Assessment of Endocrine Function - Hormonal Assays

Prof. S. Chackrewarthy

Dept. of Biochemistry & Clinical Chemistry

Faculty of Medicine

University of Kelaniya

Biological response is determined by

- Concentration of hormone
- Transport of hormone to the target tissue
- Receptor interaction
  - Number of receptors
  - Affinity of interaction
- Post receptor effects (intracellular signaling mechanisms, second messengers)

Assessment of endocrine function

(1) Assays for hormone levels of plama and urine

In vitro assays

- Radioimmunoassays
- ELISA

(2) Receptor Assays

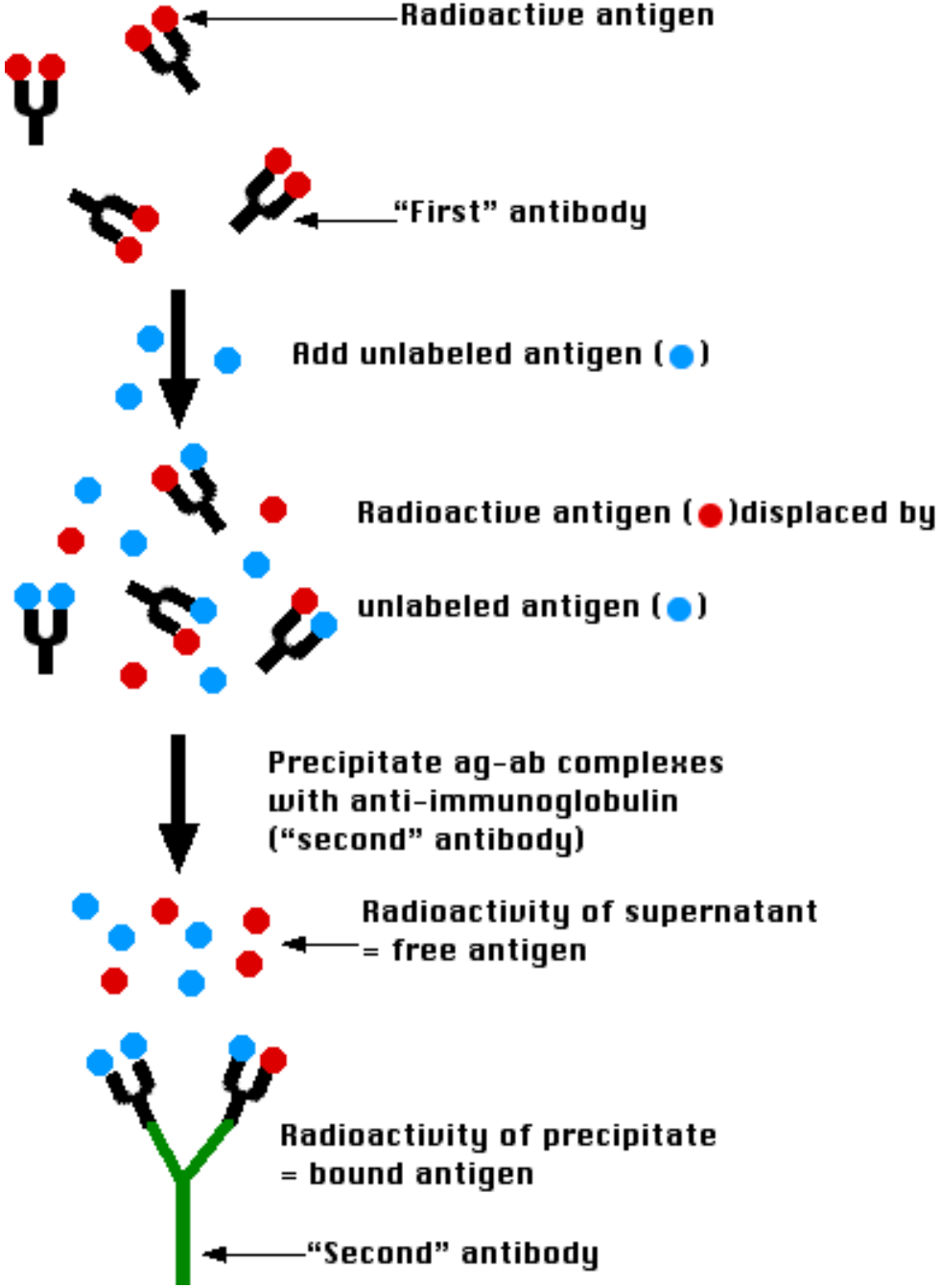
Radioimmunoassay (RIA)

- Radioimmunoassay (RIA) involves the separation of a protein (from a mixture) using the specificity of antibody - antigen binding and quantitation using radioactivity
- The technique of radioimmunoassay has revolutionized research and clinical practice in many areas, e.g.,
  - blood banking
  - diagnosis of allergies
  - endocrinology

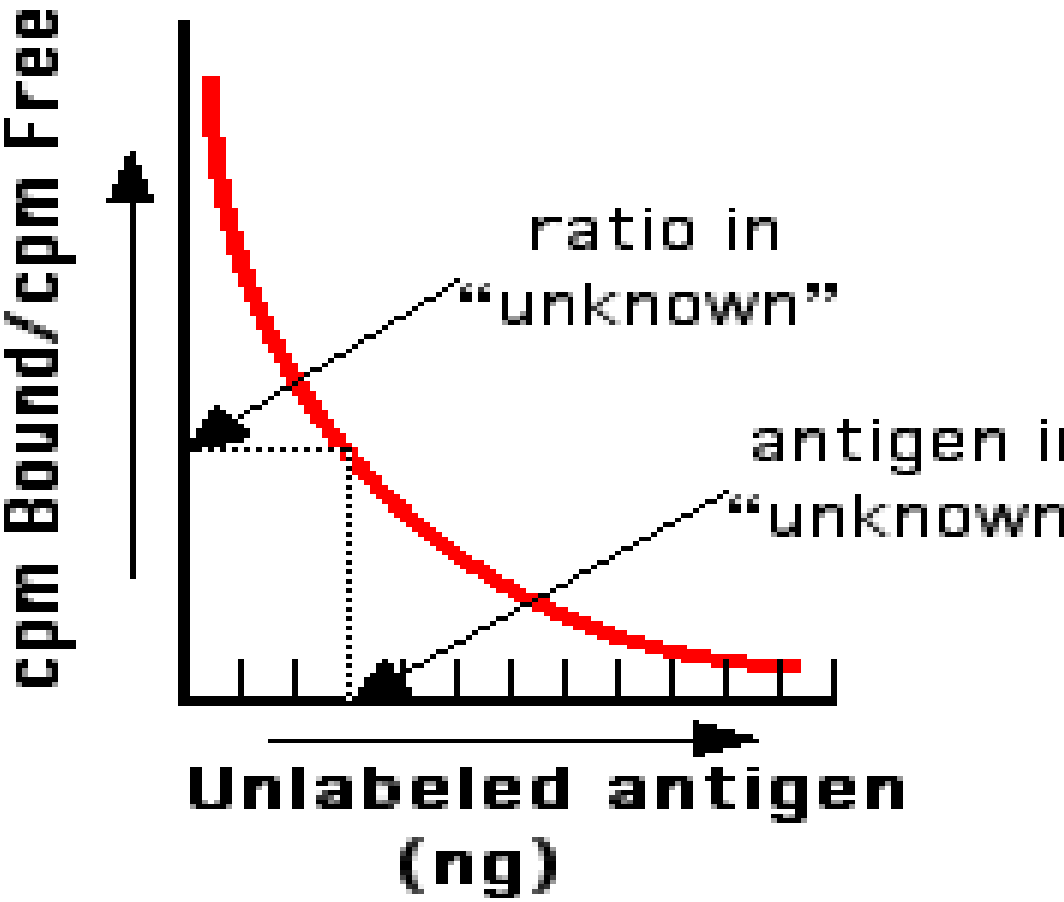
Competitive radioimmuno-assay

- A mixture is prepared of
  - radioactive antigen (radioactive-labeled hormone)
    - Because of the ease with which iodine atoms can be introduced into tyrosine residues in a protein, the radioactive isotopes ^{125}I or ^{131}I are often used.
  - antibodies against that antigen.
- Known amounts of unlabeled antigen (normal hormone) are added to samples of the mixture. These compete for the binding sites of the antibodies.

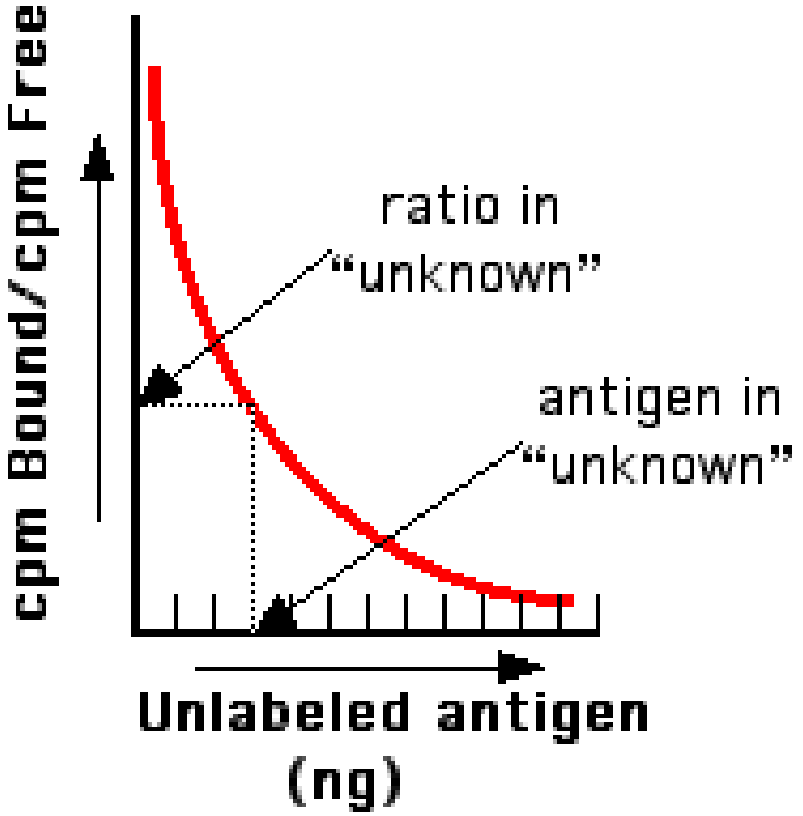
- At increasing concentrations of unlabeled antigen, an increasing amount of radioactive antigen is displaced from the antibody molecules.
- The antibody-bound antigen is separated from the free antigen in the supernatant fluid
- the radioactivity of each is measured.


Radioactive antigen
"First" antibody
Add unlabeled antigen ( )
Radioactive antigen ( )displaced by
unlabeled antigen ( )
Precipitate ag-ab complexes
with anti-immunoglobulin
("second" antibody)
Radioactivity of supernatant
= free antigen
Radioactivity of precipitate
= bound antigen
"Second" antibody

- From these data, a standard binding curve, like the one shown in red, can be drawn.



- The samples to be assayed (the unknowns) are run in parallel.
- After determining the ratio of bound to free antigen in each unknown, the antigen concentrations can be read directly from the standard curve.



Separating Bound from Free Antigen

- Precipitate the antigen-antibody complexes by adding a "second" antibody directed against the first.
  - For example, if a rabbit IgG is used to bind the antigen, the complex can be precipitated by adding an antirabbit-IgG antiserum (e.g., raised by immunizing a goat with rabbit IgG).

- Radioimmunoassay is widely-used because of its great sensitivity.
- Using antibodies of high affinity, it is possible to detect a few picograms (10^{-12} g) of antigen in the tube.
- The greater the specificity of the antiserum, the greater the specificity of the assay
- The main drawbacks to radioimmunoassay are the expense and hazards in preparing and handling the radioactive antigen.
- Both ^{125}I or ^{131}I emit gamma radiation that requires special counting equipment (gamma counter).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA)

- ELISA is a widely-used method for measuring the concentration of a particular molecule (e.g., a hormone or drug) in a fluid such as serum or urine. It is also known as enzyme immunoassay or **EIA**.
- ELISA has many of the advantages (e.g., sensitivity, ease of handling multiple samples) without the disadvantages of dealing with radioactivity (like in RIA).
- Types- Competitive (direct) Elisa

  Non-competitive (sandwich) Elisa

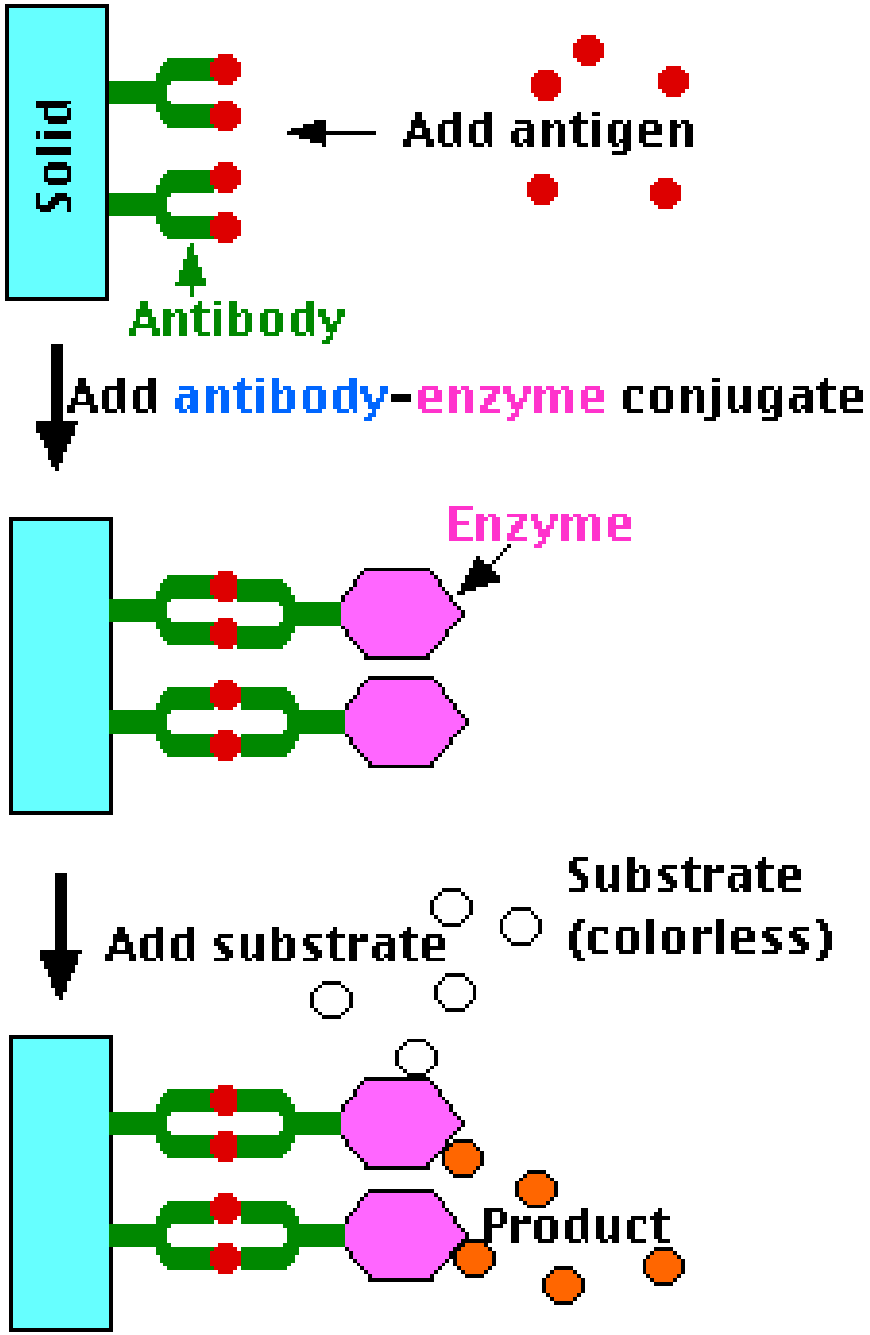
  Indirect Elisa

Non-competitive (sandwich) Elisa-

- The molecule is detected by antibodies that have been made against it; that is, for which it is the **antigen**. Monoclonal antibodies are often used.
- The test requires:
  - the antibodies fixed to a solid surface, such as the inner surface of a test tube;
  - a preparation of the same antibodies coupled to an enzyme. Usually one that produces a colored product from a colorless substrate (eg. β-galactosidase) .

Performing the Test

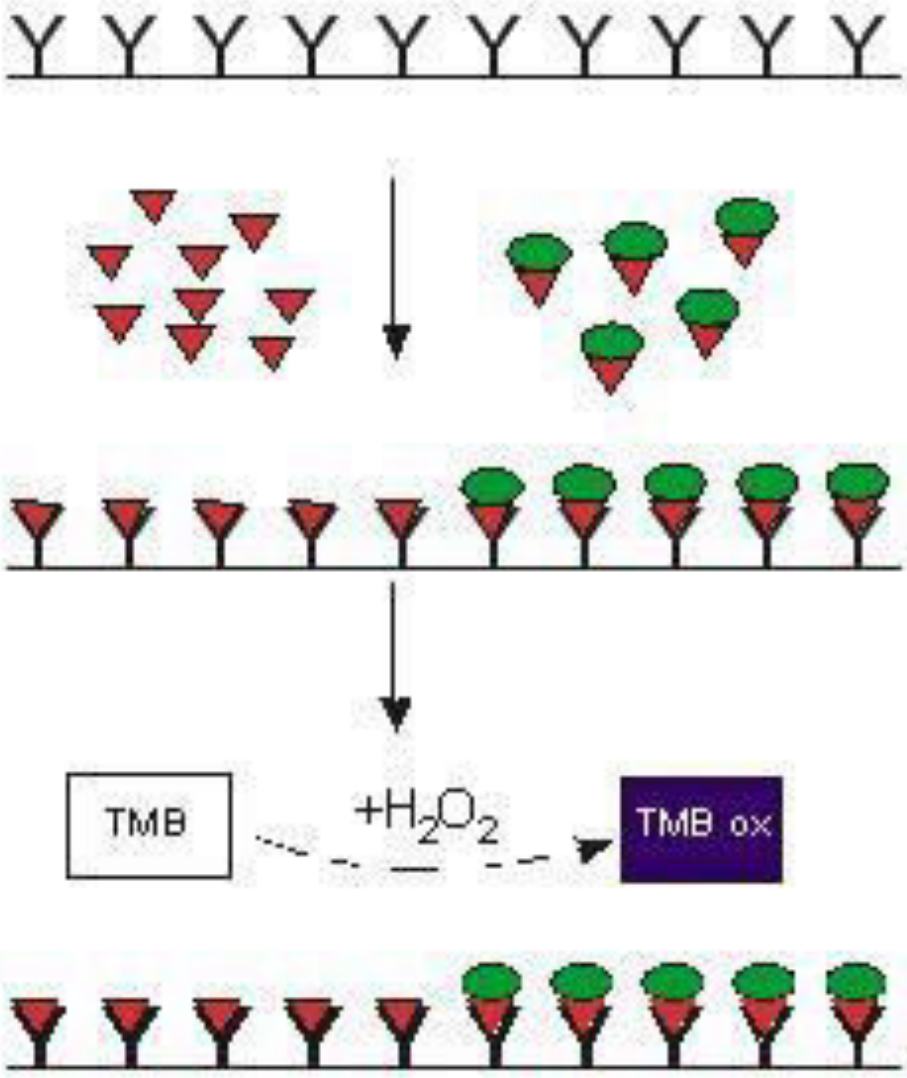
- The tubes are filled with the antigen solution (eg. urine) to be assayed. Any antigen molecules present bind to the immobilized antibody molecules.
- The antibody-enzyme conjugate is added to the reaction mixture. The antibody part of the conjugate binds to any antigen molecules that were bound previously, creating an antibody-antigen-antibody "sandwich".
- After washing away any unbound conjugate, the substrate solution is added.
- After a set interval, the reaction is stopped (e.g., by adding 1 N NaOH) and the concentration of colored product formed is measured in a spectrophotometer. The intensity of color is proportional to the concentration of bound **antigen**.


Solid
Add antigen
Antibody
Add antibody-enzyme conjugate
Enzyme
Add substrate
Substrate
(colorless)
Product

Competitive ELISA

- The labelled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The more antigen in the sample, the less labelled antigen is retained in the well and the weaker the signal).

Competitive Enzyme Immunoassay



Solid phase coated with antibody

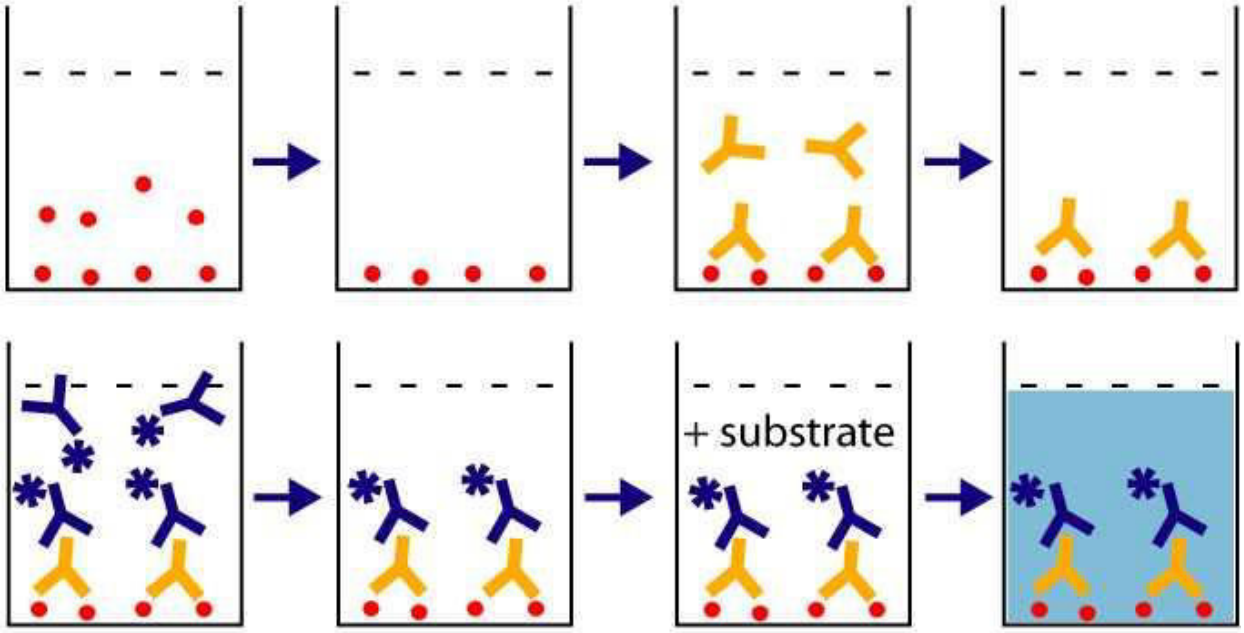
Add free and labelled antigen

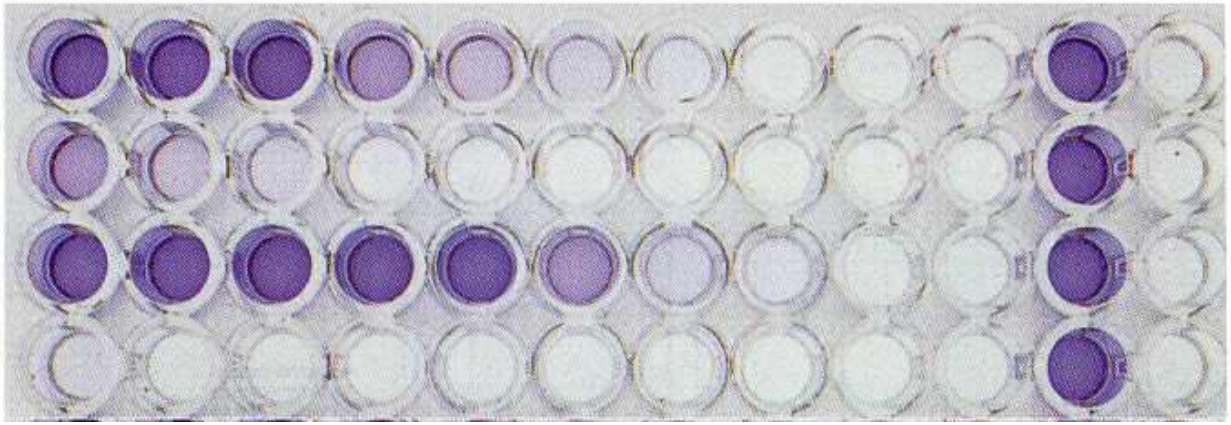
Free and labelled antigen are captured

Color formation by oxidation of substrate into a colored compound

Indirect ELISA

- The protein antigen to be tested for is added to each well of ELISA plate, where it is given time to adhere to the plastic through charge interactions.
- Then the serum is added, which contains a mixture of the serum antibodies, of unknown concentration, some of which may bind specifically to the test antigen that is coating the well.
- A secondary antibody is added, which will bind to the antibody bound to the test antigen in the well. This secondary antibody often has an enzyme attached to it.
- A substrate for this enzyme is then added. Often, this substrate changes colour upon reaction with the enzyme. The colour change shows that secondary antibody has bound to primary antibody, which strongly implies that the donor has had an immune reaction to the test antigen.


+ substrate



Applications of ELISA

- Screening donated blood for evidence of viral contamination by
  - **HIV-1 and HIV-2** (presence of anti-HIV antibodies)
  - **hepatitis C** (presence of antibodies)
  - **hepatitis B** (testing for both antibodies and a viral antigen)

- measuring hormone levels
  - **HCG** (as a test for pregnancy)
  - **LH** (determining the time of ovulation)
  - **TSH, T3 and T4** (for thyroid function)
  - **hormones** (e.g., anabolic steroids, HGH) that may have been used illicitly by athletes
- detecting infections
  - **sexually-transmitted agents** like HIV, syphilis, and chlamydia
  - **hepatitis B and C**
  - Toxoplasma gondii

- **detecting allergens** in food and house dust
- **measuring** "rheumatoid factors" and other autoantibodies in autoimmune diseases like lupus erythematosus
- **measuring toxins** in contaminated food
- **detecting illicit drugs**, eg.
  - cocaine
  - opiates
  - Δ-9-tetrahydrocannabinol, the active ingredient in marijuana

Receptor assays

Radioreceptor assays

- Similar to RIA except intact cells or membranes are used as antigen
- Tests for receptors on cells or cellular components
- Provide the information about binding affinity and receptor selectivity
- No information about biological activity

  (measures only the first step in the cascade, no information about second messengers)

Dysfunction due to change in receptor number and function

- Down regulation of insulin receptors in chronic hyperinsulinaemia in obesity
  - Caused by clustering
  - Internalization in clathrin coated vesicles
  - Degradation in lysosomes
- Up regulation of β adrenergic receptors in hyperthyroidism.

Dysfunction caused by anti-receptor antibodies

- Anti-receptor antibodies
  - Grave's disease – TSH receptor
  - NIDDM - Insulin receptor
  - Myasthenia gravis – nicotinic acetylcholine receptors

Dysfunction caused by postreceptor defects

- Defects in G protein signaling

  Eg: Activation of Gs by cholera toxin resulting in elevated levels of cAMP
- Vitamin D resistance – inherited deficiency in enzymes in metabolic pathways for vitamin D conversion
- Androgen resistance

THANK YOU