Laboratory techniques

Labeled immunoassay

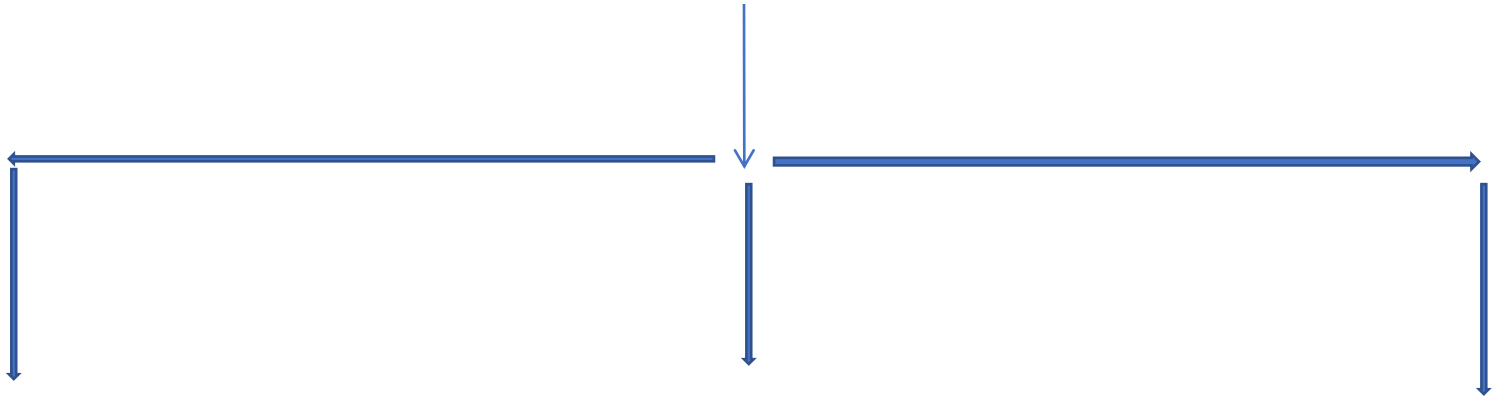
2023-2024

LEARNING OUTCOMES:

After finishing this lab, you should be able to:

- Describe major characteristics of colorimetric, radioactive, and fluorescent labeled immunoassays.
- Outline the steps of direct (to detect antigen) and indirect enzyme-linked immunosorbent assay (ELISA) (to detect an antibody) in a patient sample.
- Describe the difference between direct and indirect immunofluorescence techniques.
- Explain the principle of competitive binding in immunoassay design (RIA).
- Outline the principle of Immunohistochemistry (IHC).
- Identify an appropriate immunoassay for a particular analyte (Biomarker).
- Discuss the clinical applications for each technique.

Labeled Immunoassay



Fluorescent immunoassay

Label: fluorochrome

Fluorescent Microscope

Enzyme Immunoassay

Label: Enzyme

Photometric (Spectrophotometer)

Radioimmunoassay(RIA)

Label: radioactive substance

^{3}H - Tritiated hydrogen

^{125}I - Iodine 125

Gama Counter

Labeled Immunoassay

- labeled immunoassays are designed to improve analytical sensitivity, which allows for detecting substances at much lower concentrations using instrumentation equipped with sensitive detectors.
- Measure a wide variety of substances found in blood, urine, and tissues which is often called the analyte (or biomarker).
- Antibodies used in immunoassays must be very specific and have a high affinity for the antigen in question.
- **Specificity** helps to reduce cross-reactivity, and the **affinity** determines how stable the binding is between antigen and antibody. These two factors help to determine the **sensitivity** of such immunoassays.

[**Specificity** → **reduce cross-reactivity**]

[**Affinity** → **stable the binding**]

[**Specificity + Affinity = Sensitivity**]

FLUORESCENT ANTIBODY TESTS

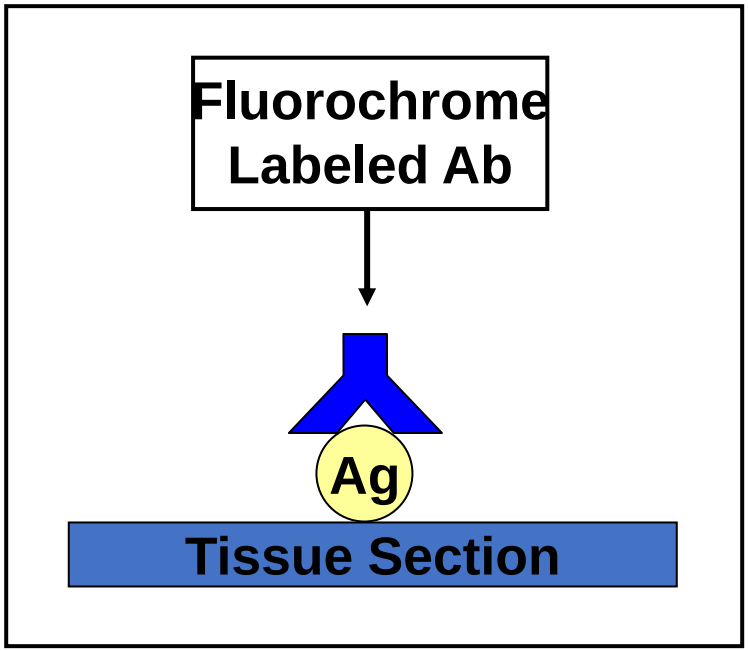
- Fluorochromes are fluorescent compounds that absorb energy from an incident light source and convert that energy to light of a longer wavelength.
- Direct immunofluorescence assays involve antigen detection through a specific antibody that is labeled with a fluorescent tag. The presence of fluorescence is detected with a fluorescent microscope that utilizes UV light.
- In indirect immunofluorescent assays, the original antibody is unlabeled. Incubation with antigen is followed by addition of a second fluorescent-labeled anti-immunoglobulin that detects antigen–antibody complexes.

FLUORESCENT ANTIBODY TESTS

- **The direct fluorescent antibody test (DFA**) is used to detect and localize antigen in the patient. The tissue sample to be tested is treated with antibodies against that particular antigen that have been labeled with a fluorescent dye. If the antigen is present in the tissues, the fluorescent-labeled antibodies will bind, and their binding can be detected with a fluorescence microscope.
- Variations of this test are used to diagnose respiratory syncytial virus, herpes simplex- 1 and -2, and Pneumocystis infections.

Immunofluorescence

- Qualitative
- Direct
  - Samples (specimens) fixed on slide
  - Ab to tissue Ag (is labeled with a fluorochrome)
  - Incubation + Washing + Visualization (fluorescent microscopy)



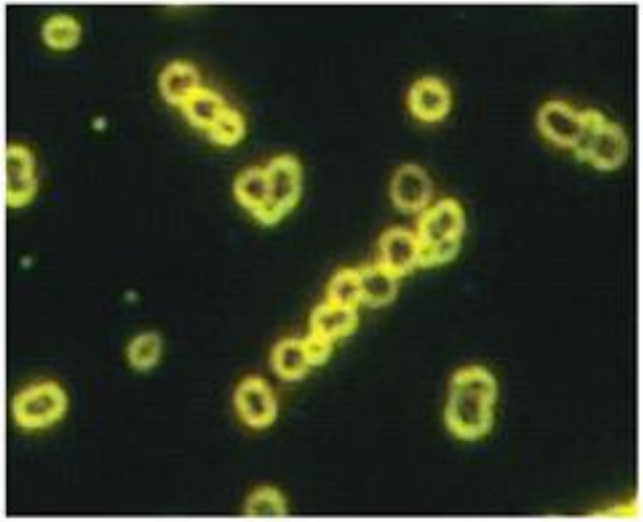
Direct Immunofluorescence

Group A streptococci from patient's throat

Fluorescent dye–labeled antibodies to group A streptococci

Fluorescent streptococci

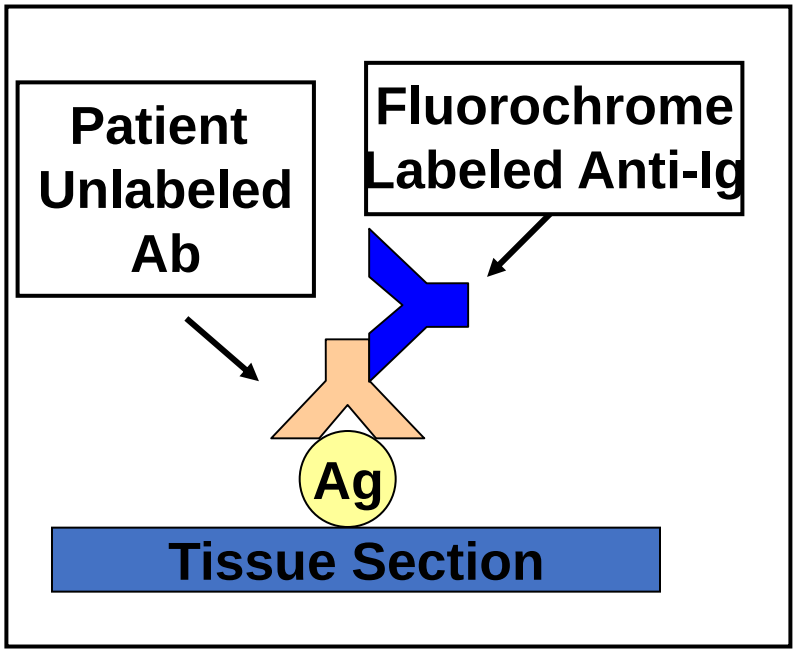
(a) Reactions in a positive direct fluorescent-antibody test



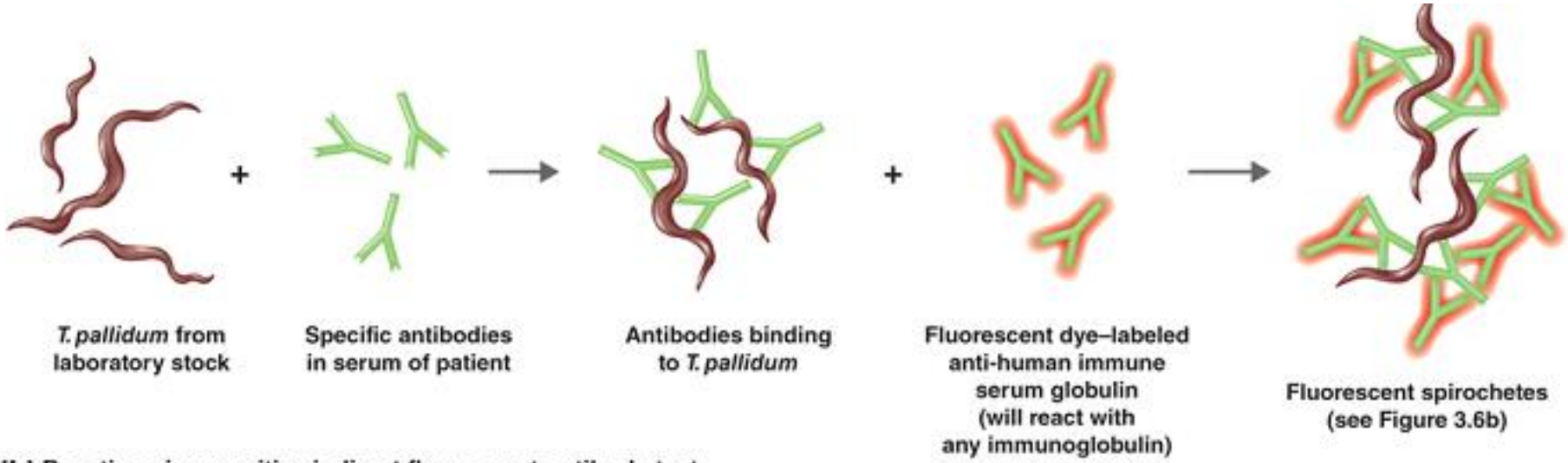
- **The indirect fluorescent antibody test (IFA)** is used to detect pathogen-specific antibodies in the patient. In this case, a laboratory-generated sample of infected tissue is mixed with serum from the patient. A fluorescent dye-labeled anti-immunoglobulin (raised in animals) is then added. If binding of antibodies from the patient to the tissue sample occurs, then the fluorescent antibodies can be bound, and fluorescence can be detected in the tissue by microscopy.
- This technique is used to detect antinuclear antibodies, anti-dsDNA antibodies, antithyroid antibodies, antiglomerular basement-membrane antibodies, and anti-Epstein-Barr virus viral-capsid antigen antibodies.

Immunofluorescence

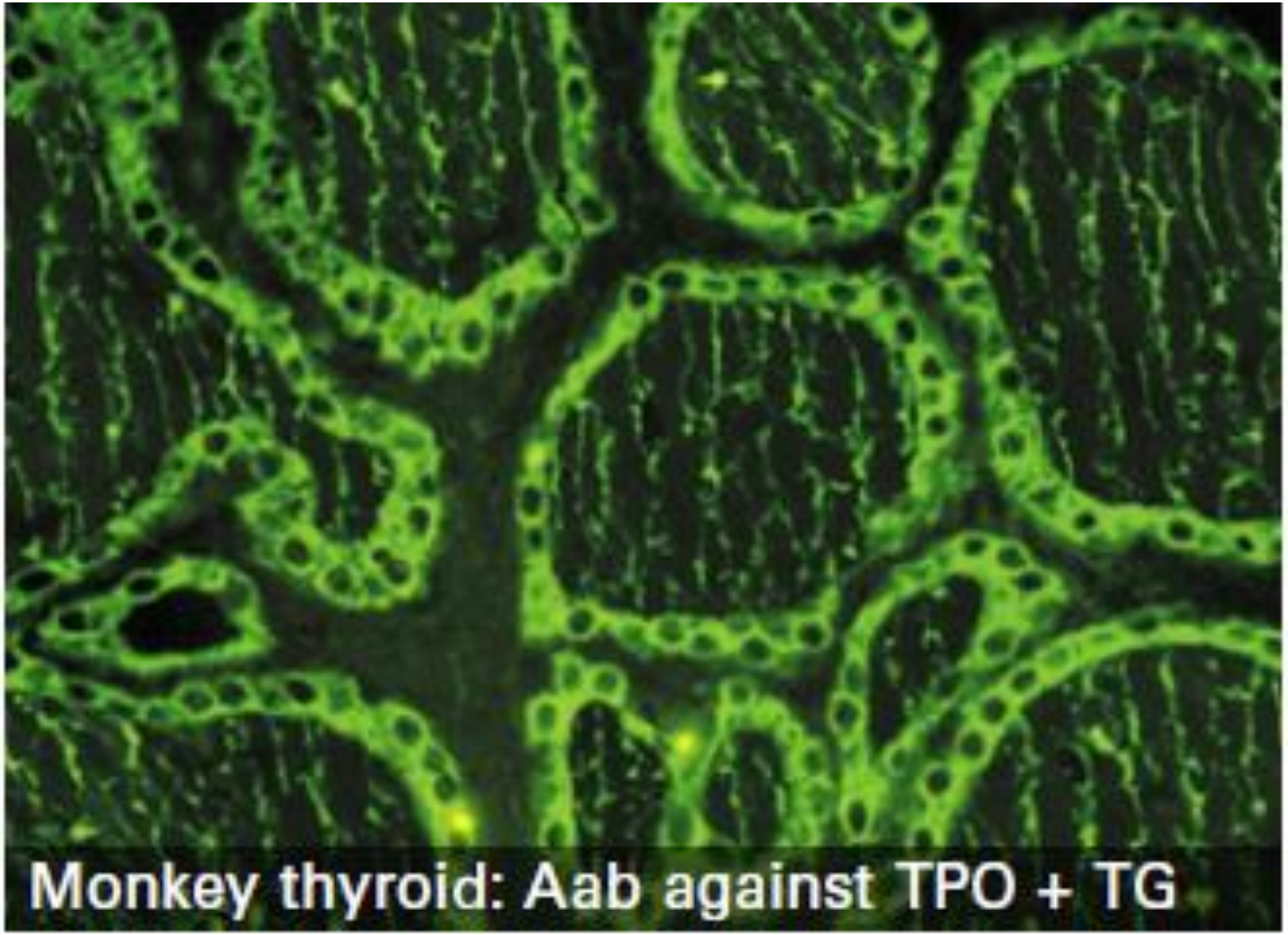
- Indirect
  - ➢Patient serum may contain antibody (primary Ab) added to……
  - ➢Slides with fixed tissue Ag
  - ➢Incubation + washing
  - ➢Secondary Fluorochrome-labeled anti-Ig is used to detect binding of the first Ab.
  - ➢Washing + visualization



Indirect Immunofluorescence



(b) Reactions in a positive indirect fluorescent-antibody test

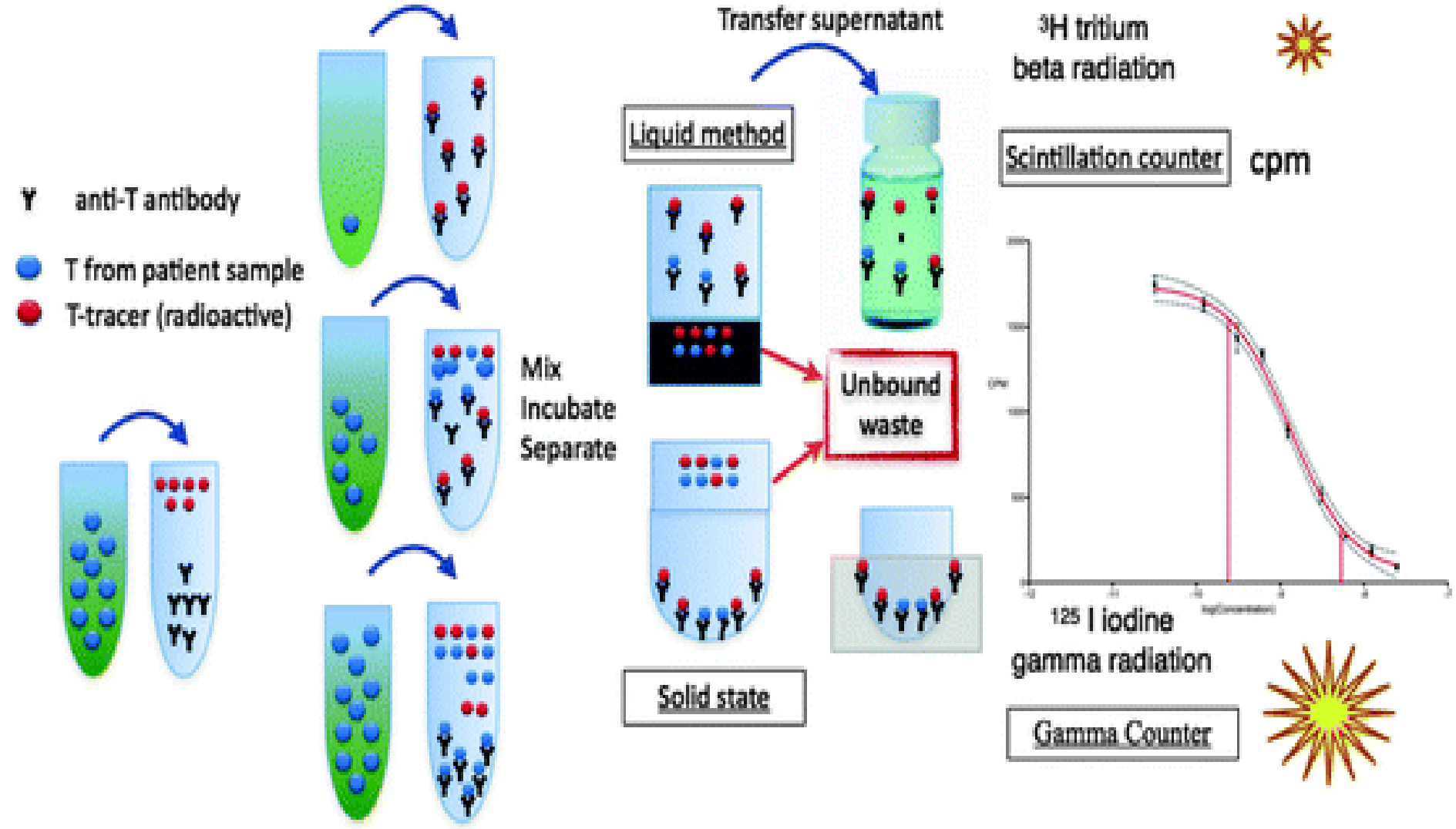

Monkey thyroid: Aab against TPO + TG

RADIOIMMUNOASSAY (RIA) AND ENZYME-LINKED IMMUNOABSORBENT ASSAY (EIA OR ELISA)

- RIA and ELISA are extremely sensitive tests that are common in medical laboratories. They can be used to detect the presence of hormones, drugs, serum proteins, infectious disease antigens, and tumor markers.
- Both tests are conducted similarly, but the RIA uses the detection of a **radiolabeled** product and the ELISA detects the presence of **enzyme-mediated color changes in a chromogenic substrate**.
- **Radioimmunoassays (RIAs)** are based on a competitive principle, using a radioisotope label. These assays have extremely high analytical sensitivity but come with the disadvantages inherent in using radioactive substances.

RIA - METHODOLOGY

Competitive binding between testosterone in sample and T-tracer (radioactive)

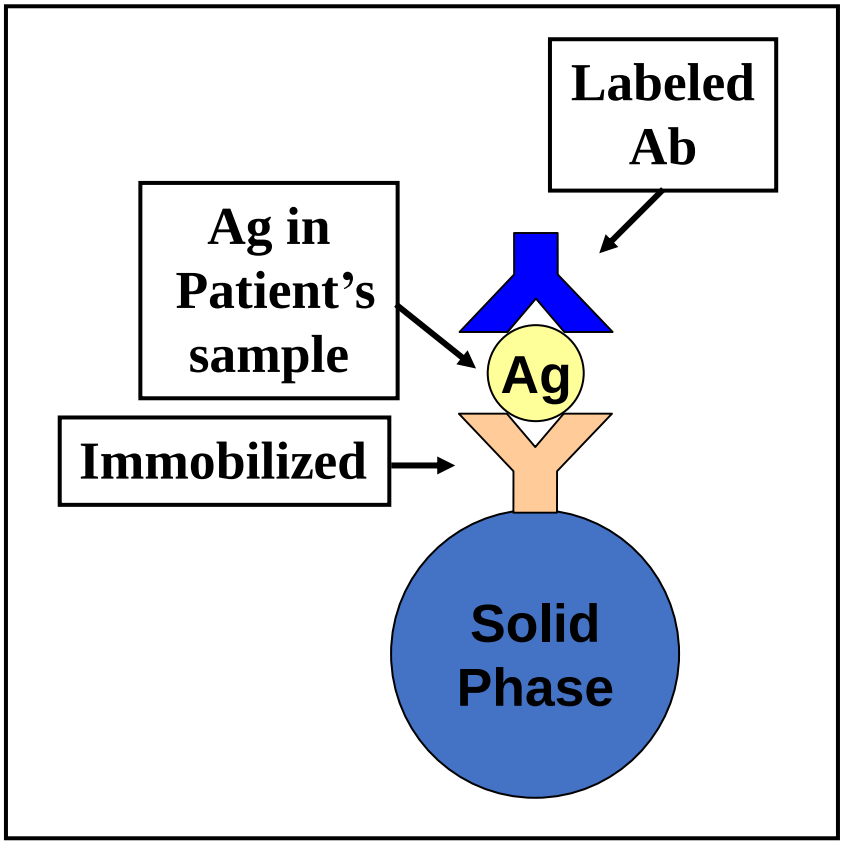


ELISA

- **Enzyme immunoassays (EIAs)** use enzymes that catalyze biochemical reactions. They convert reagent substrates to produce chemically modified products that can be detected.
- Immunoassays that detect antigen are termed capture immunoassays.
- In capture or sandwich immunoassays, the antibody is bound to a solid phase, and any patient antigen is allowed to bind or be captured.
- A second enzyme-labeled antibody is added. The final complex formed with sample antigen in between the two reagent antibodies creates the "sandwich."

Double Antibody ELISA(direct)

- Ag detection
  - Immobilize Ab
  - Incubate with sample
  - Add labeled antibody
  - Amount of labeled Ab bound is proportional to the amount of Ag in the sample
  - (Sandwich)

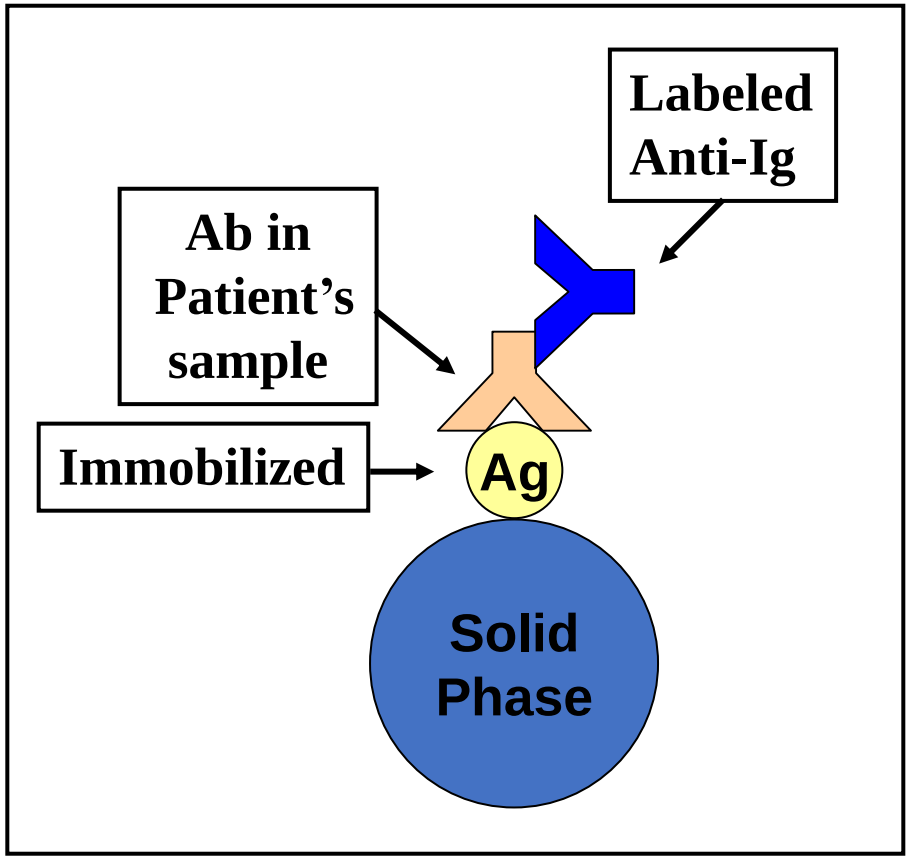


- Quantitative

- Application
  - Screening for HIV infection by detection of p24 antigens in patient serum
  - Measurement of insulin level in patient serum

Indirect ELISA

- Ab detection
  - Immobilize Ag
  - Incubate with sample
  - Add labeled anti-Ig
  - Amount of labeled Ab bound is proportional to amount of Ab in the sample



- Quantitative

- Application
  - Detection of antibodies against SARS-CoV-2 in patient serum
  - Screening for hepatitis B infection by detecting anti-HBV antibodies in patient serum.

Applications

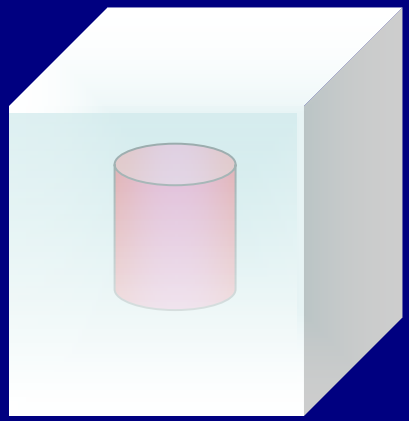
- Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool both for determining serum antibody concentrations (such as with the HIV test or West Nile Virus) and also for detecting the presence of antigen.
- It has also found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds, and eggs.

Immunohistochemistry

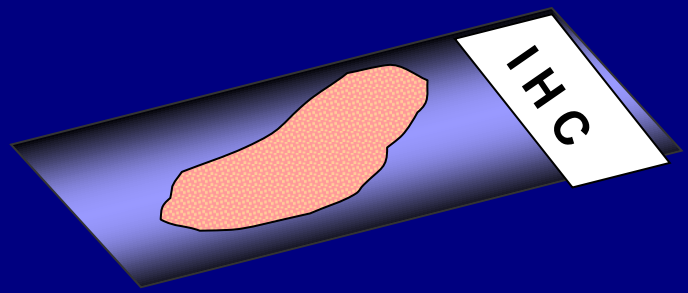
- Immunohistochemistry (IHC) combines histological, immunological, and biochemical techniques for identifying **specific tissue components** by means of a specific antigen/antibody reaction tagged with a **visible label**.
- IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue.
- Immunohistochemistry uses antibodies covalently conjugated with enzymes to visualize cells and tissues. Once the antibodies are bound, substrates are added that are converted to products, which form colored precipitates that are deposited at the site of antibody binding.

Immunohistochemistry

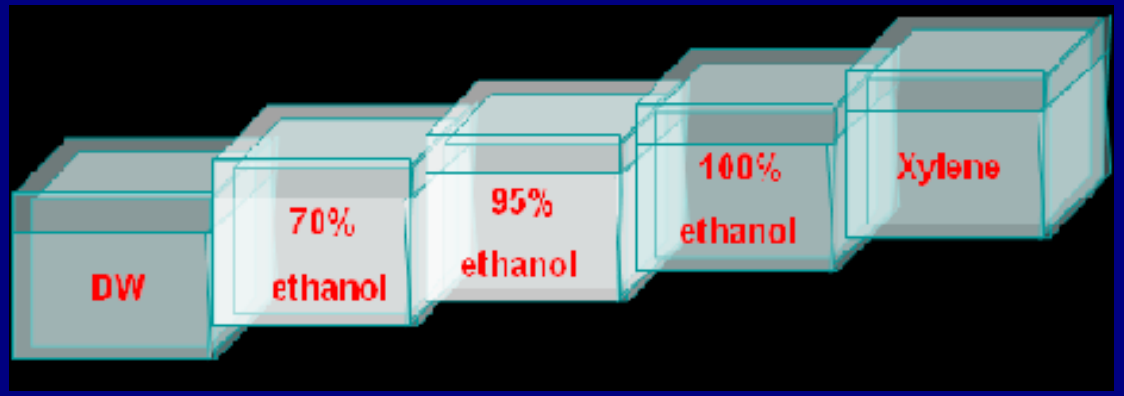
- Paraffin-embedded block



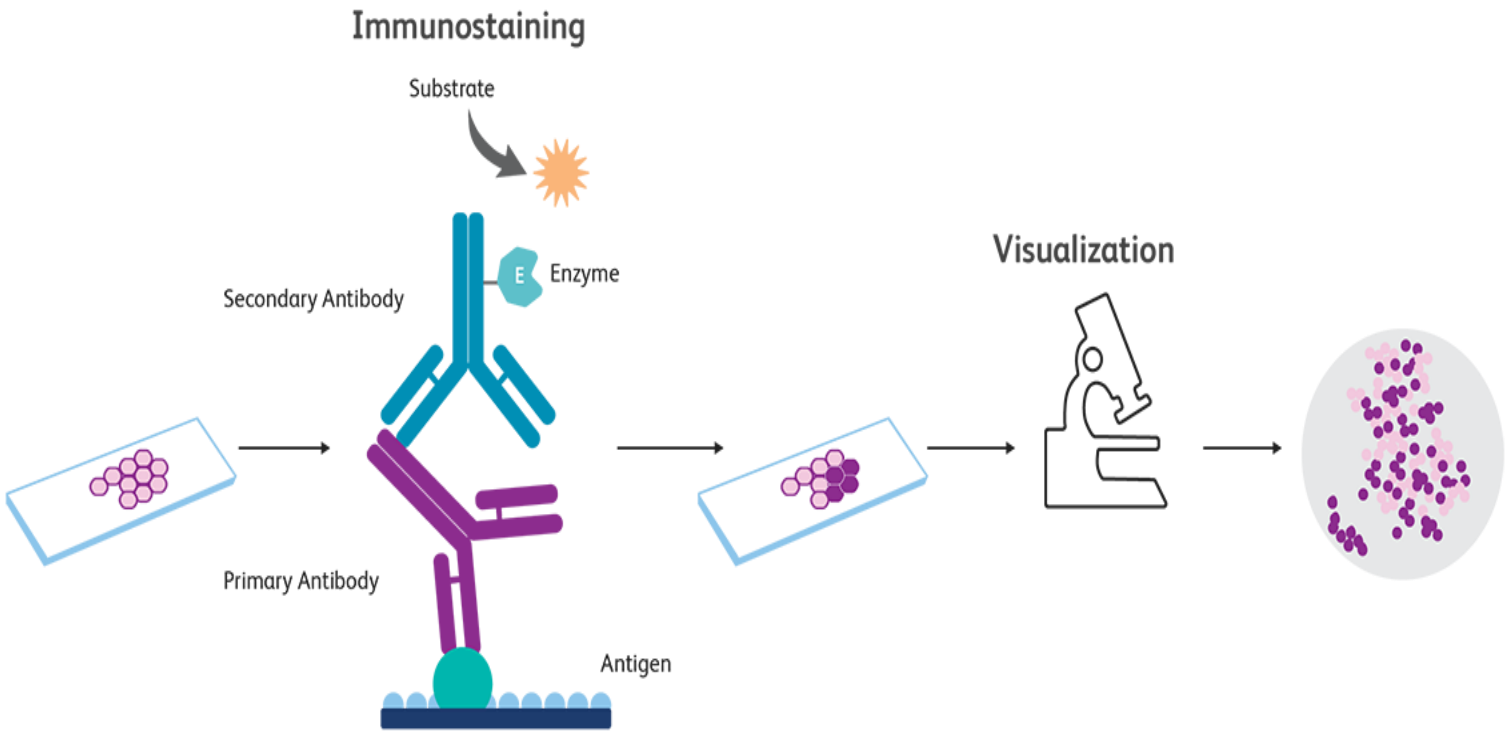
- Sectioning of the block into 5 um thick sections onto positively charged slides



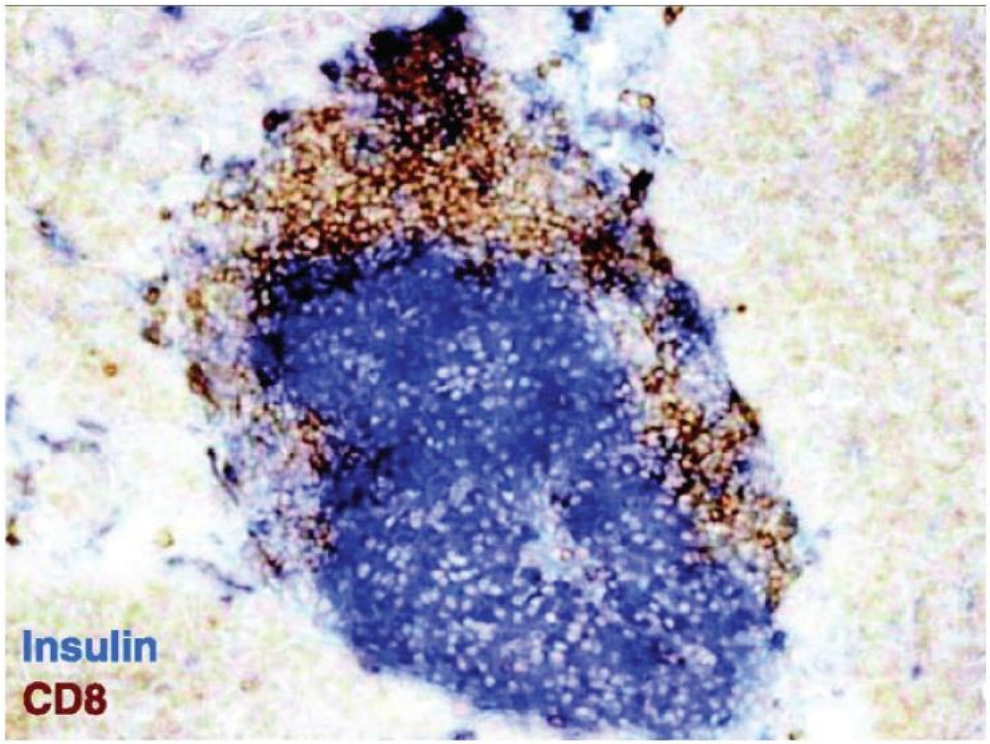
- Rehydration



Principle of the test



- **Enzyme-conjugated antibodies can be used to create immunohistochemical images.**
- The pancreas from a female mouse of the nonobese diabetic strain is stained for insulin (blue) and for CD8+ T cells (brown-red). This slide shows a pancreas close to the onset of clinical disease, when the infiltration of CD8+ T cells is significant.



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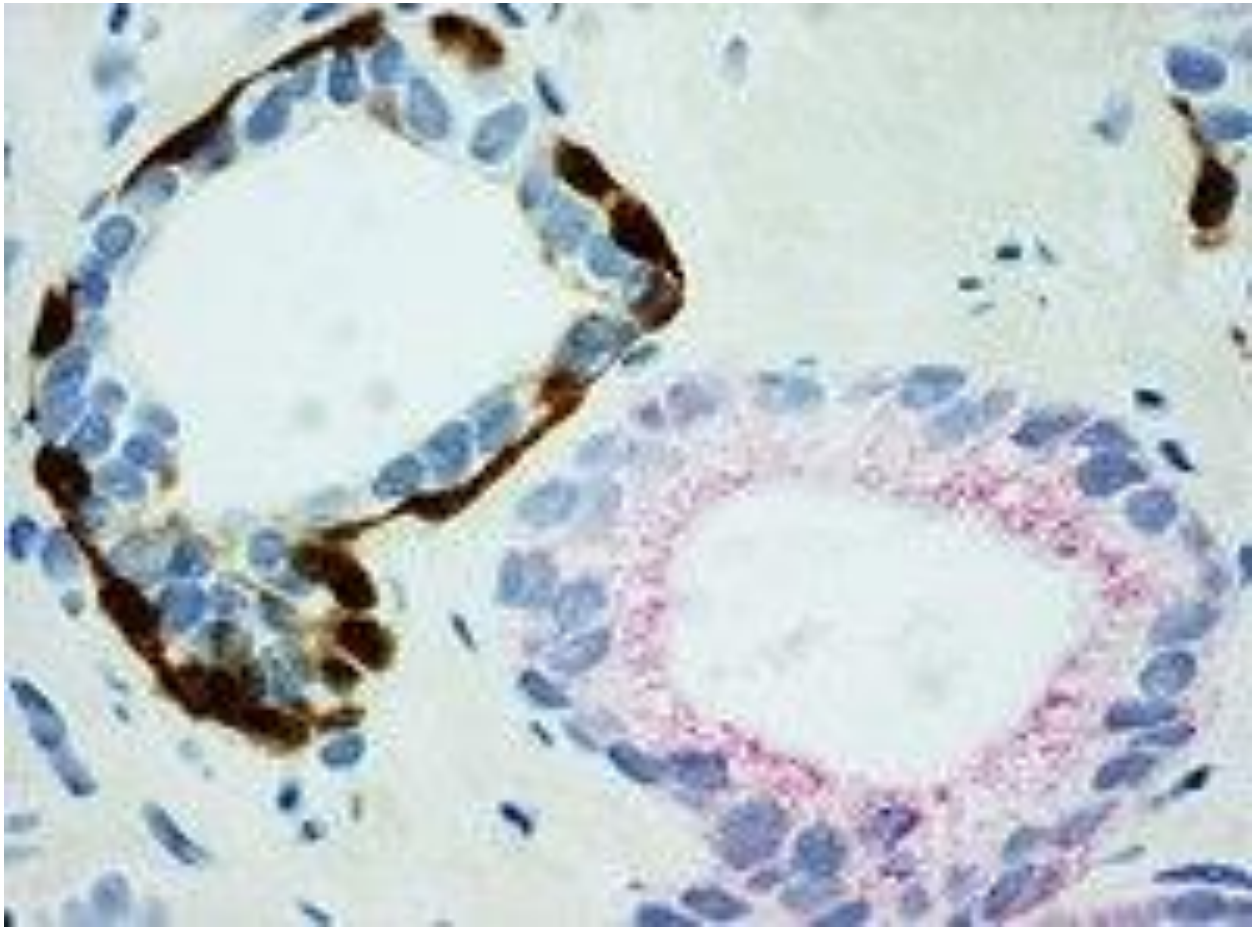

staining of a benign gland (left) and prostate adenocarcinoma (right). The adenocarcinoma lacks basal epithelial cells (stained dark brown).
stained samples, adenocarcinoma cells generally display red cytoplasm

Applications

- **Diagnostic IHC markers**

IHC is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined.

- **Directing therapy**

Immunohistochemistry can be used to assess which tumors are likely to respond to therapy, by detecting the presence or elevated levels of the molecular target.

- **Research**

  - ✓ **Biomarker Studies:** Identifying and characterizing biomarkers associated with various diseases, helping researchers understand disease mechanisms.
  - ✓ **Immunology:** Investigating immune responses by detecting the presence and distribution of immune cells, cytokines, or other immunological markers in tissues.

In an indirect ELISA, what would be the outcome of an improper wash after the antibody–enzyme conjugate is added?

a) Results will be falsely decreased.
b) Results will be falsely increased.
c) Results will be unaffected.
d) No wash step is required in the ELISA procedure.

References:

- Miller, Linda E; Stevens, Dorresteyn Christine. Clinical Immunology & Serology A Laboratory Perspective (p. 178). F.A. Davis Company. Kindle Edition.
- Kuby immunology 8th edition, 2019