Unit-5

IMMUNE TECHNIQUES: Immunocytochemistry, Antibody generation, detection of molecules using ELISA, RIA, western blot, immunoprecipitation, flowcytometry and immunofluorescence microscopy, Acquired Immuno Deficiency Syndrome (AIDS) test, hybridoma technology, radioimmuno assay.

Immunocytochemistry

Immunocytochemistry

- Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells (cultured cells, cell suspensions) by use of a specific antibody, which binds to it, thereby allowing visualization and examination under a microscope.

- It is a valuable tool for the determination of cellular contents from individual cells.

- Samples that can be analyzed include blood smears, aspirates, swabs, cultured cells, cell suspensions, and cytospin.

- Each sample is treated differently, yet all the methods are interchangeable. There is no one way to prepare these types of cell samples for immunocytochemical analysis.

Depositing/Fixing the cells

- The cells are grown on 100-mm or 150-mm diameter tissue culture dishes and that the cells are then seeded on 6-well tissue culture plates before the day of the experiment.
- Seed adherent cells on 6-well tissue culture plates in a sterile tissue culture hood.
- Sterilize glass coverslips by dipping them in 90% ethanol and carefully drying them over a flame for a few seconds.
- Place each coverslip in sterile 6-well tissue culture plates.
- Add 1-2ml of cell suspension over each coverslip in the 6-well plates.

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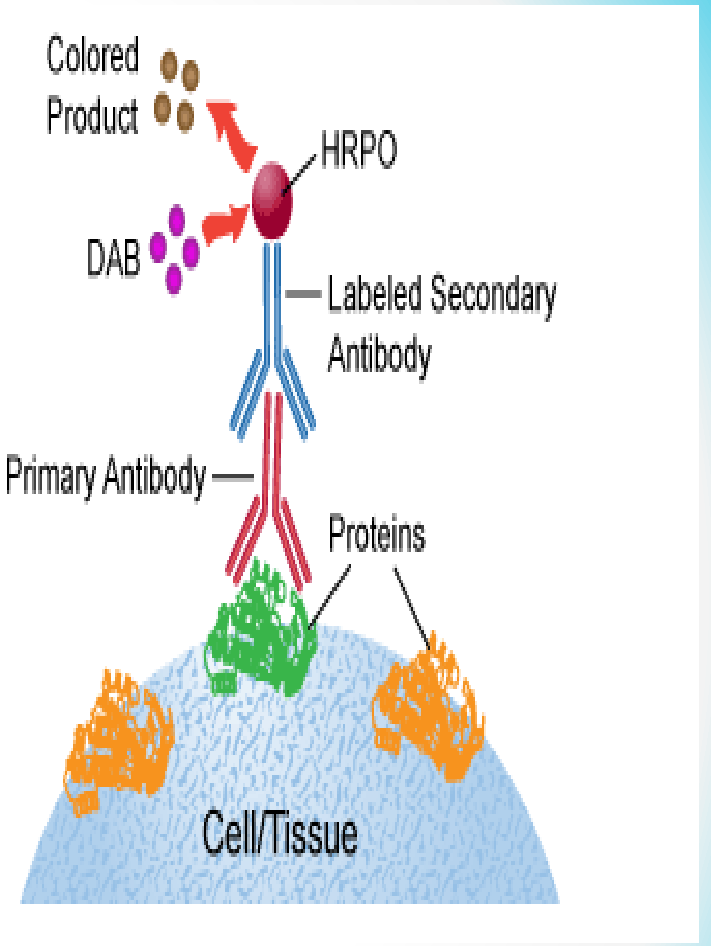
- Grow the cells at 37°C in a humidified CO2 incubator until they are 50-70% confluent.
- Aspirate the culture medium from each well and gently rinse the cells twice in PBS at room temperature. *Do not let the cells dry out.*
- Fix the cells by incubating them in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature.
- Rinse the cells three times with PBS. The cells can be stored in 0.02% (w/v) sodium azide in PBS at 4°C for several days.
- Proceed to Antigen Retrieval Protocol, if necessary



Permeabilize / Staining Procedure:

- Incubate the cells in 0.1% Triton X-100 in PBS for 15 minutes at room temperature.
- Rinse the cells 3 times in PBS.
- Incubate the cells in 10% goat serum in PBS for 1 hour at room temperature.
- Dilute the primary monoclonal antibody/antibodies to the appropriate concentration using block solution; the final volume should be sufficient to cover each coverslip (e.g. 0.5 ml-1 ml per coverslip).
- Incubate the cells in the primary antibody/antibodies at 4°C, overnight, or at room temperature for 2 hours.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA)

- ELISA is an antigen antibody reaction.
- In 1971, ELISA was introduced by Peter Perlmann and Eva Engvall at Stockholm University in Sweden.
- It is a common laboratory technique which is usually used to measure the concentration of antibodies or antigens in blood.
- ELISA is a plate based assay technique which is used for detecting and quantifying substances such as peptides, proteins, antibodies and hormones.
- An enzyme conjugated with an antibody reacts with colorless substrate to generate a colored product. Such substrate is called chromogenic substrate.

- A number of enzymes have been used for ELISA such as alkaline phosphatase, horse radish peroxidase and beta galactosidase.

- Specific substrate such as ortho-phenyldiamine dihydrochloride (for peroxidase), paranitrophenyl phosphate (for alkaline phosphatase) are used which are hydrolysed by above enzymes to give colored end product.

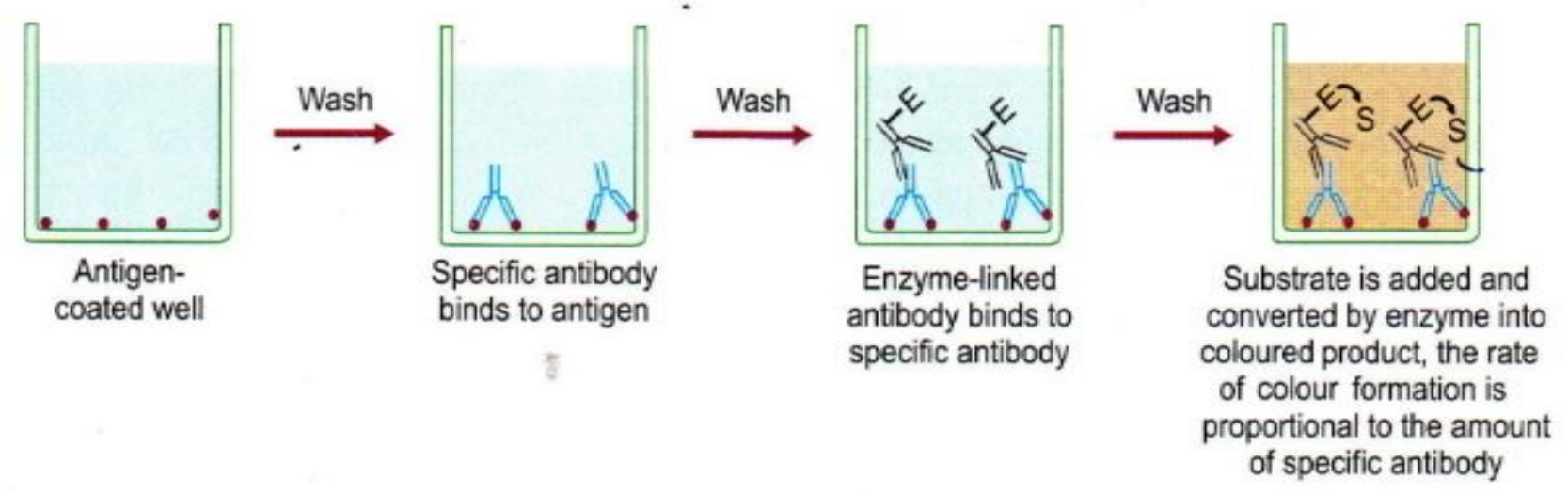
Types of ELISA

There are 3 types of ELISA on the basis of binding structure between the Antibody and Antigen.

- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA

Indirect ELISA

- Antibody can be detected or quantitatively determined by indirect ELISA. In this technique, antigen is coated on the microtiter well.
- Serum or some other sample containing primary antibody is added to the microtiter well and allowed to react with the coated antigen.
- Any free primary antibody is washed away and the bound antibody to the antigen is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody.
- Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added.
- Enzyme hydrolyzes the substrate to form colored products.
- The amount of colored end product is measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.



Advantages

- Increased sensitivity, since more than one labeled antibody is bound per primary antibody.
- A wide variety of labeled secondary antibodies are available commercially.

Maximum immunoreactivity of the primary antibody is retained because it is not labeled.

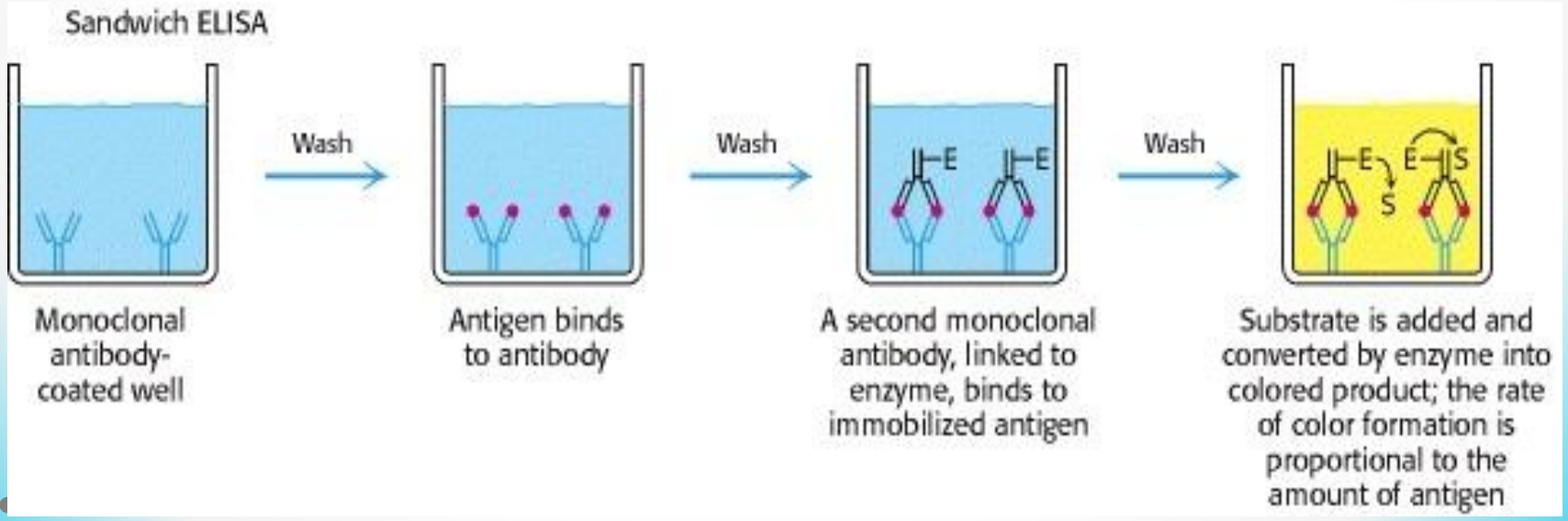
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Flexibility, since different primary detection antibodies can be used with a single labeled secondary antibody.
- Cost savings, since fewer labeled antibodies are required.
- Different visualization markers can be used with the same primary antibody.

Disadvantages

- Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.
- An extra incubation step is required in the procedure.

Sandwich ELISA

- Antigen can be detected by sandwich ELISA. In this technique, antibody is coated on the microtiter well.
- A sample containing antigen is added to the well and allowed to react with the antibody attached to the well, forming antigen-antibody complex.
- After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen.
- Then after unbound secondary antibody is removed by washing.
- Finally substrate is added to the plate which is hydrolyzed by enzyme to form colored products.

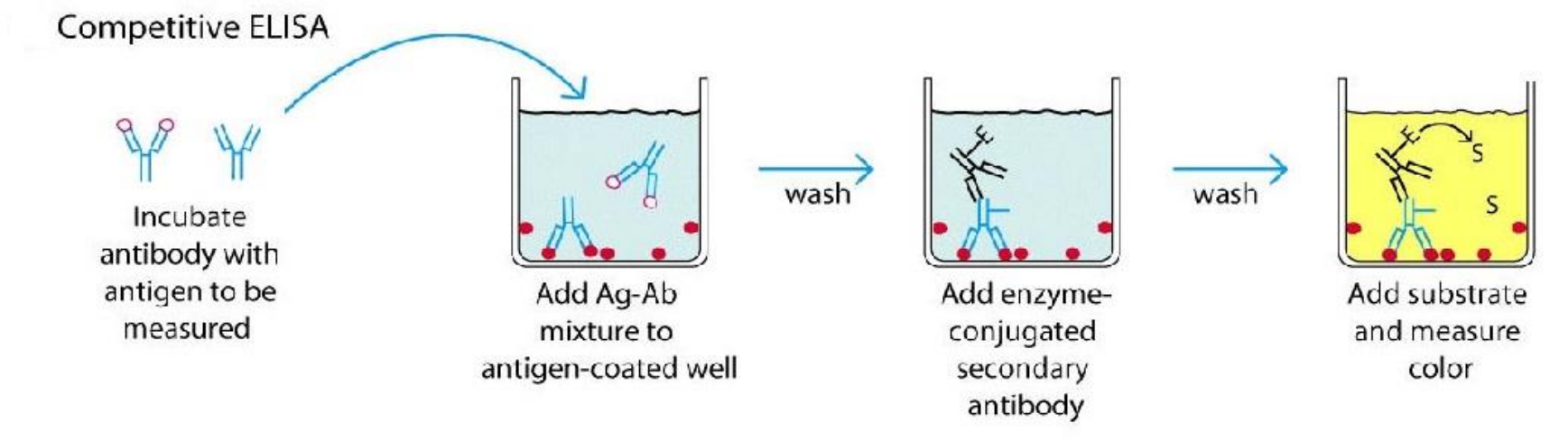


Advantages

- High specificity, since two antibodies are used the antigen is specifically captured and detected.
- Suitable for complex samples, since the antigen does not require purification prior to measurement.
- Flexibility and sensitivity, since both direct and indirect detection methods can be used.

Competitive ELISA

- This test is used to measure the concentration of an antigen in a sample.
- In this test, antibody is first incubated in solution with a sample containing antigen.
- The antigen-antibody mixture is then added to the microtitre well which is coated with antigen.
- The more the antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well.
- After the well is washed, enzyme conjugated secondary antibody specific for isotype of the primary antibody is added to determine the amount of primary antibody bound to the well.
- The higher the concentration of antigen in the sample, the lower the absorbance.



Advantages

- High specificity, since two antibodies are used.
- High sensitivity, since both direct and indirect detection methods can be used.
- Suitable for complex samples, since the antigen does not require purification prior to measurement.

Application of ELISA

- Presence of antigen or the presence of antibody in a sample can be evaluated.
- Determination of serum antibody concentrations in a virus test.
- Used in food industry when detecting potential food allergens.
- Applied in disease outbreaks- tracking the spread of disease e.g. HIV, bird flu, common, colds, cholera, STD etc.

Radioimmunoassay (RIA)

- **Radioimmunoassay** (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity.

- Basically any biological substance for which a specific antibody exists can be measured, even in minute concentrations.

- RIA has been the first immunoassay technique developed to analyze nanomolar and picomolar concentrations of hormones in biological fluids.

Method

- The target antigen is labeled radioactively and bound to its specific antibodies.

- A sample, for example a blood-serum, is then added in order to initiate a competitive reaction of the labeled antigens from the preparation, and the unlabeled antigens from the serum-sample, with the specific antibodies

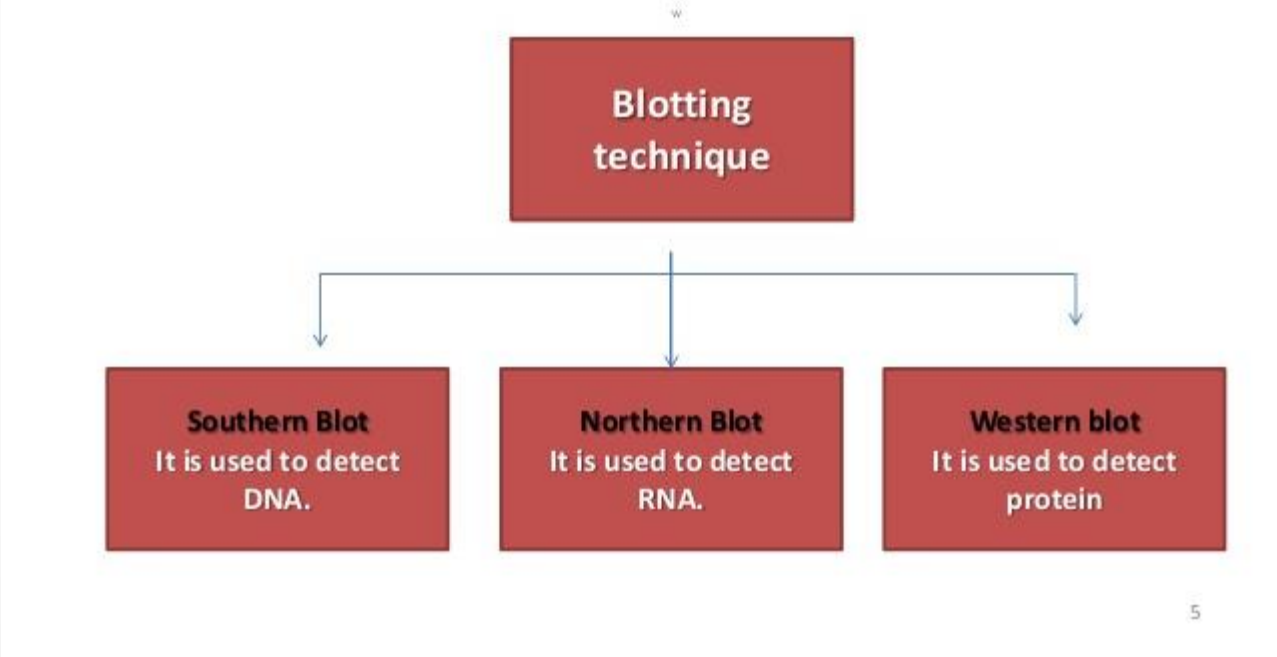
- The competition for the antibodies will release a certain amount of labeled antigen.
- This amount is proportional to the ratio of labeled to unlabeled antigen.
- A binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.
- The concentration of unlabeled antigen is increased, more of it binds to the antibody, displacing the labeled variant.
- The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured.
- A binding curve can be generated using a known standard, which allows the amount of antigens in the patient's serum to be derived.

Western Blotting

Blotting techniques

- Blots are techniques for transferring DNA , RNA and proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis.

- The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.



- Western blotting is ananalytical method thati nvolves the immobilizationof proteins on membranes before detection using monoclonal or polyclonalantibodies.

- There aredifferent blotting protocols(dot blot, 2D blot); one of themost powerful is westernblotting.

Principle of Western Blotting

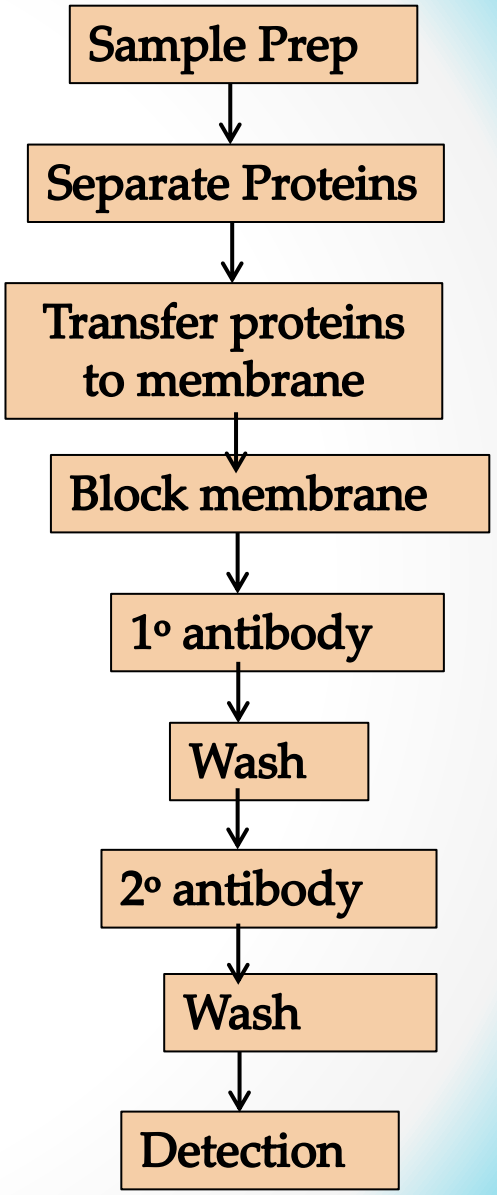
- Western blotting is an Immunoblotting technique which rely on the specificity of binding between a molecule of interest and a probe to allow detection of the molecule of interest in a mixture of many other similar molecules.

- In Western blotting, the molecule of interest is a proteinand the probe is typically an antibody raised against thatparticular protein.The SDS PAGE technique is a prerequisite for Westernblotting

- Proteins are separated by SDS-PAGE and then a specific antibody used to detect the protein of interest.

Methods

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

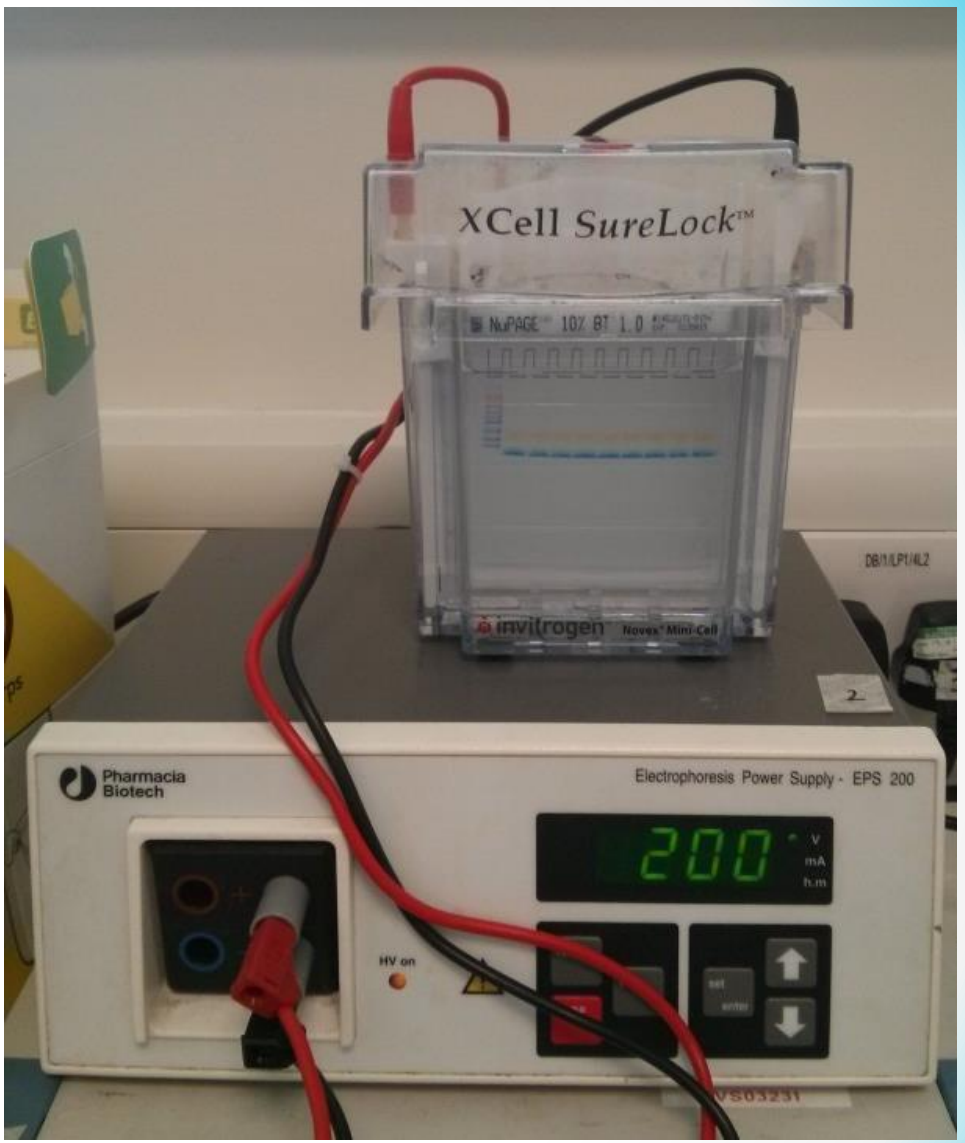


Tissue Preparations

- Samples may be taken from whole tissue or from cell culture.
- In most cases, solid tissues are first broken down mechanically using a blender.
- It should be noted that bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only.
- Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins.
- Tissue preparation is often done at cold temperatures to avoid protein denaturing.

Gel Electrophoresis

- The proteins of the sample are separated using gel electrophoresis.
- Separation of proteins may be by isoelectric point molecular weight, electric charge, or a combination of these factors.
- The principle involved is the difference in the electrophoretic mobilites of different proteins.

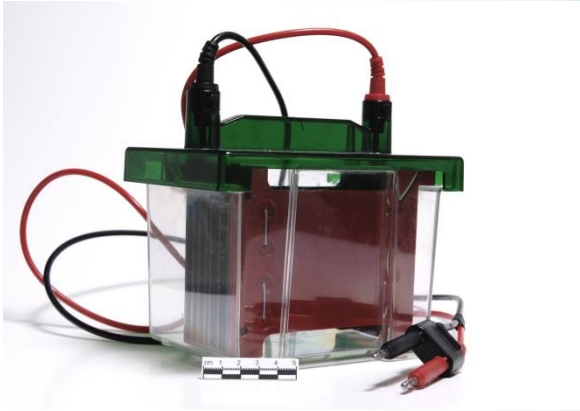


Transfer

- Transfer separated proteins onto a membrane, which can then be probed with antibodies to detect the protein of interest.
- Membrane can be Nitrocellulose or PVDF
- Nitrocellulose -> cheaper, easier to use. PVDF -> bit more faff. Binds most proteins more effectively.
- There are 3 types of transfer: wet, semi-dry, dry

Types of transfer:

Wet



Best for proteins >100kDa

Semi-dry

Quick

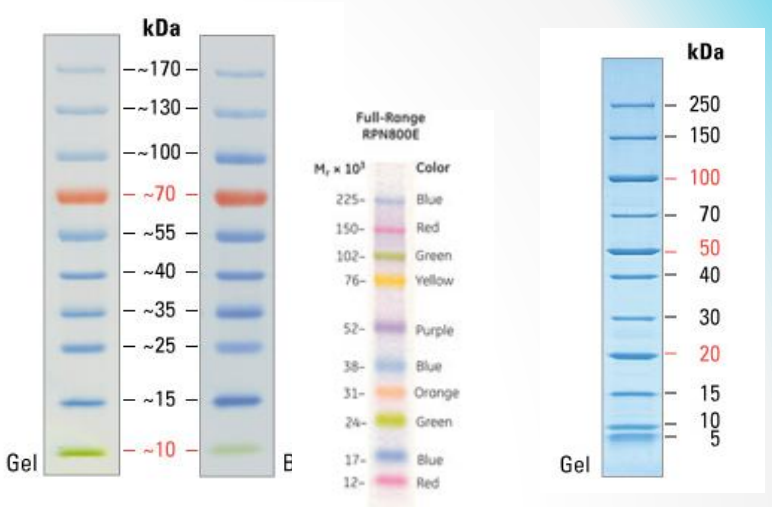
Dry

Even quicker

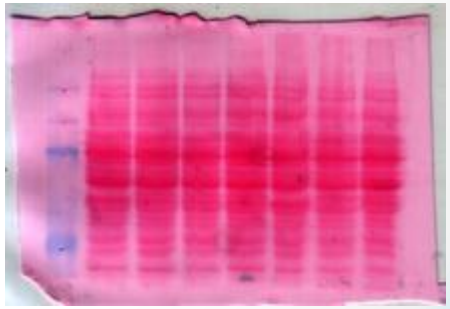
Transferring

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

Pre-stained ladder



Ponceau staining (reversible)



Blocking

- The membrane has the ability to bind to proteins in in this case both the target and antibodies are proteins and so there could be some unwanted binding.
- Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically Bovine serum albumin(BSA) with a minute percentage of detergent such as Tween 20.
- The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached.
- Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein.

Detection

- During the detection process, the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colorimetric reaction and produces a color.

Analysis

- After the unbound probes are washed away, the western blot is ready for detection of the probes that are labeled and bound to the protein of interest.
- Size approximations are taken by comparing the stained bands to that of the marker loaded during electrophoresis.
- The process is repeated for a structural protein, such as actin or tubulin that should not change between samples.

Advantages

- While ELISA being a non specific test, Western blotting is a more specific test for detection of HIV.
- It can detect one protein in a mixture of proteins while giving information about the size of the protein and so is more specific.
- Western blot test is referred to as the Gold StandardIt also tells you how much protein has accumulated in cells.

Disadvantages

- If a protein is degraded quickly, Western blotting wont detect it well. This test takes longer thatother existing testsIt might also be morecostly

Western Blot in Clinical Medicine

- The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample.
- Proteins from known HIV-infected cells are separated and blotted on a membrane then, the serum to be tested is applied in the primary antibody incubation step;f ree antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added.
- The stained bands then indicate the proteins to which the patients serum contains antibody.
- A Western blot is also used as the definitive test for Bovinespongiform encephalopathy (BSE, commonly referred to as mad cowdisease).Some forms of Lyme disease testing employ

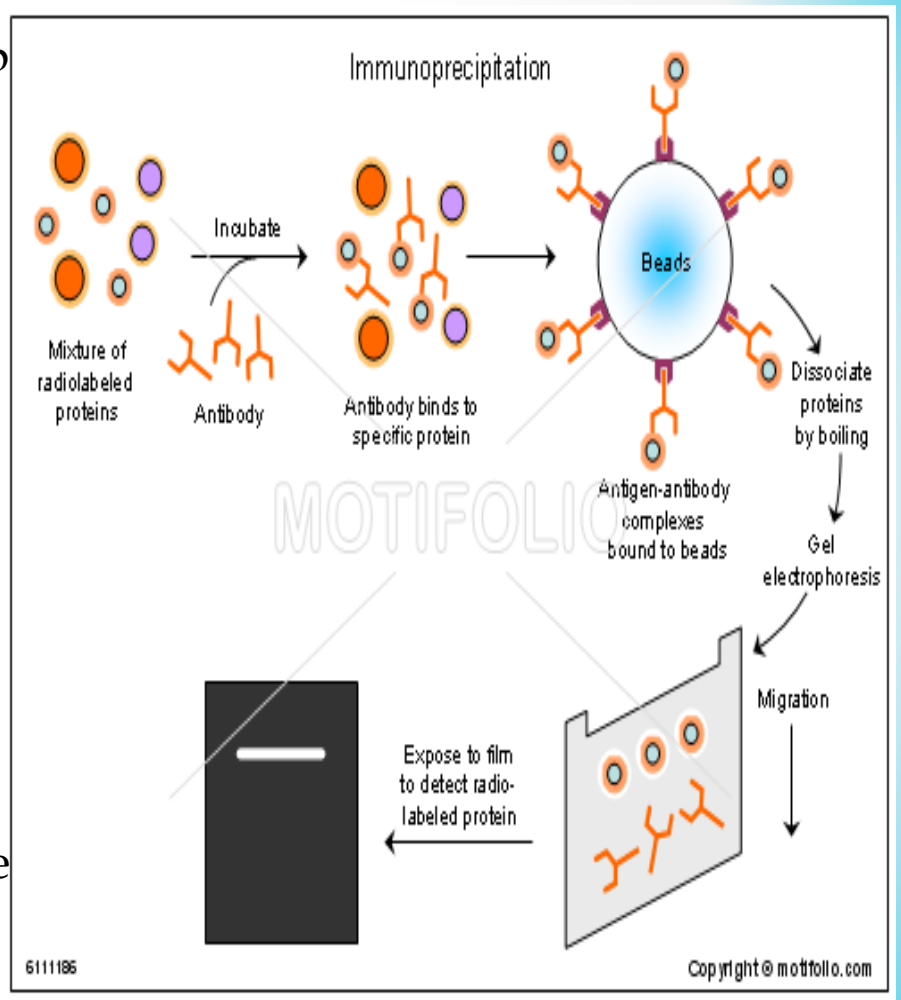
Western Blot a Confirmatory test in HIV Infection

- The virus is enveloped with different proteins.
- The detection of these proteins are useful in the detection of the presence of the virus.
- Western blotting helps in the detection of these proteins.

Immunoprecipitation

Immunoprecipitation

- General immunoprecipitation (IP) procedure with reagents and a table to help you choose the correct protein beads.
- Immunoprecipitation is a method that enables the purification of a protein.
- An antibody for the protein of interest is incubated with a cell extract enabling the antibody to bind to the protein in solution.
- The antibody/antigen complex is then pulled out of the sample using protein A/G-coupled agarose beads.
- This isolates the protein of interest from the rest of the sample.
- The sample can then be separated by SDS-PAGE for western blot analysis.



Methods

The two general methods for immunoprecipitation are the direct capture method and the indirect capture method.

Direct

- Antibodies that are specific for a particular protein (or group of proteins) are immobilized on a solid-phase substrate such as super paramagnetic microbeads or on microscopic non-magnetic) beads.

- The beads with bound antibodies are then added to the protein mixture, and the proteins that are targeted by the antibodies are captured onto the beads via the antibodies; in other words, they become immunoprecipitated.

Indirect

- Antibodies that are specific for a particular protein, or a group of proteins, are added directly to the mixture of protein.

- The antibodies have not been attached to a solid-phase support yet.

- The antibodies are free to float around the protein mixture and bind their targets.

❖As time passes, the beads coated in protein are added to the mixture of antibody and protein.

❖At this point, the antibodies, which are now bound to their targets, will stick to the beads.

Selection

- ❖ An indirect approach is sometimes preferred when the concentration of the protein target is low or when the specific affinity of the antibody for the protein is weak.
- ❖ The indirect method is also used when the binding kinetics of the antibody to the protein is slow for a variety of reasons.
- ❖ In most situations, the direct method is the default, and the preferred, choice.

Applications

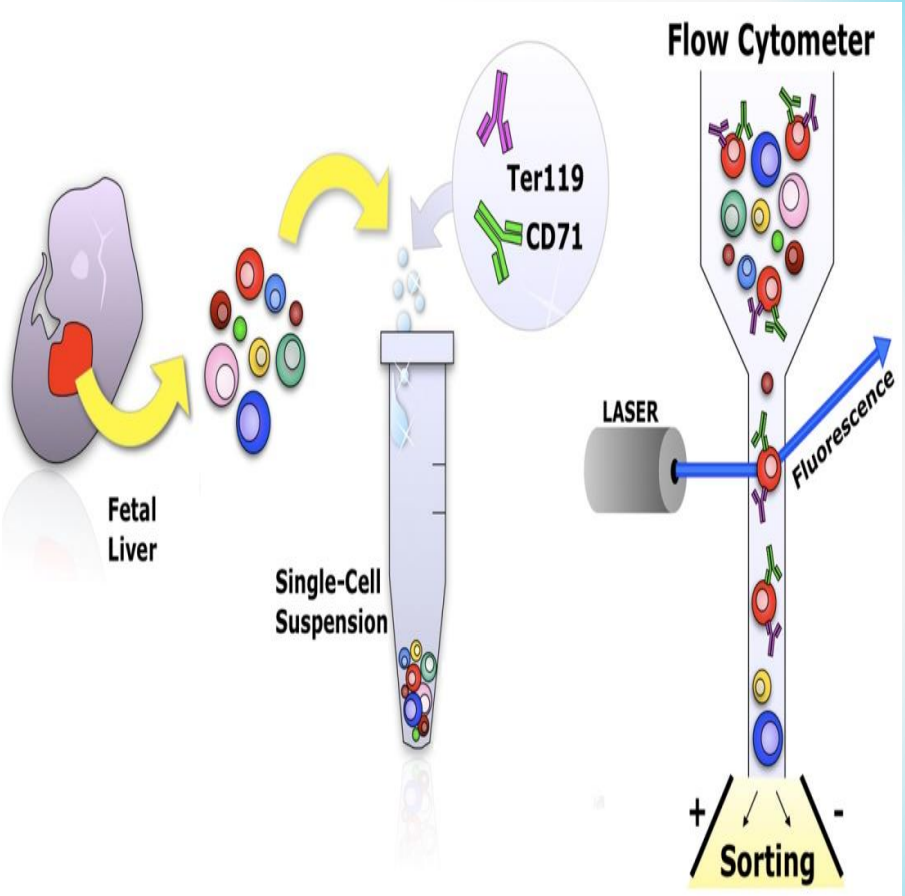
- Isolate / Detect Proteins of interest
- Enrichment of low abundant proteins
- Study protein-protein interaction and protein complexes
- Identify unknown proteins in a protein compleX
- Verify protein expression in a specific tissue.

Flow cytometry

Flow cytometry

- Flow cytometry is a popular laser-based technology to analyze the characteristics of cells or particles.

- Flow cytometry is a widely used method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations and analyzing cell size and volume.
- It allows simultaneous multi-parameter analysis of single cells.



- The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected, counted and sorted.
- Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths.
- The fluorescence can then be measured to determine the amount and type of cells present in a sample.
- Thousands of particles per second can be analysed as they pass through the liquid stream.
- A beam of laser light is directed at a hydrodynamically-focused stream of fluid that carries the cells.
- Several detectors are carefully placed around the stream, at the point where the fluid passes through the light beam.

Applications

- The technology has applications in a number of fields, including molecular biology, pathology, immunology, and marine biology .
- It has broad application in medicine especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics
- In neuroscience, co-expression of cell surface and intracellular antigens can also be analyzed.
- In marine biology, the autofluorescent properties of photosynthetic can be exploited by flow cytometry in order to characterise abundance and community structure.

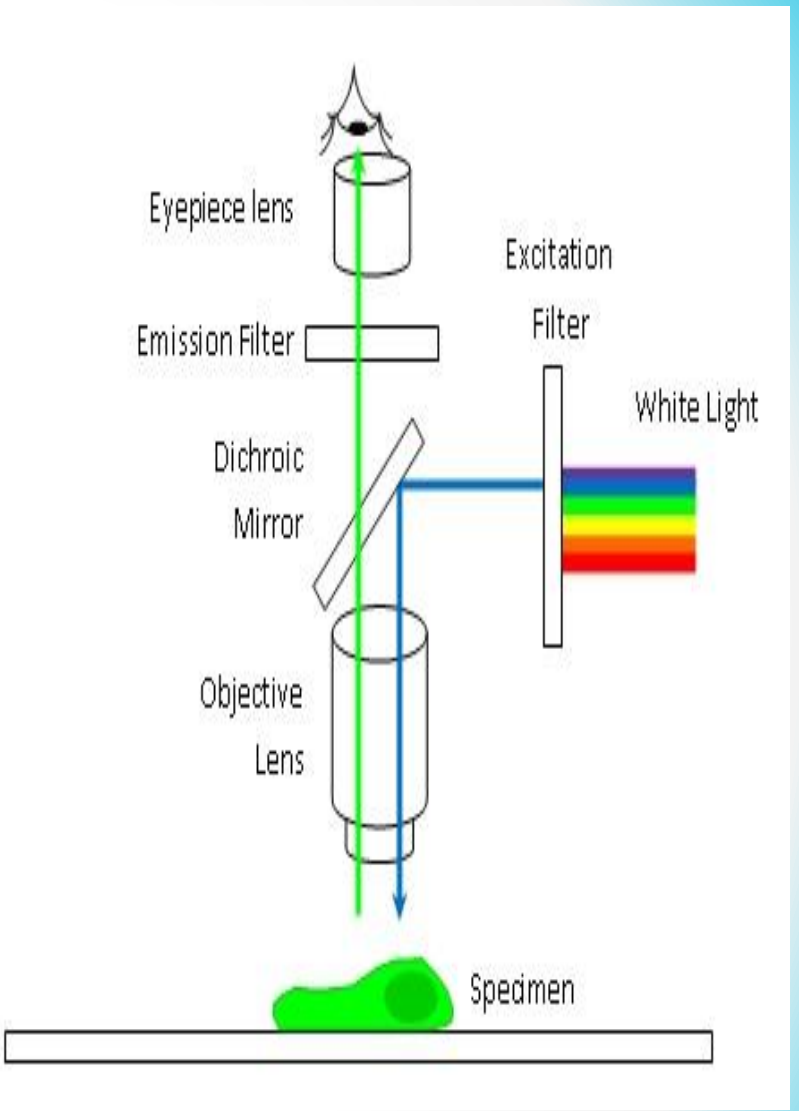
Immunofluorescence microscopy

Immunofluorescence microscopy

- Immunofluorescence (IF) microscopy is a particularly robust and broadly applicable method generally used by researchers to assess both the localization and endogenous expression levels of proteins of interest.
- Immunofluorescence microscopy is a widely used example of immunostaining and is a form of immunohistochemistry based on the use of fluorophores to visualize the location of bound antibodies.
- The effective application of this method comprises several considerations, including the nature of the antigen, specificity and sensitivity of the primary antibody, properties of the , permeabilization and fixation technique of the sample, and fluorescence imaging of the cell.
- Although each protocol will require fine-tuning depending on the cell type, the antibody, and the antigen.

Applications

- Immunofluorescence can be used on tissue sections, cultured cells or individual cells that are fixed by a variety of methods.
- Antibodies can be used in this method to analyze the distribution of proteins, glycoproteins and other antigen targets including small biological and non-biological molecules.



Acquired immunodeficiency syndrome (AIDS)

Acquired immunodeficiency syndrome (AIDS)

- AIDS stands for "acquired immunodeficiency syndrome.“
- HIV usually is spread from person to person through contact with infected sexual secretions or blood.
- People with AIDS have weakened immune systems that make them vulnerable to selected conditions and infections.
- For people infected with HIV, the risk of progression to AIDS increases with the number of years the person has been infected. The risk of progression to AIDS is decreased by using highly effective antiretroviral therapy (ART) regimens.
- In people with AIDS, ART improves the immune system and substantially increases life expectancy. Many patients who are treated with ART have near-normal life expectancies.

Symptoms and signs of AIDS

- AIDS is an advanced stage of HIV infection.
- Because the CD4 cells in the immune system have been largely destroyed, people with AIDS often develop symptoms and signs of unusual infections or cancers.
- When a person with HIV infection gets one of these infections or cancers, it is referred to as an "AIDS-defining condition.
- People with AIDS may develop symptoms of pneumonia due to *Pneumocystis jiroveci*, which is rarely seen in people with normal immune systems.

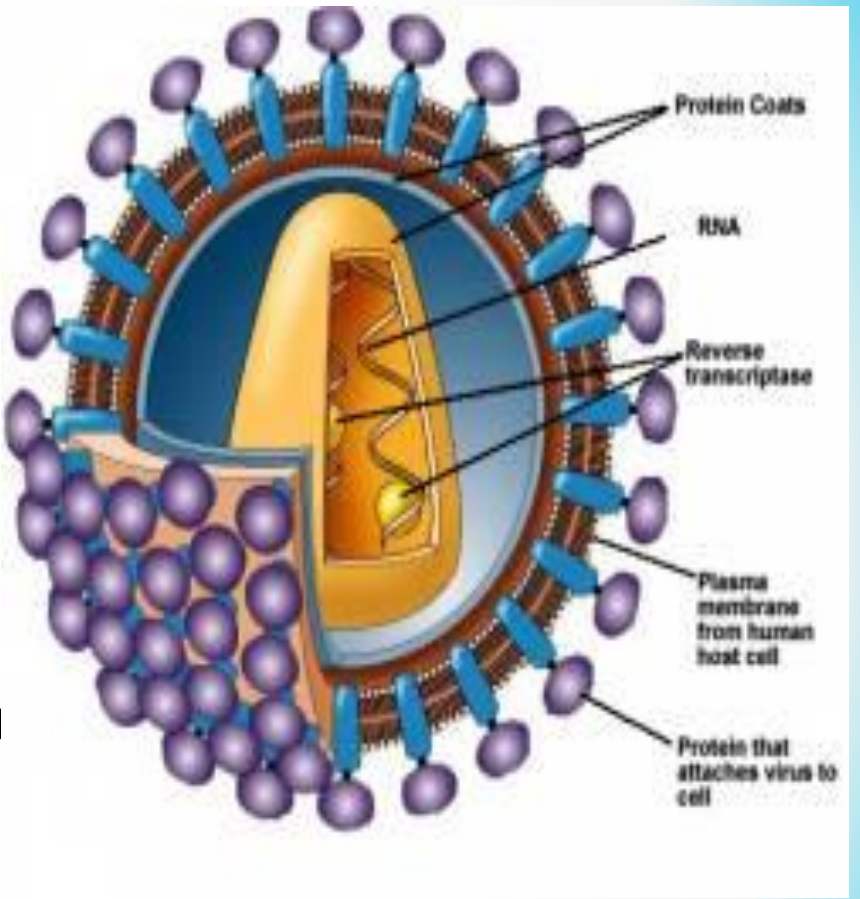
- They also are more likely to get pneumonia due to common bacteria.
- Globally, tuberculosis is one of the most common infections associated with AIDS.
- In addition, people with AIDS may develop seizures, weakness, or mental changes due to toxoplasmosis, a parasite that infects the brain.
- Neurological signs also may be due to meningitis caused by the fungus *Cryptococcus*.
- Complaints of painful swallowing may be caused by a yeast infection of the esophagus called candidiasis. Because these infections take advantage of the weakened immune system, they are called "opportunistic infections."

Diagnosis

- To diagnose AIDS,

(1) a confirmed, positive test for HIV ("HIV positive" test) and

(2) evidence of an AIDS-defining condition or severely depleted CD4 cells.



- Testing for HIV is a two-step process involving a screening test and a confirmatory test.

- The first step is usually a screening test that looks for antibodies against the HIV.

- Specimens for testing come from blood obtained from a vein or a finger stick, an oral swab, or a urine sample.

- Results can come back in minutes (rapid tests) or can take several days, depending on the method that is used.

- If the screening HIV test is positive, the results are confirmed by a special test called a Western blot or indirect immunofluorescence assay test.

- A Western blot detects antibodies to specific components of the virus.

- The confirmatory test is necessary because the screening test is less accurate and occasionally will be positive in those who do not have HIV.

Antibody generation

Antibody generation

- Antibody generation is the production of antibodies from cells or animals in sufficient quantity to be used as an experimental or therapeutic reagent.

- Polyclonal antibody mixtures are generated by injecting an animal with an agent that elicits an immune response, and monoclonal antibodies are generated from immortal immune cell lines.

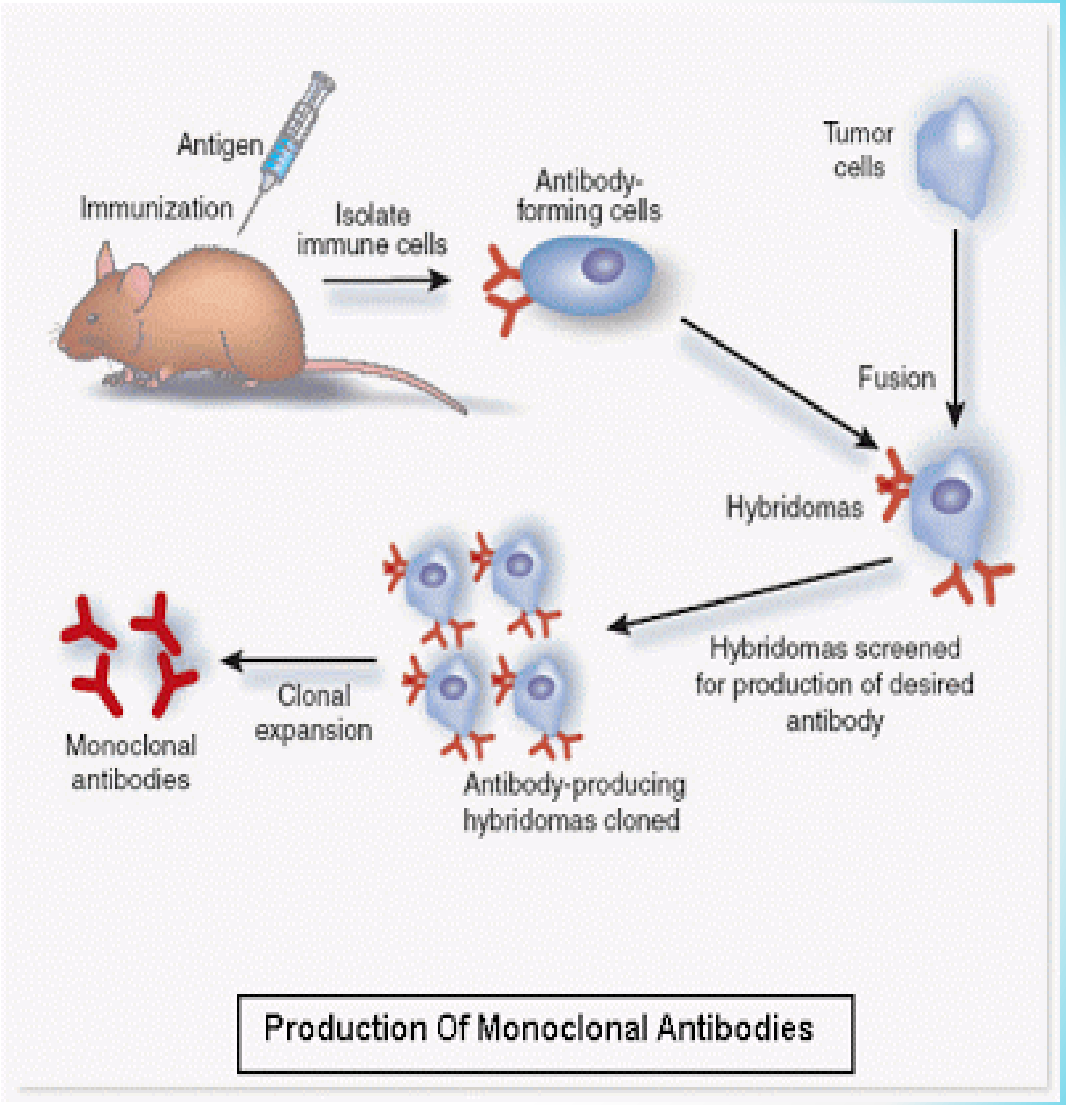
Generation of Hybridomas

- Permanent Cell Lines Secreting Monoclonal Antibodies Production of monoclonal antibodies involves in vivo or in vitro procedures or combinations thereof.
- Before production of antibodies by either method, hybrid cells that will produce the antibodies are generated.
- The generation of mAb-producing cells requires the use of animals, usually mice.
- The procedure yields a cell line capable of producing one type of antibody protein for a long period.
- A tumor from this “immortal” cell line is called a hybridoma

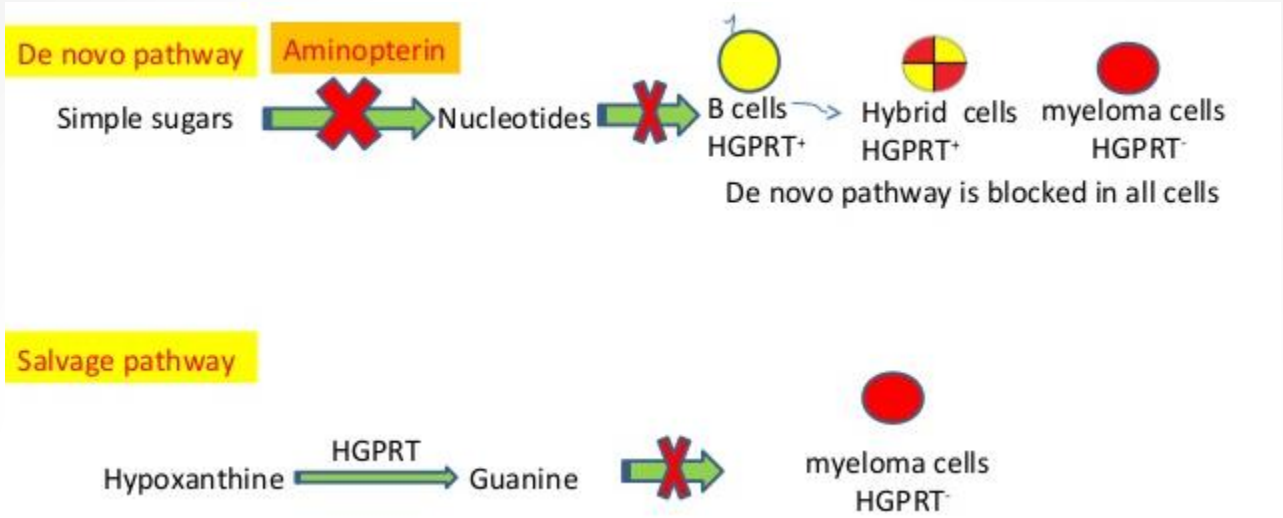
Hybridoma technology

Hybridoma technology

- Monoclonal Antibodies (MABs) MABs are antibodies that arise from a single clone of Hybridoma technology cells.
- They are homogenous E.g. in a plasma cell tumour (myeloma).
- Monoclonal antibodies are highly useful in diagnostic tests and in research.
- Hybridoma Technology developed by Kohler and Milstein has been widely used for the production of MABs



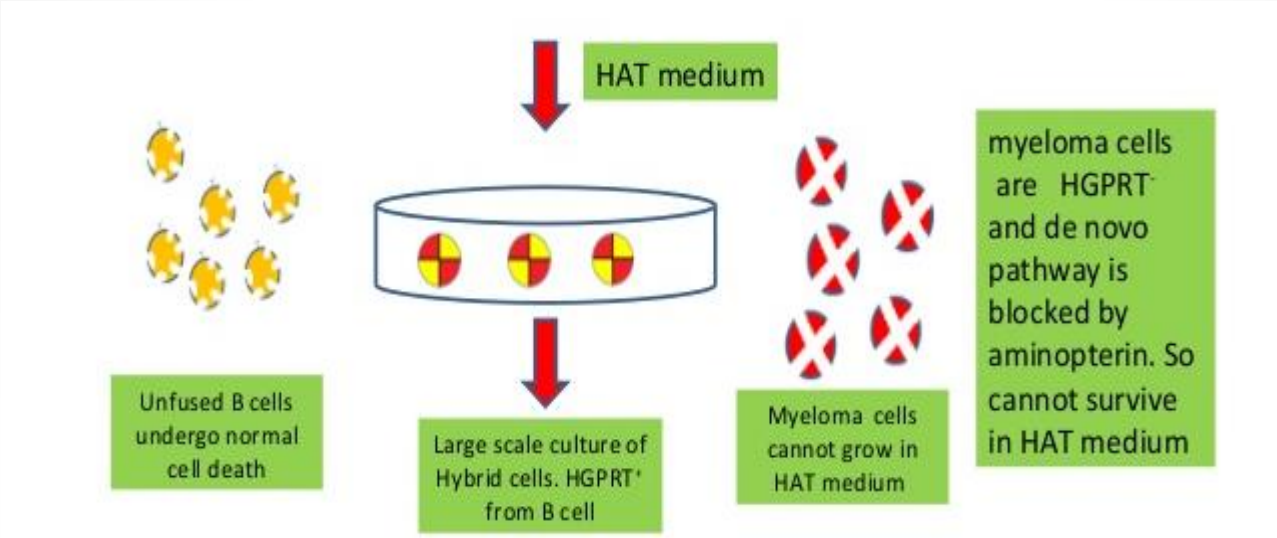
- B cells can produce antibodies of single Myeloma B cell cells specificity ie: monoclonal HGPRT+ HGPRT- antibody.
- Fusion using PEG Myeloma cells or tumor cells are capable of continuous division forming large number of cells.
- Hybrid cell Hybrid cells will be having the property of B cells HAT medium antibody production and tumour cells ability of continuous division.
- Hybrid cell ensures antibody production continuously Unfused B cells in dividing cells Myeloma cells undergo normal cannot grow in cell death Large scale HAT medium culture of Hybrid cells
- HAT medium has Hypoxanthine, Aminopterin and thymidine
- Nucleotide synthesis is essential for cell survival



- HAT medium myeloma cells are HGPRT- and de novo pathway is blocked by aminopterin.

- So Unfused B cells Myeloma cells cannot survive undergo normal Large scale culture of in HAT medium cannot grow in cell death Hybrid cells. HGPRT+ HAT medium from B cell

Hybrid cells are selected in HAT medium



- HAT medium for selection of hybrid cells: Hypoxanthine aminopterin thymidine medium
- Aminopterin blocks the cellular synthesis of purines and pyrimidines fromsimple sugars (de novo pathway). But cells can thrive by utilizing Hypoxanthine and thymidine present in the medium by salvage pathway.
- Hypoxanthine and thymidine can be utilized for the production ofnucleotides in the presence of enzyme hypoxanthine guaninephosphoribosyl transferase (HGPRT)

- Myeloma cells are HGPRT deficient . So this cells cannot survive in HAT medium as aminopterin blocks denovo pathway

- Bcells are HGPRT+ and can survive in the HAT medium after some division cells undergo normal cell death

- Hybrid cells has HGPRT enzyme from the B cells. So only hybrid cells can survive in HAT medium.

Applications

- Monoclonal antibodies are proving to be very useful as diagnostic, imaging, and therapuetic reagents in clinical medicine.

- Many monoclonal antibody diagnostic reagents now available are products for detecting pregnancy, diagnosing numerous pathogenic microorganisms, measuring blood levels of various drugs, and detecting antigens shed by certain tumors.

- A pregnant woman has the hormone human chorionic gonadotrophin (HCG) in her urine.

- Monoclonal antibodies to HCG have been produced. These have been attached to enzymes which can later interact with a dye molecule and produce a colour change.

- The test of HIV infection is based on detecting the presence of HIV antibody in the patient's blood serum.

a) HIV antigen is attached to the plate.

b) Patients serum passed over the plate. Any HIV antibody in the patients serum will attached to the antigen already on the plate.

c) A second antibody which is specific to the HIV antibody is passed over the plate. This antibody will attach to the concentrated HIV antibody on the plate. This second antibody has an enzyme attached to its structure.

d) Chromagen dye is passed over the complex of concentrated HIV antibody/conjugated antibody.

e) The enzyme will turn the chromagen to a more intense colour. The more intense the colour, the greater the HIV antibody level. This would be the a positive result for a HIV test.

- Cancer cells carry specific tumour-associated antigens (TAA) on their plasma membrane.
- Monoclonal anti-TAA antibodies have been produced.
- Drugs which kill tumour cells or inhibit key proteins in tumour cells are attached to monoclonal anti-TAA antibodies.
- Cancer cells are specifically targeted, avoiding damage to healthy host cells.

Radioimmunoassay (RIA)

Radioimmunoassay (RIA)

- Radioimmunoassay (RIA) involves the separation of a protein (from a mixture) using the specificity of antibody - antigen binding and quantify it using radioactivity

- The technique was introduced in 1960 by Berson and Yalow as an assay for the concentration of insulin in plasma.

- Here radioactive materials are not administered to the individual but are used as reagents.

- The technique of radioimmunoassay has revolutionized research and clinical practice in many areas, e.g.,
  - ✓ blood banking
  - ✓ diagnosis of allergies
  - ✓ endocrinology.

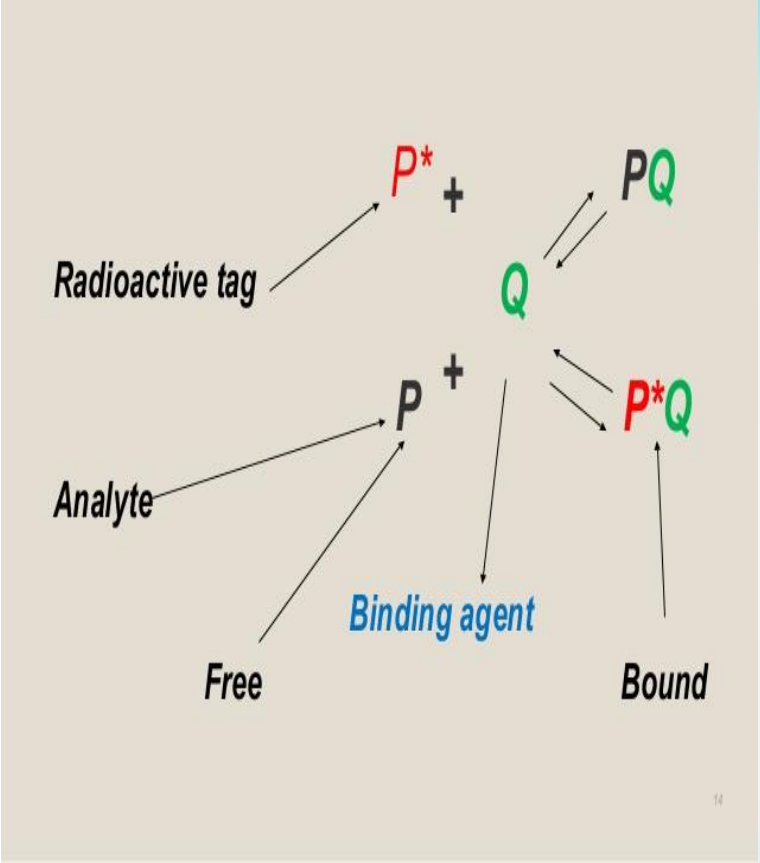
Principle of Radioimmunoassay

Uses an immune reaction [Antigen – Antibody reaction] to estimate a ligand

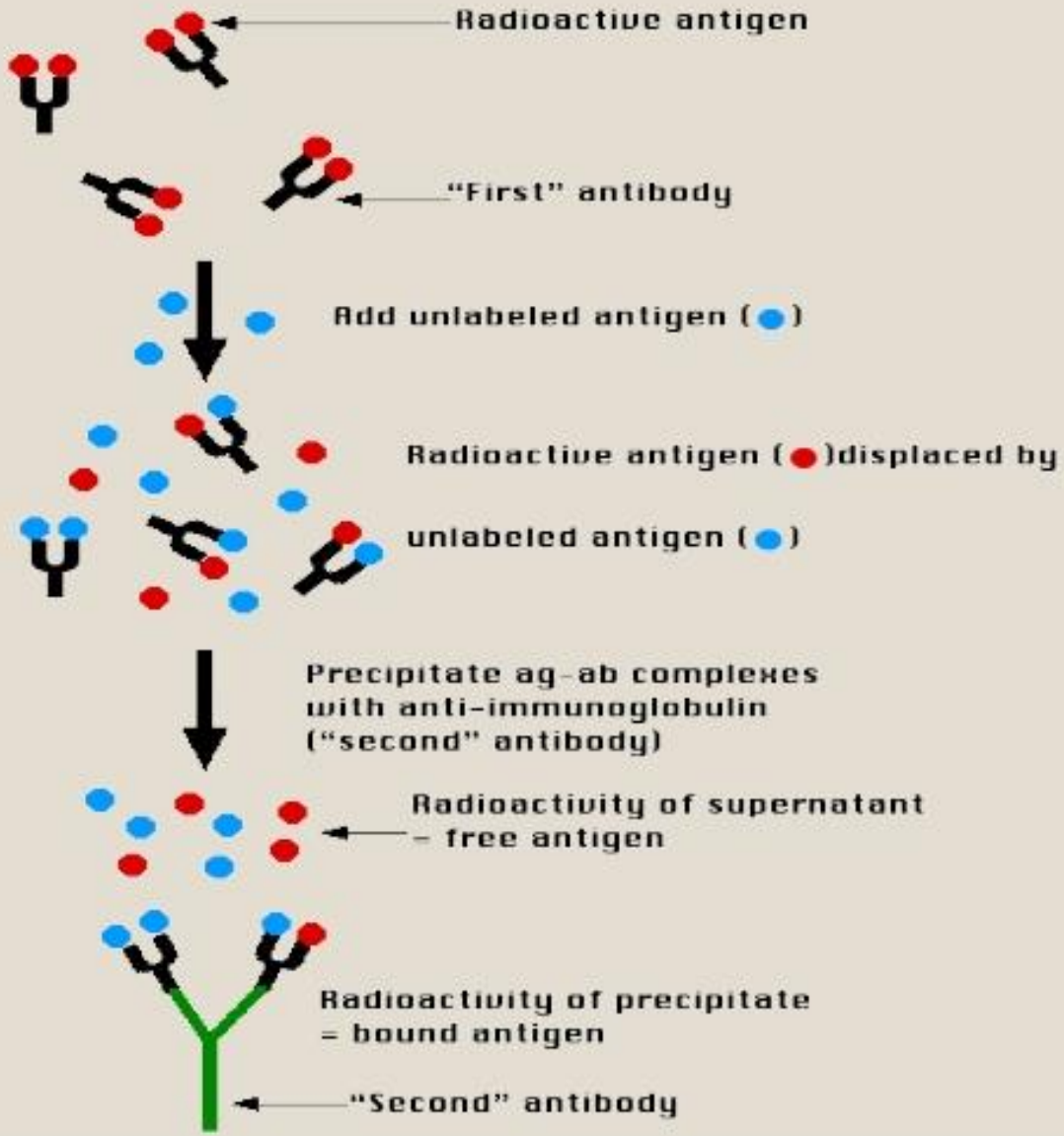
Ag + Ag* + Ab ⚜ AgAb + Ag*Ab + Ag + Ab*

- Unbound Ag* and Ag washed out
- Radioactivity of bound residue measured
- Ligand conc. is inversely related to radioactivity

[Ag : ligand to be measured ; Ag* radiolabelled ligand]



- A mixture is prepared of
  - ✓ radioactive antigen
  - ✓ antibodies against that antigen.
- Known amounts of unlabeled ("cold") antigen are added to samples of the mixture.
- These compete for the binding sites of the antibodies.
- At increasing concentrations of unlabeled antigen, an increasing amount of radioactive antigen is displaced from the antibody molecules.
- The antibody-bound antigen is separated from the free antigen in the supernatant fluidand
- The radioactivity of each is measured.


Radioactive antigen
"First" antibody
Add unlabeled antigen ( )
Radioactive antigen ( )displaced by
unlabeled antigen ( )
Precipitate ag-ab complexes
with anti-immunoglobulin
("second" antibody)
Radioactivity of supernatant
= free antigen
Radioactivity of precipitate
= bound antigen
"Second" antibody

Requirements for RIA

- Preparation & characterisation of the Antigen [Ligand to be analysed]
- Radiolabelling of the Antigen
- Preparation of the Specific Antibody
- Development of Assay System

Preparation & Radiolabelling of the Ag

- Antigens prepared by
  - ✓ Isolation from natural sources
  - ✓ Synthesis of the molecule
- Radiolabelling [Tagging procedure]
  - ✓ 3 H ,14 C, 125 I are used as radioactive tags
  - ✓ Antigens are tagged to 3 H ,14 C, 125 I
  - ✓ Tagging should NOT affect Antigenic specificity & Antigenic activity

Preparation of the Specific Antibody

- Antigen injected intradermally into rabbits or guinea pigs - antibody production
- Antibodies recovered from the serum

Development of the Assay System

- Crucial step is separation of unbound antigens
- Antibodies bind to microtitre well surface [Solid phase RIA]
- Antigens bound to the fixed antibodies remain stuck to the inner surface
- Decanting & washing the well removes unbound antigens
- Other techniques of separation: Centrifugation, Precipitation and Electrophoresis

Advantages

- Highly specific: Immune reactions are specific
- High sensitivity : Immune reactions are sensitive
- Possible to detect picograms of Ag
- Sepharose beads used in RIA are reuseable

Disadvantages

- Radiation hazards: Uses radio labelled reagents Requires specially trained persons Labs require special license to handle radioactive material Requires special arrangements for Requisition storage of radioactive material radioactive waste disposal.
- Both 125 I or 131 I emit gamma radiation that requires special counting equipment
- The body concentrates iodine atoms — radioactive or not — in the thyroid gland where they are incorporated in thyroxine (T4).

Applications of RIA

- Analysis of hormones, vitamins,metabolites, diagnostic markers

  Eg. ACTH, FSH, T3, T4, Glucagon, Insulin, Testosterone, vitamin B12, prostaglandins, glucocorticoids,

- Therapeutic drug monitoring: Barbiturates, morphine, digoxin

- ◎ Diagnostic procedures for detecting infection eg: HIV, Hepatitis A, B etc

- used to assay : plasma levels of:

  - ✓ most of our hormones;
  - ✓ digitoxin or digoxin in patients receiving these drugs;
  - ✓ certain abused drugs

- for the presence of hepatitis B surface antigen (HBsAg) in donated blood

- In Endocrinology
  - ✓ Insulin, HCG, Vasopressin
  - ✓ Detects Endocrine Disorders
  - ✓ Physiology of Endocrine Function
- In Pharmacology
  - ✓ Morphine
  - ✓ Detect Drug Abuse or Drug Poisoning
  - ✓ Study Drug Kinetics
- Epidemiology
  - ✓ Hepatitis B
- Clinical Immunology
  - ✓ Antibodies for Inhalant Allergens
  - ✓ Allergy Diagnosis
- Oncology
  - ✓ Carcinoembryonic Antigen
  - ✓ Early Cancer Detection and Diagnosis
- Narcotic drug detection
- Tracking of leukemia virus
- diagnosis and treatment of peptic ulcers
- research with Neurotransmitters