Antigen Antibody Reactions

Kusum Shrestha
Assistant Professor
School of Health and Allied Sciences
Pokhara University

Antigen

- Any substance that satisfies two distinct immunologic properties i.e. antigenicity and immunogenicity.

1. Immunogenicity: It is the ability of an antigen to induce immune response in the body both humoral and cell mediated immunity.
2. Antigenicity: The ability of an antigen to combine specifically with the final products of the above two responses i.e antibodies and cell surface receptors.

Antibody

- Antibody or immunoglobulin is a specialized glycoprotein, produced from activated B cells in response to an antigen, and in capable of combining with the antigen that triggered its production.
- Antibodies are divided into five major classes: IgM, IgG, IgA, IgD, and IgE, based on their constant region structure and immune function

Antigen- Antibody Reaction

- Bimolecular association where the antigen and antibody combine with each other specifically and in an observable manner.

❖Properties of antigen-antibody reaction:

1)Specific: involves interaction of specific epitope of antigen with its paratope of homologous antibody. However, cross reaction may occur due to sharing of antigens

2)Non-covalent interactions: involves interaction like hydrogen bonding, electrostatic interactions

contd

3) Strength : Strength is influenced by affinity and avidity of antigen-antibody interaction

- Affinity: Refers to sum total of non covalent interaction between a single epitope of an antigen with its corresponding paratope present on antibody.
- Avidity: The affinities of all the binding sites when multivalent antibody reacts with a complex antigen carrying multiple epitopes.

Diagnostic Use of Ag-Ab Reactions

- Specific and observable
- The diagnostic tests based on antigen-antibody reactions are called as immunoassays .
- Immunoassays can be broadly categorized into two types-
  - Antigen detection assays
  - Antibody detection assays
- Immunoassays can be performed by both qualitative and quantitative methods.

Qualitative assays

- undiluted specimen containing the antibody is directly mixed with the suspension of antigen or *vice* versa
- Result is read as ‘positive’ or ‘negative’ based on presence or absence of antigen or antibody in the clinical specimen.

Disadvantage

- exact amount of antigen or antibody

present in specimen will not be known

- If either antigen or antibody are present in higher quantity - false negative.
- To rule out a false negative result, it is ideal to test the series of diluted sera (quantitative test) instead of just testing the one specimen of undiluted serum.

Quantitative assays

- When the qualitative test turns positive, the exact amount of antibody in serum can be known by serial dilution of the patient's serum and mixing each dilution of the serum with a known quantity of antigen.
- The measurement of antibody is expressed in terms of units or more commonly titre.
- Quantitative tests are more reliable as they can differentiate between true negative and false negative results.
- This can be explained by Marrack's Lattice hypothesis.

Marrack’s Lattice Hypothesis

- Ag-Ab reaction is weak or fails to occur when the number of antigen and antibodies are not proportionate to each other.
- Prozone phenomenon - In the earlier test tubes, antibodies are excess, hence the Ag-Ab reaction does not occur.
- Post zone phenomenon - In the later test tubes, antigen is excess, hence the Ag-Ab reaction fails to occur.
- Marrack’s Proposed the lattice hypothesis to explain this mechanism
- Ag-Ab reaction optimally occurs when a large lattice is formed consisting of alternate antigen and antibody molecules. This is possible only in the zone of equivalence.

Marrack's or Lattice Hypothesis contd

- In prozone/post zone - Lattice does not enlarge, due to inhibition of lattice formation by the excess antibody or antigen respectively, as the valencies of the antibody and the antigen respectively, are fully satisfied.

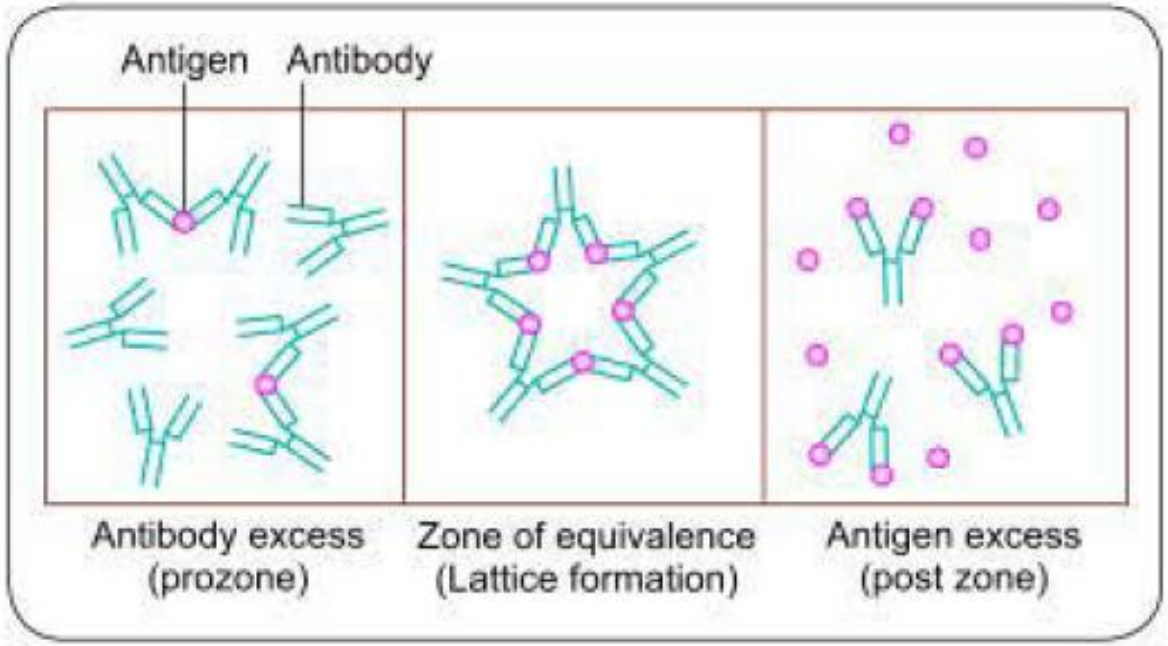


Fig.12.2: Lattice hypothesis

Evaluation of Immunoassays

- Sensitivity and specificity are the two most important statistical parameters.
- Sensitivity is defined as ability of a test to identify correctly all those who have the disease i.e. true positives.
- Sensitivity is calculated as = $\frac{\text{True positives}}{\text{True positives + False negatives}}$
- Specificity is defined as ability of a test to identify correctly all those who do not have disease i.e. true negatives.
- Specificity is calculated as = $\frac{\text{True negatives}}{\text{True negatives + False positives}}$

Types of Ag-Ab reactions

Conventional Techniques
Precipitation Reaction
Agglutination Reaction
Complement Fixation Test
Neutralization Test

Newer Techniques
Enzyme-linked immunosorbent assay (ELISA)
Enzyme linked fluorescent assay(ELFA)
Immunofluorescence Assay(IFA)
Radioimmunoassay(RIA)
Chemiluminescence-linked immunoassay(CLIA)
Rapid Tests ➢ Lateral flow assay(Immunochromatographic test) ➢ Flow through assay
Western Blot
Immunoassay using electron microscope

Precipitation Reaction

- When a *soluble antigen* reacts with its antibody in the presence of optimal temperature, pH and electrolytes (NaCl), it leads to formation of the antigen-antibody complex in the form of:

  Insoluble precipitate band when gel containing medium is used

  or

  Insoluble floccules or precipitate when liquid medium is used

Precipitation in Liquid Medium

- Ring test: In a narrow tube (e.g. capillary tube), antigen solution is layered over an antiserum; a precipitate ring appears at the junction of two liquids.

  Examples: Streptococcal grouping by Lancefield technique, and Ascoli's thermoprecipitin test done for anthrax.

- Flocculation test- When a drop of antigen is mixed with a drop of patient's serum, then floccules appear.

  Examples of slide flocculation test- VDRL and RPR tests used for diagnosis of syphilis.

  Examples of tube flocculation test- Kahn test used previously for syphilis

Precipitation in gel (Immunodiffusion)

- Using 1% soft agarose gel for precipitation reaction has many advantages over liquid medium-
  - -Results in formation of clearly visible bands instead of floccules that can be preserved for longer time.
  - -Can differentiate individual antigens from a mixture as each antigen forms a separate band after reacting with specific antibody.

Principles of Immunodiffusion

- Whether only Ag diffuses (single diffusion) or both Ag and Ab diffuse (double diffusion)

- Whether Ag or Ab diffuses in *one dimension* (i.e. vertical diffusion when test is done on a tube layered with gel) or *two dimensions* (i.e. diffusion in both X and Y axis when test is done on a slide or a petri dish layered with gel)

Types of immunodiffusions in gel

1. Single diffusion in one dimension (Oudin procedure)
2. Double diffusions in one dimension (Oakley- Fulthorpe procedure)
3. Single diffusion in two dimensions (Radial immunodiffusion)
4. Double diffusions in two dimensions (Ouchterlony procedure)

❖ Electroimmunodiffusion

- Electric current is applied to a slide layered with gel.
- Helps in identification and approximate quantitation of various proteins present in the serum with formation of precipitin lines

❖**Counter-current immunoelectrophoresis:**

- *It* involves simultaneous electrophoresis of antigen and antibody in gel in opposite directions resulting in band formation

❖**Rocket electrophoresis**

- One-dimensional single electroimmunodiffusion test.
- Was mainly used in the past for quantitative estimation of antigens.

AGGLUTINATION REACTION

When a particulate or insoluble antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.

- Advantage:
  - More sensitive than precipitation test.
  - Clumps are better visualized and interpreted than bands or floccules.
- Wide diagnostic applications of agglutination reactions.
- Classified as:
  - Direct agglutination reactions.
  - Indirect (passive) agglutination reactions.
  - Reverse passive agglutination reactions.
- Performed either on a slide, or in tube or in card or some time in microtiter plates.

contd

Direct agglutination: Antigen directly agglutinates with the antibody

1)Slide agglutination: use for blood grouping, serotyping of bacteria

2)Tube agglutination: Enteric fever (Widal), Coombs Antiglobulin test

Indirect or passive agglutination test: soluble antigen combined with carrier

- Indirect hemagglutination test (IHA): RBC as carrier
- Latex agglutination test (LAT): latex acts as carrier. Example: detection of ASO

Reverse Passive Agglutination Test for Antigen Detection

Antibody is coated on a carrier molecule which detects antigen in the patient's serum

Test	Carrier Molecule	Clinical applications
RPHA (Reverse passive hemagglutination assay)	RBCs	Detection of hepatitis B surface antigen (HBsAg) – not used now.
Latex agglutination test	Latex particles	Detection of CRP (C reactive protein), RA (rheumatoid arthritis factor
Coagglutination test	*Staphylococcus aureus*	• Stable than latex • Cryptococcal Antigen detection in CSF • Identification of *Streptococci, Neisseria meningitides, Neisseria gonorrhoeae, Hemophilus influenzae*

Direct hemagglutination test

- Serum antibodies directly agglutinate with surface antigens of RBCs to produce a matt. Examples include-
  - Paul Bunnell test – It employs sheep RBCs as antigens to detect Epstein-Barr virus antibodies in serum. Performed in tubes.
  - Coombs test or Antiglobulin test: Performed to diagnose Rh incompatibility by detecting Rh antibody from mother's and baby's serum.

COMPLEMENT FIXATION TEST

Principle: If antibody is present in test serum, it binds with antigen to form antigen-antibody complex. Complement binds to ag-ab complex and gets fixed. In such case, if RBCs are added, there will be no free complement to bind to RBCs and there will be no hemolysis.

Examples:

- Wasserman test for syphilis
- Demonstration of antibodies to *Mycoplasma pneumoniae, Bordetella pertusis*

NEUTRALIZATION TEST: Antigen antibody reaction in which the biological effect of virus and toxins are neutralized by homologous antibodies.

1. Virus neutralization test: Used for diagnosis of influenza, mumps and rubella
2. Toxin neutralization tests: Schick test for diphtheria, Nagler reaction

ELISA(Enzyme linked immunosorbent assay)

ELISA is so named because of its two components:

- **Immunosorbent:** Here, an absorbing material is used (e.g. polystyrene, polyvinyl) that specifically absorbs the antigen or antibody present in serum.
- **Enzyme** is used to label one of the components of immunoassay (i.e. antigen or antibody).

Substrate-chromogen system: A substrate-chromogen system is added at the final step of ELISA.

- The enzyme reacts with the substrate, which in turn activates the chromogen to produce a color.
- Sometime, the substrate is chromogenic in nature (e.g. pNPP). On reaction with the enzyme, it changes its color (Table 12.3).
- The color change is detected by spectrophotometry in an ELISA reader. Intensity of the color is directly proportional to the amount of detection molecule (Ag or Ab) present in test serum.

(Ag-Ab complex)-enzyme + substrate → activates the chromogen → color change → detected by spectrophotometry

contd

- Enzymes: Horseradish peroxidase, alkaline phosphatase
- Substrate:

➢O-phenyl-diamine dihydrochloride for HRP

➢P-nitrophenyl phosphate for ALP

❖Types of ELISA

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

TABLE 12.4: Principles of different ELISA types

ELISA type	Used for detection of	Enzyme is labeled with
Direct ELISA	Antigen	Primary antibody
Indirect ELISA	Antibody or antigen	Secondary antibody
Sandwich ELISA	Antigen	Primary antibody in direct sandwich ELISA Secondary antibody in indirect sandwich ELISA
Competitive ELISA	Antigen or antibody	Secondary antibody
ELISPOT	Cells producing antibody or cytokine	Primary antibody

Note: Primary antibody is directed against the antigen, secondary antibody is an antihuman (or other species) Ig directed against Fc region of any human/other species Ig

CHEMILUMINESCENCE-LINKED IMMUNOASSAY (CLIA)

Chemiluminescence refers to the emission of light (luminescence), as a result of a chemical reaction. The principle of CLIA is similar to that of ELISA; however, the chromogenic substance is replaced by chemiluminescent compounds (e.g. luminol and acridinium ester) that generate light during a chemical reaction (luxogenic). The light (photons) can be detected by a photomultiplier, also called as luminometer.

Ag-Ab-[Luminol] + [hydrogen peroxide] → Ag-Ab-[Products]+light(photons) → detected by luminometer

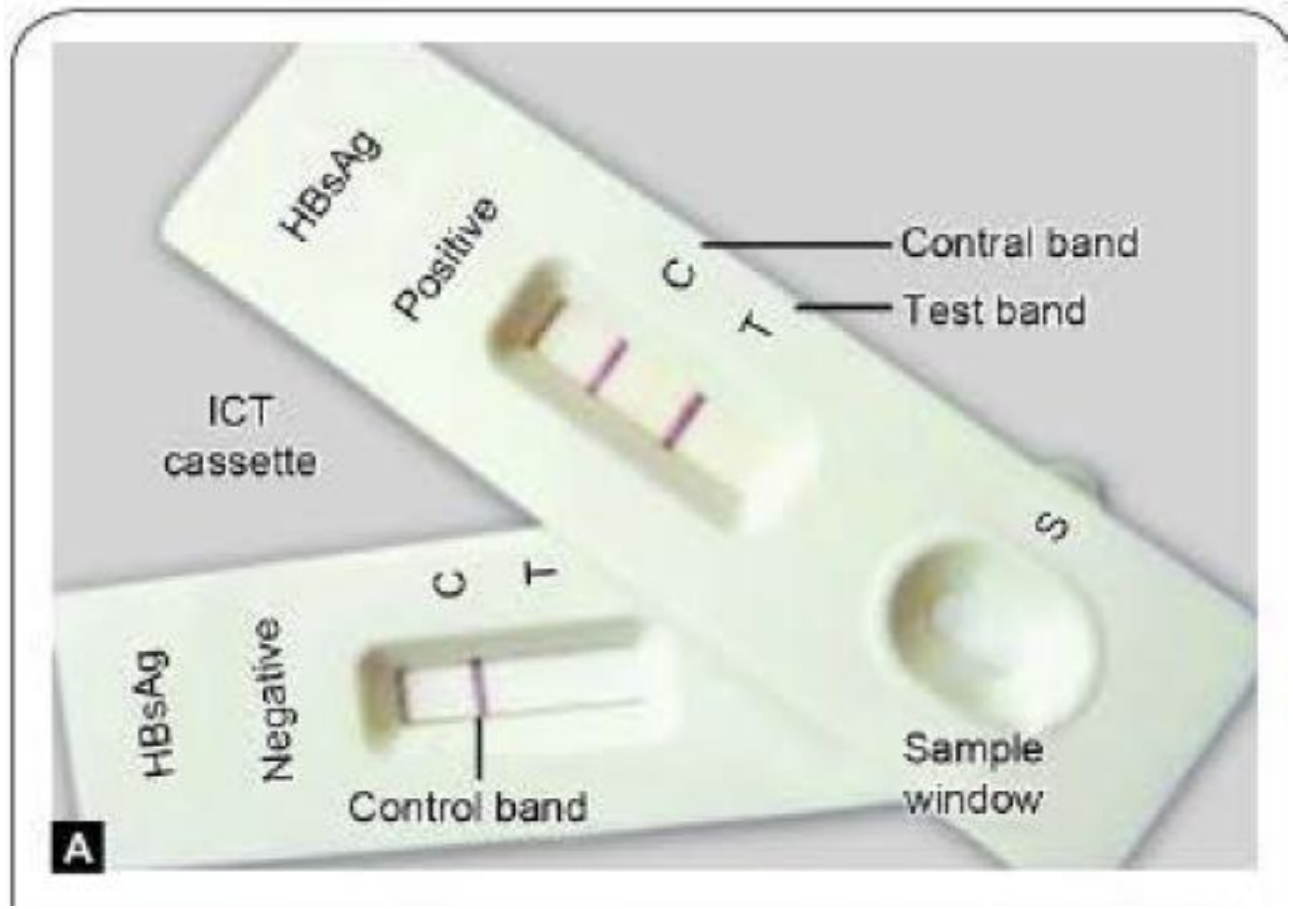
Immunohistochemistry:

- process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of using labeled antibodies binding specifically to the antigens in biological tissues.
- It can be based on principles of ELISA or IFA
- It is widely used in the diagnosis of abnormal cells (e.g. tumor cells).

❖**Rapid tests**: lateral flow assay and flow through assay

Immunochromatographic Test (Lateral flow assay)

- **Principle:**
  - Test system consists of a nitrocellulose membrane and an absorbent pad.
  - NCM coated at two places in the form of lines- A test line, coated with monoclonal antibody targeted against test antigen and a control line, coated with anti-species immunoglobulin.
  - The test antigen is added to sample well which reacts with Ab labelled with chromogenic marker present in sample pad
  - Both Ag-specific Ab-colloidal gold complex as well as free colloidal gold labelled Ab move laterally along the nitrocellulose membrane
  - Formation of Ag-Ab conjugate complex= positive reaction.
- Antigen detection- Malaria, Hepatitis B,
- Antibody detection- HIV, HCV, HAV


HBsAg
Positive
C
T
Contral band
Test band
ICT
cassette
S
C
T
HBsAg
Negative
Control band
Sample
window
A

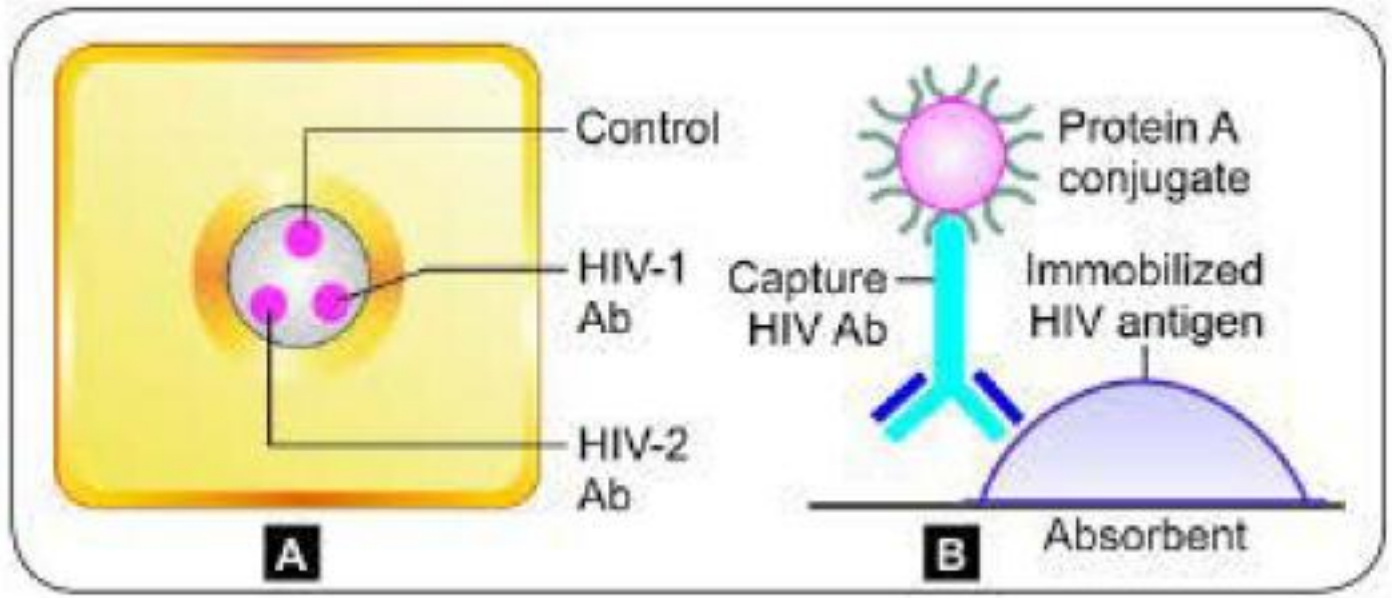
Flow Through Assay

- Protein A is used for labelling antibody instead of gold conjugate
- Sample flows vertically through the nitrocellulose membrane
- Example: HIV Tridot
- Principle:

➢ Cassete Format; consisting of a NCM and an absorbent pad

➢NCM is coated at three regions- two test regions coated antigens and third control region coated with antihuman Ig.

➢Patient’s sample pass through the membrane and if Ab present; bind to immobilized antigens.



Figs 12.24A and B: Flow-through assays. **A.** HIV TRI-DOT assay for HIV 1 and 2 antibodies detection; **B.** Principle of HIV TRI-DOT

Immunofluorescence

Principle

Fluorescence refers to absorbing high energy-shorter wavelength ultraviolet light rays by the fluorescent compounds and in turn emitting visible light rays with a low energy-longer wavelength.

- The fluorescent dye is used to conjugate the antibody and such labeled antibody can be used to detect the antigens or antigen–antibody complex on the cell surface.
- The fluorescent compounds commonly used-fluorescein isothiocyanate (FITC).

- Detects presence of specific antigen in biological specimens, eg.*T. pallidum*
- Detection of autoantibodies in autoimmune disease

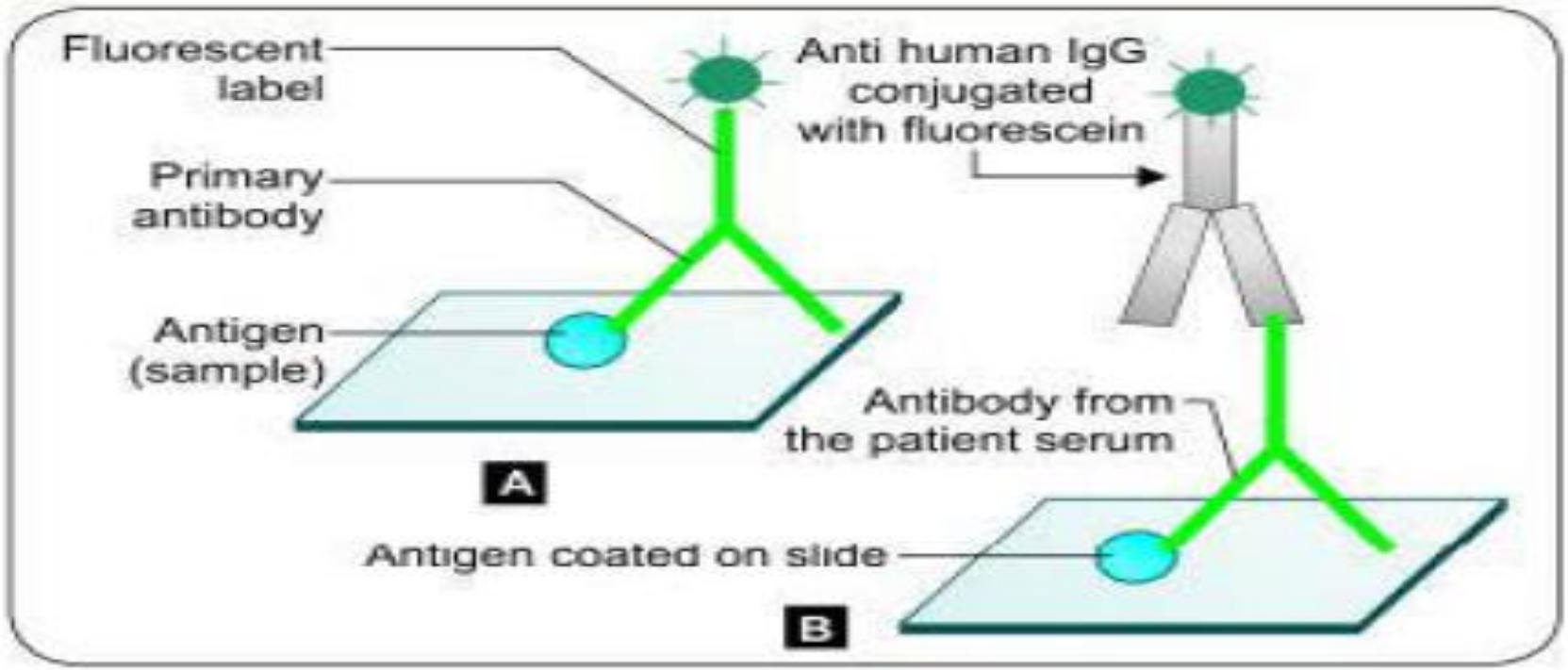


Fig. 12.20: Immunofluorescence assay. **A.** Direct; **B.** Indirect

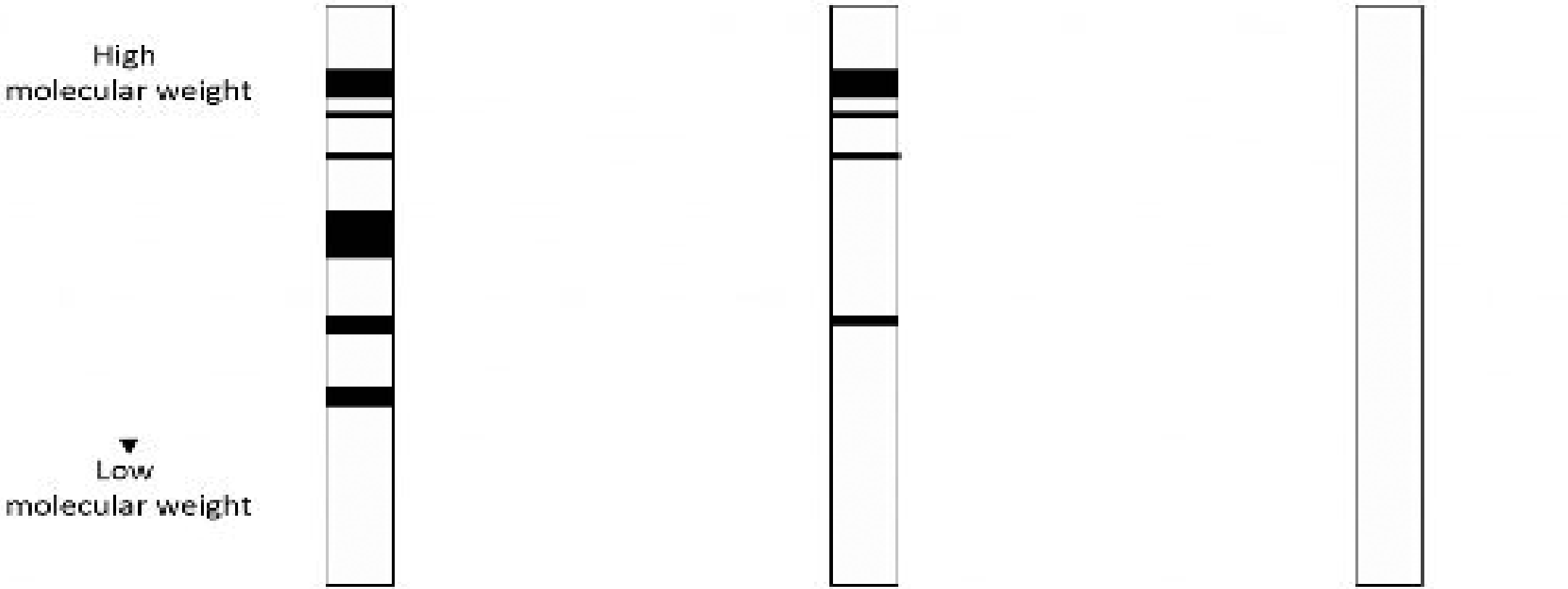
Radio-immuno assay

- Very sensitive and specific technique used for quantitative detection of antigens.
- Similar to Immunofluorescence, but tagging is by radio-isotopes to antigen or antibodies.
- Widely used in detection/quantitation of hormones, drugs, tumor markers, vitamins and proteins.

Western blotting

- Method to detect many antibodies against a composite antigen.
- Detection of antibody in various disease such as HIV, Herpes simplex virus.
- Viral antigen are electrophoretically separated in nitrocellulose strip on basis of size and shape
- Now the strip is incubated with test sample containing antibody
- After washing an enzyme labeled conjugate is added
- After another wash substrate is added
- Colored bands appear at the site of initial antibody reactivity

Drawing of a western blot result



Control
ensures the test
is working properly

Positive result
shows purified target proteins
according to molecular weight

Negative result
shows no target proteins

❖Immunoelectron microscopy:

- Viral particles appear to be clumped when mixed with specific antisera and observed under the electron microscope.
- Used for finding hepatitis A virus particles from stool specimen

❖Immunoferritin

- Ferritin particle used to label an antibody which can bind to surface antigens of cells and tissues.
- When visualized under electron microscope, the electron dense label absorbs more electrons and appears as small black dot thus confirming the presence of antigen.

Flowcytometry

- Laser based technology
- Cells (e.g; CD4 T cells) coated with specific fluorescent tagged antibodies (e.g; anti-CD4) are mixed with sheathed fluid and are passed through a special instrument called ultrasonic nozzle vibrator
- Fluorescent tagged cells are excited by laser beam and collected on separate chambers based on their electrical charge
- Used in T cells count in HIV/AIDS

TABLE 12.2: Immunoassays and the types of molecule used for labeling

Abbreviation	Immunoassay method	Molecules used for labeling	Type of visible effect
ELISA	Enzyme-linked immunosorbent assay	Enzyme	Color change is detected by spectrophotometer
IFA	Immunofluorescence assay	Fluorescent dye	Emits light, detected by fluorescence microscope
RIA	Radioimmunoassay	Radioactive isotope	Emits β and γ radiations, detected by β and γ counters
CLIA	Chemiluminescence-linked immunoassay	Chemiluminescent compounds	Emits light, detected by luminometer
IHC	Immunohistochemistry	Enzyme or fluorescent dye	Color change (naked eye) or fluorescence microscope
WB	Western blot	Enzyme	Color band (naked eye)
Rapid test	Immunochromatographic test	Colloidal gold or silver	Color band (naked eye)
	Flow-through assay	Protein A conjugate	Color band (naked eye)
IEM	Immunoferritin electron microscopy	Electron dense molecules (e.g ferritin)	Appears as black dot under electron microscope

THANK-YOU