MOLECULAR DIAGNOSTICS

Topic: Immunoassays:

BY

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DR. ANNIKA SINGH FUNDAMENTALS OF BIOCHEMISTRY

UNIT I (15 Periods)

Enzyme Immunoassays:

Comparison of enzymes available for enzyme immunoassays, conjugation of enzymes. Solid phases used in enzyme immunoassays.

Homogeneous and heterogeneous enzyme immunoassays. Enzyme immunoassays after immuno blotting. Enzyme immuno histochemical techniques. Use of polyclonal or monoclonal antibodies in enzymes immuno assays.

Applications of enzyme immunoassays in diagnostic microbiology

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ELISA - an acronym for Enzyme-Linked Immuno Sorbent Assay.

The **enzyme-linked immunosorbent assay** (**ELISA**) is a commonly used analytical biochemistry assay, first described by Eva Engvall and Peter Perlmann in 1971.

The assay uses a solid-phase type of enzyme immunoassay (EIA) to detect the presence of a ligand (commonly a protein) in a liquid sample using antibodies directed against the protein to be measured. ELISA has been used as a diagnostic tool in medicine, plant pathology, and as a quality control check in various industries.

In the most simple form of an ELISA, antigens from the sample to be tested are attached to a surface.

A matching antibody is applied over the surface so it can bind the antigen. This antibody is linked to an enzyme and then any unbound antibodies are removed. In the final step, a substance containing the enzyme's substrate is added.

If there was binding, the subsequent reaction produces a detectable signal, most commonly a color change.

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Antigens are any foreign substance in the body.

Antigens can be “not-self” molecules and cells, such as:

a. foreign proteins
b. viruses
c. environmental pollutants and other foreign substances like asbestos, tattoo ink, and cigarette smoke
d. bacteria and parasites (Protista, Fungi, Plantae, and Animalia cells)
e. foreign transplanted tissue
f. cancerous cells

Antibodies are large glycoprotein molecules produced by B-lymphocytes during the humoral immune response to antigens introduced into the body.

Lymphocytes include B-lymphocytes (B-cells) and T-lymphocytes (T-cells)

The immune system is made of two parts – humoral (antibody-mediated) and cellular (cell-mediated).

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- B-lymphocytes produce large glycoproteins called antibodies in response to antigens (any foreign substance) and then mark those antigens-antibody complex to be destroyed by the T-lymphocytes.
- Each B-cell makes its own distinct antibody in response to a specific antigen which comes in contact with it. Each antibody is designed to bind to a specific surface binding site or epitope on the antigen.
- There are millions of different types of antibodies circulating in an individual's bloodstream and they are based on exposure to antigens in his/her environment.
- Over 80% of human glycoprotein antibodies are in the immunoglobulin class IgG. They are shaped like a Y and are found in the blood, lymph, and intestine.
- IgG molecules have a molecular weight of 150,000 Daltons and are made of 2 long (heavy) chains coded from chromosome 14, and 2 short (light) chains coded from either chromosome 2 or 22, and then all connected by disulphide bonds.
- Most of the molecule is composed of a constant region that doesn't change from one IgG molecule to another. However, the ends of the Y are variable, which accounts for each IgG molecule binding only to a specific antigen.

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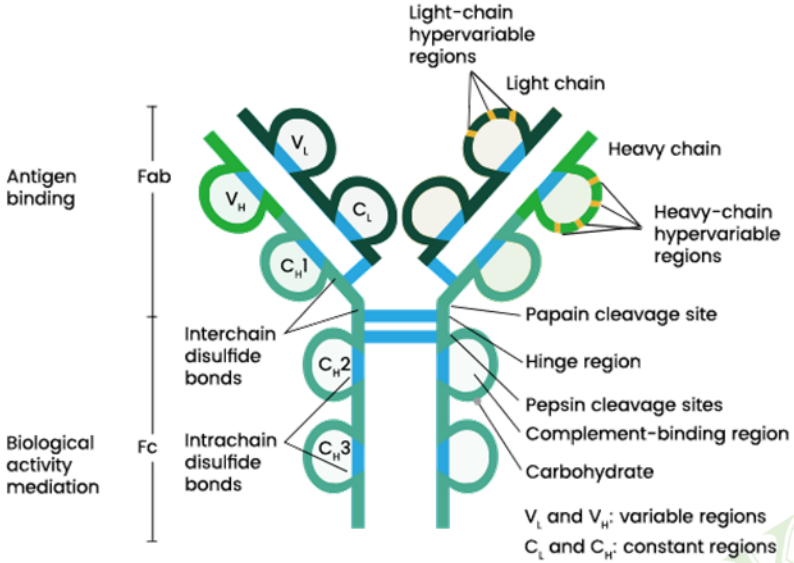

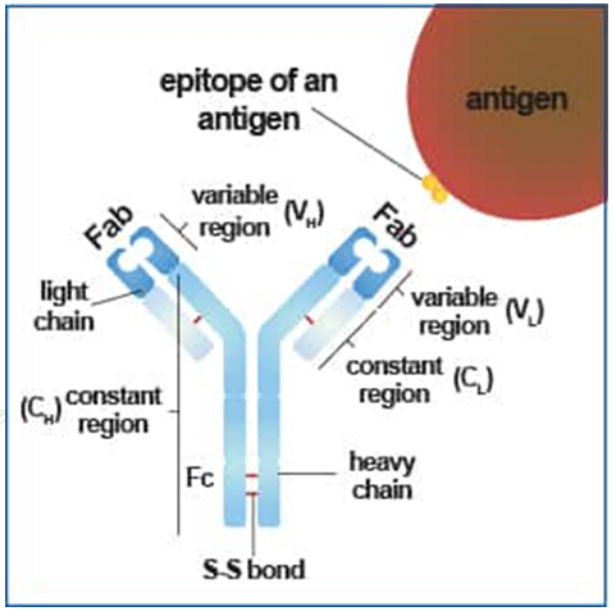

- The ELISA assay is a widely used biochemical assay to detect in a sample the presence of and quantity of proteins, such as hormones and antibodies and bacteria or viruses.
- The ELISA assay uses the coupling of antigens and antibodies and relies on the specificity and affinity of antibodies for antigens. Specificity is the ability to discriminate among diverse proteins. Affinity is the ability to tightly bind to molecules.
- One can determine how much antibody is present by starting with an antigen, or one can determine how much antigen or hormone is present by starting with an antibody.

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There are five types of ELISA methods which include:

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA
- Multiplex ELISA

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- **Direct ELISA**
- is used to quantify an antigen in solution.

The steps of direct ELISA follows the mechanism below:

•A buffered solution of the antigen to be tested for is added to each well of a microtiter plate, where it is given time to adhere to the plastic through charge interactions.

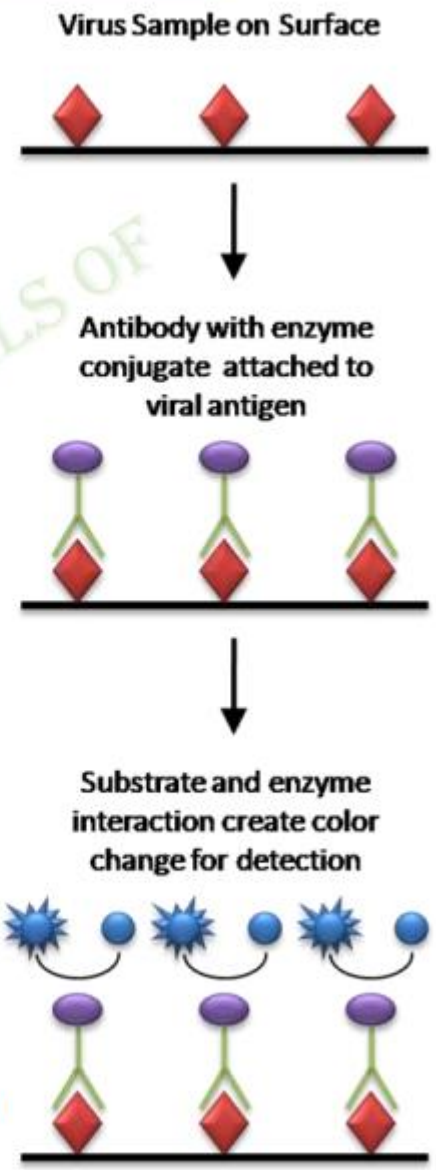
•A solution of nonreacting protein, such as bovine serum albumin or casein, is added to each well in order to cover any plastic surface in the well which remains uncoated by the antigen.

•The primary antibody with a conjugated enzyme is added, which binds specifically to the test antigen coating the well.

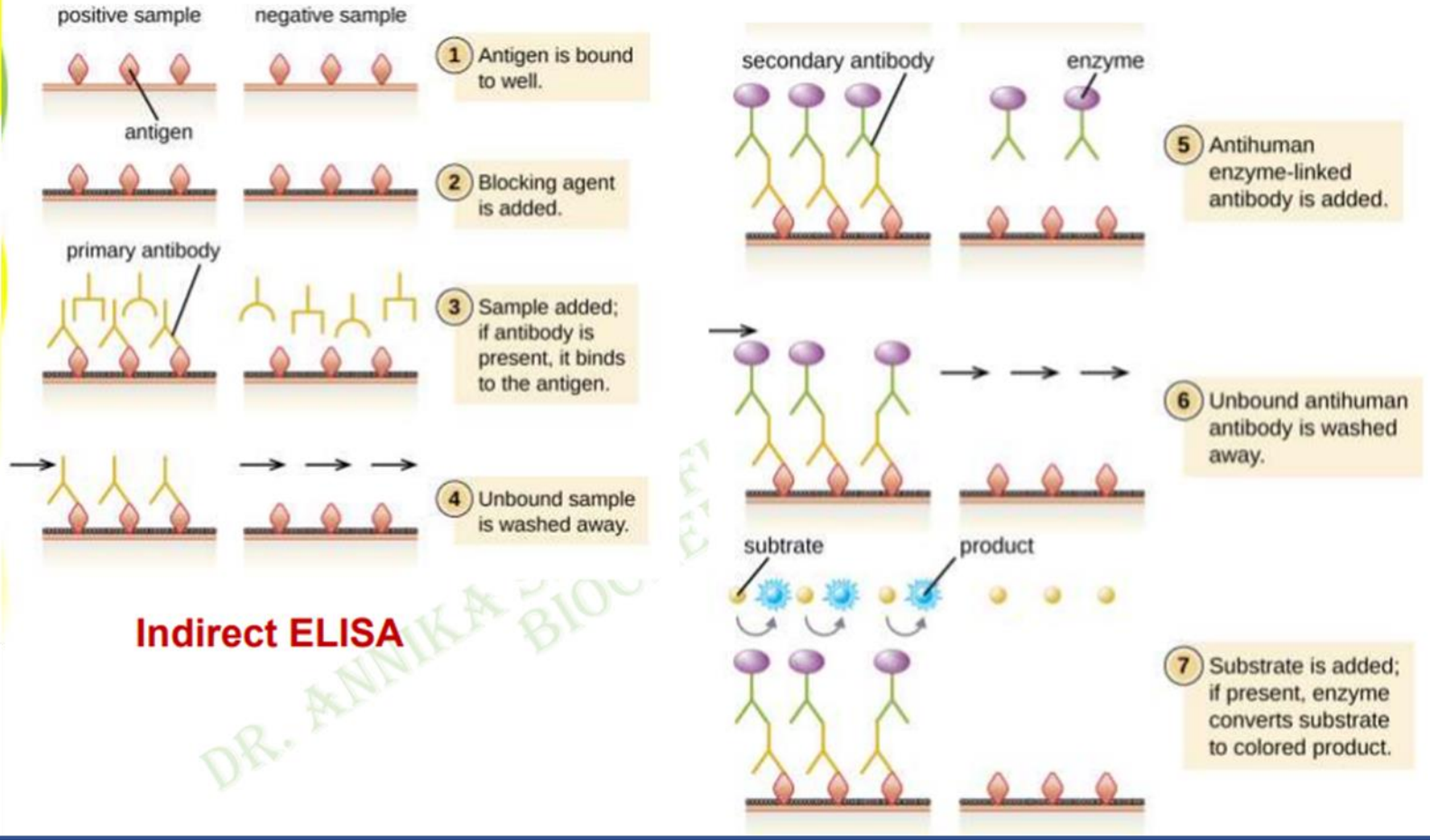
•A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme.

•The higher the concentration of the primary antibody present in the serum, the stronger the color change.

References



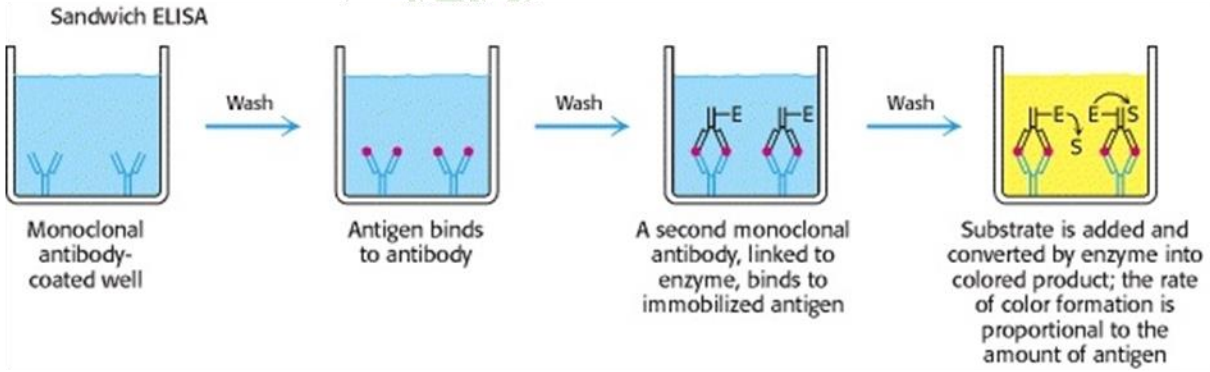
- **Indirect ELISA** is used to detect antibodies in patient serum by attaching antigen to the well of a microtiter plate, allowing the patient (primary) antibody to bind the antigen and an enzyme conjugated secondary antibody to detect the primary antibody



Indirect ELISA

➢ SANDWICH ELISA

- An antibody to a target protein is immobilized on the surface of microplate wells and incubated first with the target protein and then with another target protein-specific antibody, which is labeled with an enzyme.
- After washing, the activity of the microplate well-bound enzyme is measured. The immobilized antibody (orange) and the enzyme-labeled antibody (green) must recognize different epitopes of the target protein.
- Compared to direct ELISA, the sandwich ELISA (combining antibodies to two different epitopes on the target protein) has a higher specificity.
- Sandwich ELISA is useful for applications that require a high accuracy



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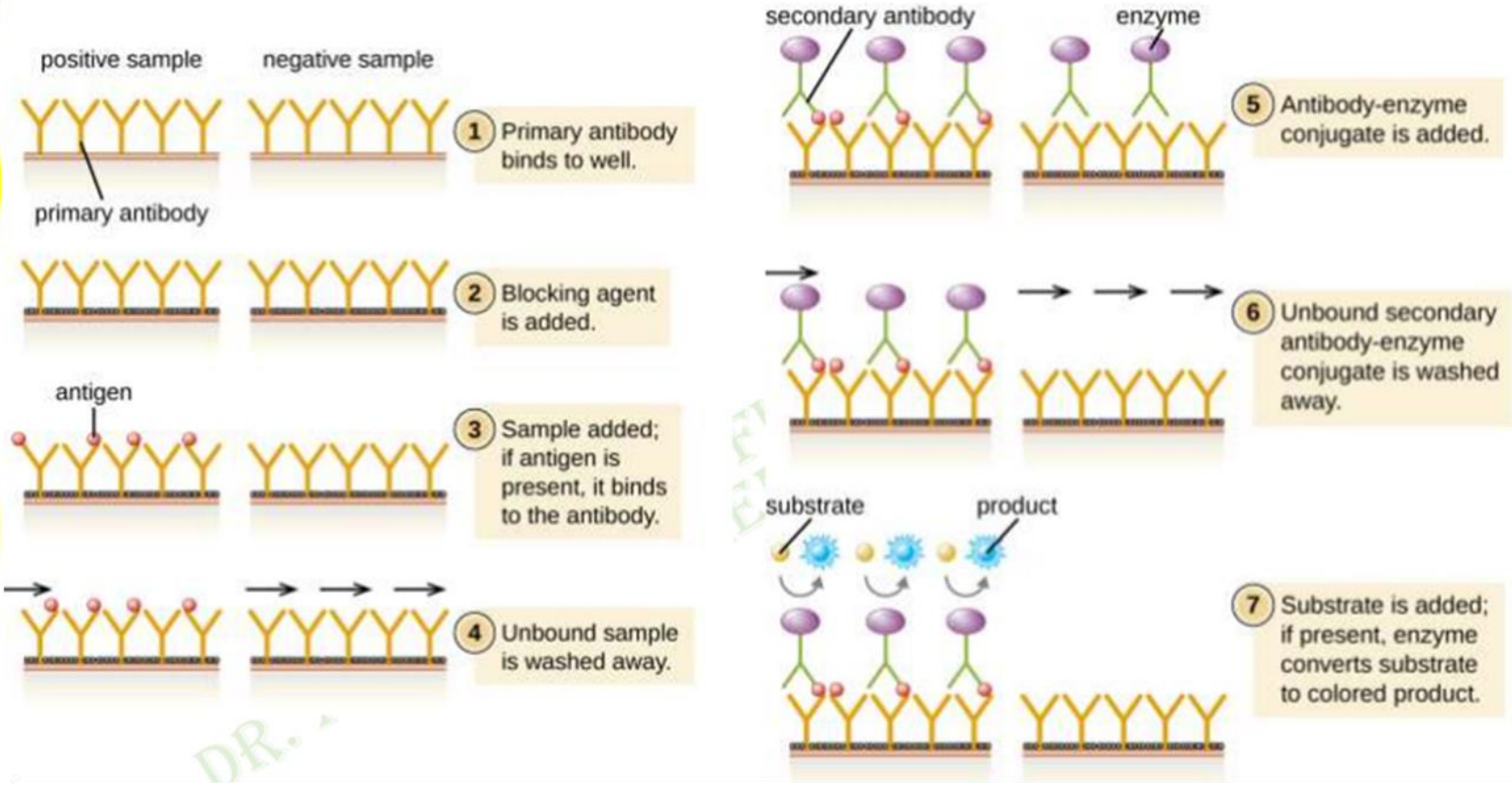

positive sample
negative sample
primary antibody
1 Primary antibody binds to well.
2 Blocking agent is added.
antigen
3 Sample added; if antigen is present, it binds to the antibody.
4 Unbound sample is washed away.
secondary antibody
enzyme
5 Antibody-enzyme conjugate is added.
6 Unbound secondary antibody-enzyme conjugate is washed away.
substrate
product
7 Substrate is added; if present, enzyme converts substrate to colored product.

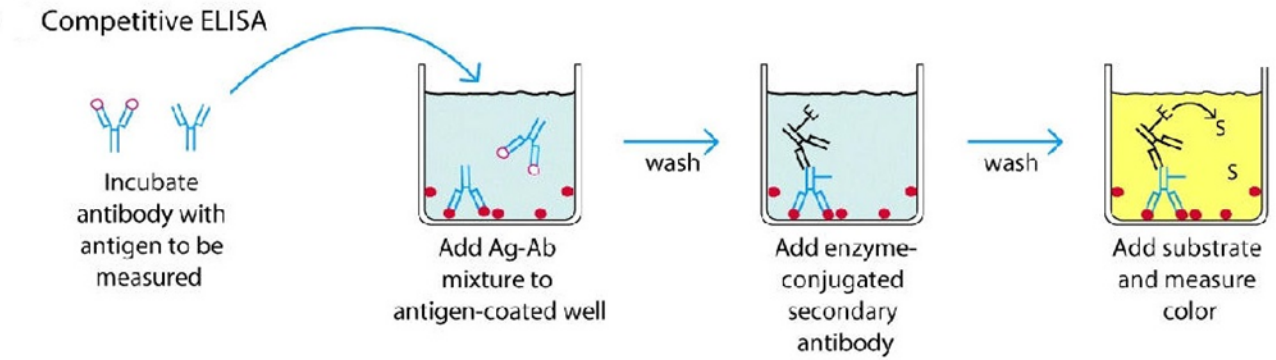
Competitive ELISA

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.



Multiplex assays

Multiplex assays are a type of immunoassay capable of simultaneous measurement of multiple analytes. Multiplex assays are derived from the ELISA format, but instead of producing a single signal measurement, they produce multiple signal measurements. In multiplex microsphere assays, microspheres in different colors are coated with different antibodies. After incubation, the results are read using flow cytometry which distinguishes the different colored beads by their varying levels of fluorescence.

Multiplex measurement offers two main advantages over single assay measurement:

•Measuring the properties of several analytes using single measurement assays such as ELISA requires multiple assays to be performed in parallel, which is expensive and time-consuming. However, parallel analyses also increase the risk of error when interpreting data collected at different time points and during different experiments.

•Multiplex measurement also allows the user to collect a large amount of data using smaller samples. In single parallel assays, a separate sample containing the analyte is used for each test. In multiplex assays, one single sample is used to measure multiple analytes.

•They allow us to see how different analytes interact with each other.

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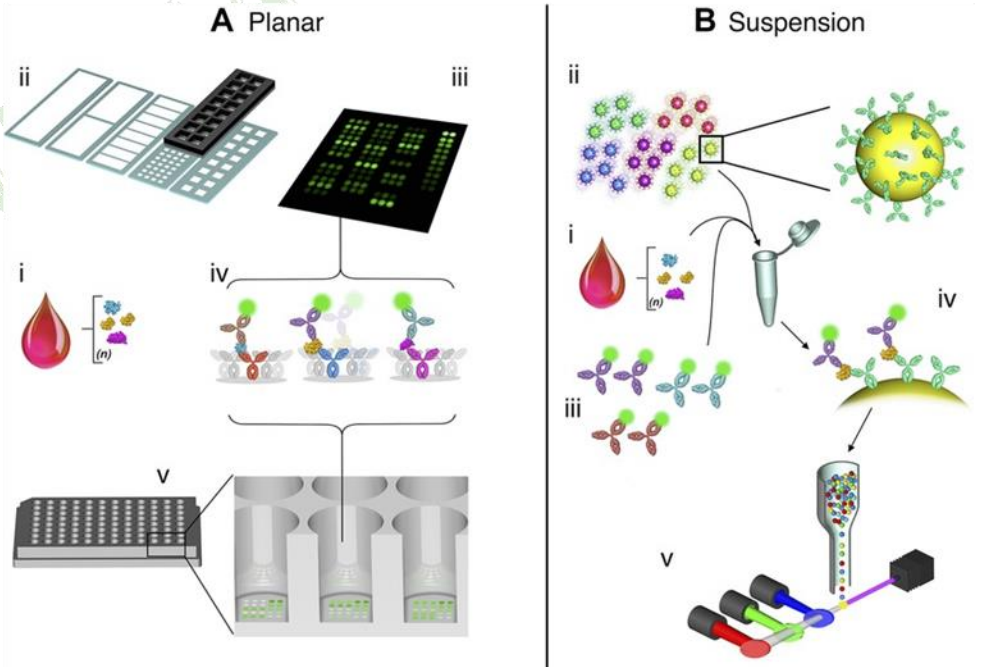
Applications of multiplex measurement

As multiplex assay techniques are highly sensitive, yet highly versatile, they are well suited to clinical applications such as immunology, cancer diagnosis and immuno-oncology. They are particularly useful for biomarker discovery where a panel of analytes are measured together as an indicator for a specific disease, a response to medical treatment, or for stratifying treatments at the patient level.

As they require samples in far smaller quantities than single assay testing, they are particularly valuable where sample volumes are limited. This includes research that uses mice models, research and clinical treatment of neonates and analysis of tumor biopsy samples.



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Multiplex ELISA analysis is intended to simultaneously look for multiple targets in one sample. This approach has been largely adopted in genomics and progressively expands to various domains of laboratory investigation.

In protein analysis, immunoassays are the fundamental methods and their multiplexing and miniaturization is of great applicability to both basic and applied research.

There are two Main strategies of immunoassay multiplexing

planar and bead-based arrays.

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Immunoblotting techniques

- Immunoblotting techniques use antibodies (or other specific ligands in related techniques) to identify target proteins among a number of unrelated protein species. They involve identification of protein target via antigen-antibody (or protein-ligand) specific reactions.
- A laboratory procedure, in which proteins that have been separated by electrophoresis are transferred to a membrane of nitrocellulose or another material and are identified by their reaction with labeled antibodies.
- Immunoblotting allows detection of a protein antigen immobilized on the protein-retaining membrane support such as nitrocellulose or polyvinylidene fluoride (PVDF). The detection of the protein of interest relies on the binding of an antibody that specifically recognizes the protein of interest exposed on the membrane

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Dot immunobinding assay

Immunoblotting procedures in which proteins are transferred from a gel after electrophoretic separation on nitrocellulose membranes and are detected by an immunological assay were introduced by Towbin et al. (1979). Subsequently, it was shown that peptides could be immobilized on nitrocellulose by crosslinking them to the membrane using glutaraldehyde or formaldehyde.

Western blotting

The western blot (protein immunoblot), is a widely used analytical technique in molecular biology and immunogenetics to detect specific proteins in a sample of tissue homogenate or extract. Besides detecting the proteins, this technique is also utilized to visualize, distinguish, and quantify the different proteins in a complicated protein combination.

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METHODOLOGY OF WESTERN BLOTTING

There are six steps involved in western blot.

1. Sample preparation
2. Gel electrophoresis
3. Proteins transfer
4. Blocking
5. Antibody incubation and
6. Proteins detection & Visualization

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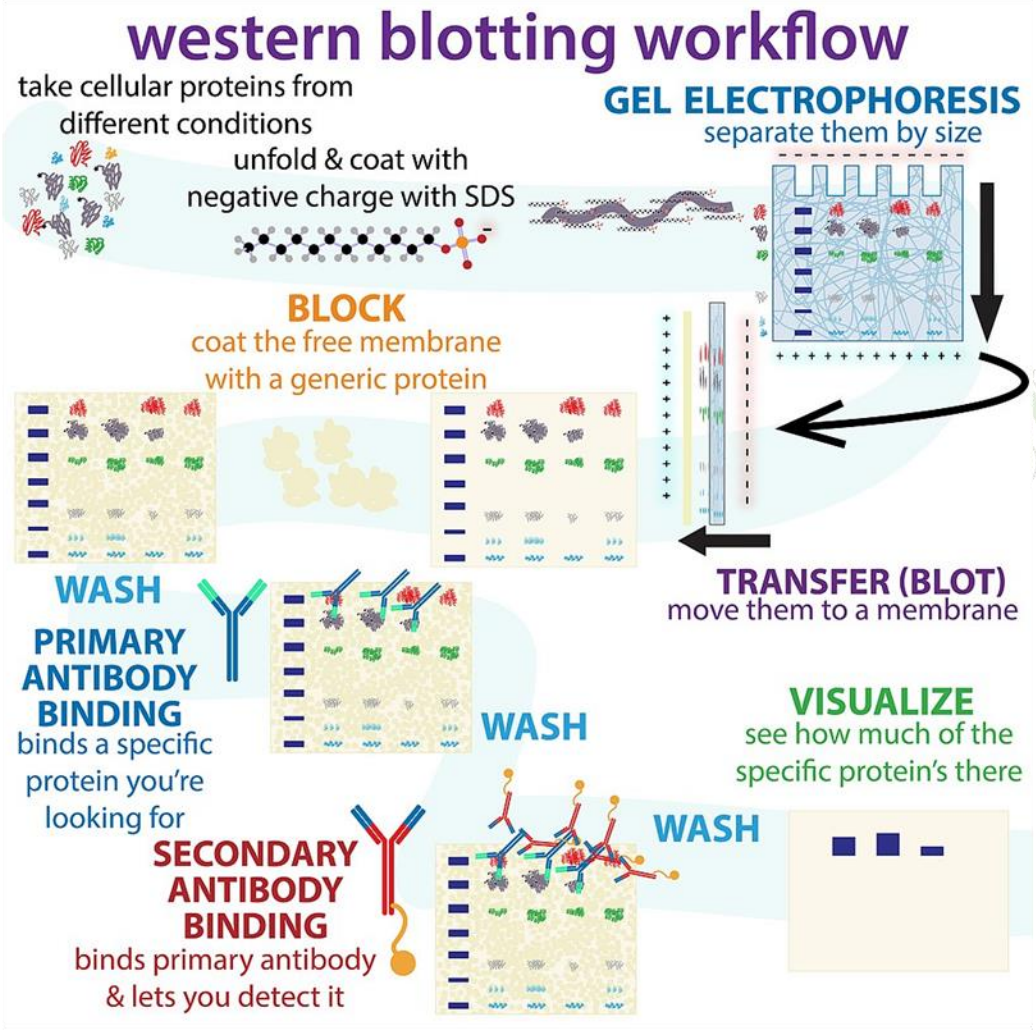
western blotting workflow
take cellular proteins from different conditions
unfold & coat with negative charge with SDS
GEL ELECTROPHORESIS
separate them by size
BLOCK
coat the free membrane with a generic protein
TRANSFER (BLOT)
move them to a membrane
WASH
PRIMARY ANTIBODY BINDING
binds a specific protein you're looking for
WASH
VISUALIZE
see how much of the specific protein's there
SECONDARY ANTIBODY BINDING
binds primary antibody & lets you detect it
WASH