B.Sc. Biotechnology Part II

Introduction to ELISA

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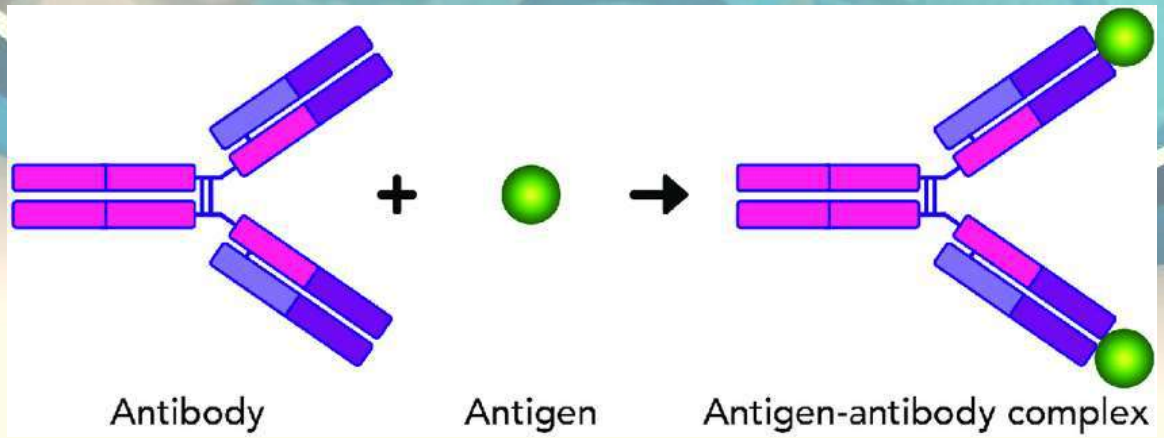
Introduction

- ELISA is a common quantitative technique based on Ag – Ab interaction which is used to measure the concentration of an analyte in the solution.
- The term ELISA was coined by Eva Engvall and Peter Perlmann at Stockholm University of Sweden in 1971.
- However, Radioimmunoassay (RIA) was first described by Rosalyn Sussman Yalow and Solomon Berson in 1960.
- It is a widely used biochemical assay to detect the presence of proteins, such as hormones and antibodies, and pathogenic microbes like bacteria and viruses.

Basic principle of ELISA

ELISA is based on antigen-antibody interaction. Enzyme-linked antibody (Ab) binds to the antigen (Ag) and the enzyme bound to the antibody converts a colorless substrate (chromogen) to a colored product which indicates the presence of Ag : Ab interaction or binding.



Basic principle of ELISA

Antigen (Ag)

- Any molecule that induces the production of antibodies when introduced into the body.
- It is a foreign particle or non-self. Sometimes, self Ag also induces antibody production, known as auto-antigen (condition is due to break of tolerance leading to autoimmune disorders),
- It has an **epitope** that interacts with the complementary paratope on the antibodies.
- Examples are
  - **Protein molecules**
  - **Carbohydrate molecules**
  - **Microorganisms**
  - **Allergens**
  - **Viruses, etc.**

Graphical representation of antigens

Basic principle of ELISA

Antibody (Ab)

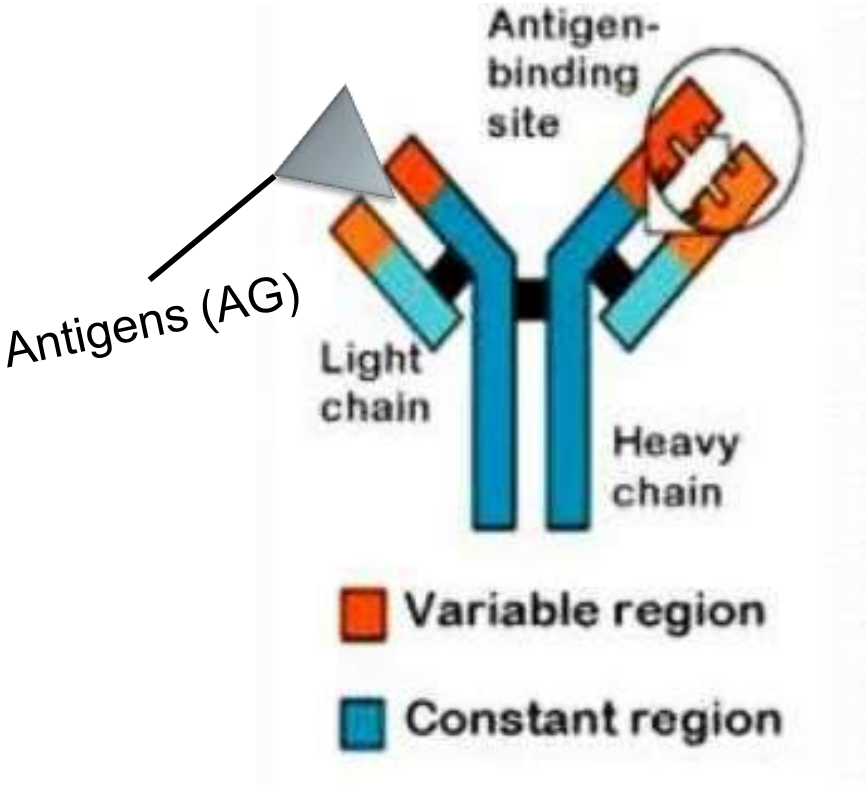
- It is a protein molecule produced by the immune system which helps in protection against pathogens by neutralizing them.
- ***Antobody isotypes:*** Classes of antibodies is known as Ab isotypes. Human antibodies have five isotypes; IgA, IgD, IgG, IgE, and IgM. It depends on the variable region.
- ***Antibody allotype:*** It is the allelic form of the immunoglobulin genes leading to variation in Ab.
- ***Antibody idiotype:*** It is due to the presence of antigen determinants present in the variable region of the antibodies.
- ***Monoclonal Ab (mAb):*** An antibody that is made in the laboratory that can bind to certain targets in the body, such as antigens on the surface of cancer cells.
- ***Polyclonal Ab (pAb):*** It is a mixture of antibodies that are secreted by different B cell lineages but bind to a specific antigen.:

Basic principle of ELISA

Antibody (Ab)

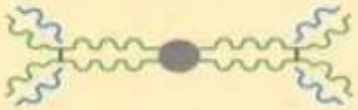
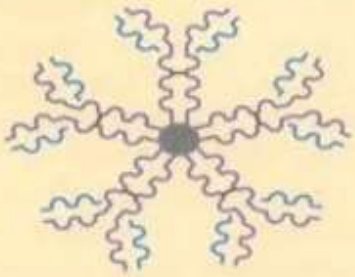
Y

Symbol of antibody (Ab)

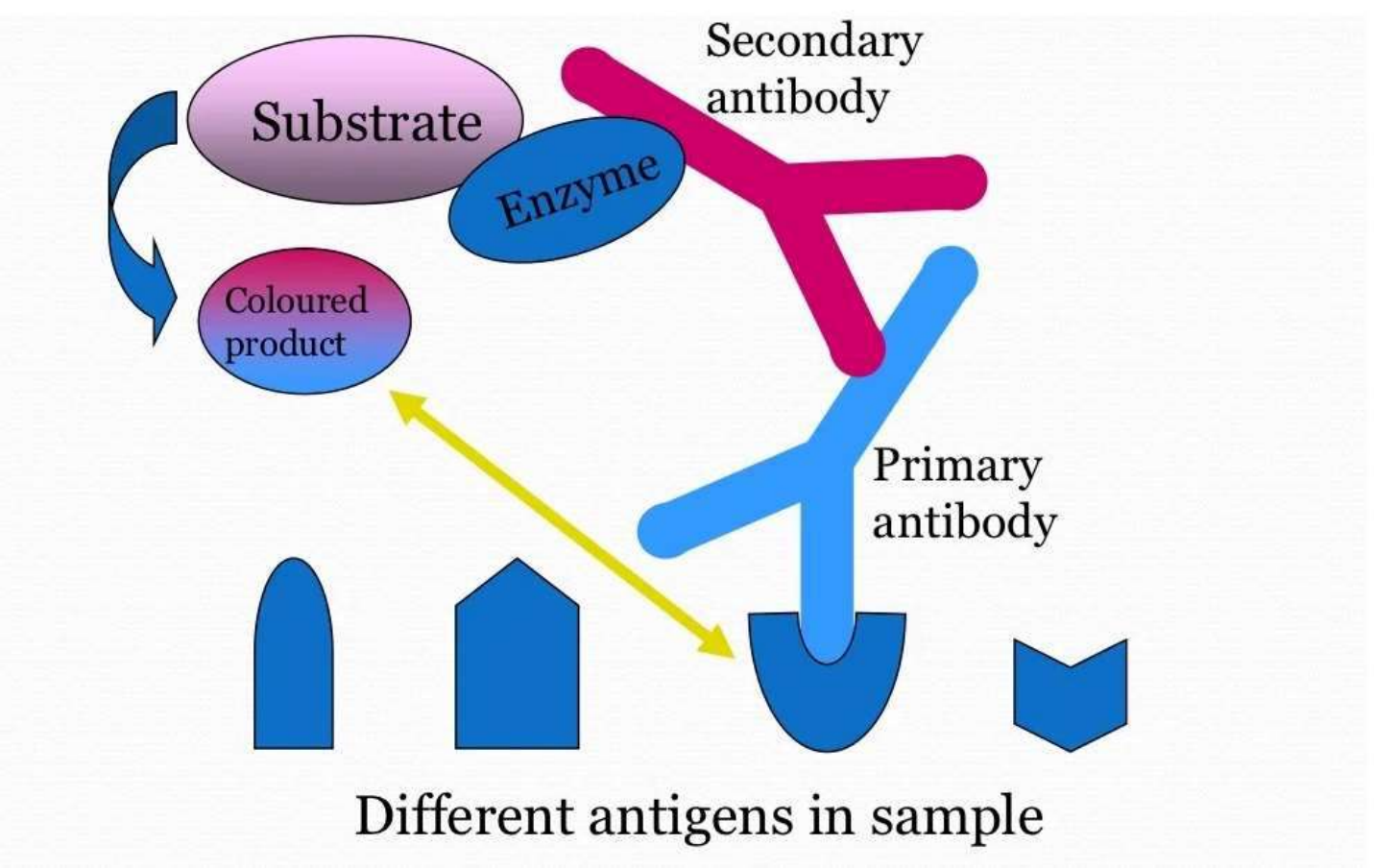


Antibody Isotypes

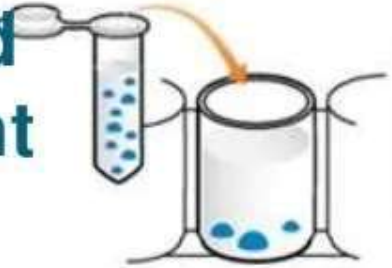
CLASSES OF IMMUNOGLOBULINS (Igs)

Name and Structure		Characteristics and Functions
IgG		Most abundant, about 75% of all antibodies in the body; found in blood, lymph, and the intestines; monomer (one unit) structure. Protect against bacteria and viruses by enhancing phagocytosis, neutralizing toxins, and triggering the complement system. The only antibodies to pass the placenta from mother to fetus and thereby confer some immune protection in newborns.
IgA		Make up about 15% of all antibodies in the body; occur as monomers and dimers (two units). Found in tears, saliva, mucus, milk, gastrointestinal secretions, blood, and lymph. Levels decrease during stress, lowering resistance to infection. Provide localized protection on mucous membranes.
IgM		About 5 to 10% of all antibodies; occur as pentamers (five units); first antibodies to be secreted by plasma cells after an initial exposure to any antigen; found in blood and lymph. Cause agglutination and lysis of microbes. Also present as monomers on the surfaces of B cells, where they serve as antigen receptors. A and B agglutinins, which bind to A and B agglutinogens on the surface of red blood cells, are IgM antibodies.
IgD		Less than 1% of all antibodies; occur as monomers; found in blood, in lymph, and on the surfaces of B cells as antigen receptors. Involved in activation of B cells.
IgE		Less than 0.1% of all antibodies; occur as monomers; located on mast cells and basophils and are involved in allergic reactions.

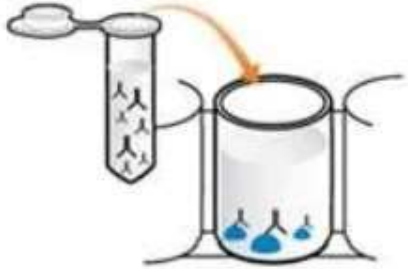
Basic principle of ELISA



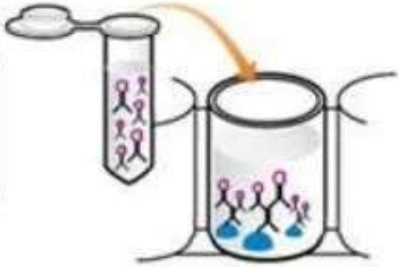
Basic Steps Of Enzyme-Linked Immunosorbant Assay



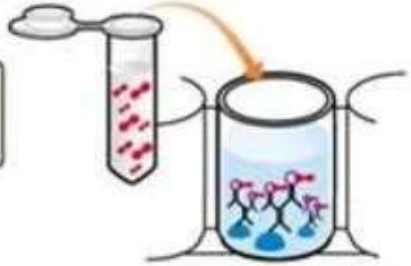
1. Load student samples (unknowns) in triplicate into wells of the microplate strips. Incubate for 5 minutes. Rinse.



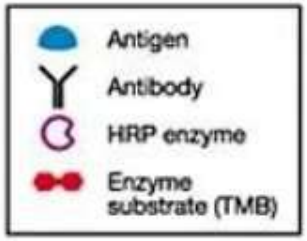
2. Add primary antibody to all wells. Incubate for 5 minutes. Rinse.



3. Add enzyme-linked secondary antibody to all wells. Incubate for 5 minutes. Rinse.



4. Add enzyme substrate to all wells. Incubate for 5 minutes.



Materials required

- Sample (It may be serum, CSF, sputum, urine, semen, culture supernatant, stool, etc.)
- ELISA reader
- Antibodies (Primary and Secondary/enzyme-linked)
- Polystyrene microtiter plate
- Standard (generally BSA is used)
- Blocking buffer
- Washing buffer
- Substrate

Materials required

Multipipette
Micropipette

Incubator

ELISA Reader
(Microplate Reader)

Reagents required

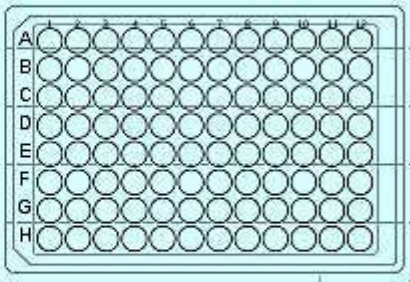
Reagent	Composition
Coating Buffer	0.01 M Phosphate Buffer + 0.15 M NaCl (PBS)
Diluting/Washing Buffer	0.01 M Phosphate Buffer + 0.50 M NaCl + 0.1% Tween 20
Blocking Buffer	Bovine Serum Albumin (BSA)
Enzyme	Horse-redish peroxidase (HRPO)
Chromogenic Substrate	Trimethyl benzidine (TMB)
Stop Solution	0.5 M H_2SO_4

Microtiter plate

ELISA plate: It is usually a PVC (polyvinylchloride) or polystyrene or polypropylene or polycarbonate microliter plate having 6, 12, 24, 48, 96, 384 or 1536 wells of varied capacity arranged in a 2:3 rectangular matrix. Some are developed with 3456 or 9600 well or strip of microplate. It may be flat bottom (F-bottom), bottom with minimal rounded edges (C-bottom), V-shaped bottom (V-bottom), and U-shaped bottom (U-bottom).

The earliest microplate was created in 1951 by a Hungarian, Dr. Gyula Takátsy (6 x 12).

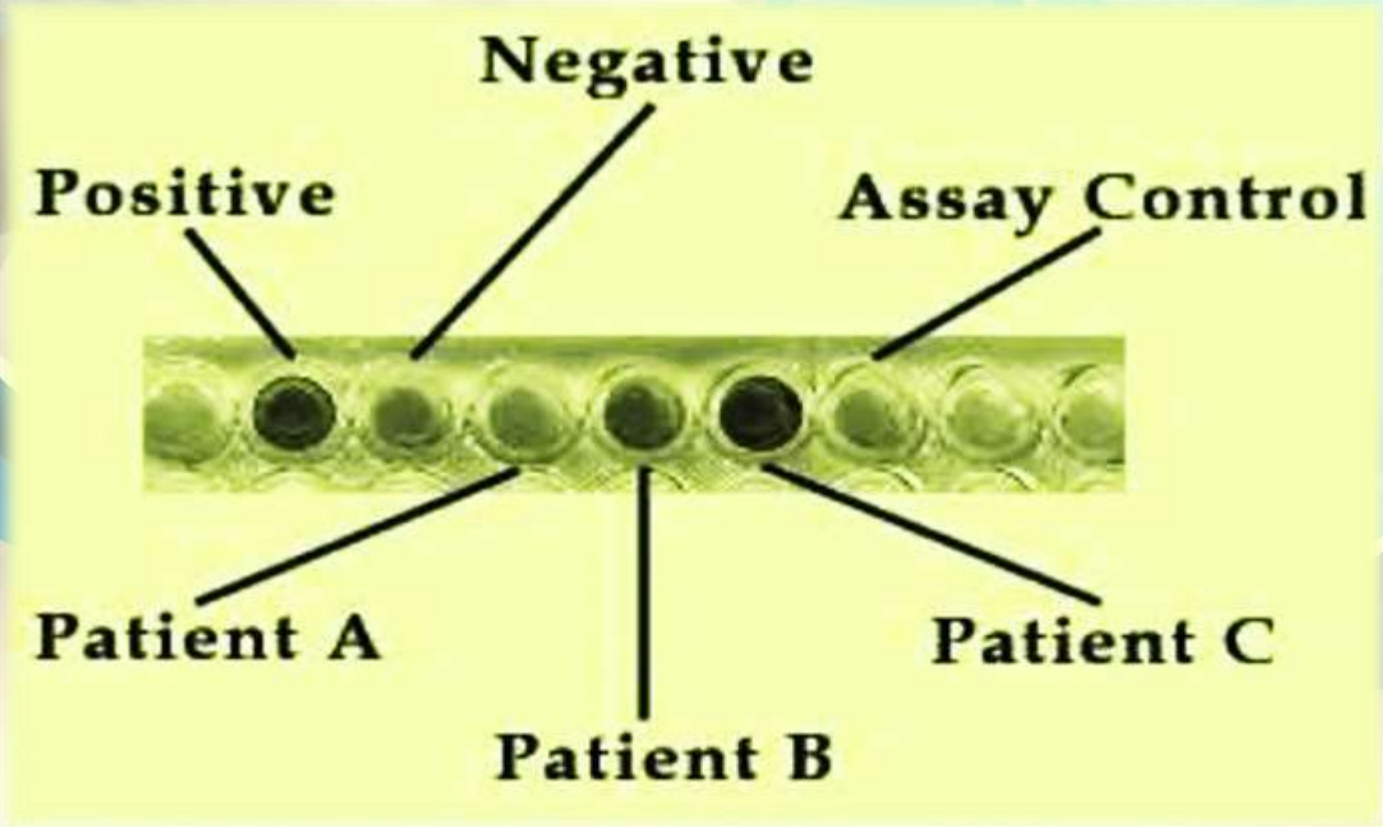
wells		volume [ml]
number	arrangement	
6	2×3	2–5
12	3×4	2–4
24	4×6	0.5–3
48	6×8	0.5–1.5
96	8×12	0.1–0.3
384	16×24	0.03–0.1
1536	32×48	0.005–0.015; Usage in UHTS (Ultra HTS)
3456	48×72	0.001–0.005; Usage in UHTS (Ultra HTS).



96-well

Microtiter is a registered trademark in the United States, so it should be used with proper atribution.

Results Interpretation



Types of ELISA

There are two types of ELISA:

1. ***Qualitative ELISA:*** The method of ELISA which is performed to only determine the presence or absence of an antigen in the sample. It does not tell about the concentration of antigens in the solution.

2. ***Quantitative ELISA:*** The method of ELISA which is performed to determine the quantity of the antigen in the sample. It is a type of titer in which a measurement of the amount or concentration of an antigen in a solution is done.

Types of ELISA

Competitive ELISA or Inhibition ELISA: A type of ELISA in which the concentration of an antigen is measured by detection of signal interference caused due to competitive binding.

Non-competitive ELISA: A type of ELISA in which the analyte is allowed to bind with an excess amount of labeled antibody. It is considered more sensitive than competitive ELISA. It may be classified as a two-site immunometric assay and a single-antibody immunometric assay. It can be classified into three:

Direct Elisa: In this type, the antigen is immobilized and only an enzyme-labeled primary antibody is used; the secondary antibody is not used.

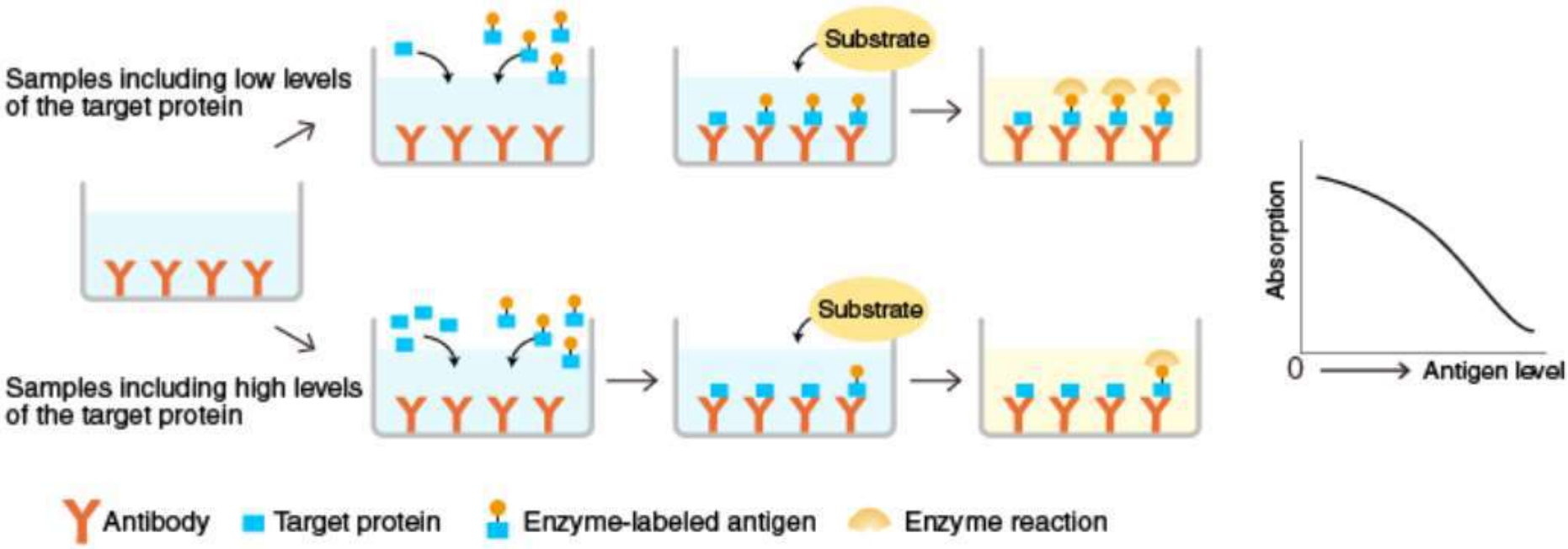
Indirect ELISA: In this type, the antigen is immobilized, and both a primary antibody and a secondary antibody are used. A secondary antibody is labeled with an enzyme in this case.

Sandwich ELISA: In this type, the antibody is immobilized to the plate called a capture antibody. In addition, the detection antibody is used which generally includes the unlabelled primary antibody and the enzyme-linked secondary antibody.

Competitive ELISA

- As discussed, the reference antigen is pre-coated on a multi-well plate.
- The sample is pre-incubated with labeled antibodies and added to the standard antigen-coated wells.
- Unbound antibody is removed by washing the plate (the more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence “competition”).
- The secondary antibody that is specific to the primary antibody and conjugated with an enzyme is added.
- A substrate is added, and the remaining enzymes elicit a chromogenic or fluorescent signal.
- Appearance of color indicates a negative test (absence of antibodies), while the absence of color indicates a positive test (presence of antibodies).

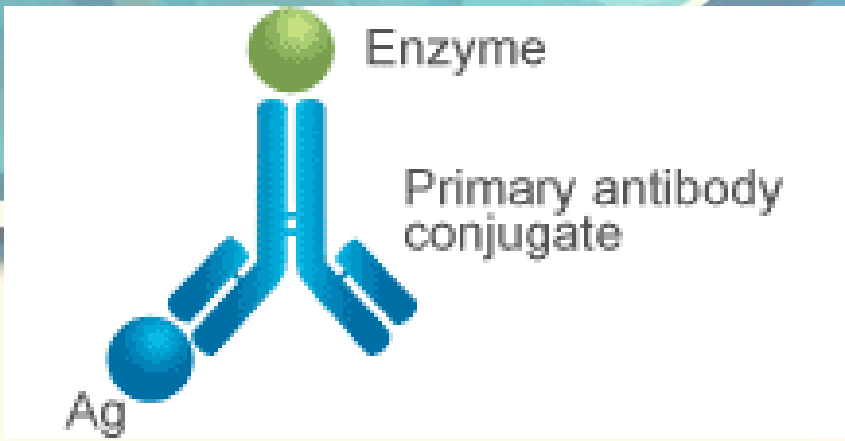
Competitive ELISA



https://ruo.mbl.co.jp/bio/e/support/method/elisa.html

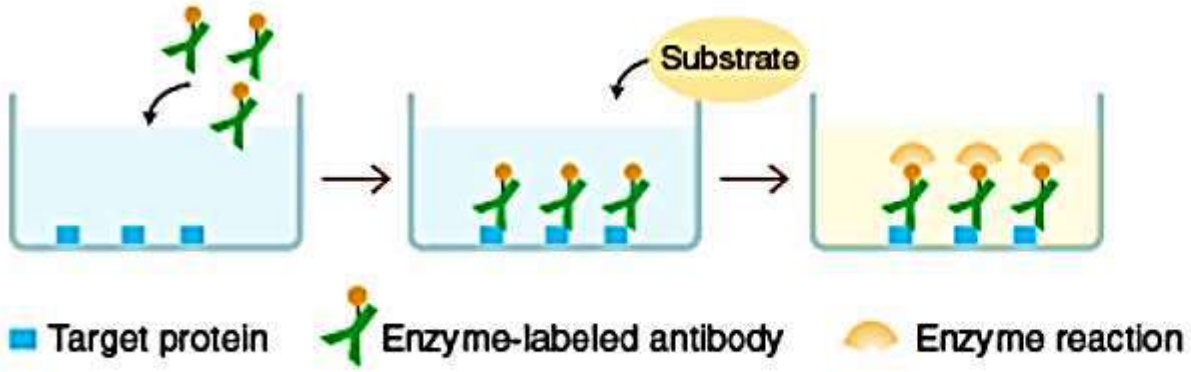
Direct ELISA

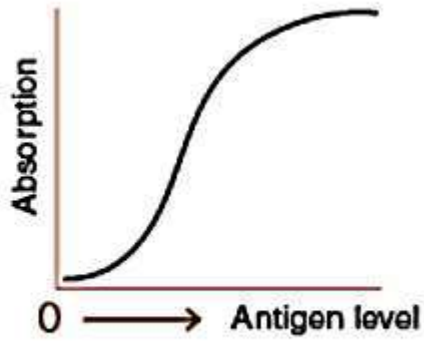
- Antigen is immobilized to the microtiter plate wells.
- Primary labeled antibody is used.
- No secondary antibody.
- Primary antibody directly binds to the Ag.
- Enzyme-linked to the primary Ab reacts with substrate to produce a visible signal that can be measured.
- In this way, the antigen is detected.



Direct ELISA

Steps of direct ELISA





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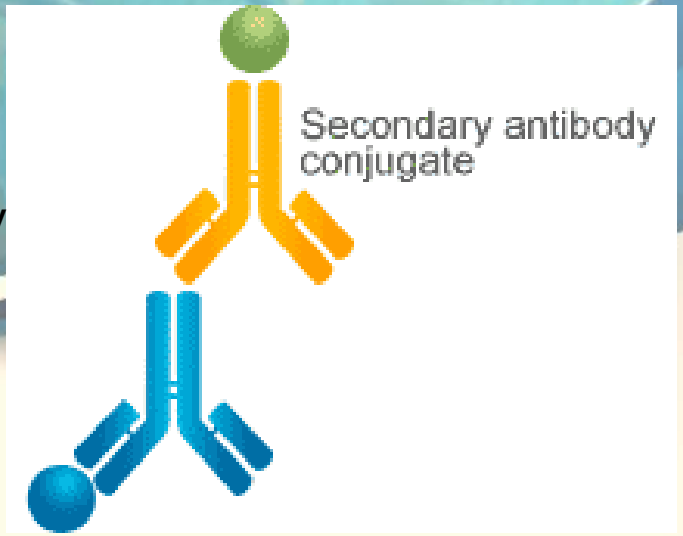
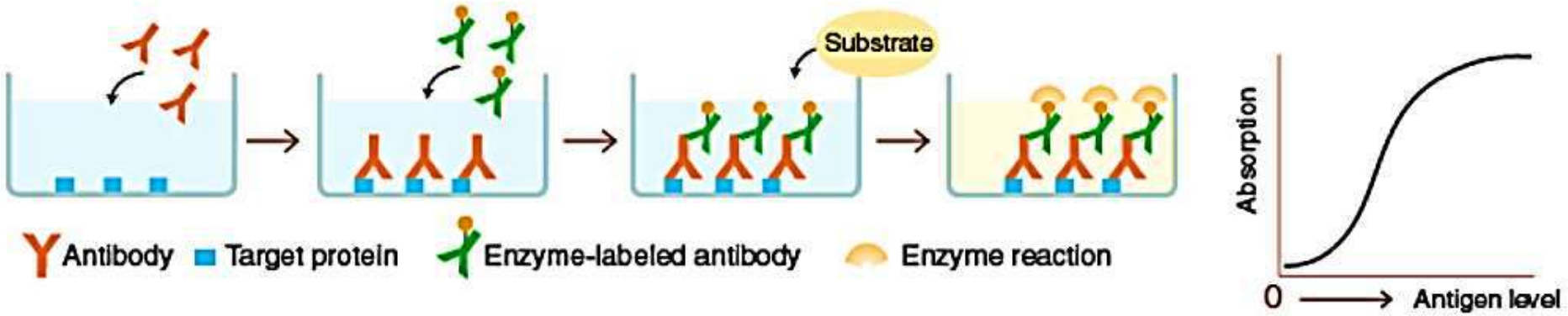
Indirect ELISA

- Antigen is immobilized to the microtiter plate wells.
- Both a primary antibody and a secondary antibody are used.
- Secondary antibody is labeled with an enzyme.
- Primary antibody binds to the Ag that is immobilized to the plate.
- Enzyme-labeled secondary Ab binds to the primary Ab which is bound to the Ag.
- Finally, the enzyme-linked to the secondary Ab reacts with its substrate to produce a visible signal that can be measured.
- In this way, the antigen is detected.



Indirect ELISA

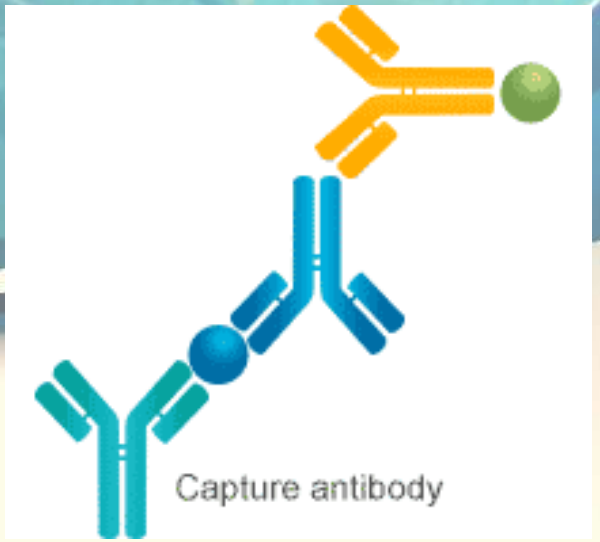
Steps of direct ELISA



https://ruo.mbl.co.jp/bio/e/support/method/elisa.html

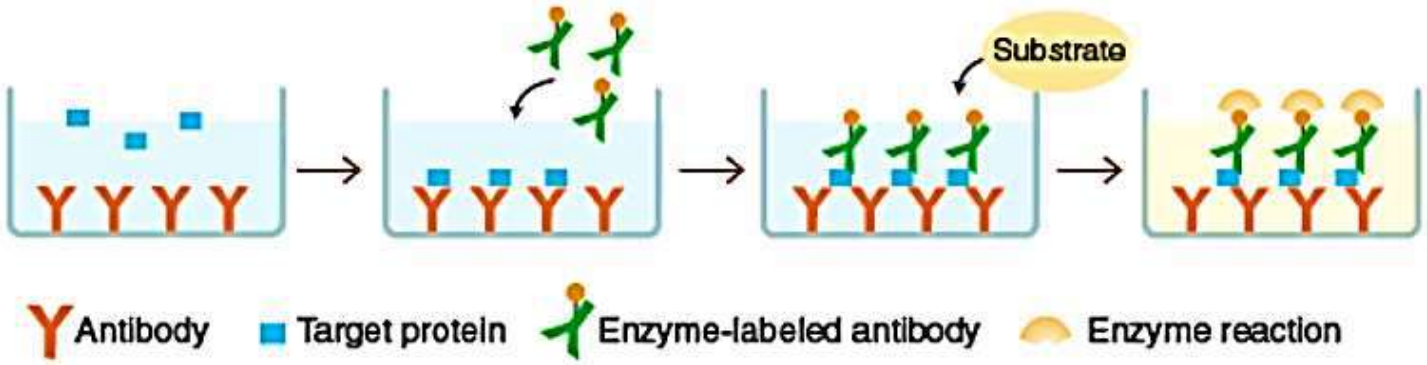
Sandwich ELISA

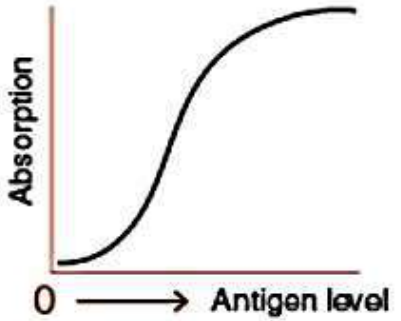
- Capture antibody is immobilized to the microtiter plate wells.
- Capture antibody binds to the antigen added to the wells.
- In addition to capture Ab, primary and secondary detection antibodies are added.
- Unlabelled primary detection antibody binds to the Ag that is bound to capture Ab.
- Then secondary detection Ab labeled with an enzyme is added to the wells which bind to primary detection Ab.
- Finally, the enzyme-linked to the secondary detection Ab reacts with its substrate to produce a visible signal.
- In this way, the antigen is detected.



Sandwich ELISA

Steps of direct ELISA





https://ruo.mbl.co.jp/bio/e/support/method/elisa.html

Enzymes of ELISA

- Most commonly used is horseradish peroxidase.
- Alkaline phosphatase
- β-galactosidase
- Lactoperoxidase
- Tetra methyl benzidine

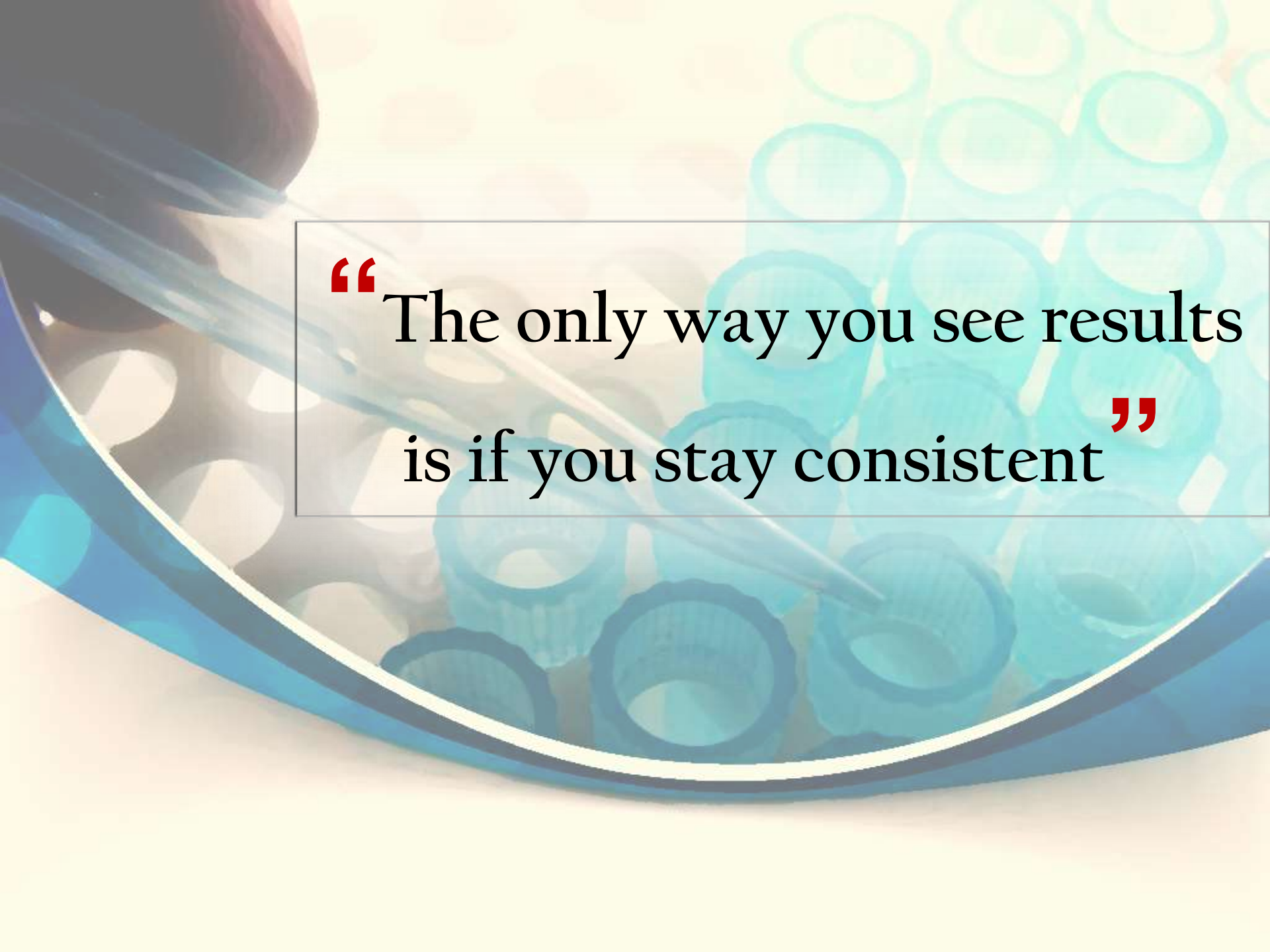
In the case of peroxidase, the substrate hydrogen peroxidase is converted into water and O_2 in the presence of electron donors like diaminobenzidine or 4-chloronaphthol which themselves oxidized in the reaction.

Oxidation of diaminobenzidine produces dark brown color while that of 4-chloronaphthol yields purple color which is the basis of ELISA.

Enzyme substrate of ELISA

Enzyme	Substrate	Chromogen	Stopping
Horse radish peroxidase	p-NPP	p-NPP + diethandamine + $MgCl_2$	1M NaOH
Horse radish peroxidase	H_2O_2	Tetramethylbenzidine + Phosphate-citrate buffer	1M H_2SO_4
Alkaline phosphatase	H_2O_2	O-phenylenediamine + HCl	1M HCl

The substrate should be initially colorless, and after degradation by the enzyme, it should be strongly colored or fluorescent.


“The only way you see results
is if you stay consistent”