Immunoassays

Dr Esam Ibraheem Azhar

(BSc, MSc, Ph.D Molecular Medical Virology)

Asst. Prof. Medical Laboratory Technology Department

Immunoassays

- ✓ Introduction
- ✓ Antigen
- ✓ Antibody
- ✓ Factors affecting immunoassays *in vitro*
- ✓ Enzyme immunoassay
- ✓ Immunofluorescence
- ✓ Radio immunoassay
- ✓ Sensitivity
- ✓ Specificity

Introduction

- **Immunoassays are a diverse group of analytical techniques which are found throughout clinical, biopharmaceutical, and environmental fields. More precisely, they are analytical methods based on the specific immuno-reaction between an antibody (Ab) and an antigen (Ag), for the determination of the amount of either reactant in the solution.**

- **All immunoassays are directly dependent on the complex formation that enables an Ag and Ab to fit together hand-in-glove.**

Introduction – Cont.

- Their unique selectivity, extremely low limits of detection, and applicability to a wide variety of compounds of interest make them a very powerful tool even in complex biological samples such as blood, plasma or urine.

- The high selectivity results from various labelling techniques such as radioactivity (**RIA**), fluorescence (**FIA**), or enzyme amplification (**EIA**).

- Immunoassay techniques are especially suited for the analysis of compounds at low concentration and in samples with little or no preparation, since their detection limits are usually within nanogram to picogram range.

Definition

- **Antigen**

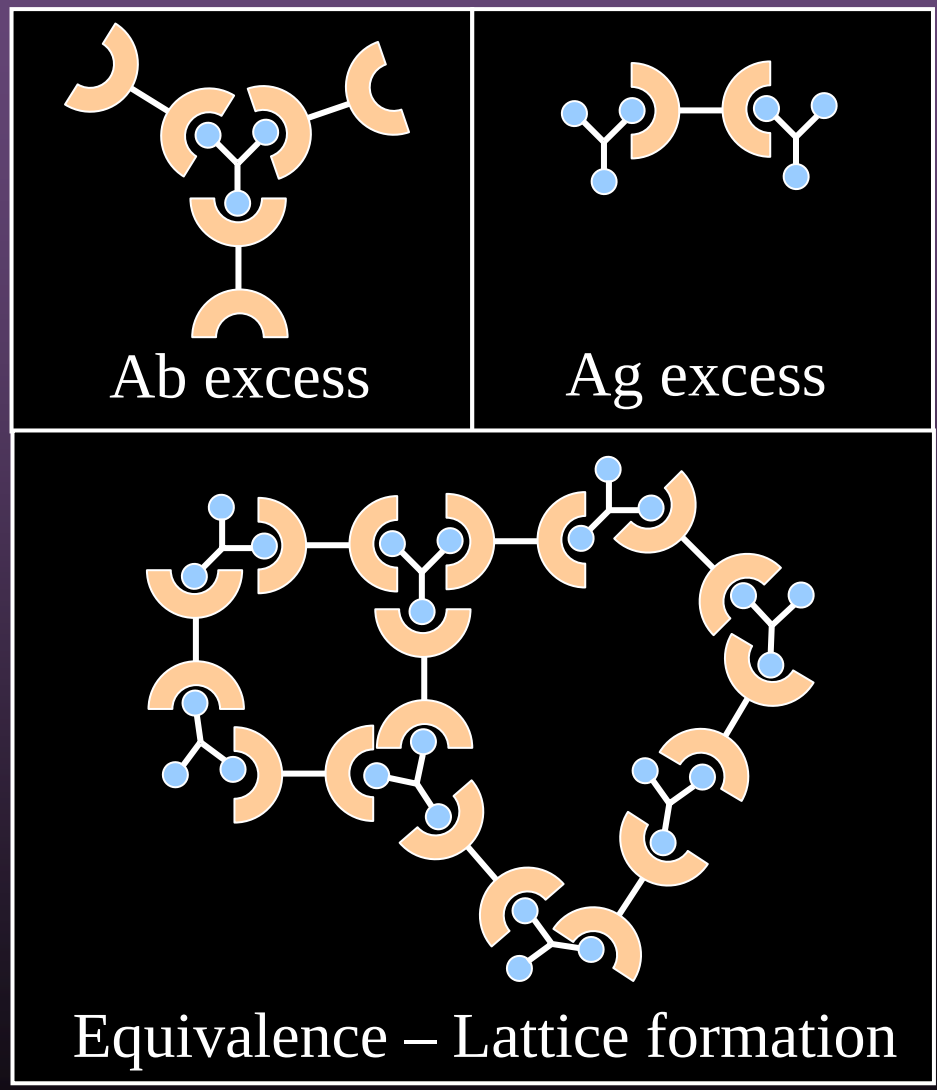
  Substance that can be bound by an antibody, or whose peptide fragment can be recognized by a T cell receptor via MHC molecule presentation.

- **Antibody**

  immunoglobulin protein with specific affinity for target molecule (antigen). Can be polyclonal if produced by antigen injection into experimental animal, or monoclonal if produced by cell fusion and cell culture techniques. Monoclonal have better specificity and more reproducible properties from batch to batch.

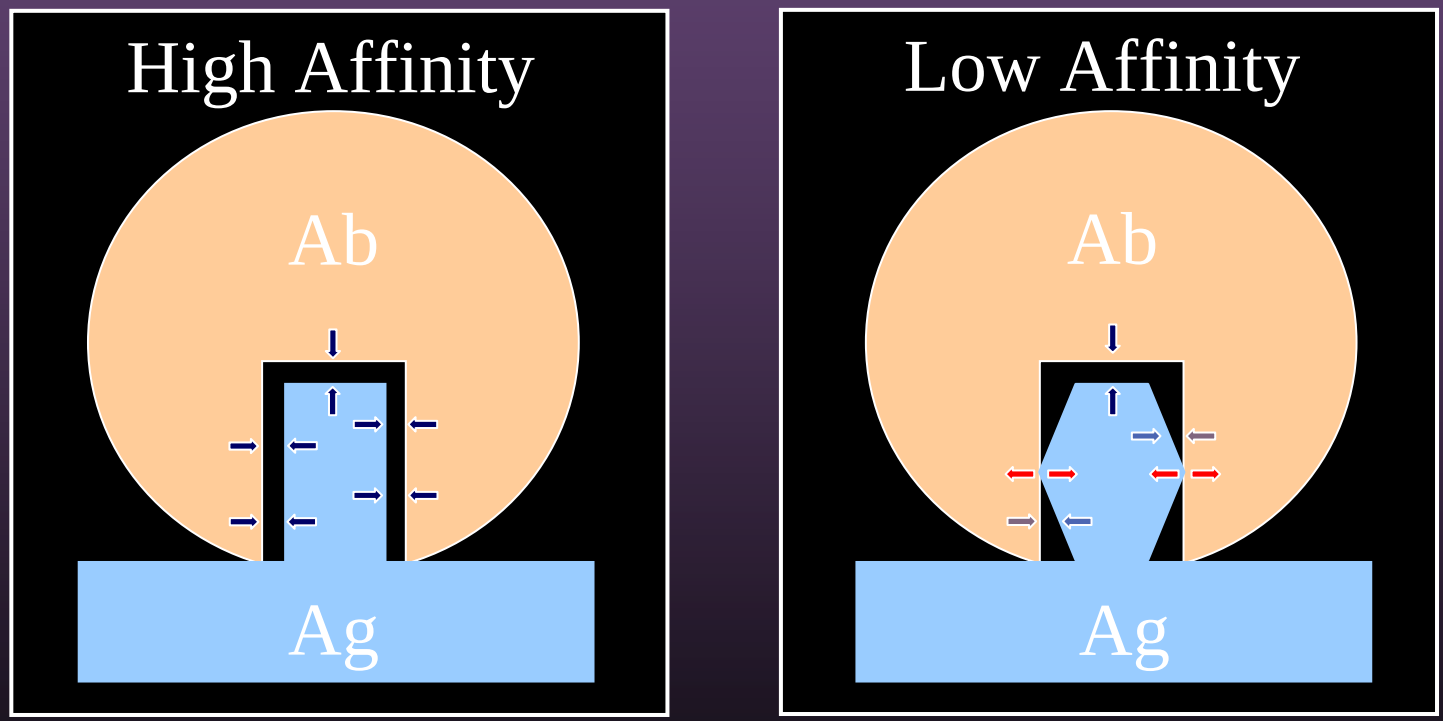
Factors affecting immunoassays *in vitro*

- Affinity
- Avidity
- Ag:Ab ratio
- Physical form of Ag



Affinity

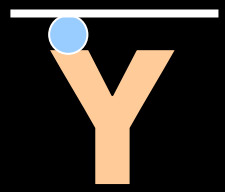
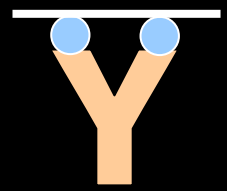
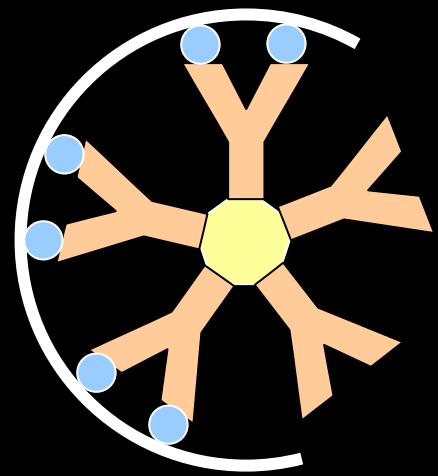
- Strength of the reaction between a single antigenic determinant and a single Ab combining site



Affinity = ☜ attractive and repulsive forces

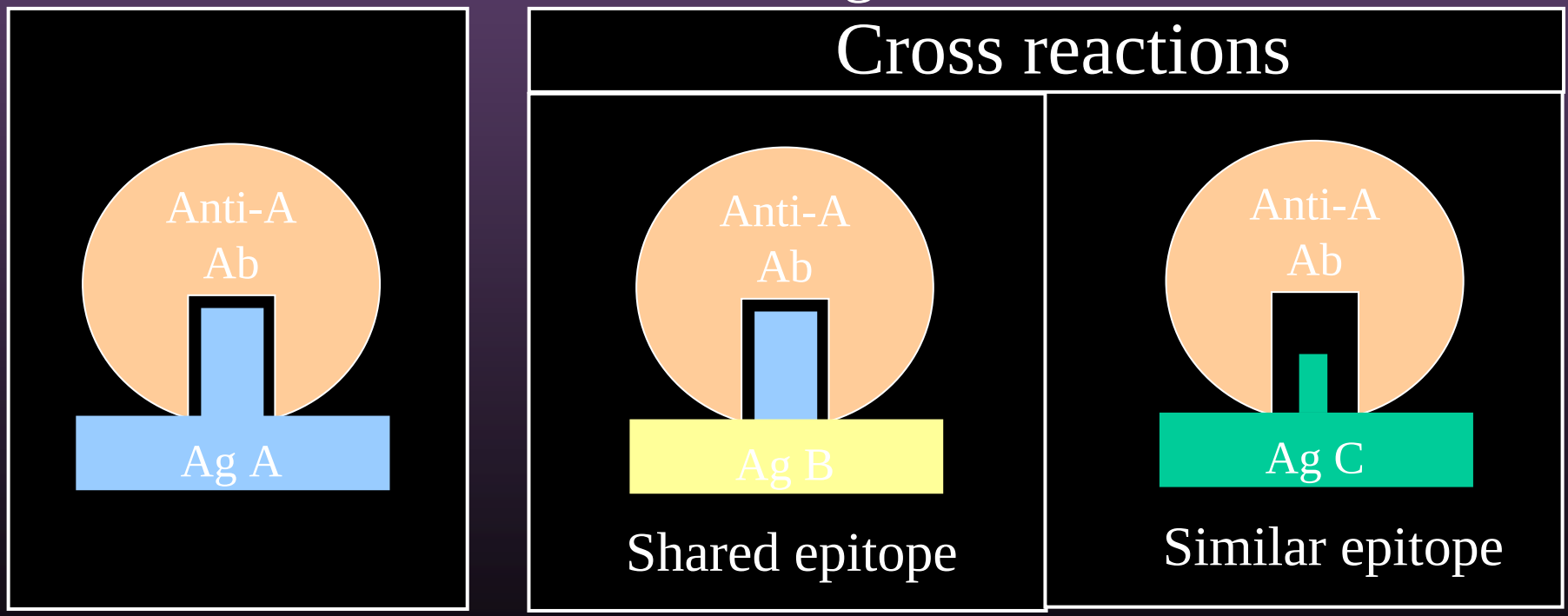
Avidity

- The overall strength of binding between an Ag with many determinants and multivalent Abs

			
K_{eq} =	10^4 Affinity	10^6 Avidity	10^{10} Avidity

Cross Reactivity

- The ability of an individual Ab combining site to react with more than one antigenic determinant.
- The ability of a population of Ab molecules to react with more than one Ag



Type of Conventional Immunoassays

- **The most common type of conventional immunoassays is to immobilize the Ab or Ag onto a solid surface. Samples containing the analyte are then poured onto the immobilized agent. After the separation of bound and free forms of analyte, quantification is achieved by measuring:**

1) the activity of an enzyme bound to the analyte, as in Enzyme-Linked Immunosorbent Assay (ELISA);

2) the radioactivity, as in Radioimmunoassay (RIA);

3) fluorescence, as in Fluorescence immunoassays (FIA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay

- **It was first described by Engvall and Perlmann in 1971.**
- **It measures the amount of an Ab present in a solution.**
- **Although it was first developed for the measurement of Abs, it has since been modified for measurements of other molecules including hormones.**
- **Both a competitive and a double Ab sandwich ELISA are available for performing Ag measurements, while Abs can be quantified by an indirect ELISA.**

Sandwich ELISA

- **In this method;**

  - The surface is first coated with a solid phase Ab.

  - The test sample, containing the Ag being measured, is then added and allowed to react with the bound Ab.

  - Any unbound Ag is washed away.

  - A known amount of enzyme-labelled Ab is then allowed to react with the bound Ag.

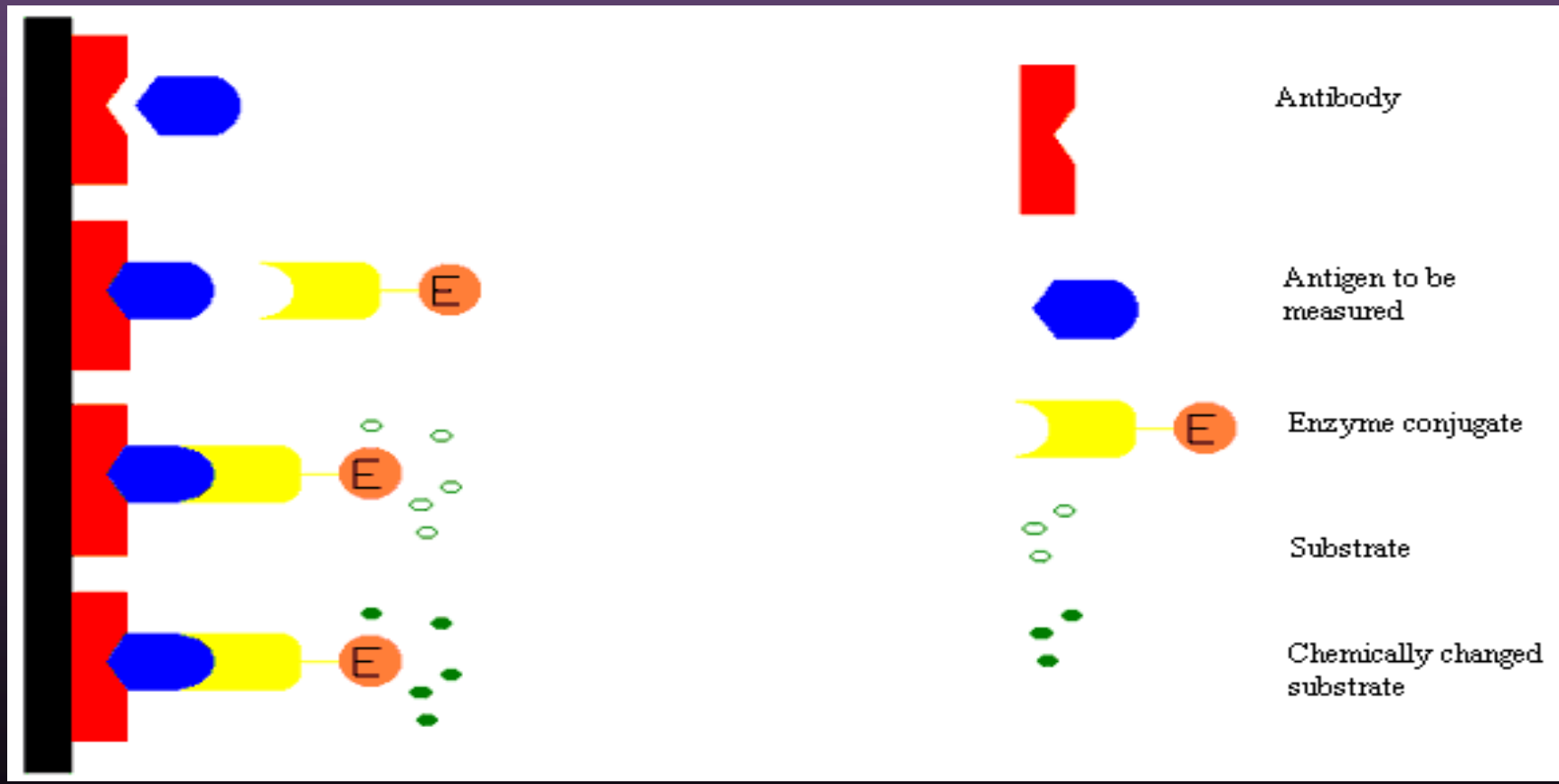
  - Any excess unbound enzyme-linked Ab is washed away after the reaction.

  - The substrate is then added and the reaction between the substrate and the enzyme produces a color change.

  - The amount of visual color change is a direct measurement of specific enzyme-conjugated bound Ab, and consequently Ag present in the specimen tested.

Sandwich ELISA – Cont.

The "sandwich" technique is so called because the Ag being assayed is held between two different Abs.



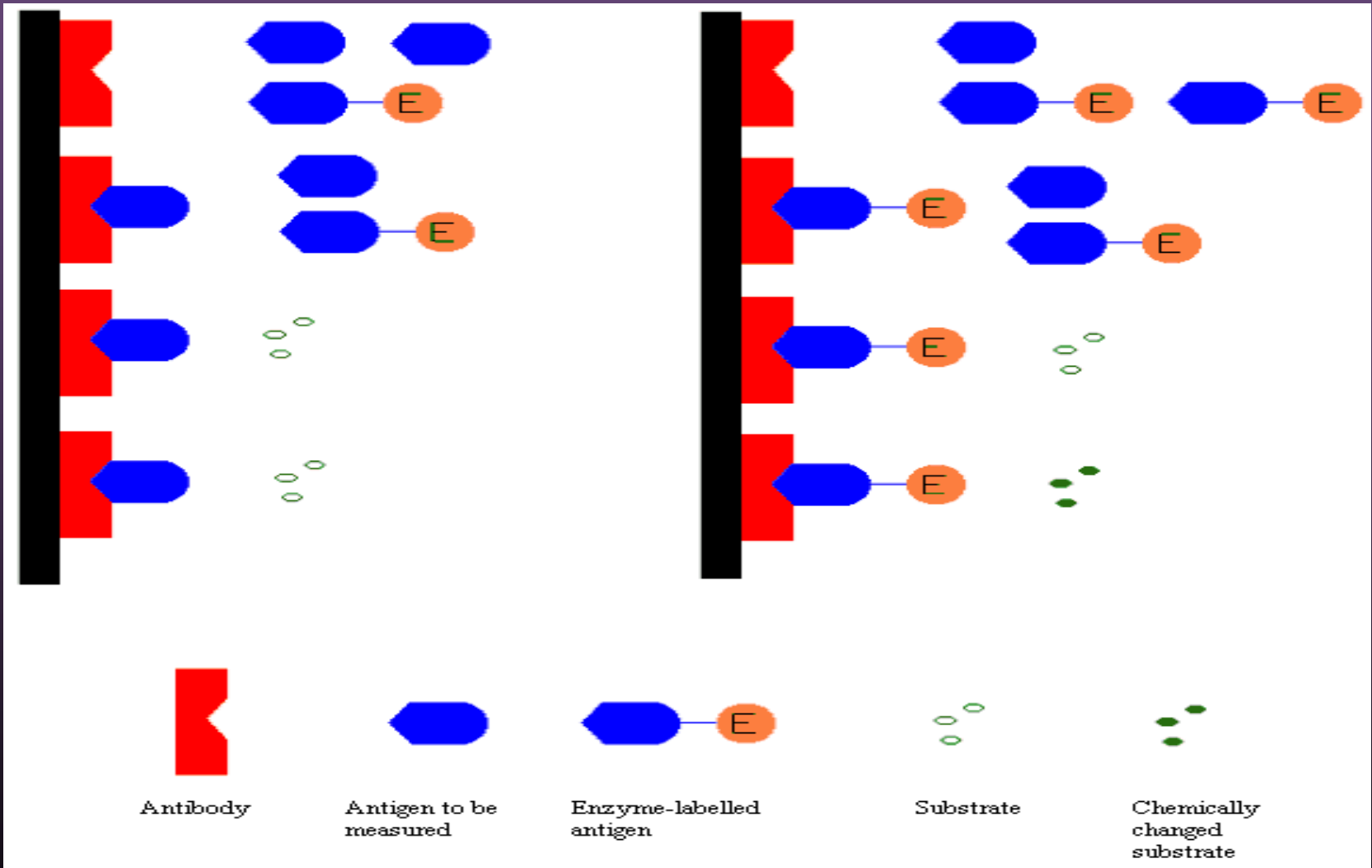
Competitive ELISA For Ag

- **The test specimen containing the Ag to be determined is mixed with a precise amount of enzyme-labelled Ag and both compete equally for a limited number of binding sites on an Ab found attached to the surface.**

- **Excess free enzyme-labelled Ag is washed off before the substrate is added.**

- **The key element in this competitive situation is the amount of color intensity resulting from substrate addition; the stronger the color intensity the less the Ag found in the sample.**

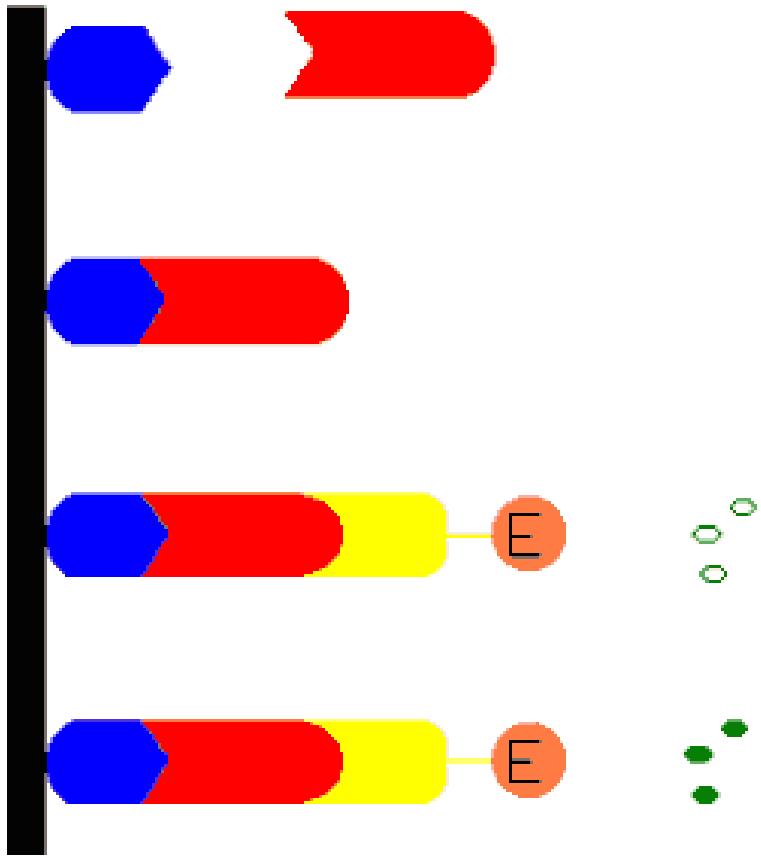
Competitive ELISA For Ag

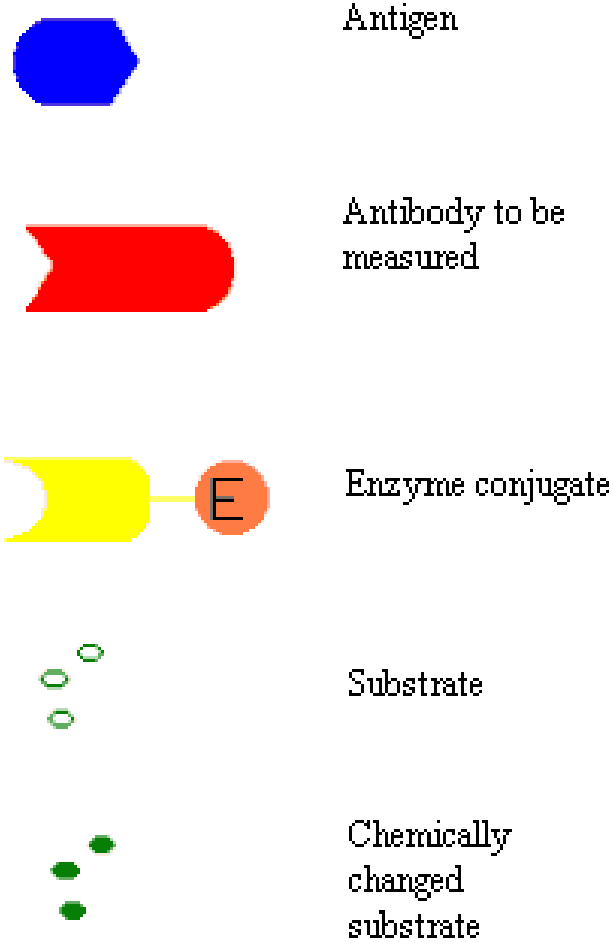


ELISA for Ab Detection (Indirect ELISA)

- **The ELISA for the Ab detection is very similar to the "sandwich Ag" technique, only instead of the Ab being adsorbed to the surface it is the Ag that is adsorbed.**
- **The test sample containing the Ab is then added and allowed to bind to the attached Ag.**
- **Unbound Ab is removed by several washings, after which enzyme-labelled Ag is added.**
- **The enzyme-labelled Ag complex binds to a specific site on the bound Ab.**
- **When a substrate is added, the enzyme-labelled Ag causes a color change by acting on the substrate.**
- **The amount of color change is an indirect measure of specific Ab present in the test sample.**

ELISA for Ab Detection





Advantages of Enzyme Immunoassay

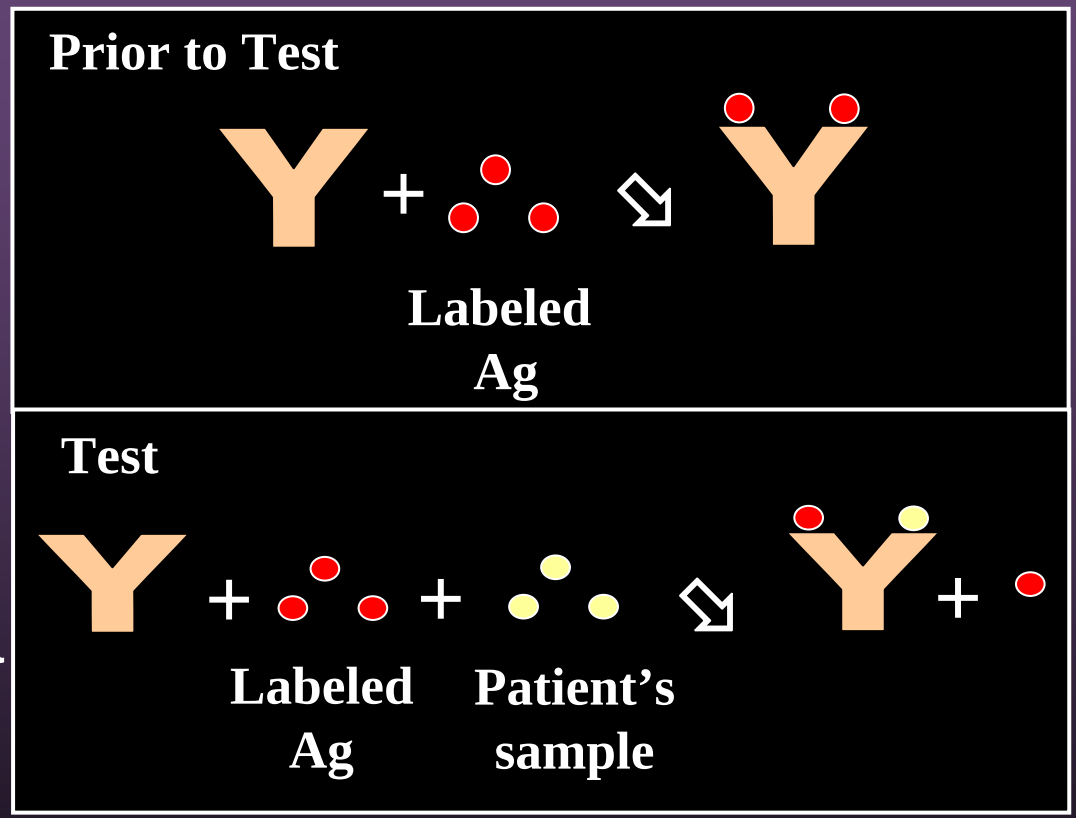
- Labels cheap and plentiful.
- Labels have a long shelf life
- Easily adapted to automation
- Reaction measured using inexpensive equipment
- Very sensitive
- No health hazards associated with reagents
- Can be used for qualitative OR quantitative procedures.

Disadvantages of Enzyme Immunoassay

- Some samples may have natural inhibitors.
- Size of enzyme label limiting factor in designing some assays.
- Nonspecific protein binding may occur.
- Enzyme reactions very sensitive to temperature.

Competitive RIA/ELISA for Ag

- **Method**
  - Determine amount of Ab needed to bind to a known amount of labeled Ag
  - Use predetermined amounts of labeled Ag and Ab and add a sample containing unlabeled Ag as a competitor



Competitive RIA/ELISA for Ag

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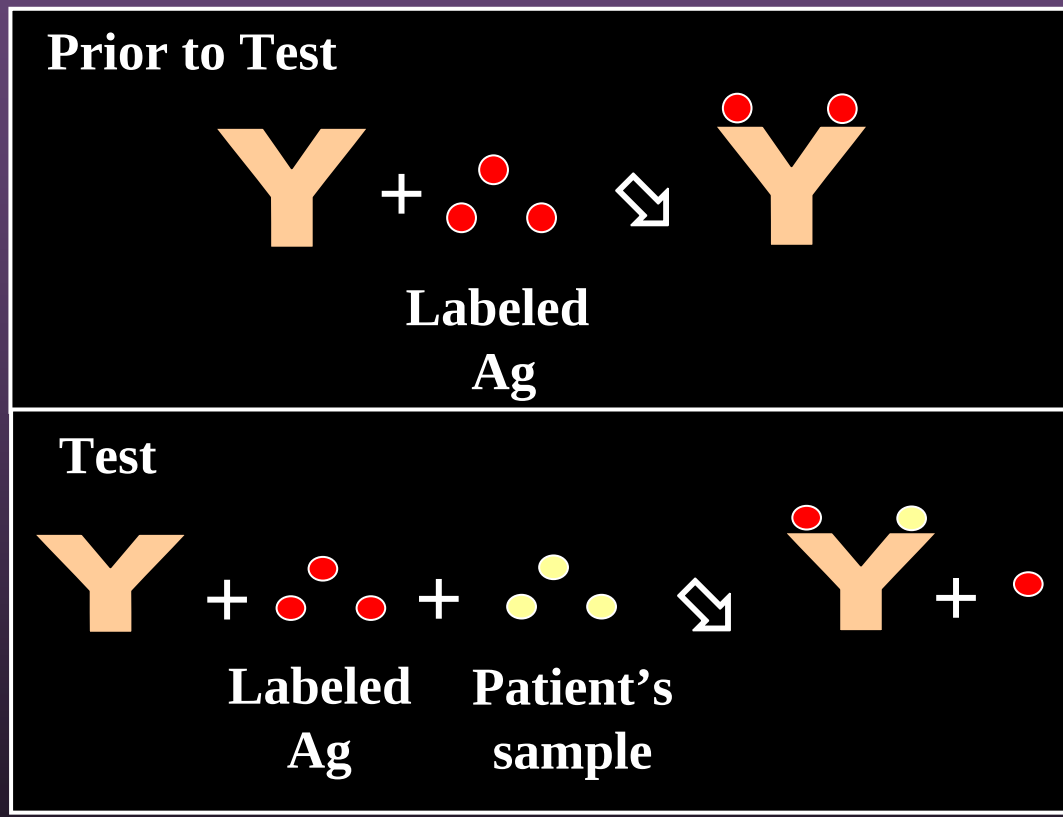
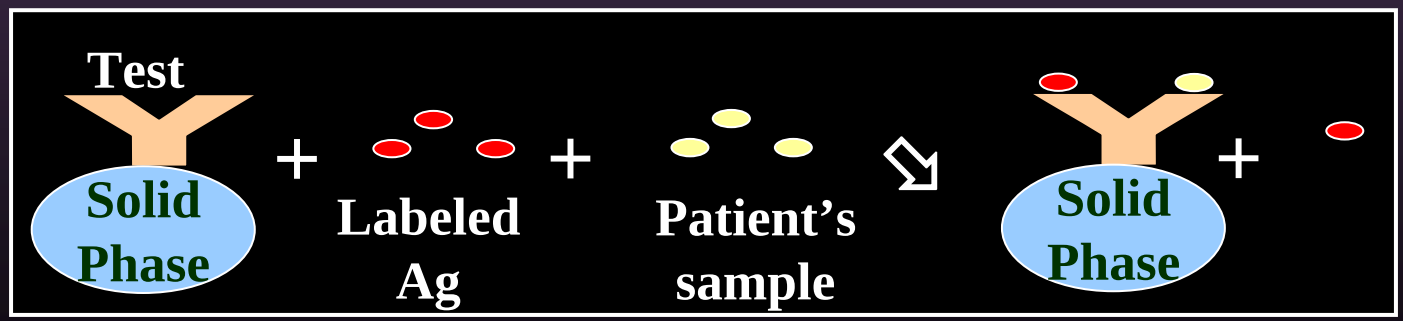


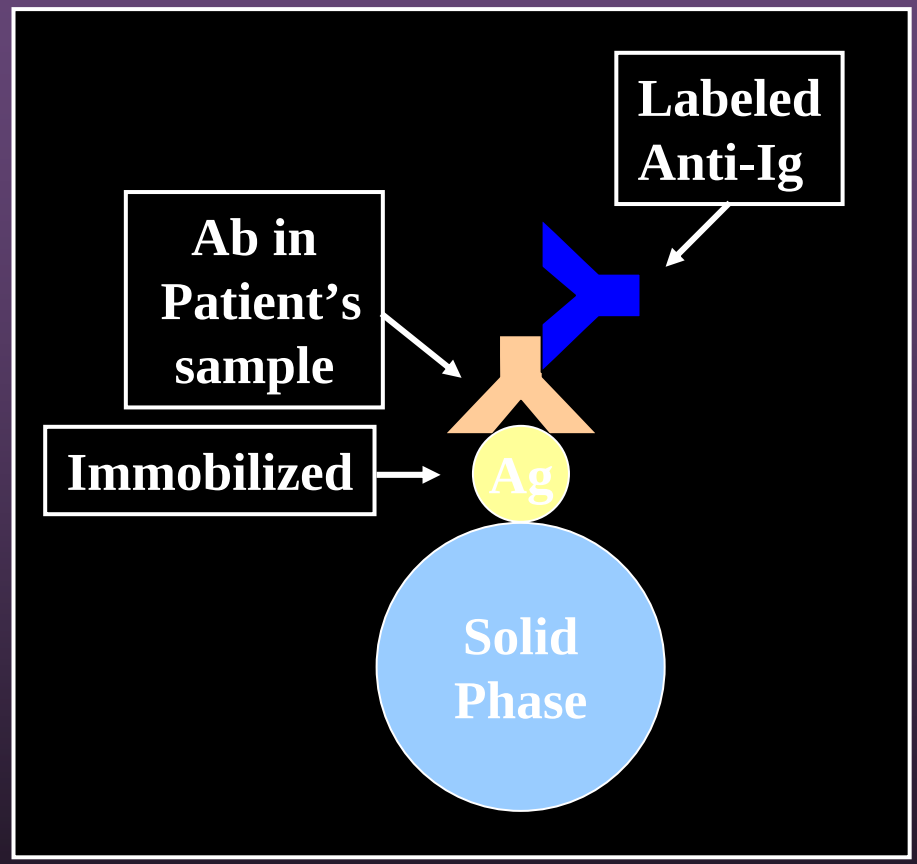
Competitive RIA for Ag

- Uses radioactive substance as a label, usually I^{125}.
- Antibody is bound to a tube or other solid matrix.
- A measured amount of patient sample is added to a measured amount of radiolabeled analyte or ligand.
- The antigen in the patient sample and the radiolabeled antigen compete for the binding to the antibody.
- If there is no antigen in the sample then there will be a high level of radiation since the radiolabeled antigen can bind to all antibody sites.
- If antigen is present in the patient sample then radioactivity will be decreased proportionally to the amount of antigen present.
- Standards are run to create a standard curve.
- Controls are run to ensure proper technique and reagent reactivity.



Solid Phase Non-competitive RIA

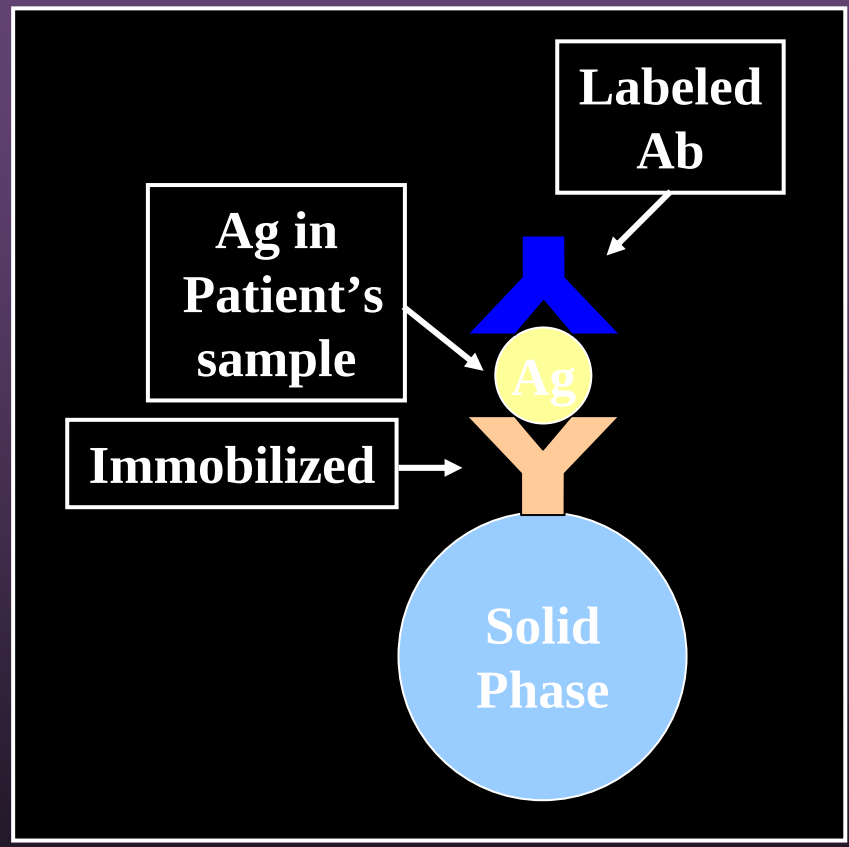
- Ab detection
  - Immobilize Ag
  - Incubate with sample
  - Add labeled anti-Ig
  - Amount of labeled Ab bound is proportional to amount of Ab in the sample



- Quantitative

Solid Phase Non-competitive RIA

- 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
- Quantitative



Advantages of RIA

- Faster reaction
- High sensitivity and specificity.

Disadvantages of using RIA

- Health hazard
- Disposal of radioactive waste
- Short shelf life
- Expensive equipment
- Require pure antigen and antibody

Sensitivity & Specificity

- Sensitivity →Proportion of true positives correctly identified by test (positive results / true positives)

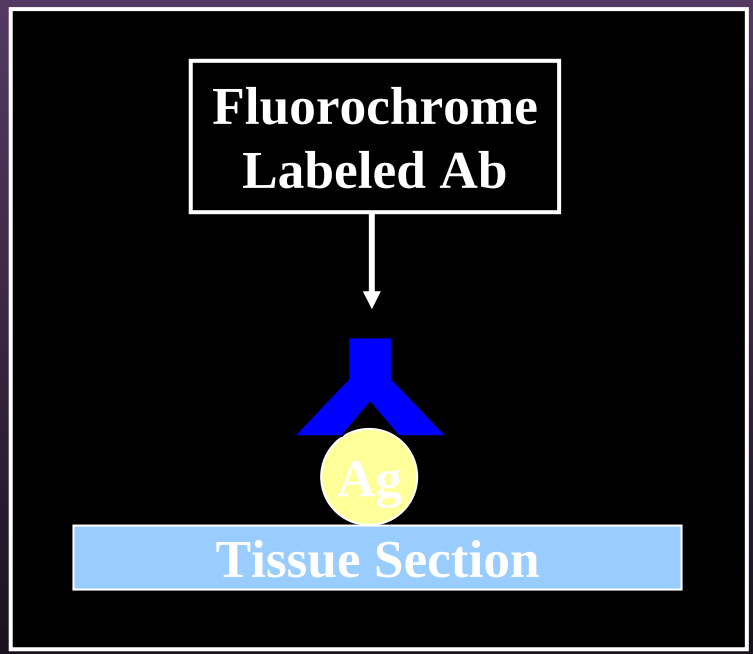
- Specificity → Proportion of true negatives correctly identified by test (negative results / true negatives)

Specificity

- The ability of an individual antibody combining site to react with only one antigenic determinant.
- The ability of a population of antibody molecules to react with only one antigen.

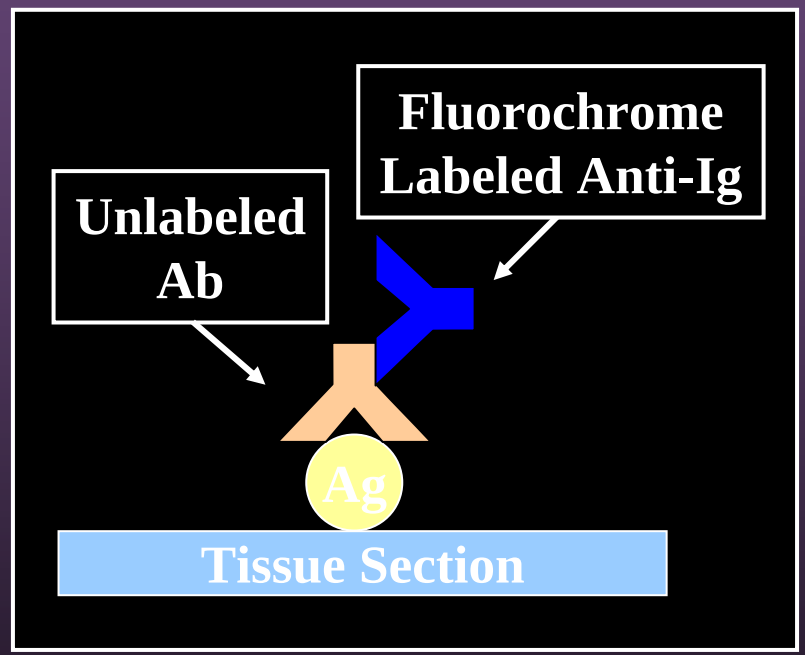
Immunofluorescence

- **Direct**
  - **Ab to tissue Ag is labeled with fluorochrome**



Immunofluorescence

- Indirect
  - Ab to tissue Ag is unlabeled
  - Fluorochrome-labeled anti-Ig is used to detect binding of the first Ab.
- Qualitative to Semi-Quantitative



Detection of the labeled analyte.

- There must be an accurate system for detecting and measuring the labeled product produced.
- For radioimmunoassay, radioactivity is measured.
- For labels such as enzymes, fluorescence, changes in absorbency on a spectrophotometer are used.

Quality control procedures must always be performed to ensure the accuracy of the results obtained

- "***Blanks***" are tubes filled with a clear solution or, if the solutions added have color, with the solution only, to determine the "background", any substances present in the original solution must be calculated out.
- ***Controls*** are substances with a known range of values and generally three levels are run: normal, high and low.
- If controls do not give the expected values the results cannot be reported out.