Lab 4

RIA,ELISA,IMMUNOCYTO/HISTO CHIMESTRY

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radio- immune assay (RIA) and ELISA

- When the antigen or antibody is labeled with a radioisotope, it may be quantified by instruments that detect radioactive decay events; the assay is called a radioimmunoassay (RIA).

- When the antigen or antibody is covalently coupled to an enzyme, the rate at which the enzyme converts a clear substrate to a colored product may be quantified by a spectrophotometer and then blotting the wavelength (absorbance) with a corresponding concentration of target antigen on a slop (different pattern for each antigen) ; the assay is called an enzyme-linked immunosorbent assay (ELISA).

Radio- immune assay (RIA) and ELISA applications

- Measure amount quantities of antigens or antibody (protein) :

  - Hormones

  - Drugs

  - Tumor markers

  - Abs

  -Viral and bacterial antigens

Enzyme Immunoassays

- Enzyme immunoassays (EIAs) can be used for detection of either antigens or antibodies in serum and other body fluids of the patient.
- In EIA techniques, antigen or antibody labeled with enzymes are used. Alkaline phosphatase, horseradish peroxidase, and galactosidase.
- Following the antigen–antibody reaction, chromogenic substrate specific to the enzyme peroxidase, alkaline phosphatase, etc.) is added.
- The reaction is detected by reading the optical density.
- Usually, a standard curve based on known concentrations of antigen or antibody is prepared from which the unknown quantities are calculated.

There are four main general steps to completing an ELISA immunoassay. These steps are:

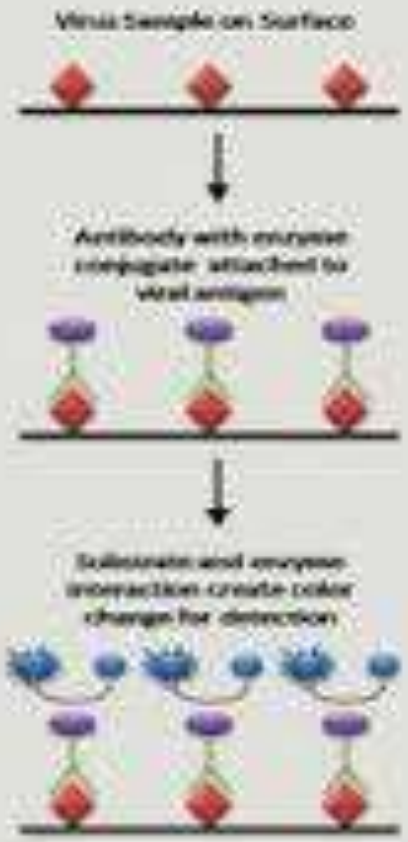
1.Coating (with either antigen or antibody)
2.Blocking (typically with the addition of bovine serum albumin [BSA])
3.Detection
4.Final read

There are four major types of ELISA:

- Direct ELISA (antigen-coated plate; screening antibody)
- Indirect ELISA (antigen-coated plate; screening antigen/antibody)
- Sandwich ELISA (antibody-coated plate; screening antigen)
- Competitive ELISA (screening antibody

DIRECT ELISA

- Apply a sample of known antigen to a surface.
- Enzyme linked primary antibody is applied to the plate.
- Washed, After this wash, only the antibody-antigen complexes remain attached.
- Apply a substrate which is converted by the enzyme to elicit a chromogenic signal.



Antibody detection

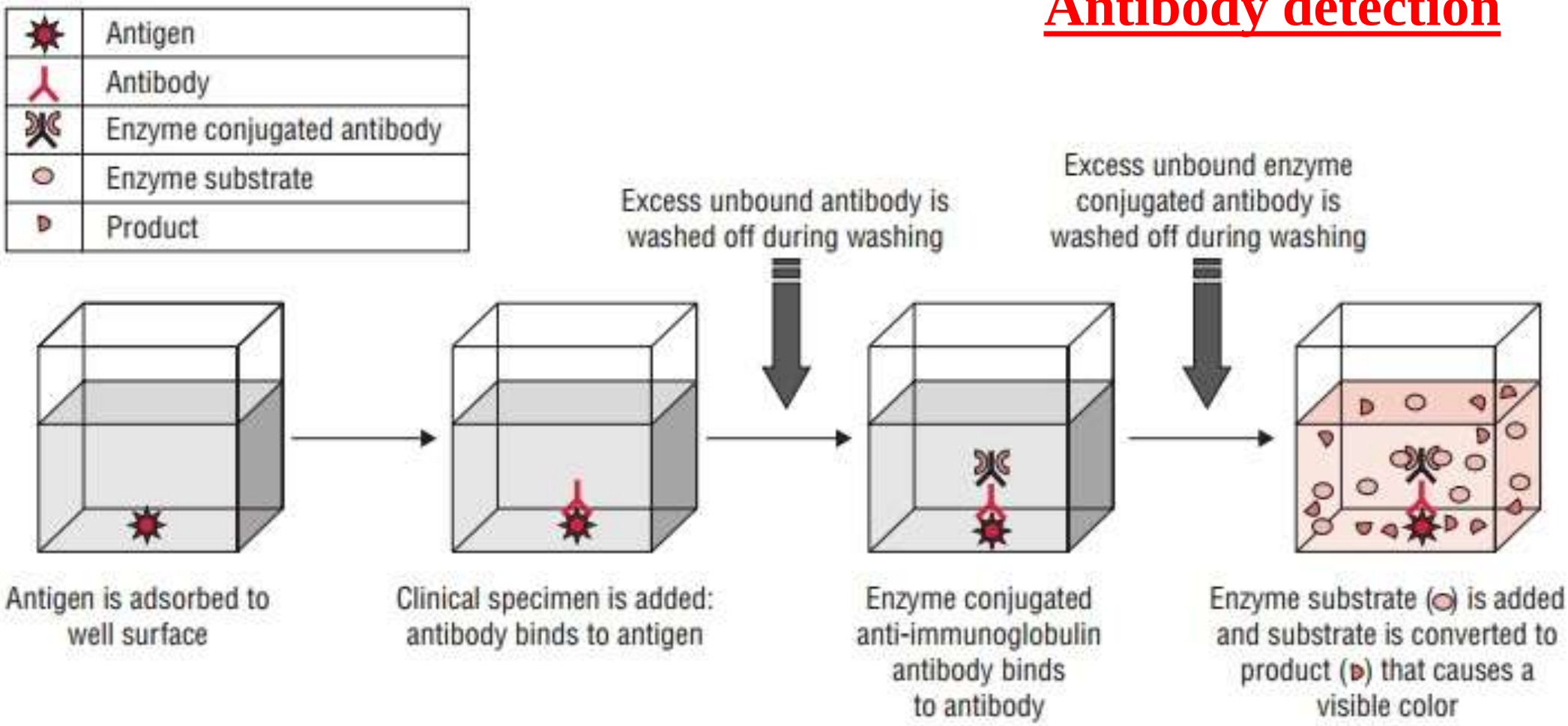


FIG. 14-14. Indirect ELISA test.

Antigen Detection

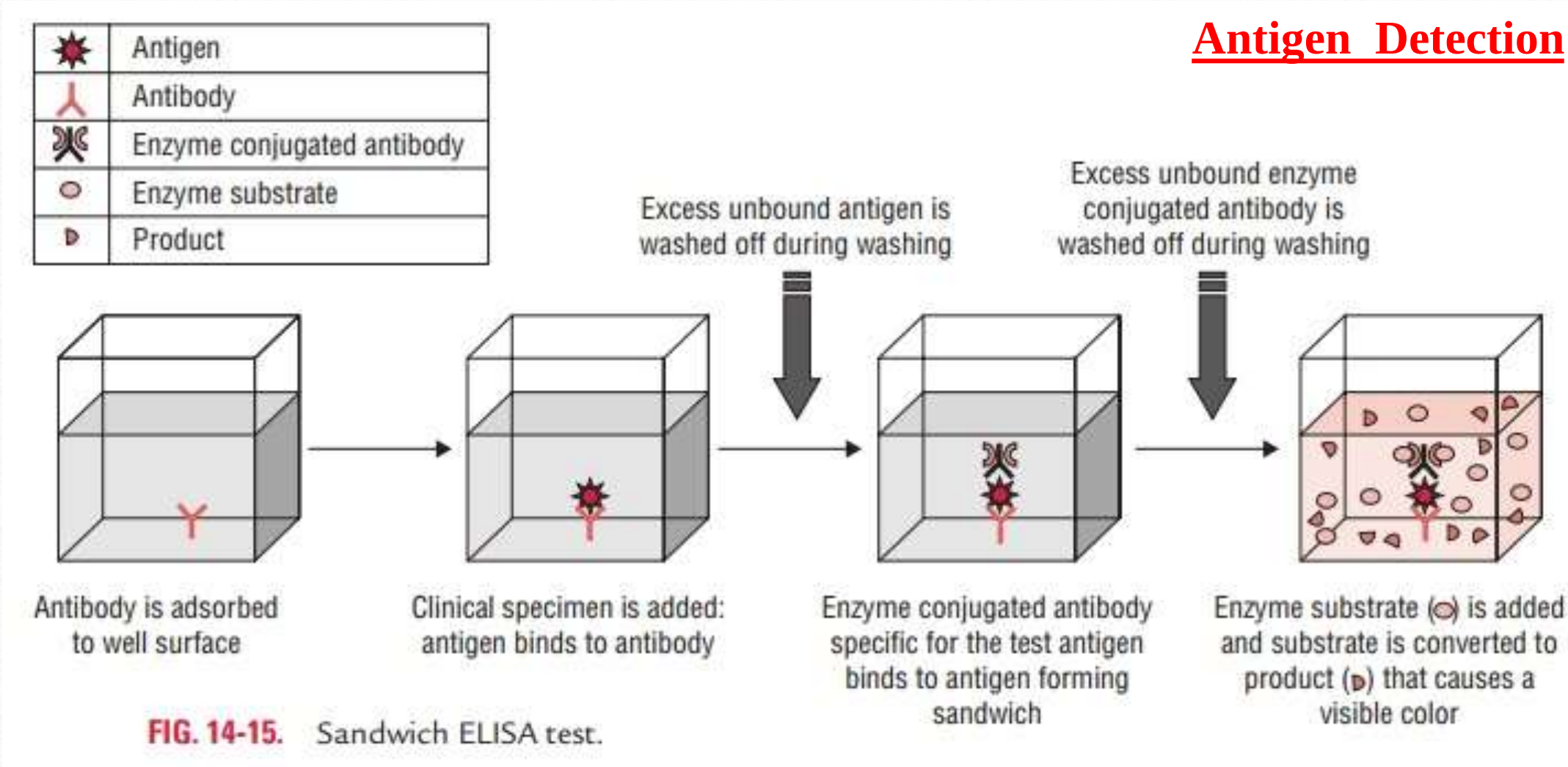
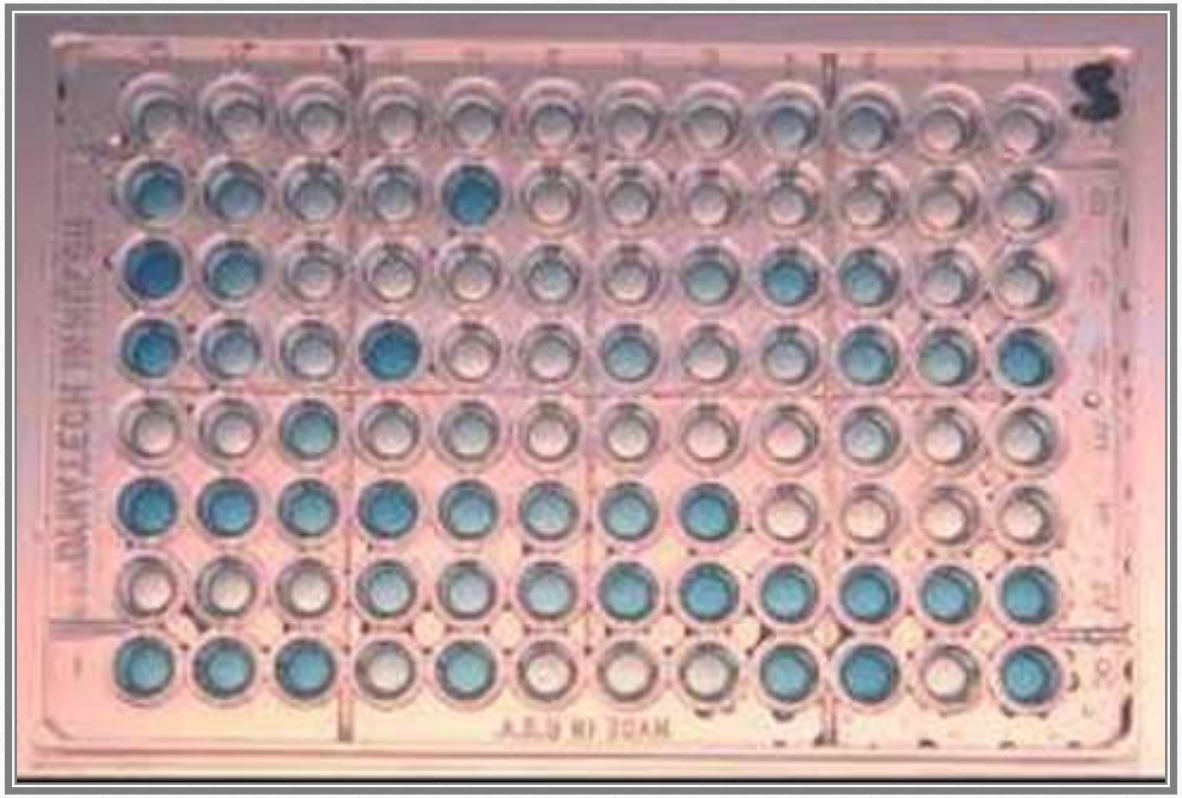


FIG. 14-15. Sandwich ELISA test.

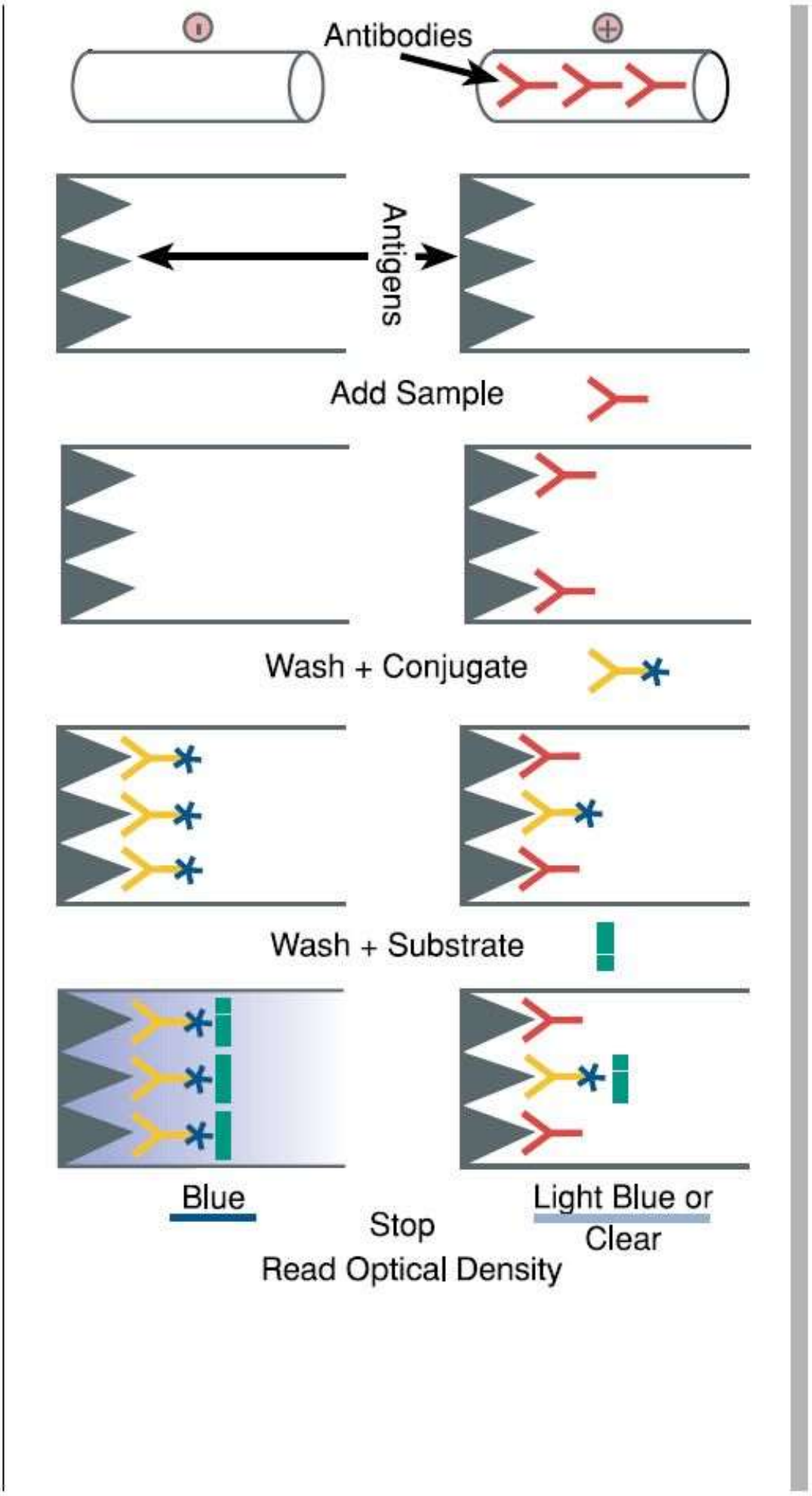
ELISA plate



Competitive ELISA

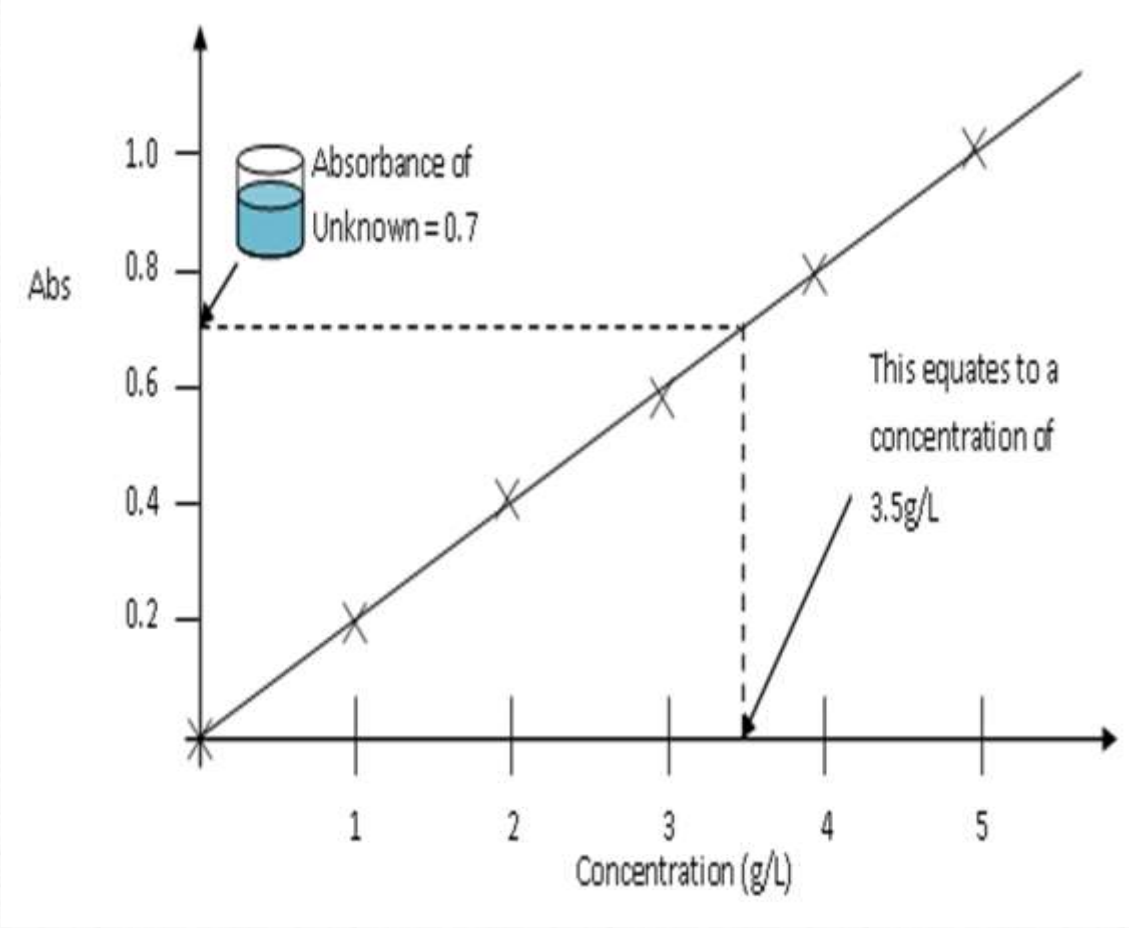
- Used for the estimation of **antibodies** present in a specimen, such as serum.
- Principle of the test is that two specific antibodies, one conjugated with enzyme and the other present in test serum (if serum is positive for antibodies), are used.
- Competition occurs between the two antibodies for the same antigen.
- Appearance of color indicates a negative test (absence of antibodies), while the absence of color indicates a positive test (presence of antibodies).

Competitive ELISA



Spectrophotometer for reading the color

Spectrophotometry absorbance and antigen concentration slop



Western Blotting Techniques

- Blotting is a technique by which a macromolecule such as DNA, RNA, or protein is resolved in a gel matrix, transferred to a solid support, and detected with a specific probe.
- These powerful techniques allow us to identify and characterize specific molecules in a complex mixture of related molecules
- Some of the more common techniques include:

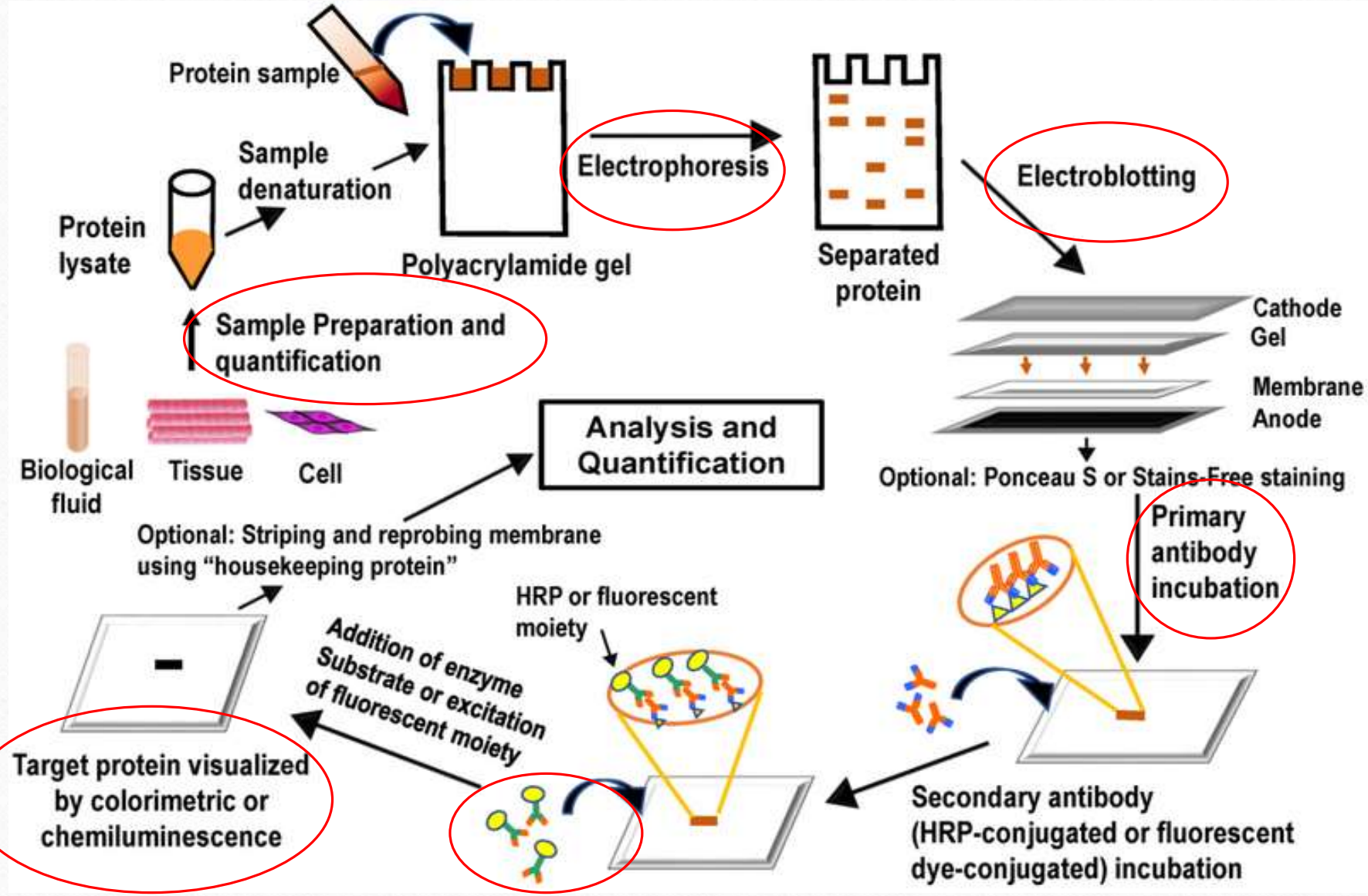
✓ Southern blotting (DNA)
✓ Northern blotting (RNA)
✓ Western blotting (for protein)

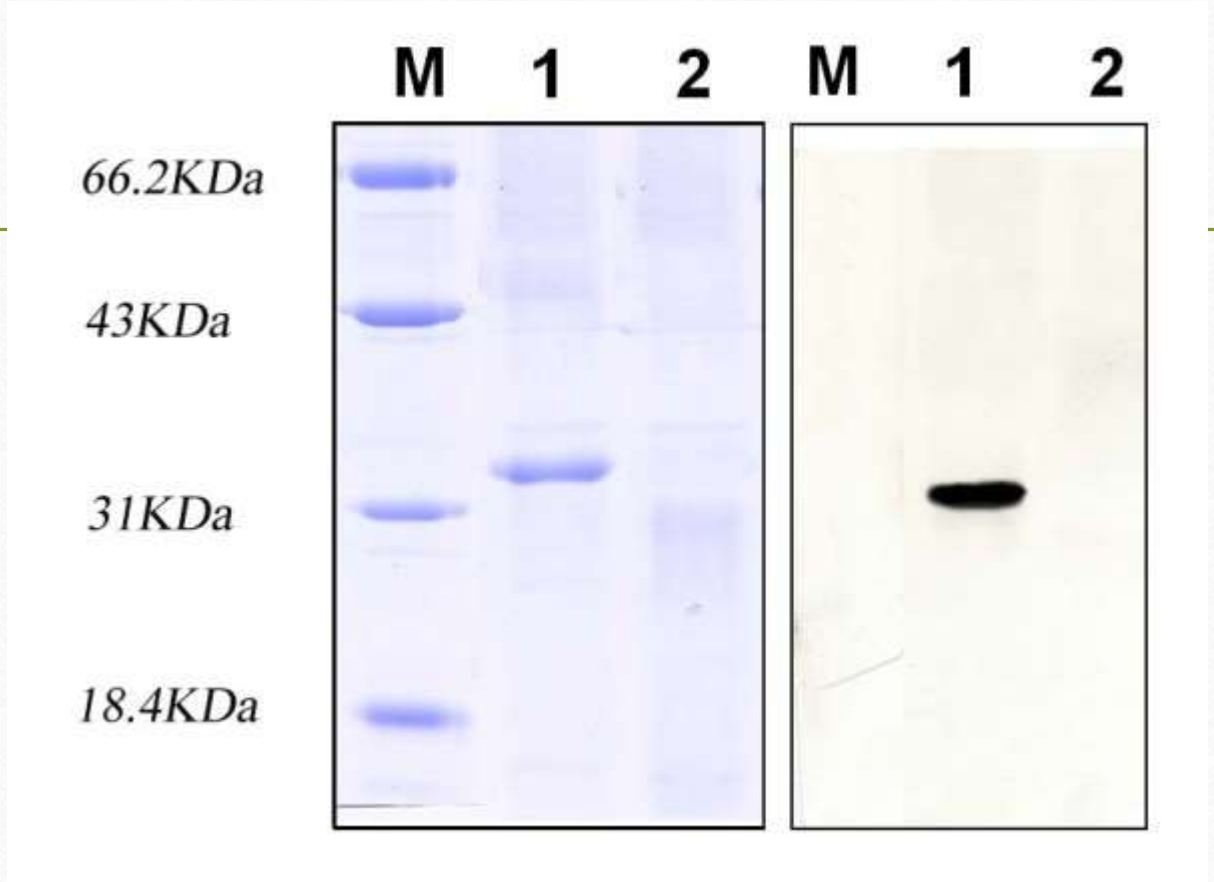
Western blot

- In this technique a mixture of proteins is separated based on molecular weight through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein, the specific protein is identified by binding specific radiolabeled or enzyme linked antibody. Identification of HIV antibodies
- When the proteins transferred from gel to membrane## and The position of the protein antigen on the membrane is depending on molecular weight,
- then it can be detected by an antibody that may be conjugated to an enzyme such as horseradish peroxidase, that generate signals and leave images on photographic film. Or by radiolabeled antibody

Steps involved in western blotting

1. Sample preparation
2. Gel Electrophoresis
3. Blotting (or transfer)
4. Blocking
5. Antibody Probing
6. Detection


Protein sample
Sample denaturation
Protein lysate
Polyacrylamide gel
Electrophoresis
Separated protein
Electroblotting
Cathode
Gel
Membrane
Anode
Optional: Ponceau S or Stains-Free staining
Primary antibody incubation
Secondary antibody (HRP-conjugated or fluorescent dye-conjugated) incubation
HRP or fluorescent moiety
Addition of enzyme Substrate or excitation of fluorescent moiety
Target protein visualized by colorimetric or chemiluminescence
Optional: Striping and reprobing membrane using "housekeeping protein"
Analysis and Quantification
Sample Preparation and quantification
Biological fluid
Tissue
Cell


M
1
2
M
1
2
66.2KDa
43KDa
31KDa
18.4KDa

Tests for Cell/ tissue Associated Antigens.Immuno-cyto/histo-chemistry

Detection of cell surface or intracellular antigens

1. **Immunofluorescence**
   Immunofluorescence is a technique whereby an antibody labeled with a fluorescent molecule (fluorescein) Determine the anatomic distribution of antigen in tissues using the fluorescence emitted by the bound antibody. Uses in tumor antigen detection, ANA in SLE and Rheumatoid arthritis and in autoimmune diseases.
   - **Direct Immunofluorescence**
     attaching fluorescein to a specific mouse antibody directed against the antigen of interest,
   - **Indirect Immunofluorescence**
     In indirect immunofluorescence, fluorescein can be attached to a second anti-antibody (e.g., rabbit anti-mouse Ig antibody) that is used to bind to the first unlabeled antibody. Indirect fluorescence is more sensitive than direct immunofluorescence since there is amplification of the signal.

Direct immunofluorescence test
Is widely used for detection of bacteria, parasites, viruses, fungi, or other antigens in CSF, blood, stool, urine, tissue biopsy, and other specimens.

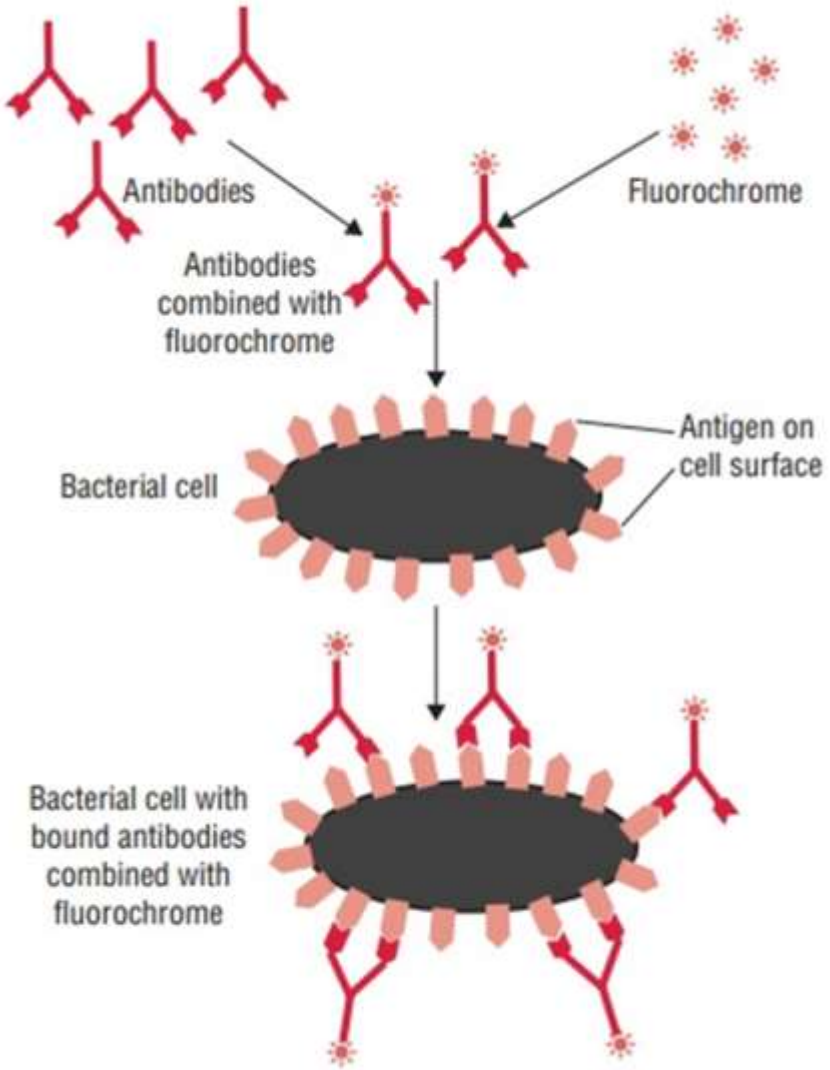


FIG. 14-13. Direct fluorescent antibody test.

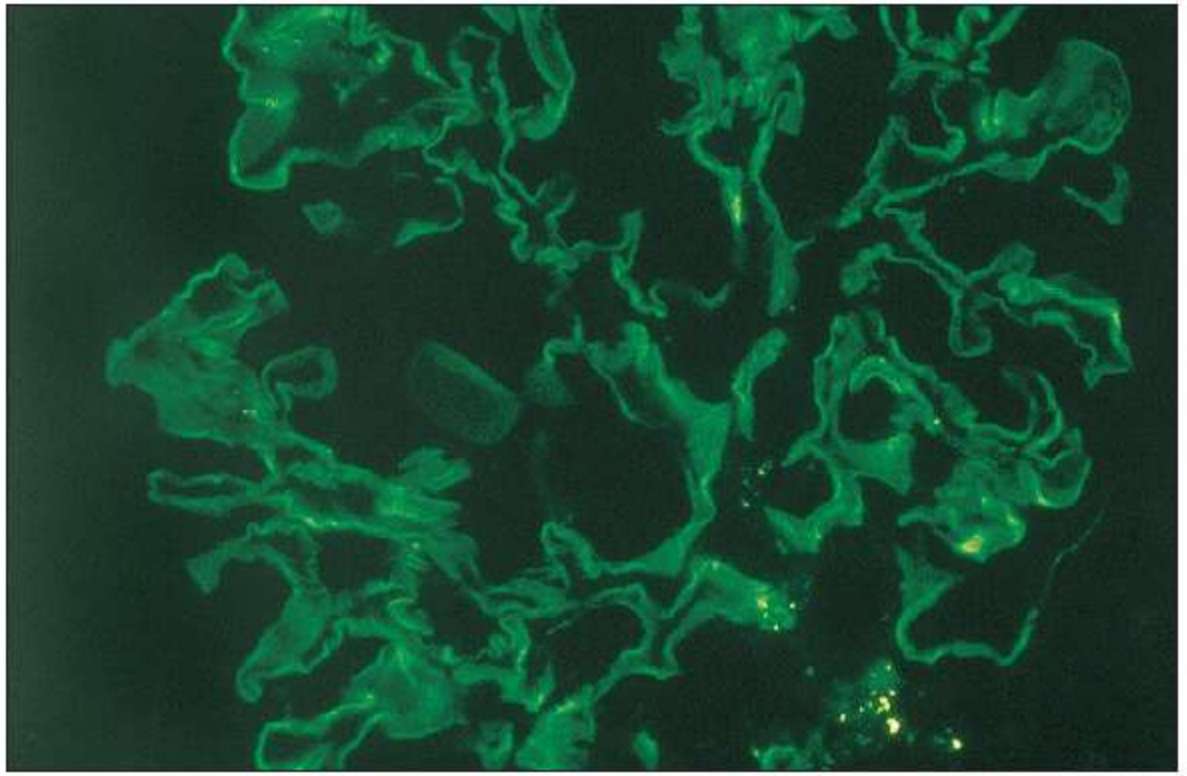
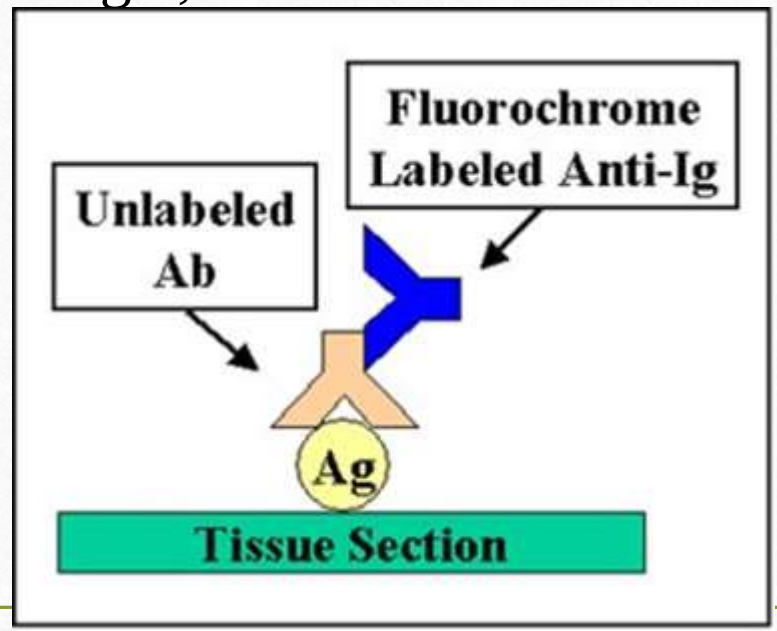


Figure 53-10 Direct immunofluorescence microscopy reveals global and linear glomerular basement membrane immunofluorescence in anti–glomerular basement membrane disease (×400).

Indirect immunofluorescence test

Indirect immunofluorescence is a two-stage process.

- **First stage**, a known antigen is fixed on a slide. Then the patient's serum to be tested is applied to the slide, followed by careful washing. If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide.
- **Second stage**, the combination of antibody with antigen can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope.

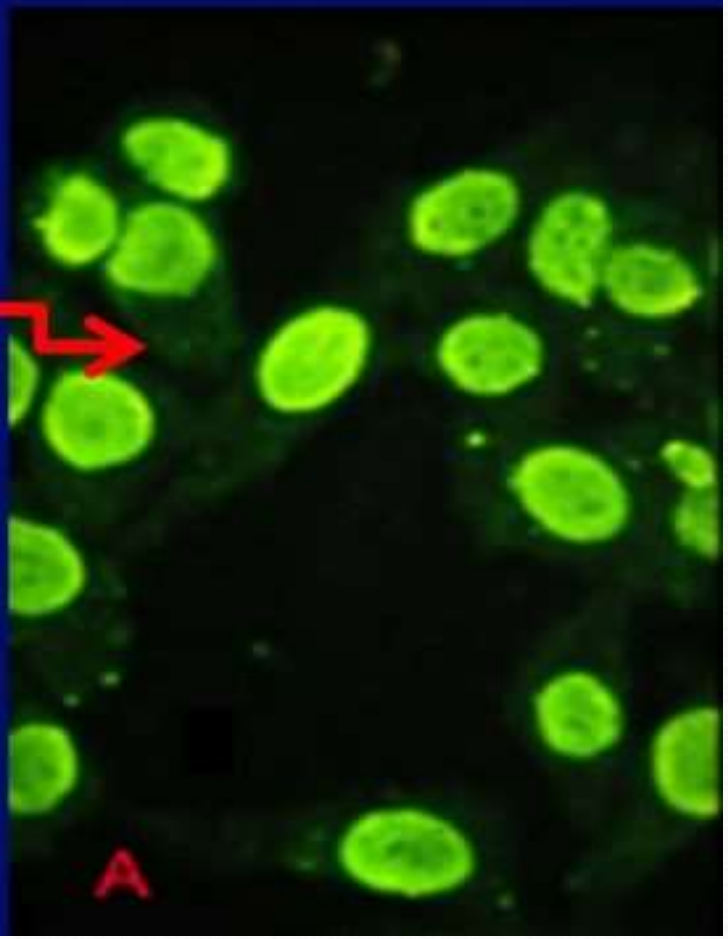


Test ANA in autoimmune diseases

- Antinuclear antibodies (ANA) are a category of antibodies that targets the **nucleus** of other cells.
- The ANA blood test used to help diagnose certain autoimmune conditions, particularly **lupus**.
- Patient serum is added to a slide containing cells. If the patient has autoantibodies to the nuclei of the cells, they bind to the slide.
- After washing away any antibodies that don't bind, an antibody against human antibody is added.
- This antibody has radiolabeled molecules attached to it which, when viewed under Immunofluorescent microscope (light-up green).

Auto antibodies

- The anti-nuclear antibody (ANA) test is the best screening test for SLE and is determined by **immunofluorescence** or **ELISA** tests
- The ANA is positive in significant titer (usually 1:160 or higher) in virtually all patients with SLE



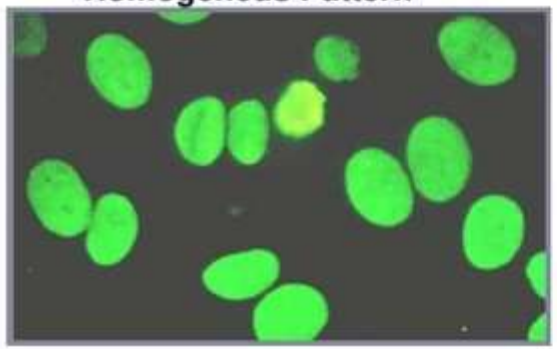
Systemic Lupus Erythematosus (SLE)

Patterns of ANA Test

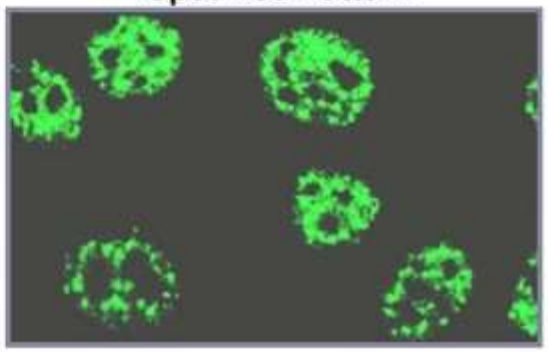
Screening Techniques

IIF (Indirect Immunofluorescence)

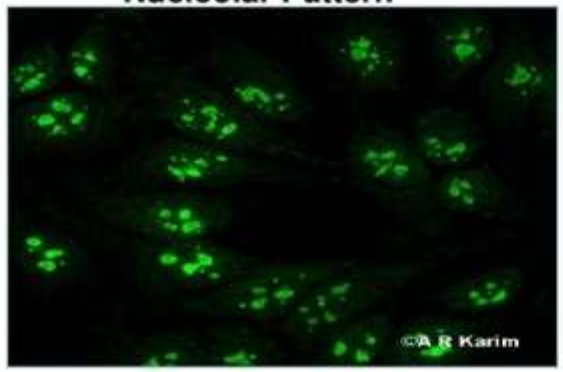
Homogeneus Pattern



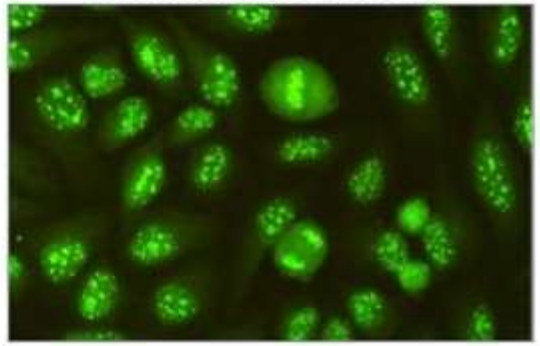
Speckled Pattern

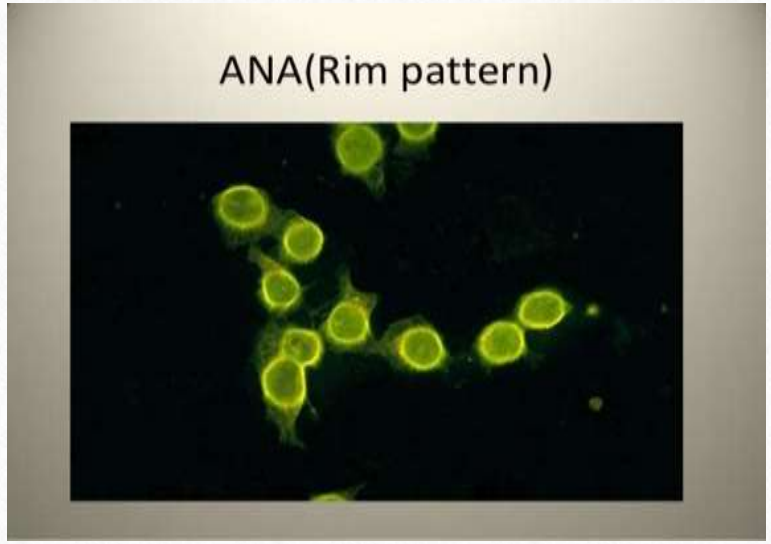


Nucleolar Pattern



Centromere-B Pattern





Example

HOMOGENEOUS PATTERN

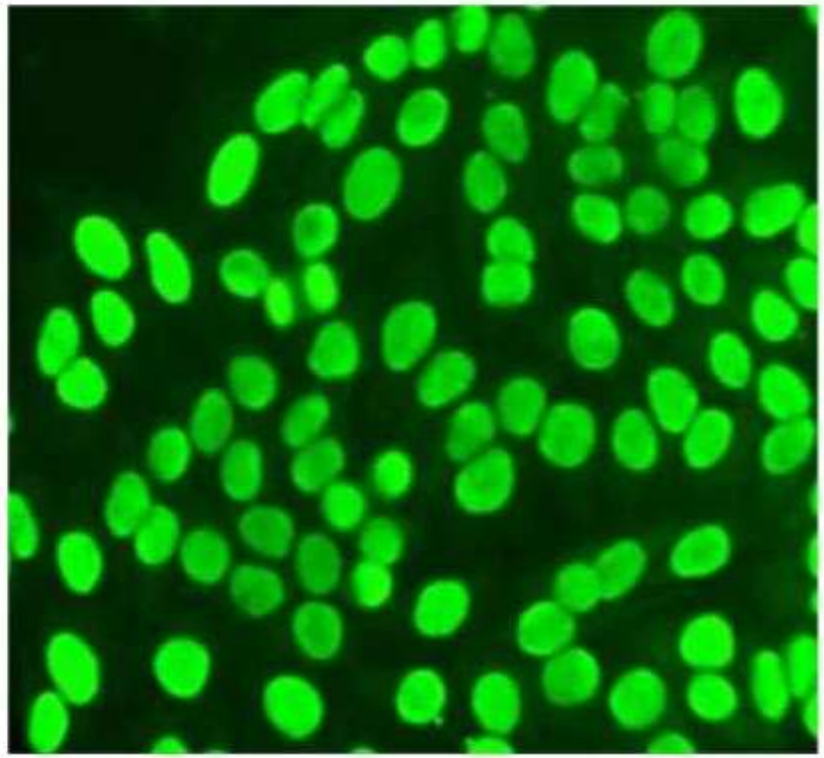
Uniform diffuse fluorescence staining of the entire nucleus in interphase cells.

ANTIGENS:

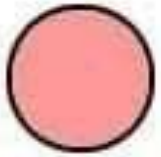
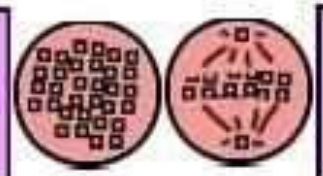
- DNA, DNA-histone

DISEASES:

- SLE (very specific)
- Drug-induced lupus
- RA **(Rheumatoid Arthritis)**
- Juvenile chronic arthritis
- Systemic sclerosis



ANA Patterns

Peripheral (rim)		Anti-DNA (not seen on HEP-2)	SLE
Homogeneous (diffuse)		Anti-DNA Anti-histone Anti-DNP (nucleosomes)	RA & SLE Misc. Disorders (anti-ssDNA)
Speckled		Anti-Sm & RNP Anti-Ro & La Anti-jo-1 & Mi-2) Anti-Sci-70	SLE & SS PM/DM PSS (Systemic)
Centromere		Anti-centromere	PSS (CREST)
Nucleolar		Anti-nucleolar	SLE & PSS

- Although sensitive, the fluorescence microscope is not an ideal tool to identify the detailed structure of the cell or tissue because of a low structural details.
- This problem has been overcome by new technologies including **confocal microscopy**.

- Antibody can be coupled to an electron-dense probe such as colloidal gold, and the location of antibody can be determined subcellular by means of an **electron microscope**.