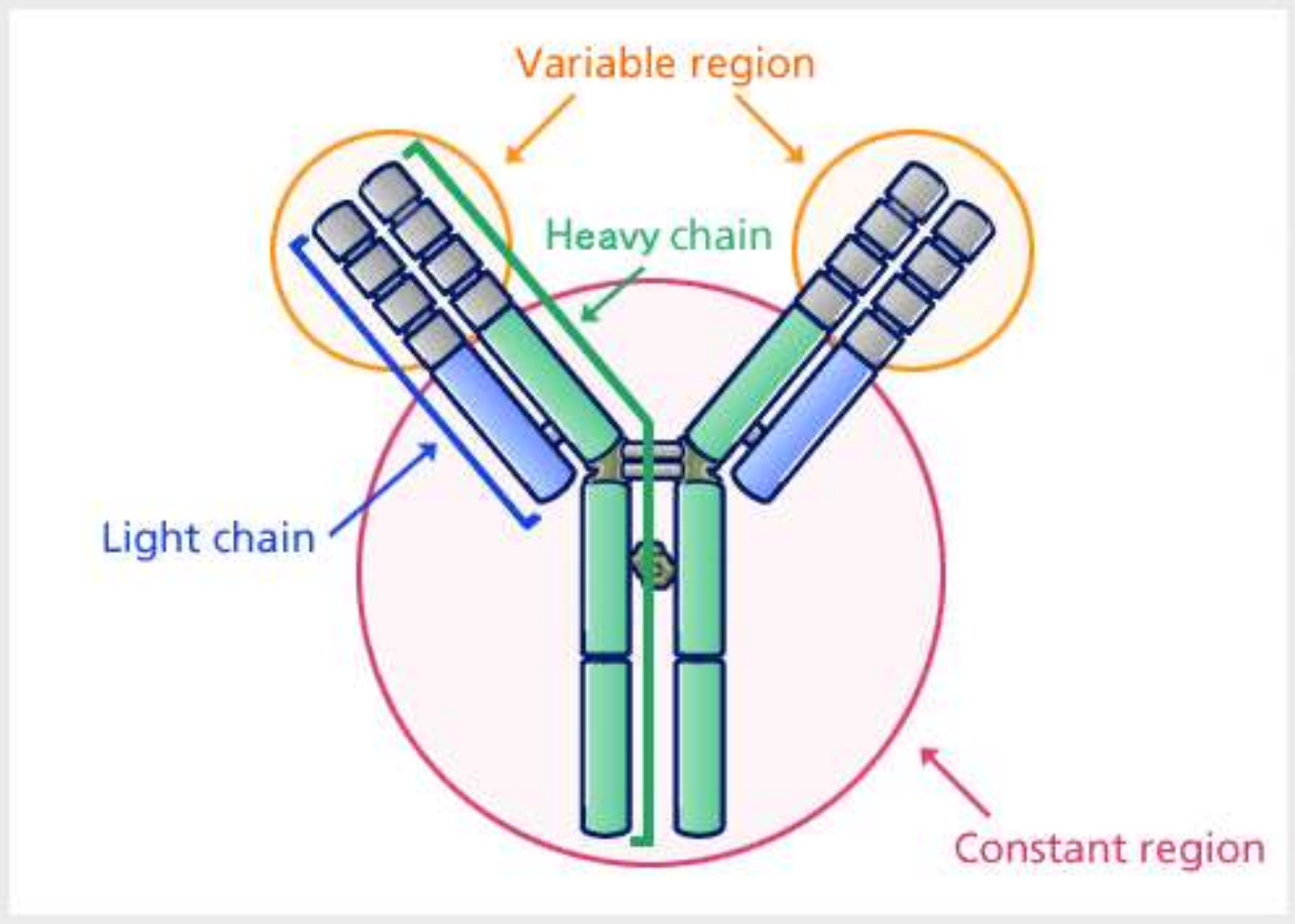
- Antibodies, also known as immunoglobulins, are secreted by B cells (plasma cells) to **neutralize antigens such as bacteria and viruses.**
- The classical representation of an antibody is a Y-shaped molecule composed of four polypeptides- **two heavy chains and two light chains.**



Binding of Antigens and Antibodies

- Each tip of the "Y" contains a paratope (a structure analogous to a lock) that is specific for one particular epitope (similarly analogous to a key) on an antigen, allowing these two structures to bind together with precision.
- **The ability of binding to an antigen has led to their ubiquitous use in a variety of life science and medical science.**

Structures of an Antibody

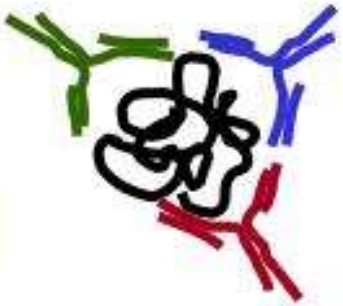


Antibodies

Polyclonal

Antibodies that are collected from sera of exposed animal

recognize
multiple antigenic sites
of injected biochemical.



Monoclonal

Individual B lymphocyte hybridoma is cloned and cultured. Secreted antibodies are collected from culture media

recognize
ONE antigenic site
of injected biochemical

Polyclonal Antibodies

- Polyclonal antibodies (pAbs) are mixture of heterogeneous which are usually produced by different B cell clones in the body.
- **They can recognize and bind to many different epitopes of a single antigen.**

Production of Polyclonal Antibodies

- Polyclonal antibodies are produced by injecting an immunogen into an animal. After being injected with a specific antigen to elicit a primary immune response, the animal is given a secondary even tertiary immunization to produce higher titers of antibodies against the particular antigen.
- After immunization, polyclonal antibodies can be obtained straight from the serum (blood which has had clotting proteins and red blood cells removed) or purified to obtain a solution which is free from other serum proteins.

Producing Polyclonal Antibodies

Polyclonal antibodies are often obtained by injecting a lab animal such as a rabbit or a goat with a specific antigen. Within a few weeks, the animal's immune system will produce high levels of antibodies specific for the antigen. These antibodies can be harvested in an antiserum, which is whole serum collected from an animal following exposure to an antigen. Because most antigens are complex structures with multiple epitopes, they result in the production of multiple antibodies in the lab animal. This so-called polyclonal antibody response is also typical of the response to infection by the human immune system. Antiserum drawn from an animal will thus contain antibodies from multiple clones of B cells, with each B cell responding to a specific epitope on the antigen.

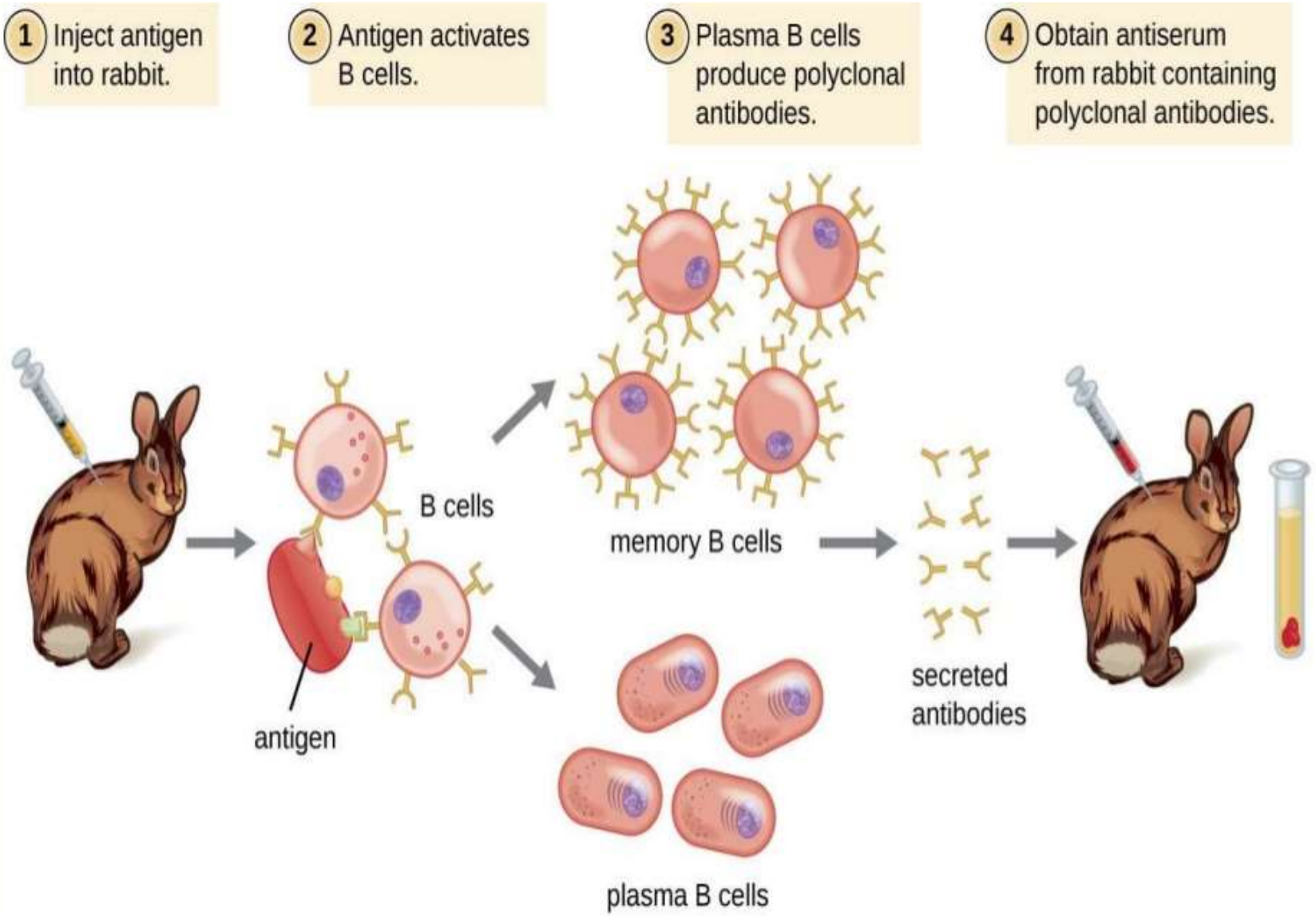


Figure: The process for harvesting polyclonal antibodies produced in response to an antigen.

Monoclonal Antibodies

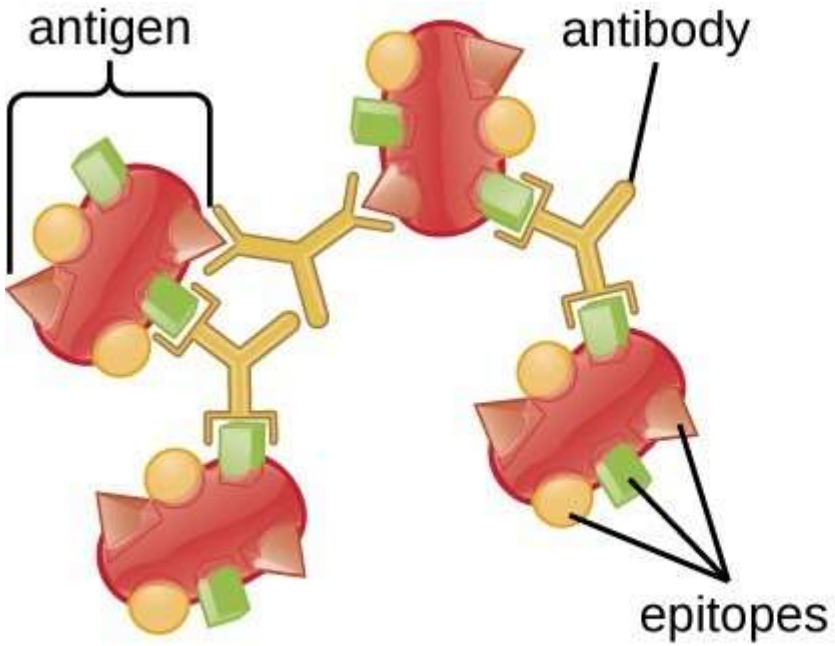
- Monoclonal antibodies (mAbs) are generated by identical B cells which are clones from a single parent cell.
- This means that the monoclonal antibodies have monovalent affinity and only recognize the same epitope of an antigen.

Characters of Monoclonal Antibodies

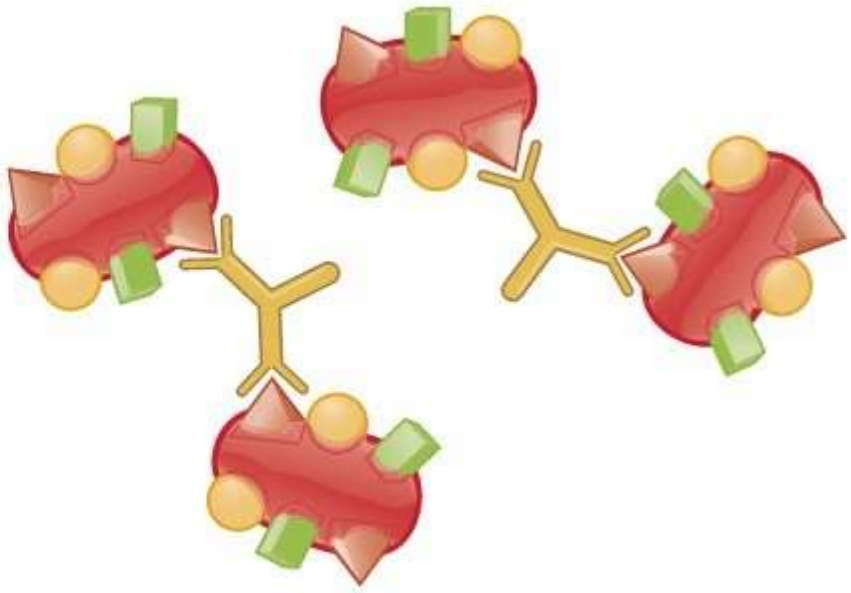
- **Monoclonal antibodies (mAb)** are a single type of antibody that are identical and are directed against a specific epitope (antigen, antigenic determinant) and are produced by B-cell clones of a single parent or a single hybridoma cell line.
- A hybridoma cell line is formed by the fusion of a one B-cell lymphocyte with a myeloma cell.
- Some myeloma cells synthesize single mAb antibodies naturally

- Unlike polyclonal antibodies, which are produced in live animals, monoclonal antibodies are produced *ex vivo* using tissue-culture techniques.
- The process begins with an injection of the desired antigen into an animal, often a mouse, multiple times. Once the animal develops an immune response, the B-lymphocytes are isolated from the animal's spleen and fused with a myeloma cell line, creating immortalized B cell-myeloma hybridomas.
- The hybridomas, which are able to grow continuously in culture while producing antibodies, are then screened for desired mAb.

Polyclonal vs Monoclonal



polyclonal antiserum



monoclonal antibodies

Monoclonal Antibodies

Advantages:

- **Highly specific recognition of only one epitope of an antigen**
- Immortal hybridoma cell lines have the ability to produce unlimited quantities of antibodies
- High consistency among experiments
- Minimal cross-reactivity
- Excellent for affinity purification

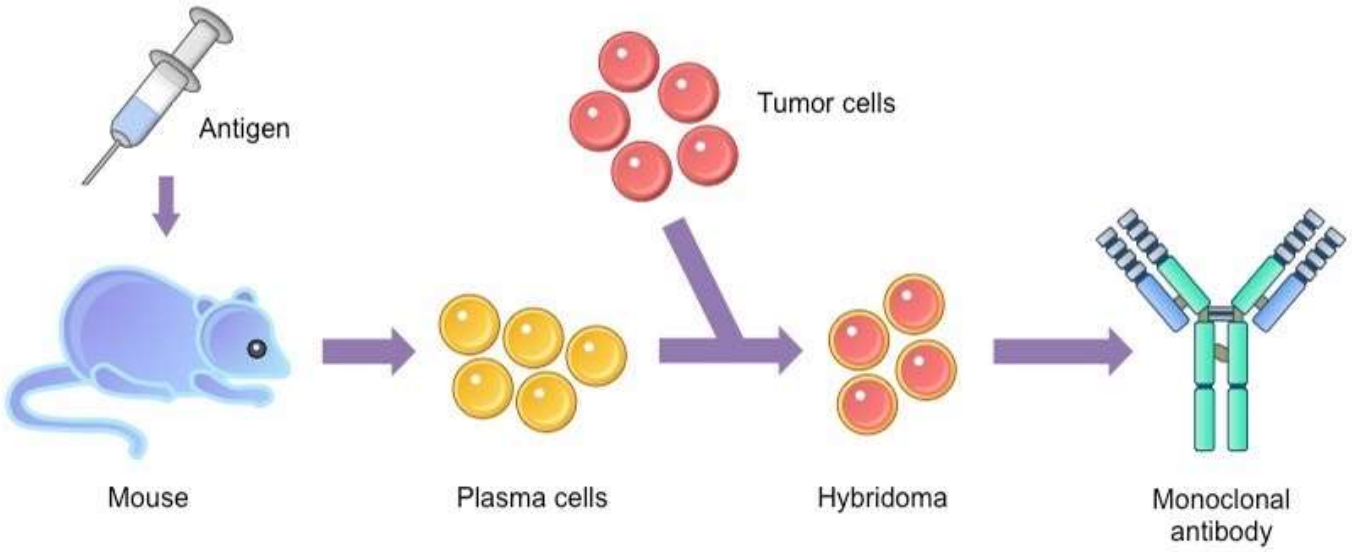
Disadvantages:

- Developing a monoclonal **takes time and requires high technical skills.**
- They can produce large amounts of specific antibodies but **may be too specific to detect in across a range of species.**
- **Vulnerable to the change of epitope.** Even a slight change in conformation may lead to dramatically reduced binding capacity.

	Polyclonal antibodies	Monoclonal Antibodies
Produced by:	Many B cell clones	A single B cell clone
Bind to:	Multiple epitopes of all antigens used in the immunization	A single epitope of a single antigen
Antibody class:	A mixture of different Ab classes (isotypes)	All of a single Ab class
Ag-binding sites:	A mixture of Abs with different antigen-binding sites	All Abs have the same antigen binding site
Potential for cross-reactivity:	High	Low

Hybridoma

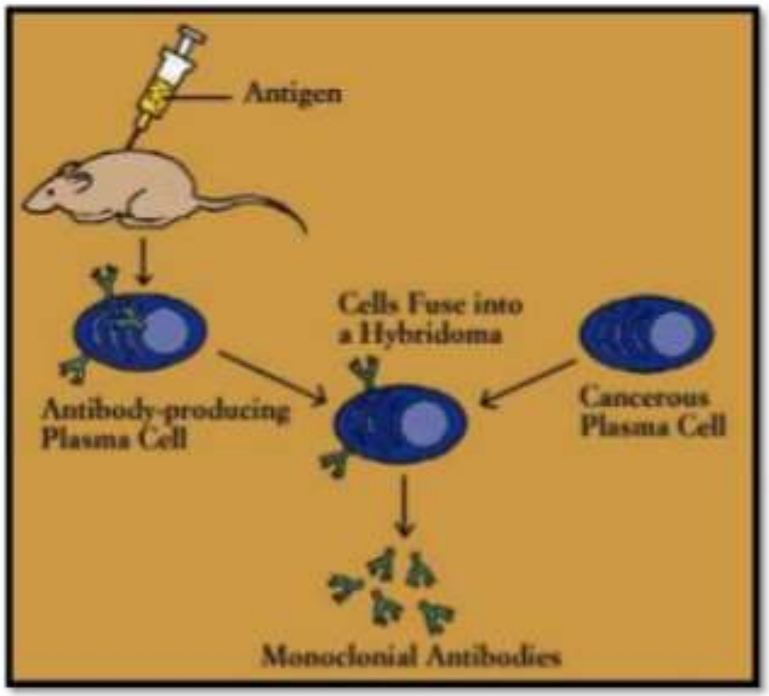
- A hybridoma is a hybrid cell obtained by fusion of B lymphocyte with usually a tumor cell of antibody forming system or B lymphocyte (these are called myelomas)



PRINCIPLE

- The hybrid cell has the capacity of **antibody production derived from B cells**
- At the same time it can divide continuously by the quality derived from Myeloma cells
- By combining the desired qualities of both the cells, **the technology ensures large scale Antibody production of single specificity**

- Specific hybridomas are either cultured in vitro or passed through mouse peritoneal cavity to obtain monoclonal antibodies, this is called as hybridoma technology



STEPWISE PROCEDURE

Isolation of B cells

-Mice,2-4 weeks old, are immunized with the antigen against which monoclonal antibodies are to be raised by subcutaneous injection

-Later B cells are isolated from the spleen of an immunized mouse

Isolation of myeloma cells

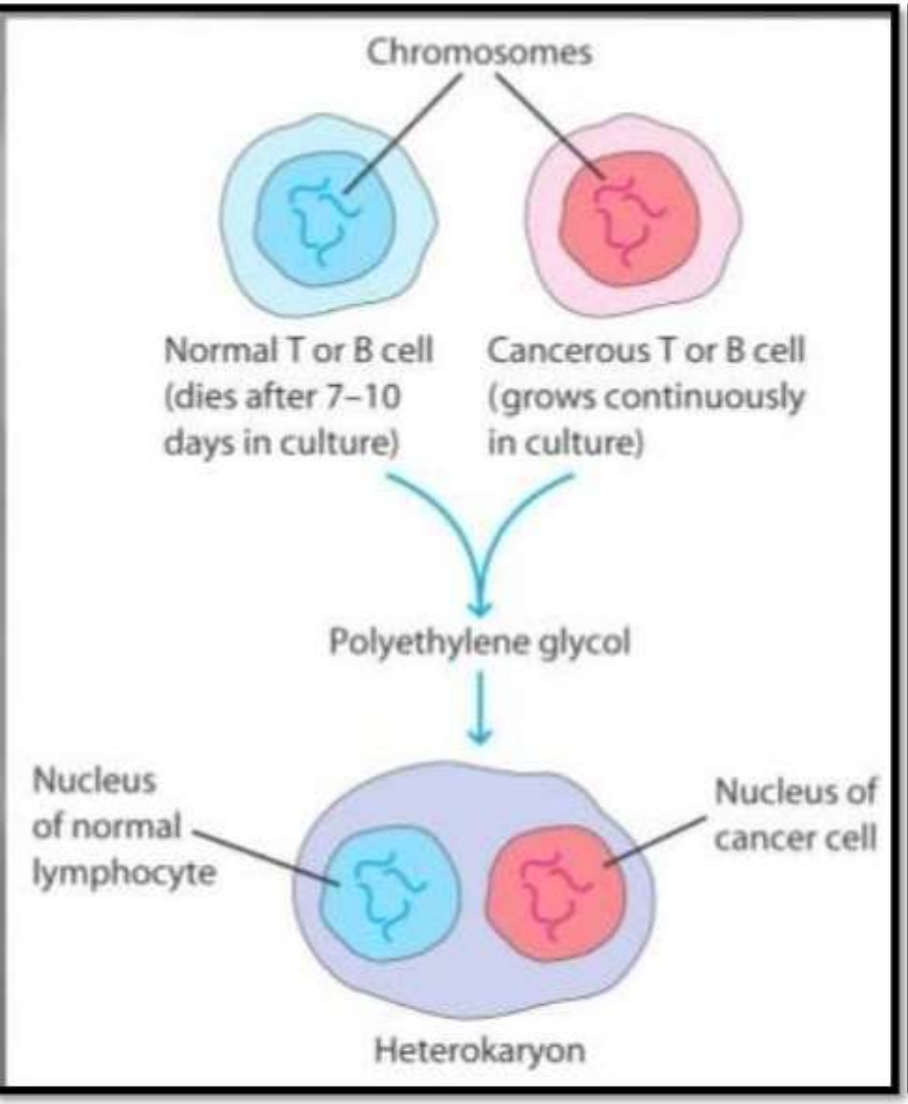
- Myeloma cells are isolated from bone marrow
- The myeloma cells used are **HGPRT(Hypoxanthine-guanine phosphoribosyl transferase) mutant cells** (raised by mutations using 8- azaguanine)

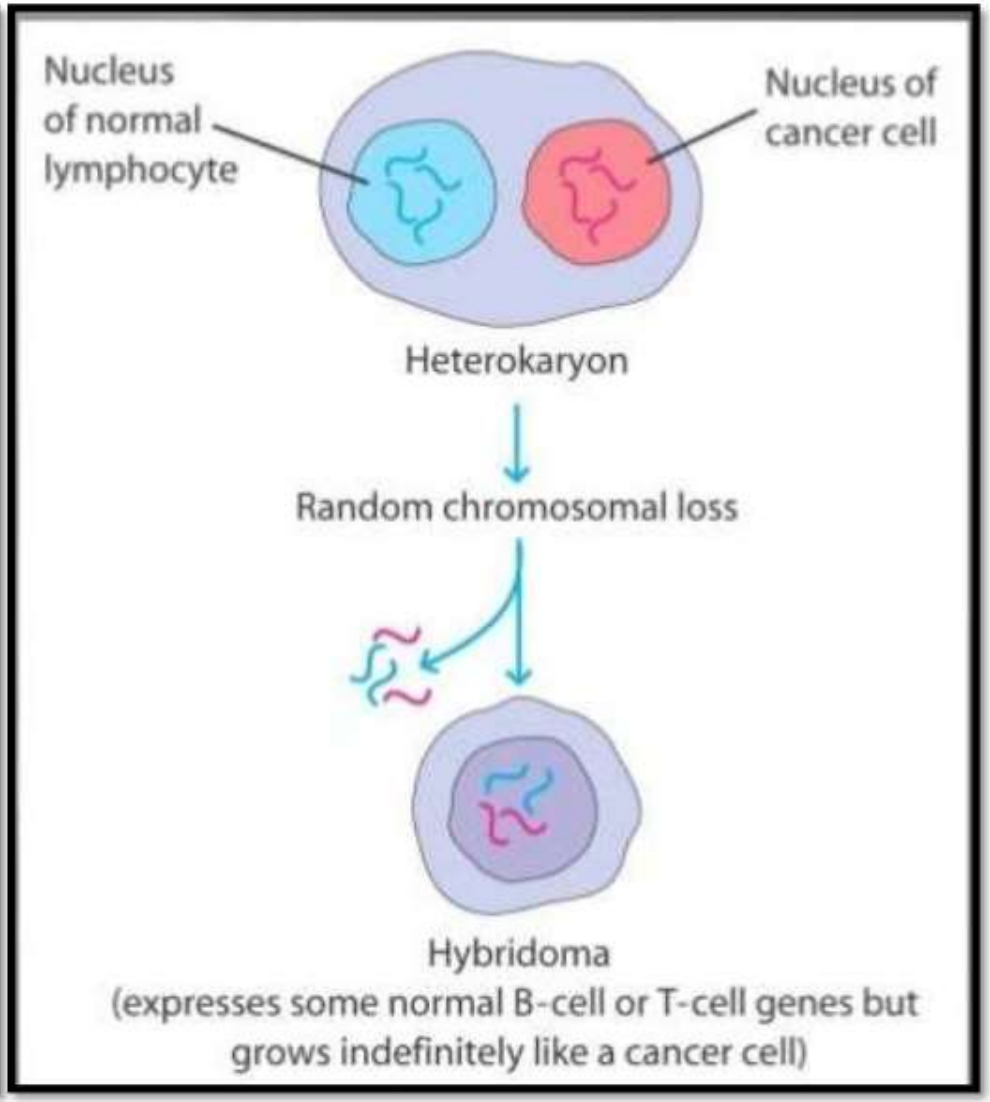
- Somatic cell fusion

  -Electrofusion : cells are allowed to fuse with the application of an electric field

  -Done by using PEG medium

  -PEG stands for Poly Ethylene Glycol


Chromosomes
Normal T or B cell (dies after 7–10 days in culture)
Cancerous T or B cell (grows continuously in culture)
Polyethylene glycol
Nucleus of normal lymphocyte
Nucleus of cancer cell
Heterokaryon


Nucleus of normal lymphocyte
Nucleus of cancer cell
Heterokaryon
Random chromosomal loss
Hybridoma
(expresses some normal B-cell or T-cell genes but grows indefinitely like a cancer cell)

- Selection of hybrid cells

  -HAT medium is used for the selection of hybrid cells

  -HAT stands for Hypoxanthine Aminopterine Thymidine

- Selection of hybrid cells

  -HAT medium is used for the selection of hybrid cells

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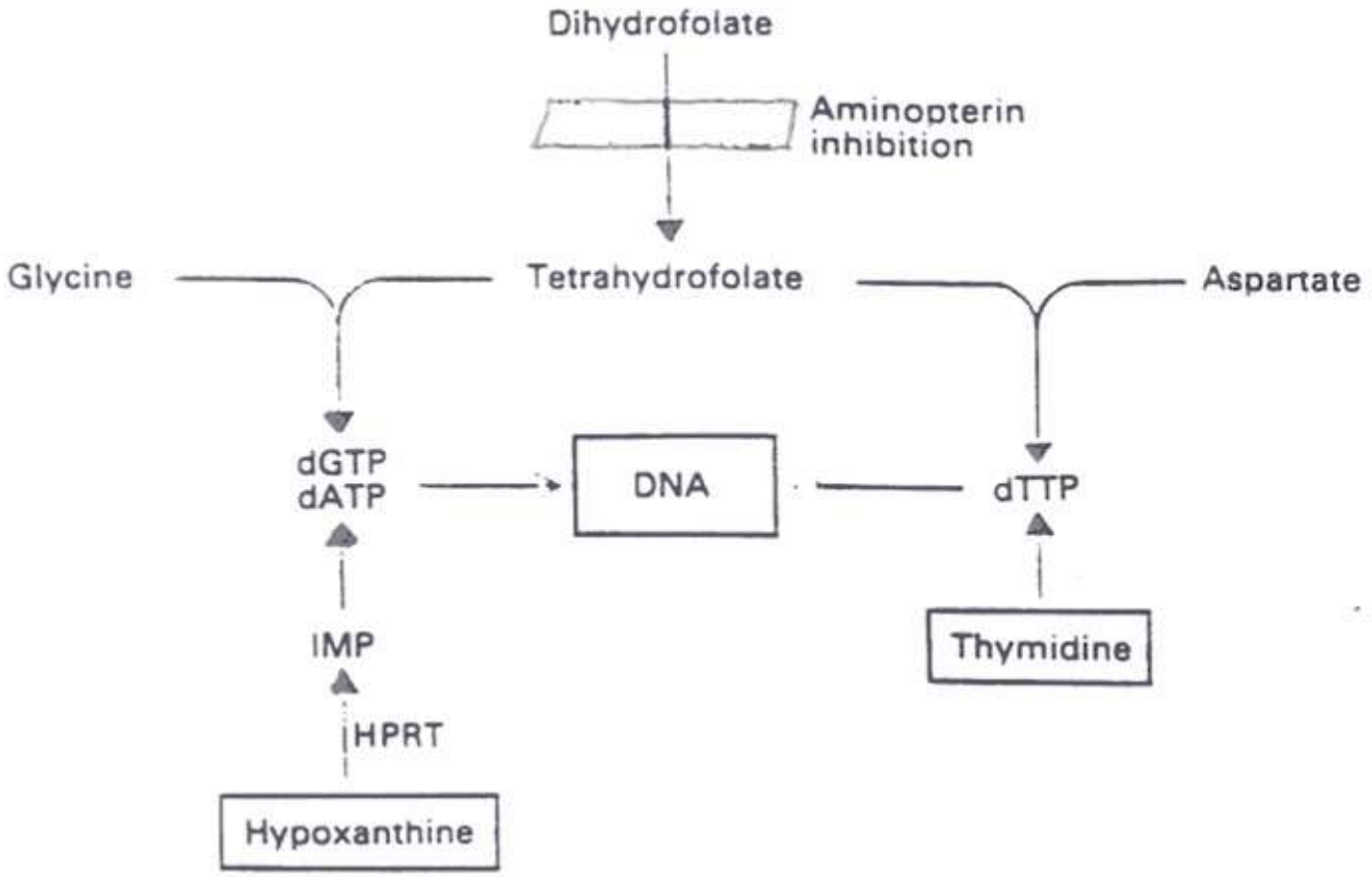
- Nucleotide synthesis is essential for cell survival
- In HAT medium, aminopterine blocks the cellular synthesis of purines and pyramidines from simple sugars (denovo pathway)
- But cells can thrive by using hypoxanthine and thymidine present in the medium by salvage pathway using the enzyme HGPRT


Dihydrofolate
Aminopterin
inhibition
Glycine
Tetrahydrofolate
Aspartate
dGTP
dATP
DNA
dTTP
IMP
Thymidine
HPRT
Hypoxanthine

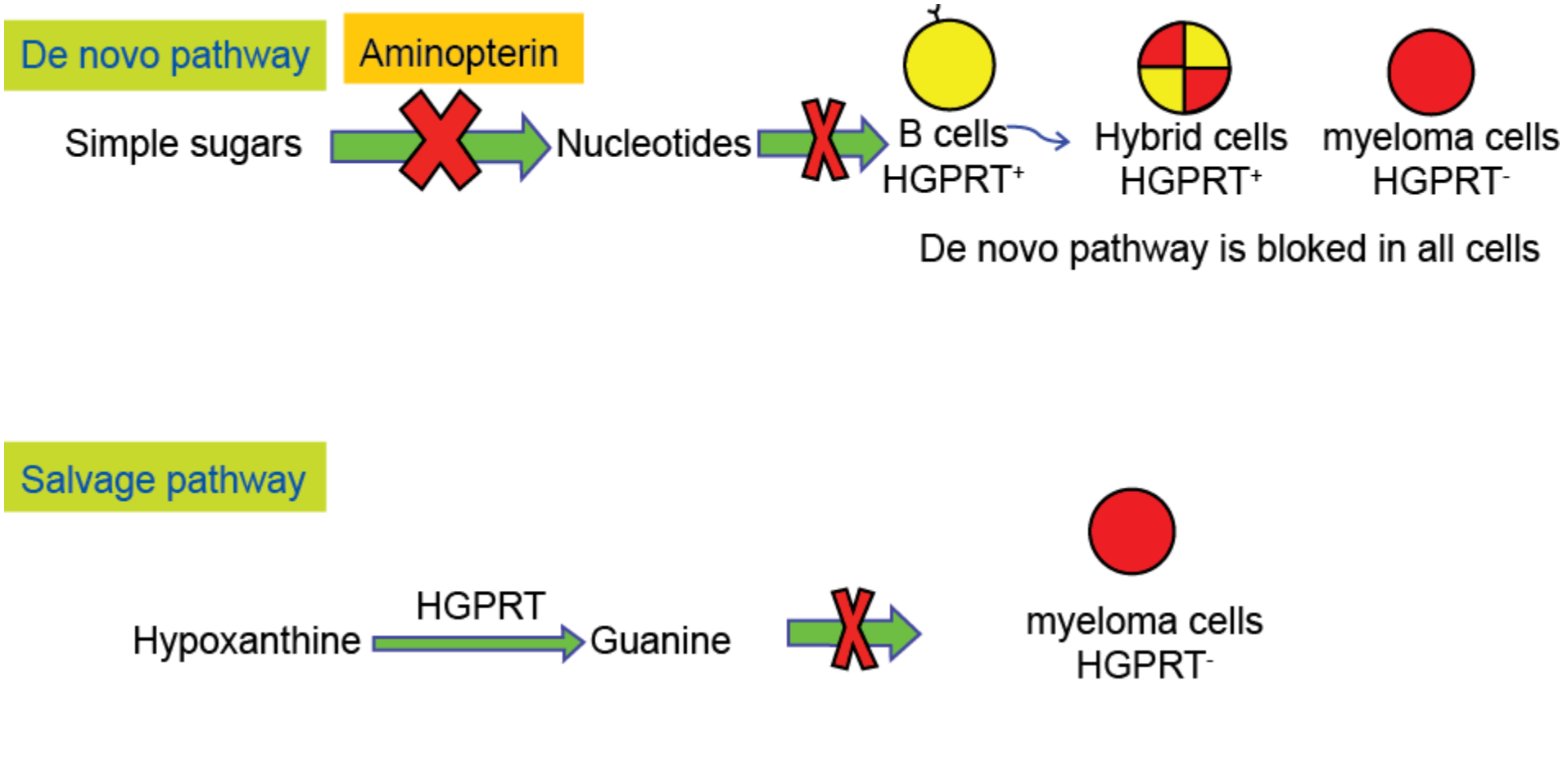
- How HAT medium works in the selection of hybrid cells

-B cells are HGPRT+ and can survive in the HAT medium, but they undergo normal cell death after some division

- In hybridoma technology, the myeloma cells used are HGPRT deficient

-So these cells can't survive in HAT medium as Aminopterine blocks the Denovo pathway

- Hybrid cells has HGPRT enzyme from the B cell as well as they have the ability to multiply repeatedly as myeloma cells
- So only hybrid cells can survive in HAT medium


De novo pathway
Aminopterin
Simple sugars
Nucleotides
B cells
HGPRT+
Hybrid cells
HGPRT+
myeloma cells
HGPRT-
De novo pathway is bloked in all cells
Salvage pathway
HGPRT
Hypoxanthine
Guanine
myeloma cells
HGPRT-

Myeloma Cell

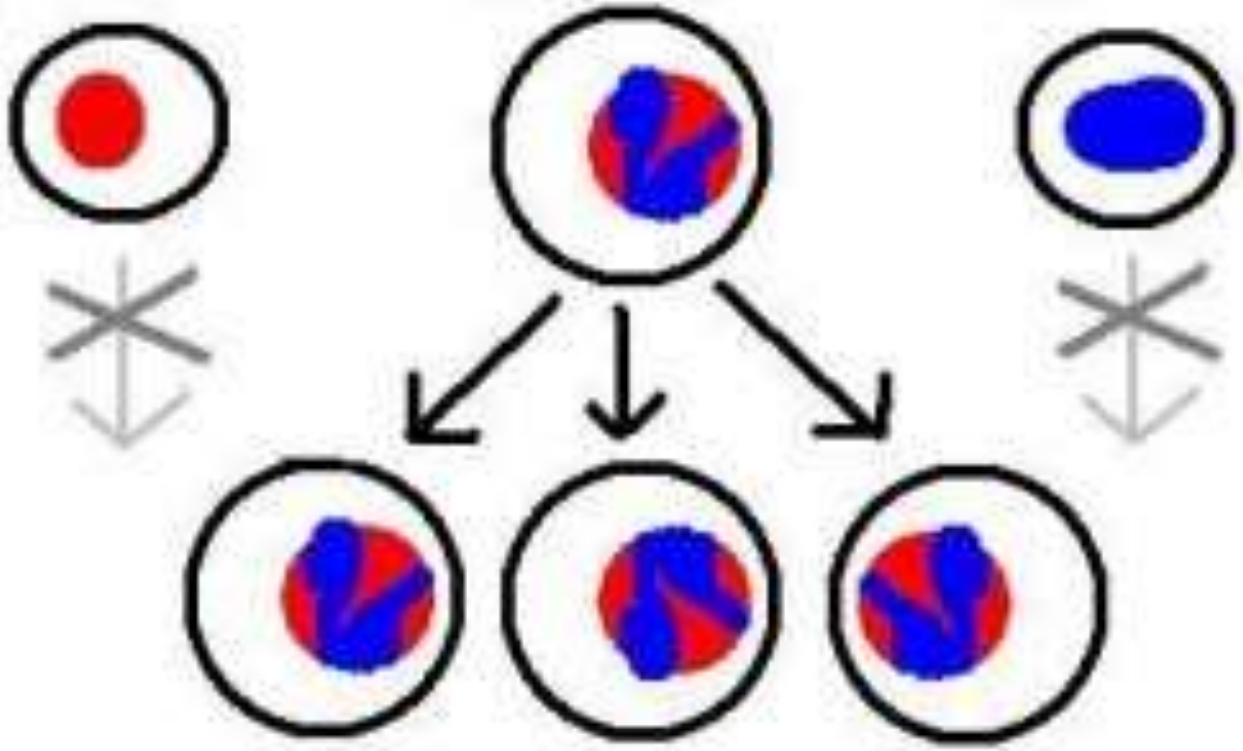
- Can grow in normal cell culture
- Lack salvage pathway enzymes
- DIE

Hybridoma

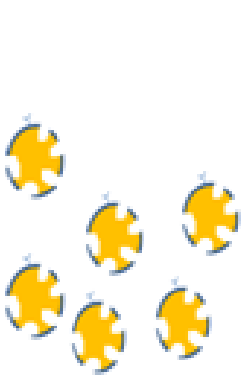
- Can grow in normal cell culture
- Possess salvage pathway enzymes
- PROLIFERATE

B-Lymphocyte

- Cannot grow in cell culture
- Possess salvage pathway enzymes
- DIE

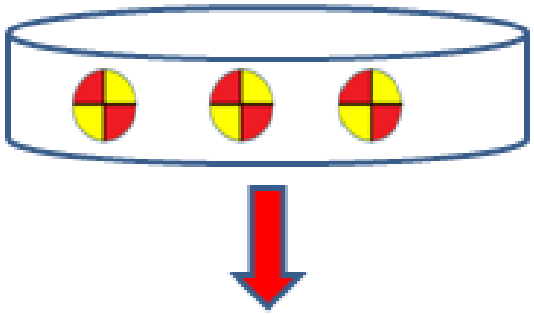


How hybrid cells are selected in HAT medium?

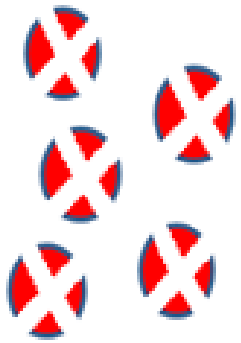


Unfused B cells undergo normal cell death

HAT medium



Large scale culture of Hybrid cells. $HGPRT^{+}$ from B cell



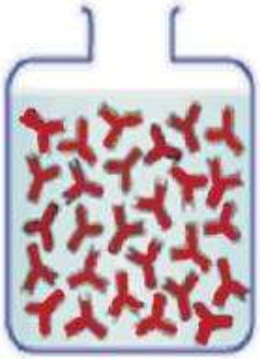
Myeloma cells cannot grow in HAT medium

myeloma cells are $HGPRT^{-}$ and de novo pathway is blocked by Aminopterin. So cannot survive in HAT medium

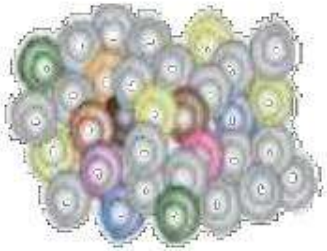
- HAT medium (Hypoxanthine Aminopetrin Thymidine) is used for preparation of monoclonal antibodies.
- Laboratory animals (eg. mice) are first exposed to an antigen to which we are interested in isolating an antibody against.
- Once splenocytes are isolated from the mammal, the B cells are fused with immortalized myeloma cells - which lack the HGPRT(hypoxanthine-guanine phosphoribosyl transferase) gene - using polyethylene glycol or the Sendai virus.
- Fused cells are incubated in the HAT (HypoxanthineAminopetrin Thymidine) medium.
- Aminopterin in the myeloma cells die, as they cannot produce nucleotides by the de novo or salvage medium blocks the pathway that allows for nucleotide synthesis. Hence, unfused D cell die.
- Unfused B cells die as they have a short life span.
- Only the cell-myeloma hybrids survive, since the HGPRT gene coming from the B cells is functional. These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells).

- The incubated medium is then diluted into multiwell plates to such an extent that each well contains only 1 cell.
- Then the supernatant in each well can be checked for desired antibody.
- Since the antibodies in a well are produced by the same B cell, they will be directed towards the same epitope, and are known as monoclonal antibodies
- Multi-well plates are used initially to grow the hybridomas and after selection, are changed to larger tissue culture flasks.
- This maintains the well being of the hybridomas and provides enough cells for cryopreservation and supernatant for subsequent investigations.

only one
B-lymphocyte clone



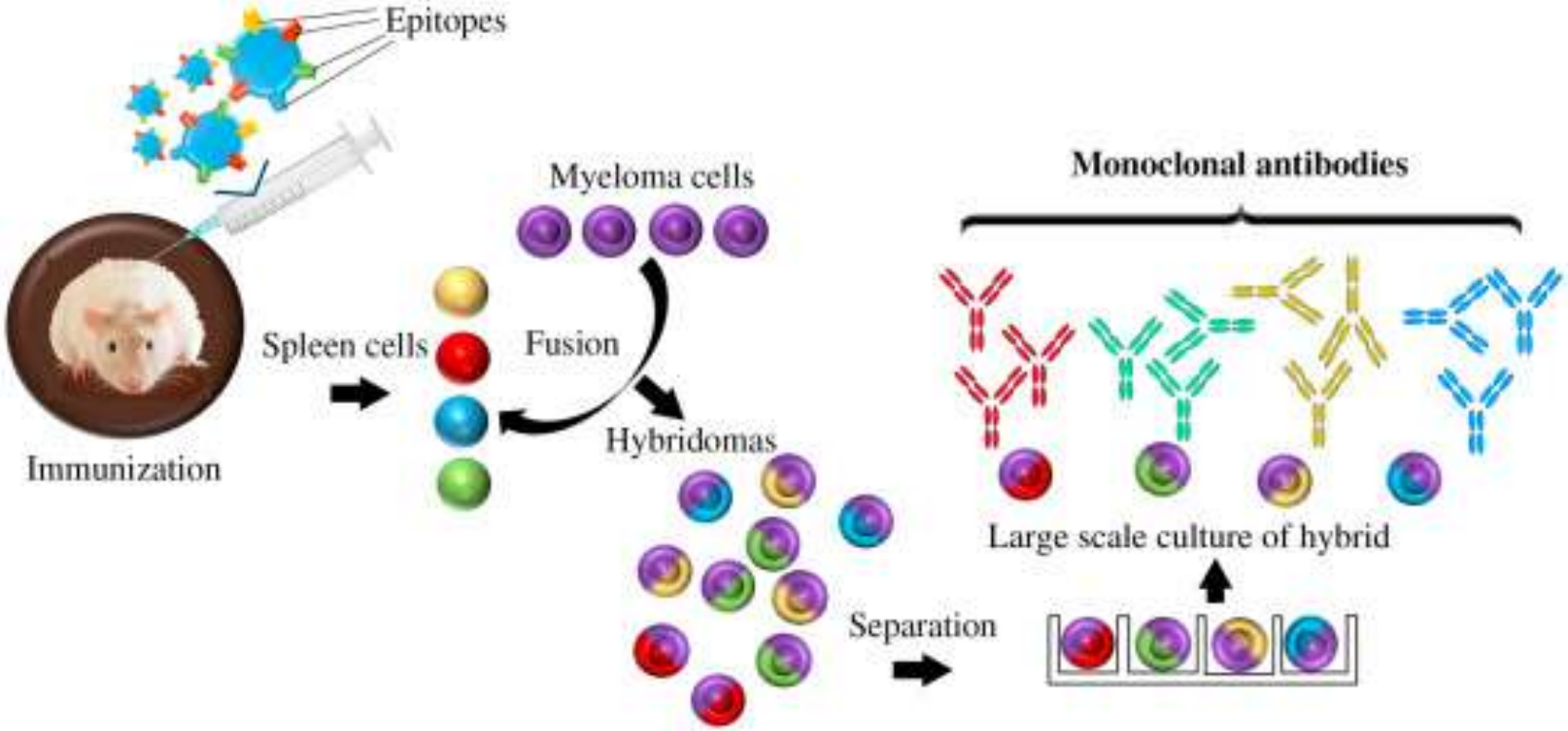
monoclonal antibody



more
B-lymphocyte clone

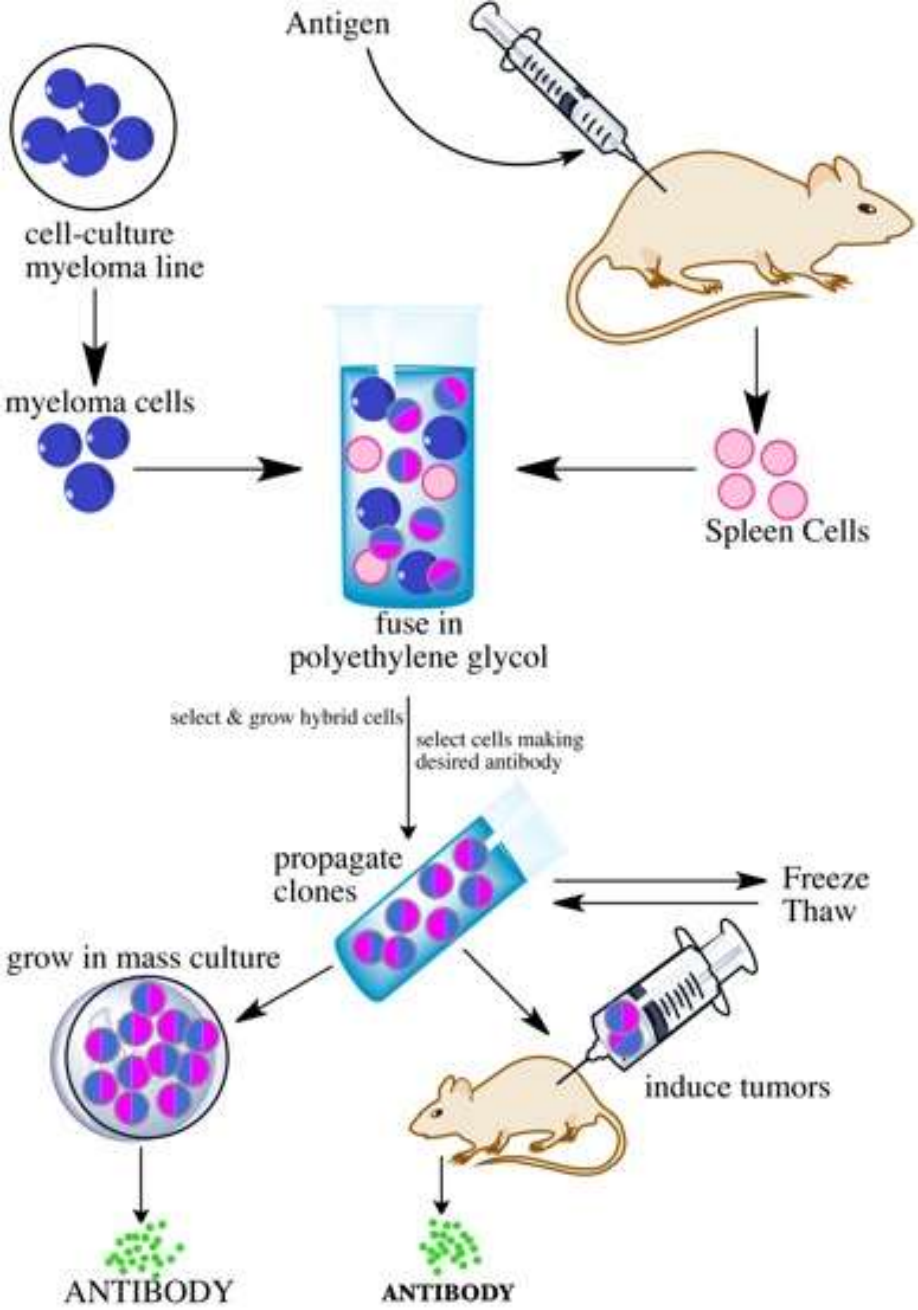
Polyclonal antibody

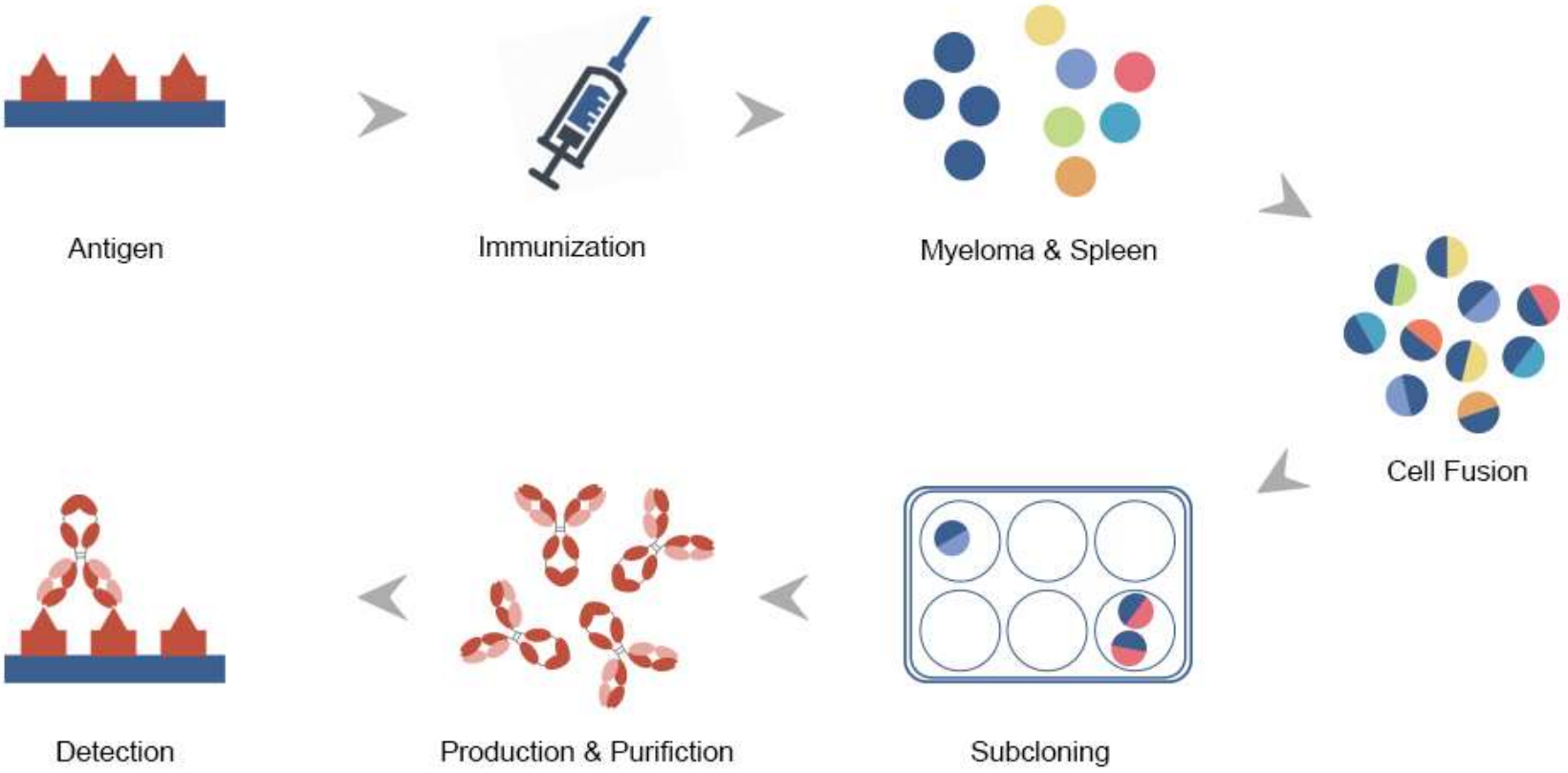
- Multiwell plates are used initially to grow the hybridomas
- After selection, they are changed to tissue culture flasks
- This provides enough cells for cryopreservation and supernatant for subsequent investigations
- The supernatant can yield 1 to 60micrograms per ml which can be maintained at lower temperatures for future use

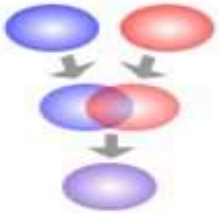
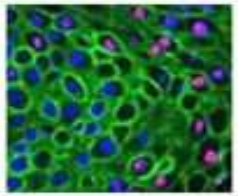
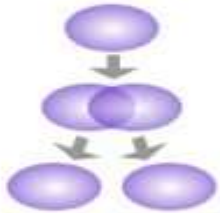

Epitopes
Immunization
Spleen cells
Myeloma cells
Fusion
Hybridomas
Separation
Large scale culture of hybrid
Monoclonal antibodies

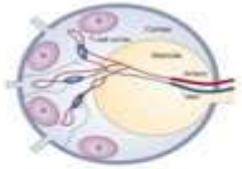
- Two methods have been used for multiplying the hybridoma cells
    1. In-vivo
    2. In-vitro

- Iv-vivo procedure involves introduction of hybridoma cells into the peritoneal cavity of the animal , then ascetic fluid is isolated and then antibodies are isolated from it
- In-vitro method involves culturing of hybridoma cells in suitable culture media and then antibodies are isolated and purified


Antigen
cell-culture
myeloma line
myeloma cells
Spleen Cells
fuse in
polyethylene glycol
select & grow hybrid cells
select cells making
desired antibody
propagate
clones
Freeze
Thaw
grow in mass culture
induce tumors
ANTIBODY
ANTIBODY


Antigen
Immunization
Myeloma & Spleen
Cell Fusion
Subcloning
Production & Purifiction
Detection

Phase I	Phase II	Phase III	Phase IV
			
IMMUNIZATION	CELL FUSION	SCREENING	SUBCLONING



Lymph node

B cells Fusion helper

&

Fusion

ELISA

What is an ELISA?

- Enzyme-linked immunosorbent assay
- Name suggests three components
  - Antibody
    - Allows for specific detection of analyte of interest
  - Solid phase (sorbent)
    - Allows one to wash away all the material that is not specifically captured
  - Enzymatic amplification
    - Allows you to turn a little capture into a visible color change that can be quantified using an absorbance plate reader

What is ELISA?

- Technique used to detect (*assay*) specific molecules (e.g. proteins & carbohydrates) in samples.
- Immunological technique: uses antibodies.
- Quantitative.
- Very sensitive.
- Commonly used in medicine and scientific research.

Definition :-

The **enzyme-linked immunosorbent assay** (**ELISA**) is a biochemical technique that uses antibodies and color change to identify a substance.

ELISA) is one of the most sensitive and reproducible technologies available. These assays are rapid, simple to perform and easily automated.

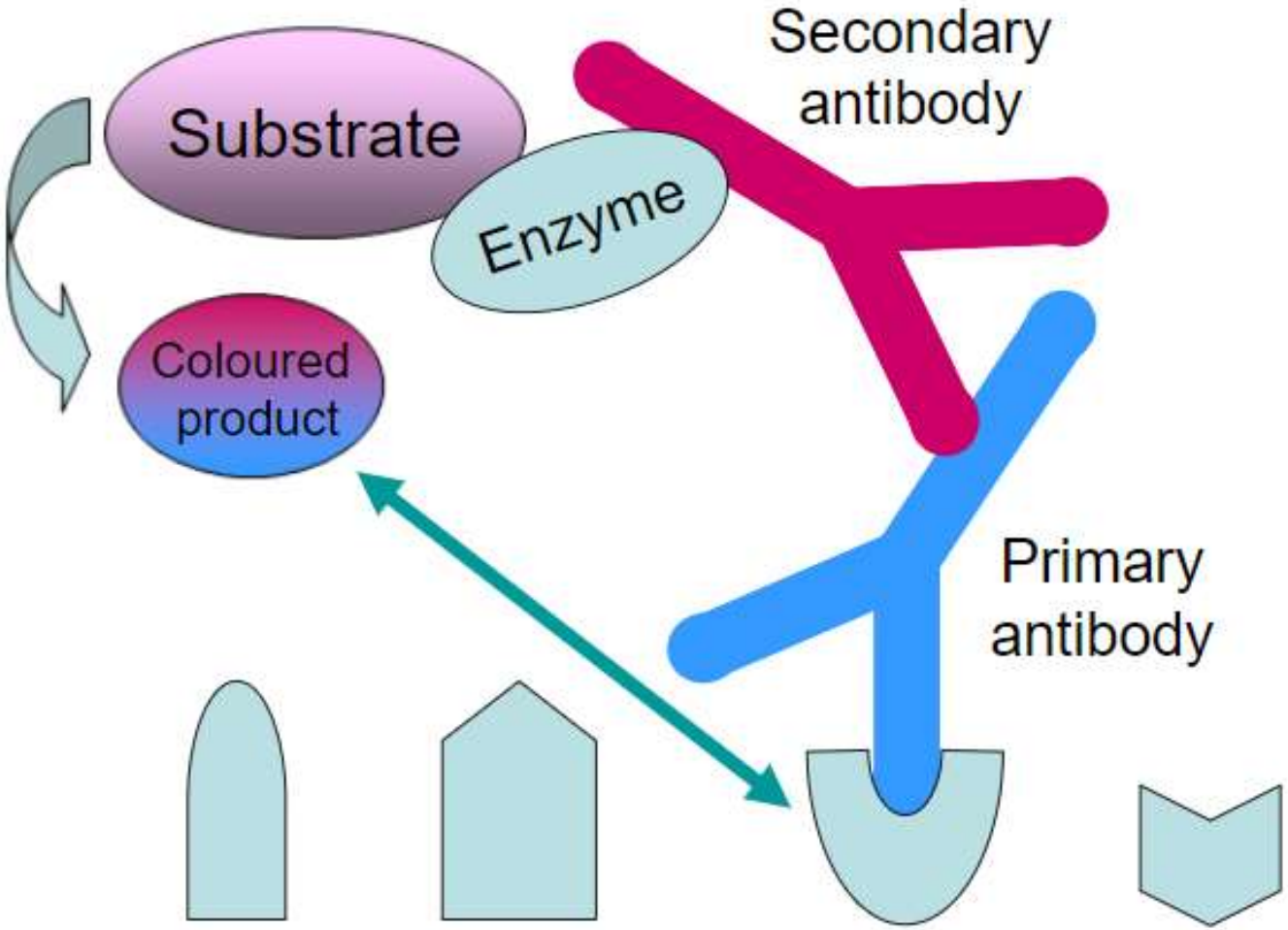
Principle :-

ELISA combine the specificity of antibodies with the sensitivity of simple enzyme assays. using antibodies or antigens coupled to an easily-assayed enzyme. ELISA can provide a useful measurement of antigen or antibody concentration

Basic steps of ELISA

Enzyme Linked Immunosorbent Assay

1. Antigen of interest is absorbed on to plastic surface ('*sorbent*').
2. Antigen is recognised by specific antibody ('*immuno*').
3. This antibody is recognised by second antibody ('*immuno*') which has enzyme attached ('*enzyme-linked*').
4. Substrate reacts with enzyme to produce product, usually coloured.


Secondary antibody
Substrate
Enzyme
Coloured product
Primary antibody
Different antigens in sample

The steps of "indirect" ELISA follows the mechanism below:-

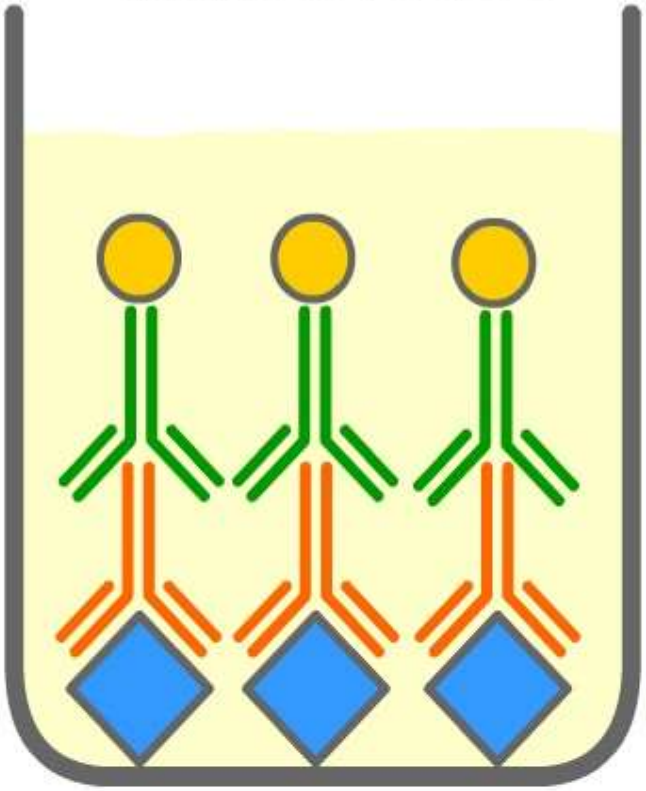
- Antigens are coated in the wells
- The primary antibody is added, which binds specifically to the test antigen coating the well.
- A secondary antibody is added, which will bind the primary antibody. This secondary antibody often has an enzyme attached to it, which has a negligible effect on the binding properties of the antibody.
- A substrate for this enzyme is then added
- The color change shows the secondary antibody has bound to primary antibody.
- The higher the concentration of the primary antibody present in the serum, the stronger the color change. Often, a spectrometer is used to give quantitative values for color strength.

Sandwich ELISA (Direct) :

Antigen-Capture (Direct) Format

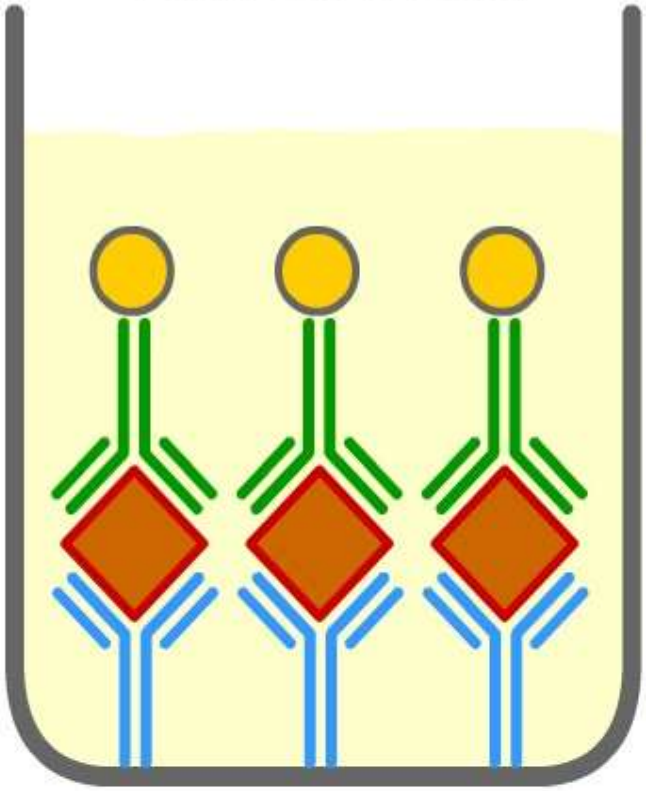
- In the antigen-capture format, the antigen in the sample is sandwiched between antibodies coated on the plate and an enzyme-labeled conjugate.
- The antibody conjugate can be either monoclonal or polyclonal.
- The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is directly proportional to the amount of the target antigen present in the sample
- Plate is coated with a capture antibody. Sample is added, and any antigen present binds to capture antibody.
- Detecting antibody is added, and binds to antigen.
- Enzyme-linked secondary antibody is added, and binds to detecting antibody.
- Substrate is added, and is converted by enzyme to detectable form.

Indirect ELISA

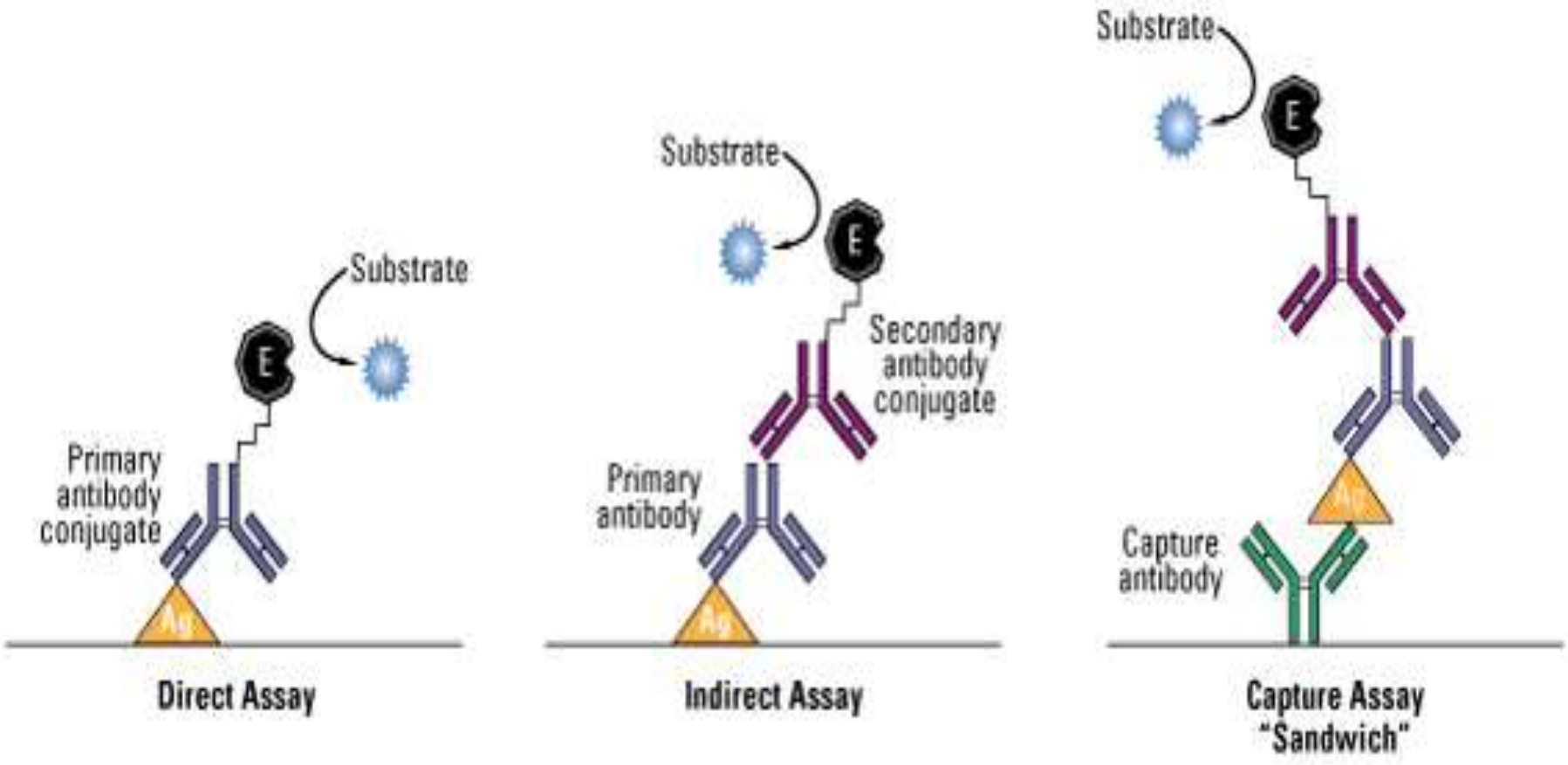


These reporter antibodies bind to the bound autoantibodies from the sample, forming a complex consisting of immobilised antigen, autoantibody and labelled reporter antibody. The wells are again rinsed to remove any unbound antibodies.

Direct ELISA



The secondary antibody attaches to the bound protein from the sample, forming a complex consisting of immobilised antibody, protein and labelled secondary antibody.

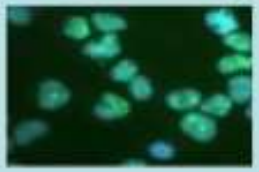

Substrate
E
Primary antibody conjugate
Ag
Direct Assay
Substrate
E
Secondary antibody conjugate
Primary antibody
Ag
Indirect Assay
Substrate
E
Capture antibody
Ag
Capture Assay "Sandwich"

Monoclonal antibody production

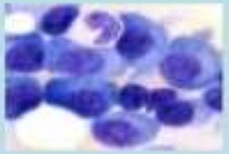
(hybridoma technology)

Inject mouse with **antigen**

Obtain Mouse spleen **B-lymphocytes**



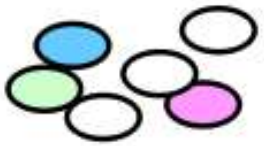
Grow mouse **myeloma** (tumour) cells in culture



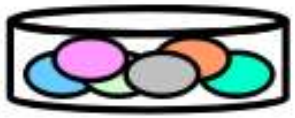
Fuse B-lymphocytes with myeloma cells

Antibody-producing **hybridoma** cells

B-lymphocyte and myeloma mixture

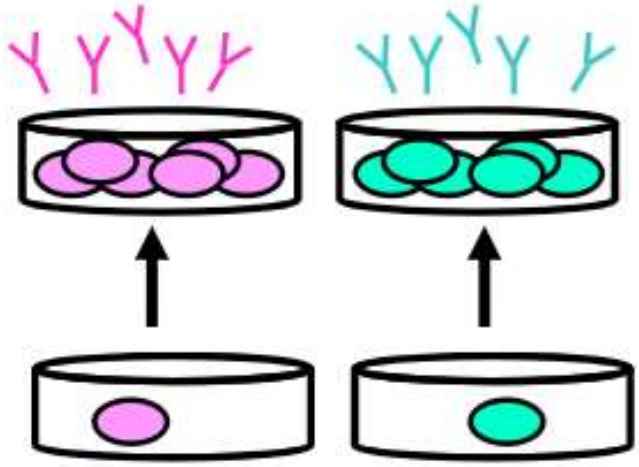


Select fused and reproducing hybridoma cells via growth medium

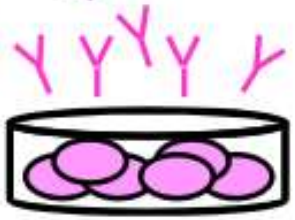


Screen hybridomas for antibody production

Make clones from **individual** antibody-producing cells



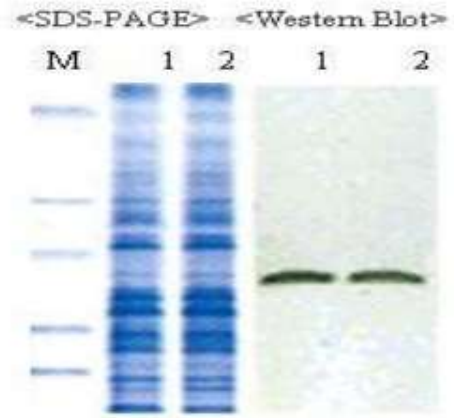
Keep clone producing antibody which best detects antigen



Unlimited supply of antibody specific for single epitope

Monoclonal – Diagnostic use

- A monoclonal antibody can be used to **detect pregnancy** only 14 days after conception. Other monoclonal antibodies allow rapid **diagnosis of hepatitis, influenza, herpes, streptococcal, and Chlamydia infections.**
- They can be used to detect for the presence and quantity of this substance, for instance in a Western blot test (to detect a substance in a solution) or an immunofluorescence test.
- Monoclonal antibodies can also be used to purify a substance with techniques called **immunoprecipitation** and **affinity chromatography.**



Monoclonal antibodies for cancer treatment

- Possible treatment for **cancer** involves monoclonal antibodies that bind only to cancer cells specific **antigen** and induce immunological response on the target cancer cell (naked antibodies). mAb can be modificated for delivery of [toxin], radioisotope, cytokine.

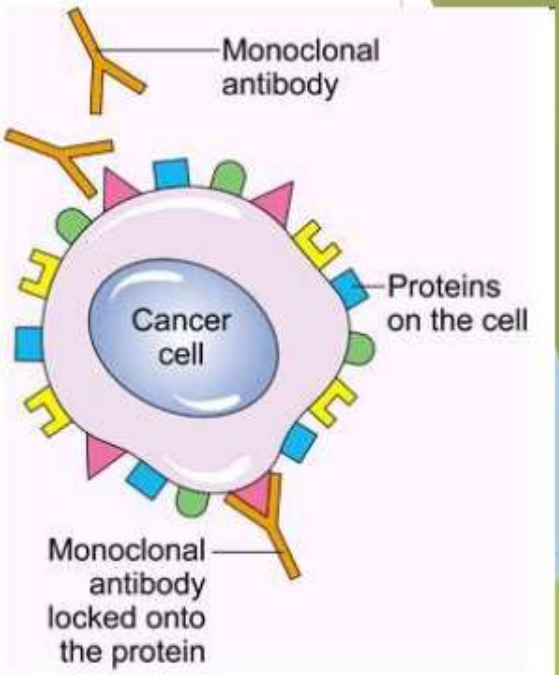


Diagram showing a monoclonal antibody attached to a cancer cell
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