APPLICATIONS OF IMMUNOLOGY

Monoclonal Antibodies in Diagnostics

BCH 4047 TD/TP. #2

By

Dr Palmer Masumbe Netongo (PhD)

Principles of Immunoassays

- Immunoassays are based on **highly specific** binding between an **antigen** and an **antibody**.
- An epitope (immunodeterminant region) on the antigen surface is recognized by the antibody's binding site.
- The type of antibody and its affinity and avidity for the antigen determines assay sensitivity and specificity.
- Depending on the assay format, immunoassays can be qualitative or quantitative.
- They can be used for the detection of antibodies or antigens specific for bacterial, viral, and parasitic diseases as well as for the diagnosis of autoimmune diseases.
- Immunoassays can measure low levels of disease biomarkers and therapeutic or illicit drugs in patient's blood, serum, plasma, urine, or saliva.
- Immunostaining is an example of an immunochemical technique, which combined with fluorescent labels allows direct visualization of target cells and cell structures.
- **Immunochromatography**

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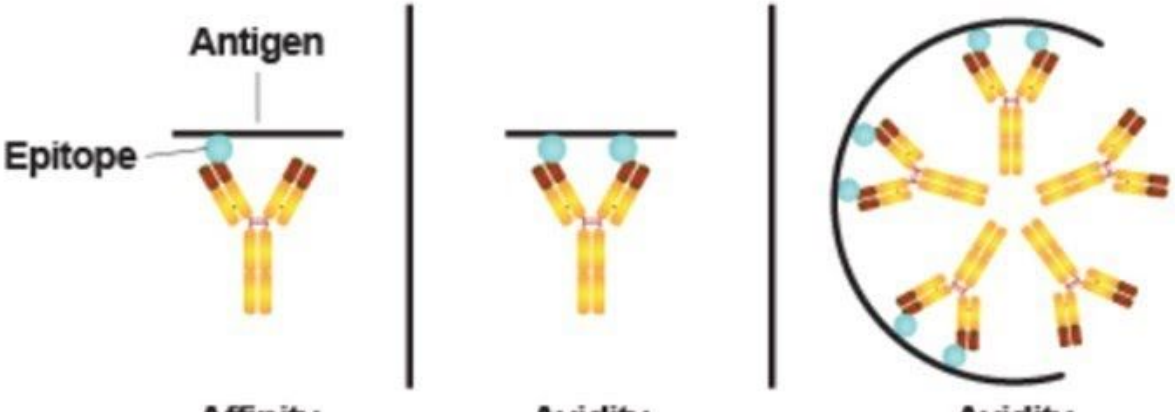
Principles of Immunoassays

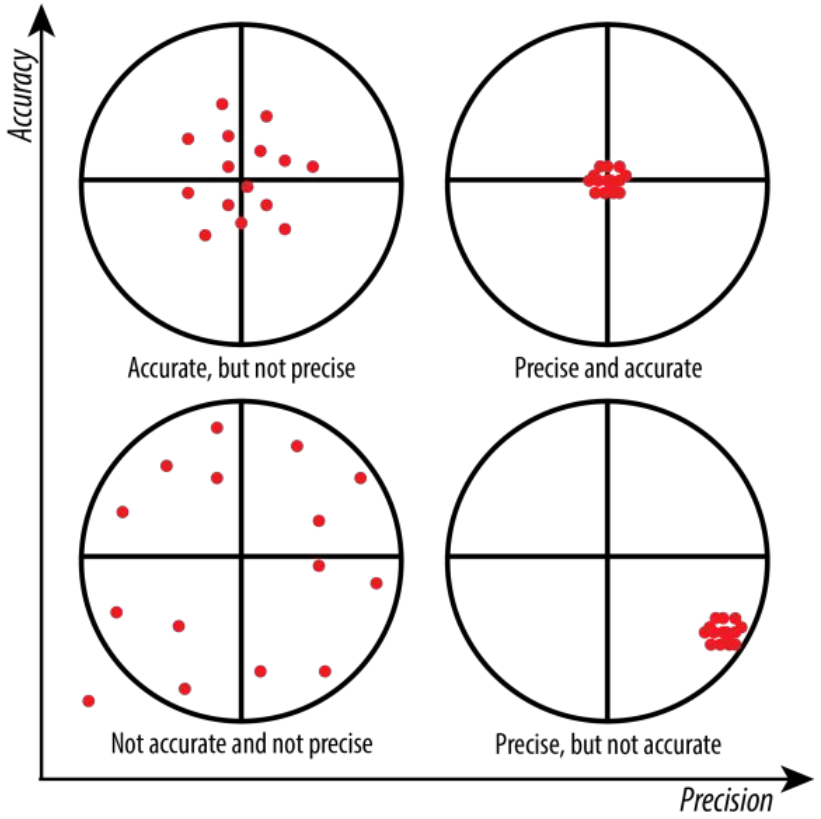
- Affinity and avidity are both measures of binding strength.
- Affinity is the measure of the binding strength at a single binding site
- Avidity is a measure of the total binding strength.

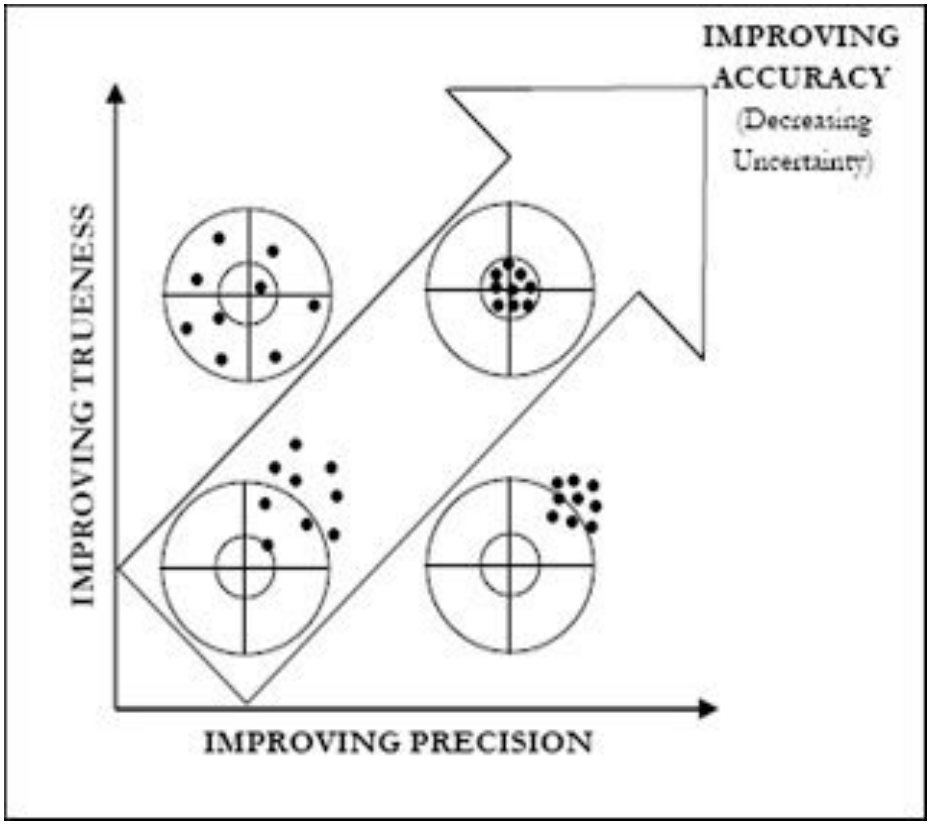
Affinity vs Avidity

Differences



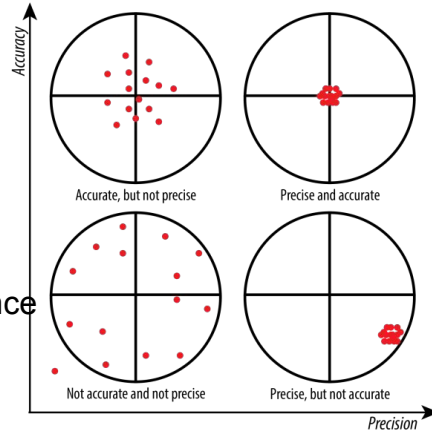
Accuracy Vs Precision





- Accuracy and Precision are independent
  - Accuracy: How close your data is to the true data
  - Precision: How close your data agrees with each other.

Accuracy Vs Precision



Determine which student has the most accurate and precise experiments if the reference measure of the amount of protein Y in the blood measured by ELISA is 2.7mg/L.

Kamdem	Zogo	Edube	Rainatou
2.924	2.316	2.649	2.701
2.923	2.527	2.731	2.699
2.925	2.941	2.695	2.702
2.926	2.136	2.742	2.698

NA	NA	A	A
P	NP	NP	P

Agglutination Reactions

- To detect particulate antigens in solution
- Antibodies cause clumping (agglutination) of their specific antigens (e.g. Hemagglutination for blood typing: detects surface antigens on RBCs)

Direct agglutination tests

- To detect if patient has antibodies to particular antigen (antibodies present = exposure or infection by the agent)
- known infectious agents are bound to a microtiter dish
- patient serum is added
- if patient has antibodies to agent agglutination will occur

Indirect agglutination tests

- Indirect = uses latex particles

- -two ways:

1. known antigen is bound to latex particles
   - ? assay for patient sample for antibody
2. known antibodies are bond to latex particles
   - ? assay for antigenic agent

Fluorescent Antibody Labeling

- Use antibody chemically linked to florescent dye that is visible with UV light
- Direct FA Test
  - identify or visualize microbes in clinical specimens
  - probe specimen with fluorescent antibodies
- Indirect FA Test
  - detect the presence of antibodies in serum
  - fix known antigen to slide, probe with patient serum, tag with fluorescent antihuman immune serum globin (αHISG)

Antihuman immune serum globin (αHISG)

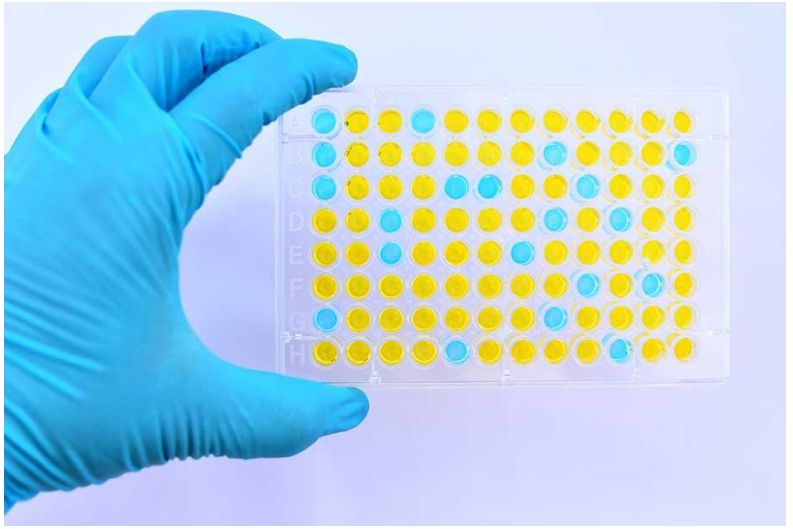
- Antibody that will bind to human antibodies at Fc region
- αHISG antigen binding sites are specific for any human IgG (or IgM) molecules as their epitope
- Fc region of αHISG will have fluorescent molecules or enzymes attached to allow detection/visualization of binding

Enzyme Linked ImmunoSorbant Assay (ELISA)

- The term **enzyme-linked** refers to an enzyme's covalent binding to an antibody.
- It is a highly sensitive assay that can detect proteins at the picomolar to nanomolar range (10^{-12} to 10^{-9} moles per liter).
- It is the mainstay (Confirmatory Test) for the diagnosis of infections by many different viruses, including HIV-1, adenovirus, and cytomegalovirus.

Enzyme Linked ImmunoSorbant Assay (ELISA): Principle

- One of the reaction components is nonspecifically adsorbed or covalently bound to the surface of a solid phase, e.g.:
  - microtiter well,
  - magnetic particle,
  - plastic bead.



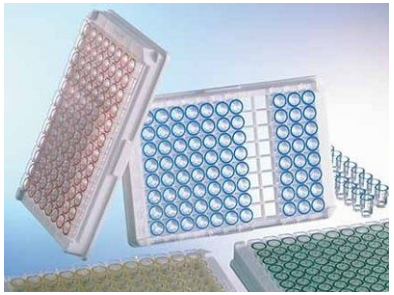
- This attachment facilitates the separation of bound and free-labeled reactants.

Enzyme Linked ImmunoSorbant Assay (ELISA): Principle

- An aliquot of sample or calibrator containing the antigen (Ag) to be quantified is added to and allowed to bind with a solid-phase antibody (Ab).
- After washing, an enzyme-labeled antibody is added and forms a “sandwich complex” of solid-phase Ab-Ag-Ab enzyme.
- Unbound antibody is then washed away, and enzyme substrate is added.
- The amount of product generated is proportional to the quantity of antigen in the sample.

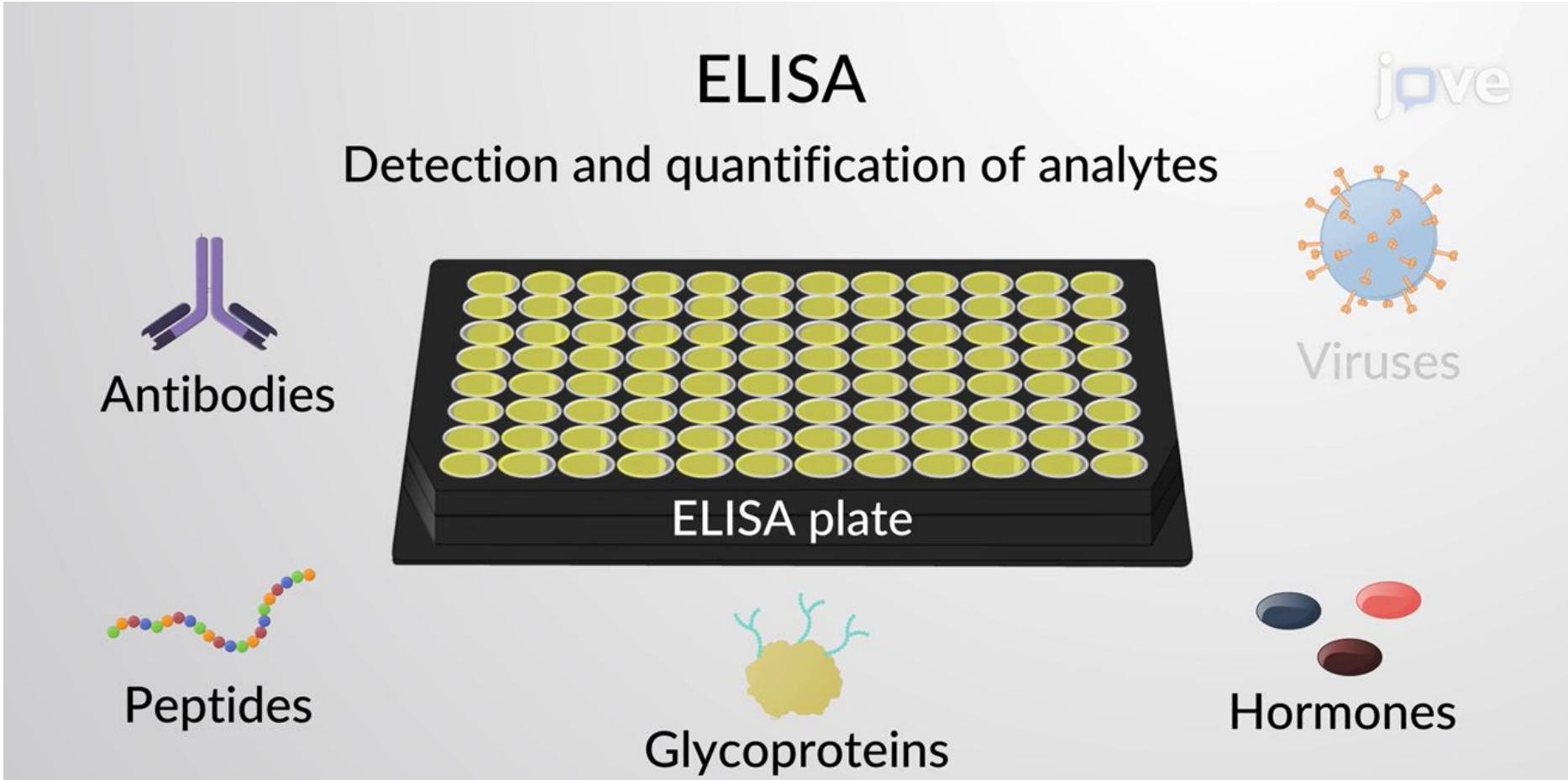
Enzyme Linked ImmunoSorbant Assay (ELISA)

- Used to detect either antigens (*antigen ELISA*) or antibodies (*antibody ELISA*) in patient sample
  - targets the protein directly on the bacteria or virus,
  - target is the animal's antibody response to the bacteria or virus
- Performed in microtiter plate
- Positive reaction produces color change
- Process can be automated: computer readout of many samples at once
- **Disadvantage**: Risk of false positives



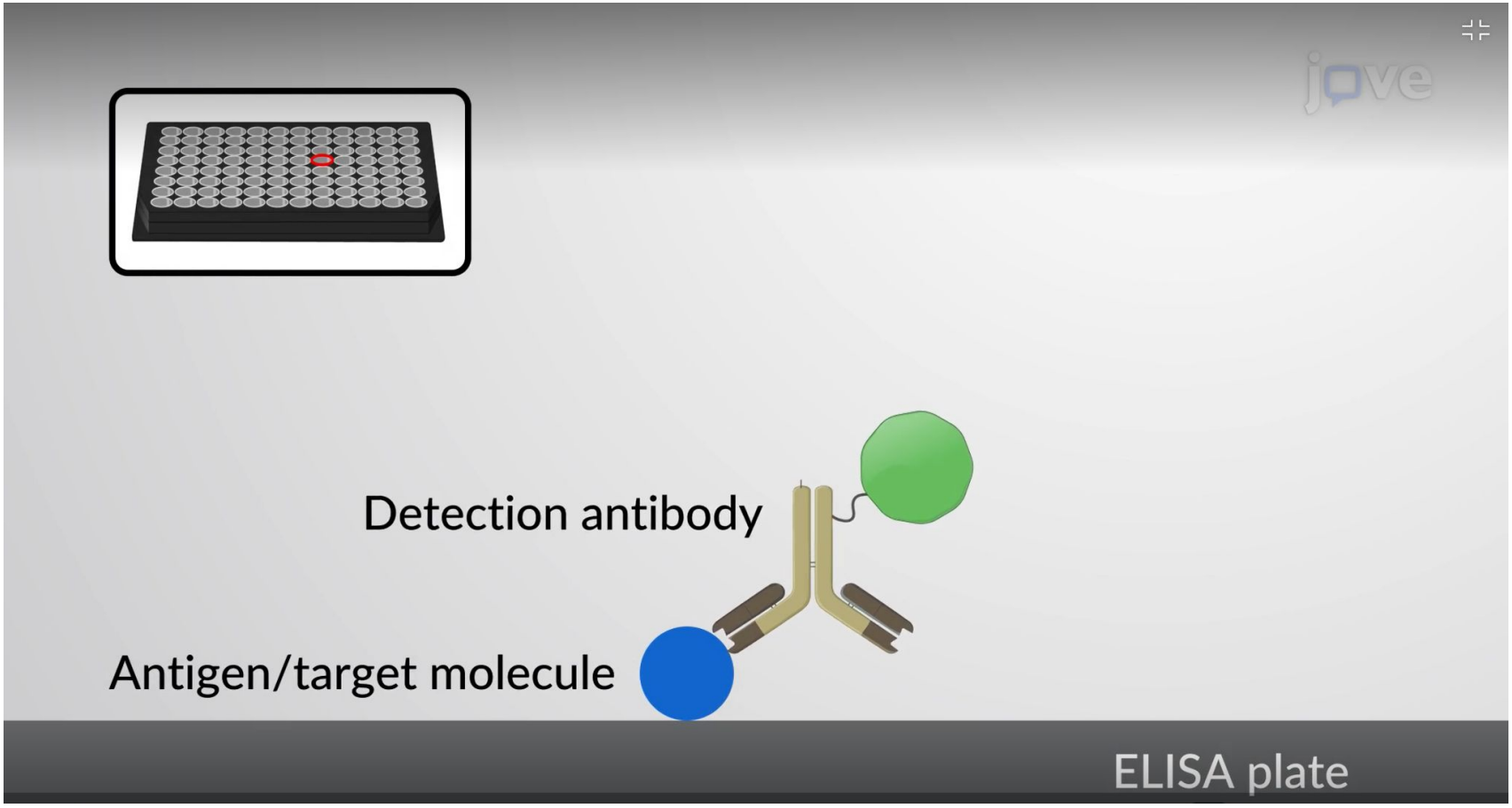
Enzyme Linked ImmunoSorbant Assay (ELISA)

kEY CONCEPT: It is based on the property of proteins to readily bind to a plastic surface.

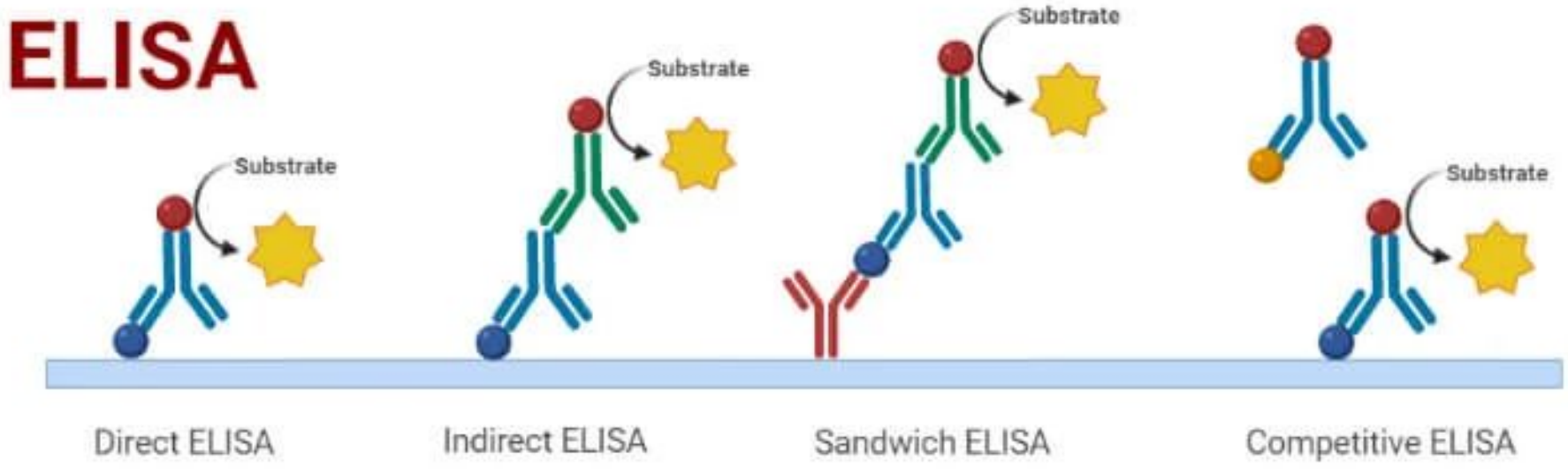


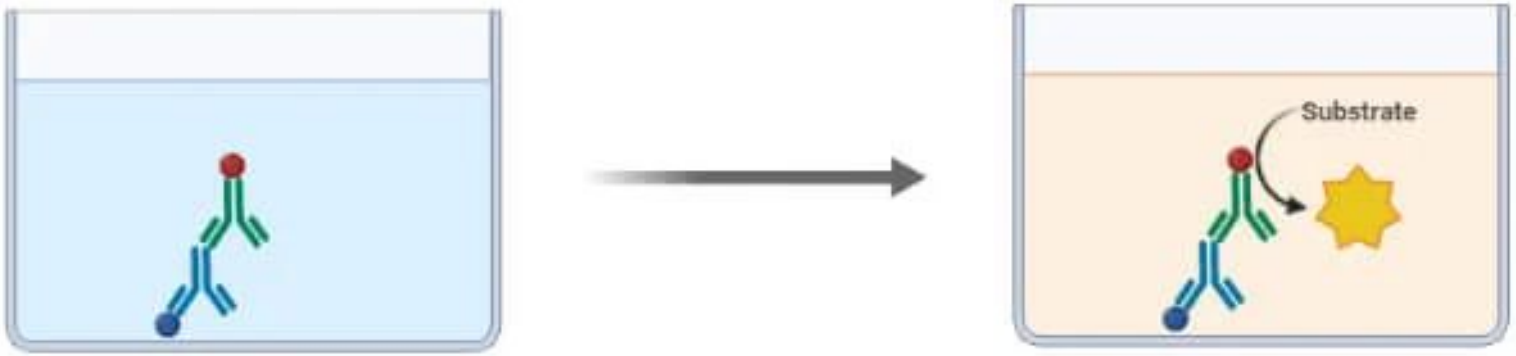
Enzyme Linked ImmunoSorbant Assay (ELISA)

Detection Antibody is key and pay attention to its origin (Mouse, Human, Rabbit, Goat etc)



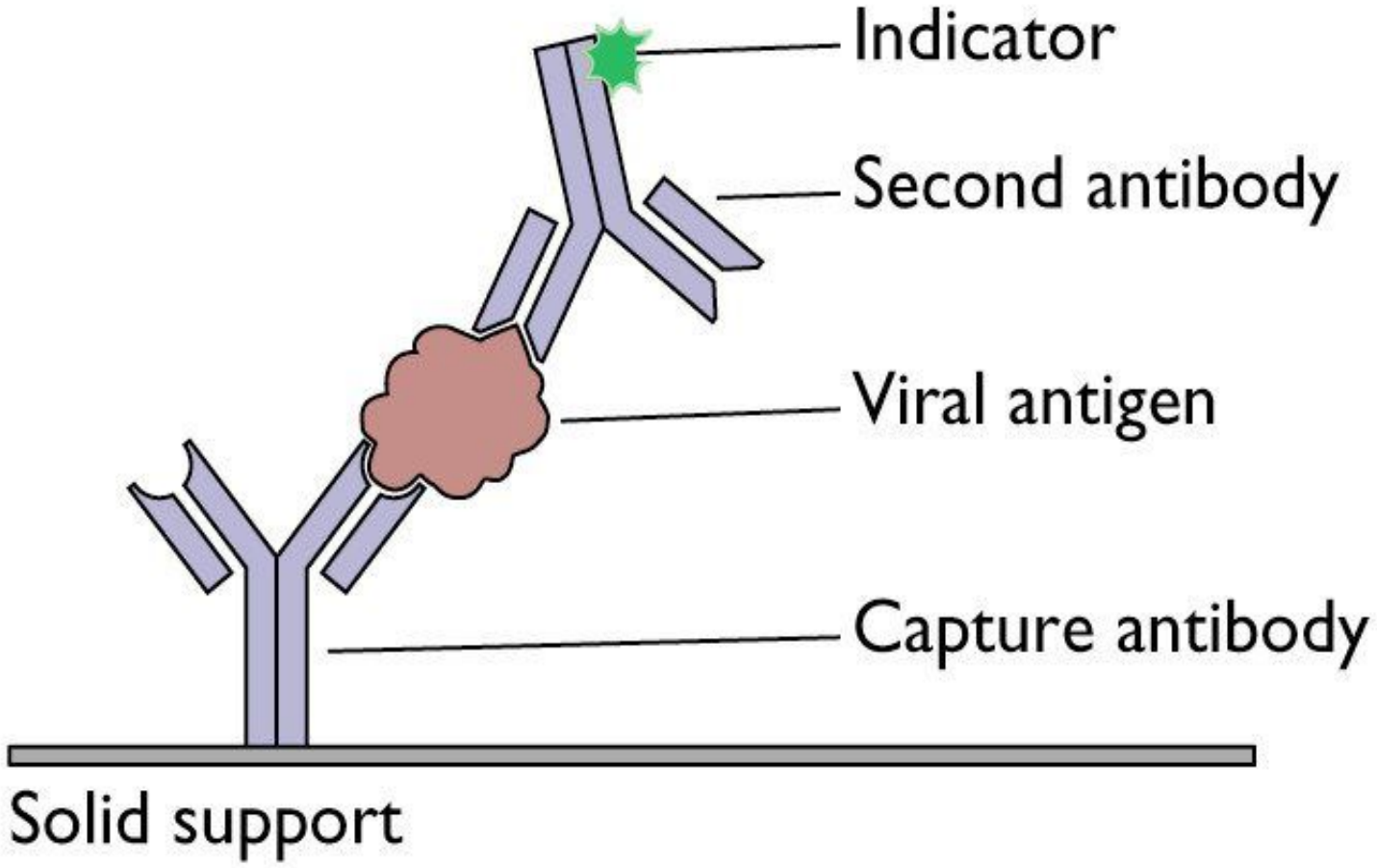
ELISA: Four Types



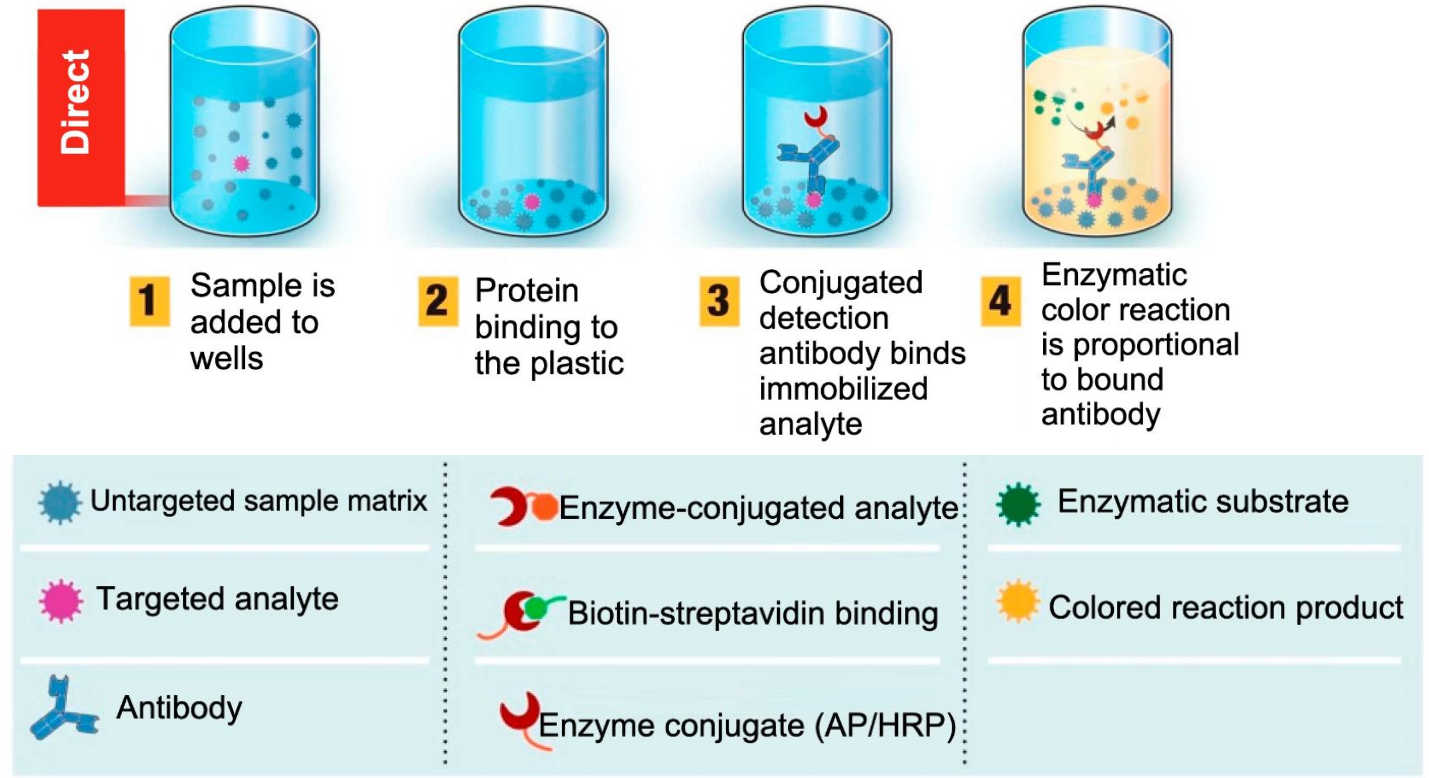


ELISA

A. Direct ELISA ("Sandwich ELISA")
   - antibody bound to dish
   - assay for presence of antigen in sample



Enzyme Linked ImmunoSorbant Assay (ELISA) Direct

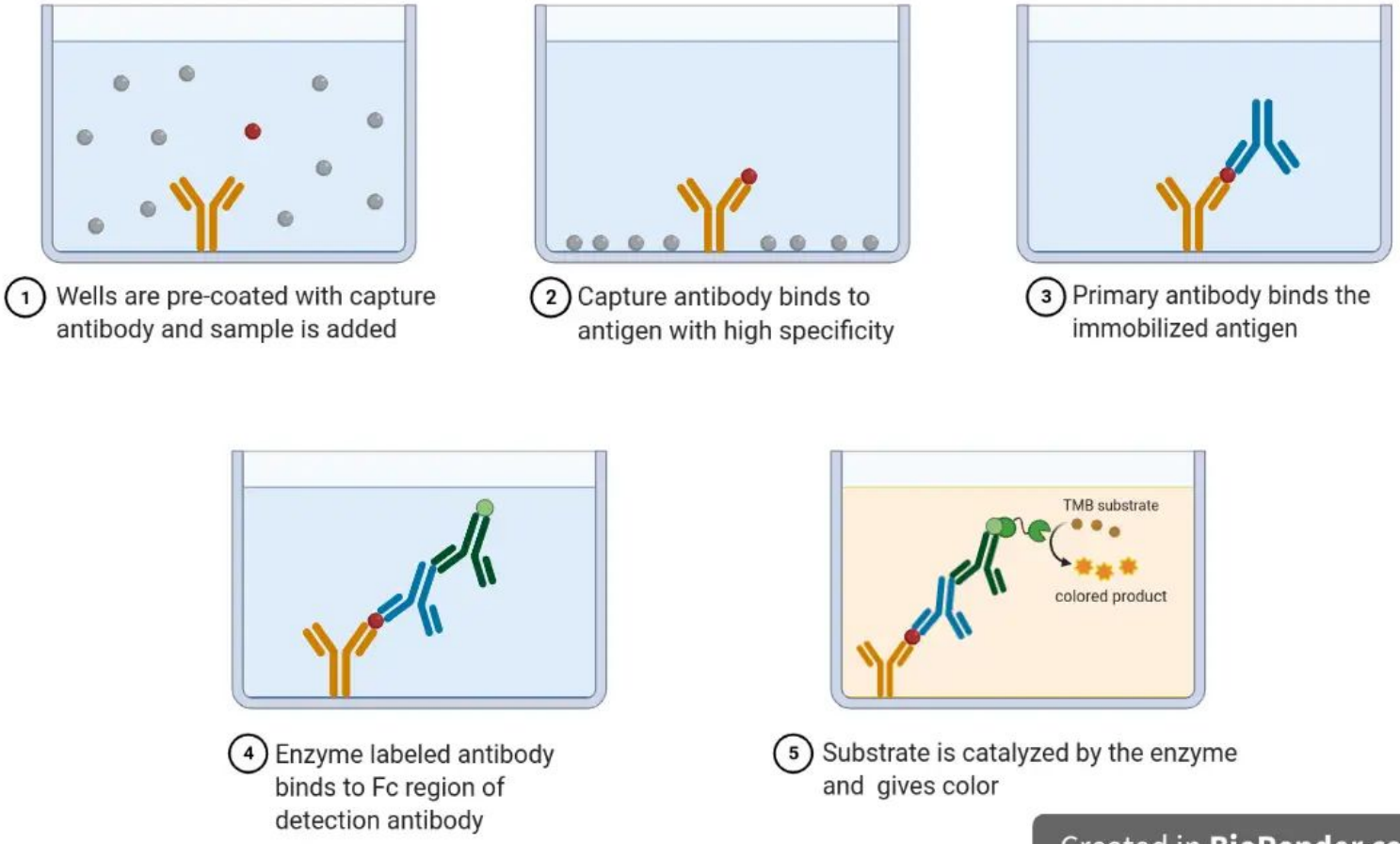


- https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

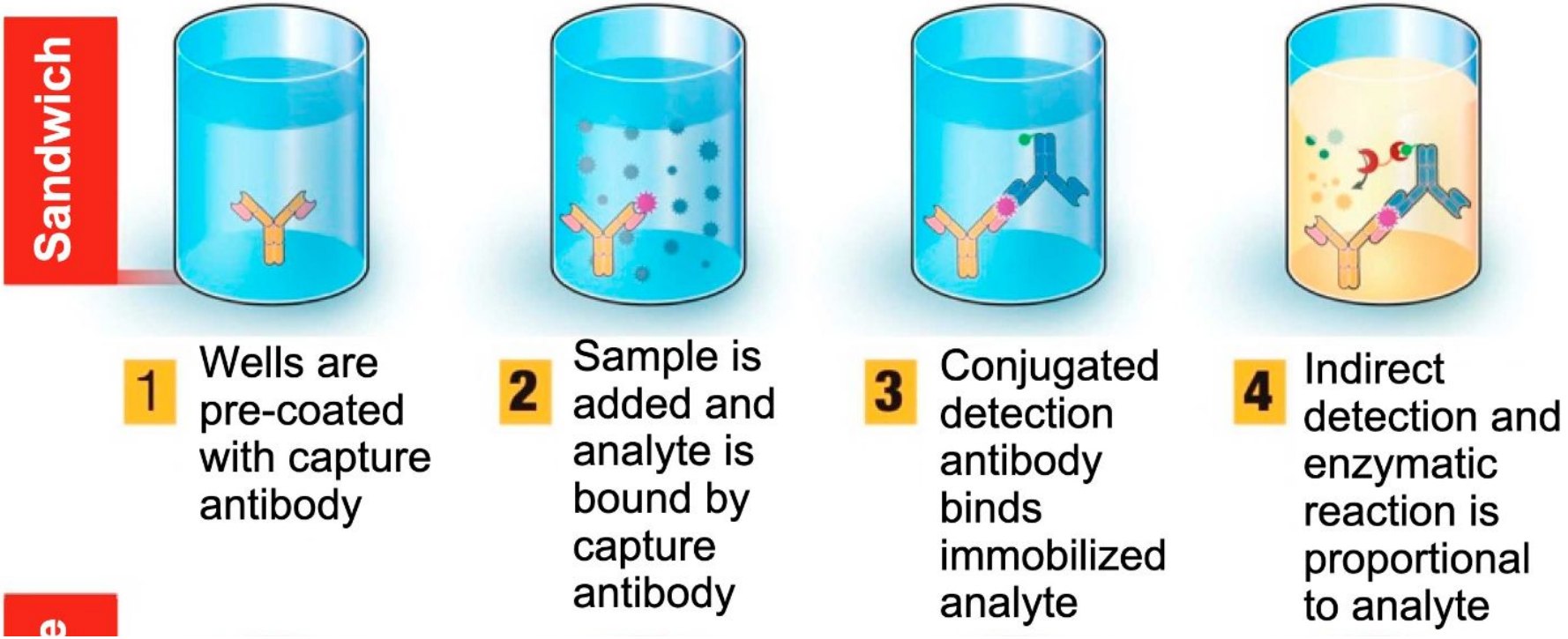
B. Direct ELISA ("Sandwich ELISA")

ELISA

- antibody bound to dish
- assay for presence of antigen in sample

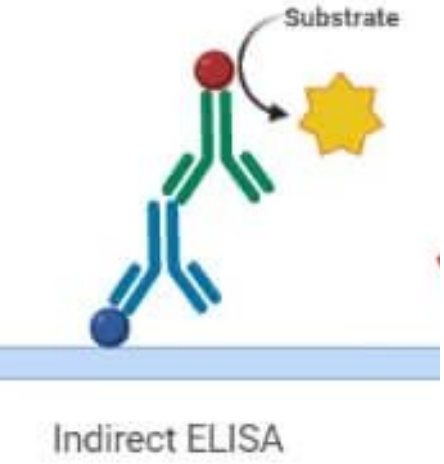


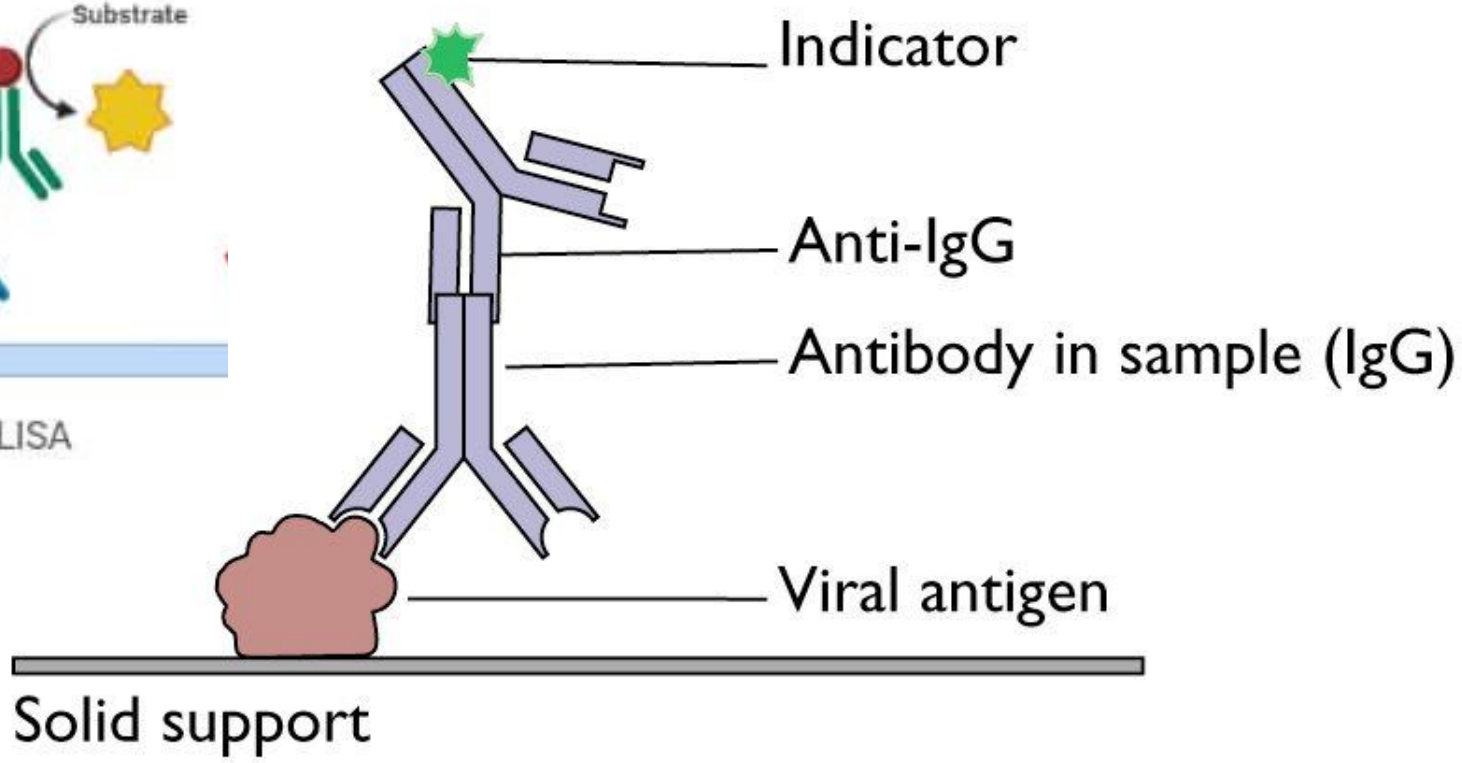
Enzyme Linked ImmunoSorbant Assay (ELISA) Sandwich



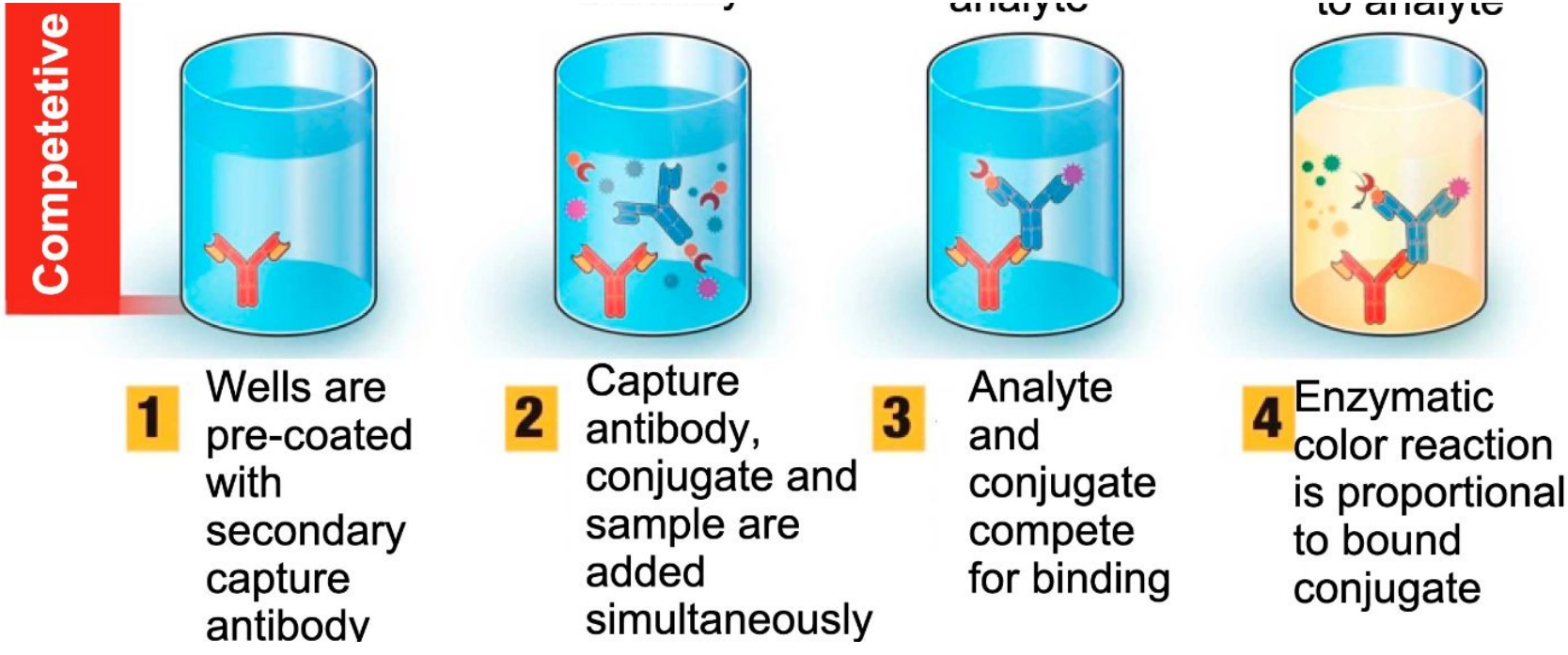
C. Indirect ELISA

- antigen bound to dish
- assay for presence of antibody in sample





D. Enzyme Linked ImmunoSorbant Assay (ELISA) Competitive



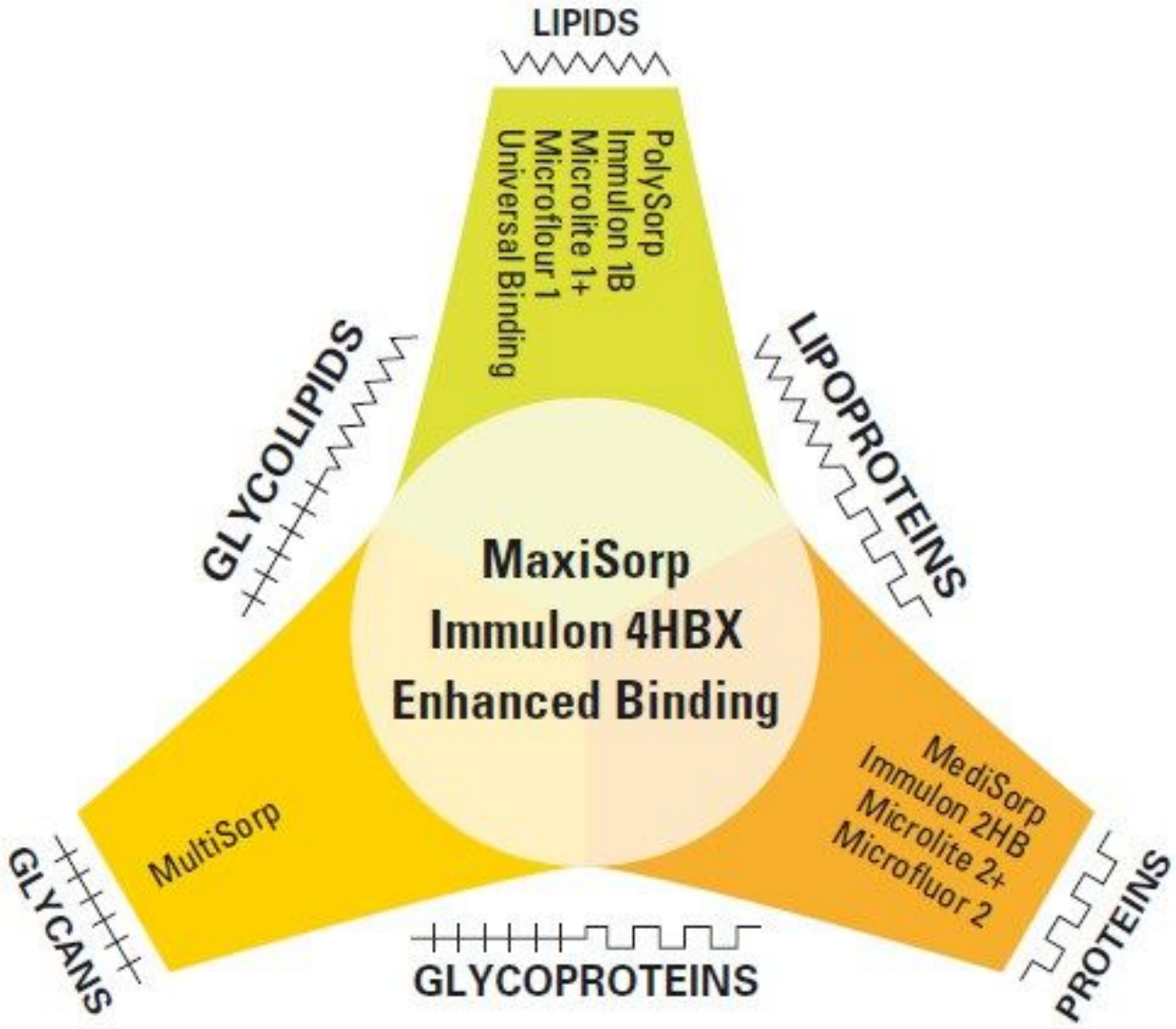
D. Competitive ELISA

Crude samples can be directly used. The experimental setup is highly flexible, wherein direct, indirect, or sandwich ELISAs can be adapted to a competitive format. The sample analyte concentration is determined by the signal interference. The "competition" comes from the fact that if a sample antigen is being tested, then the incubation of the sample with a primary antibody will result in lesser antibodies available to bind to the wells coated with the same antigen. Thus, the intensity of the signal produced in the well, due to the competitive binding, which is concentration dependent, becomes inversely correlated to the amount of sample antigen.

ELISA: Requirements

- **Coated plates (Microtitre plates):** Commonly used ones are 96 well polystyrene plates. Coated with antigens or antibodies at the bottom of the well.
- **Sample diluents:** To dilute the sample before application in some cases of ELISA test.
- **Wash Buffers:** Help to wash away the unrequired contaminants and unbound antigens or antibodies. Eg. Triphosphate buffer (ph 7.40) and detergents such as Tween-20.
- **Enzyme-linked Antibodies:** For this, most common enzymes used are
  - AP(Alkaline Phosphatase)
  - HRP(Horseradish Peroxidase)
- **Substrates:** Specific chromogenic substrates are used for the respective enzymes used. Commonly used substrates are o-phenylene diamine for HRP enzyme and p-nitrophenyl phosphate for AP enzyme.
- **Stop solution:** It stops the enzyme and substrate reaction. It can be acids such as sulphuric acid.

ELISA: What Plate is Best for your Experiment?



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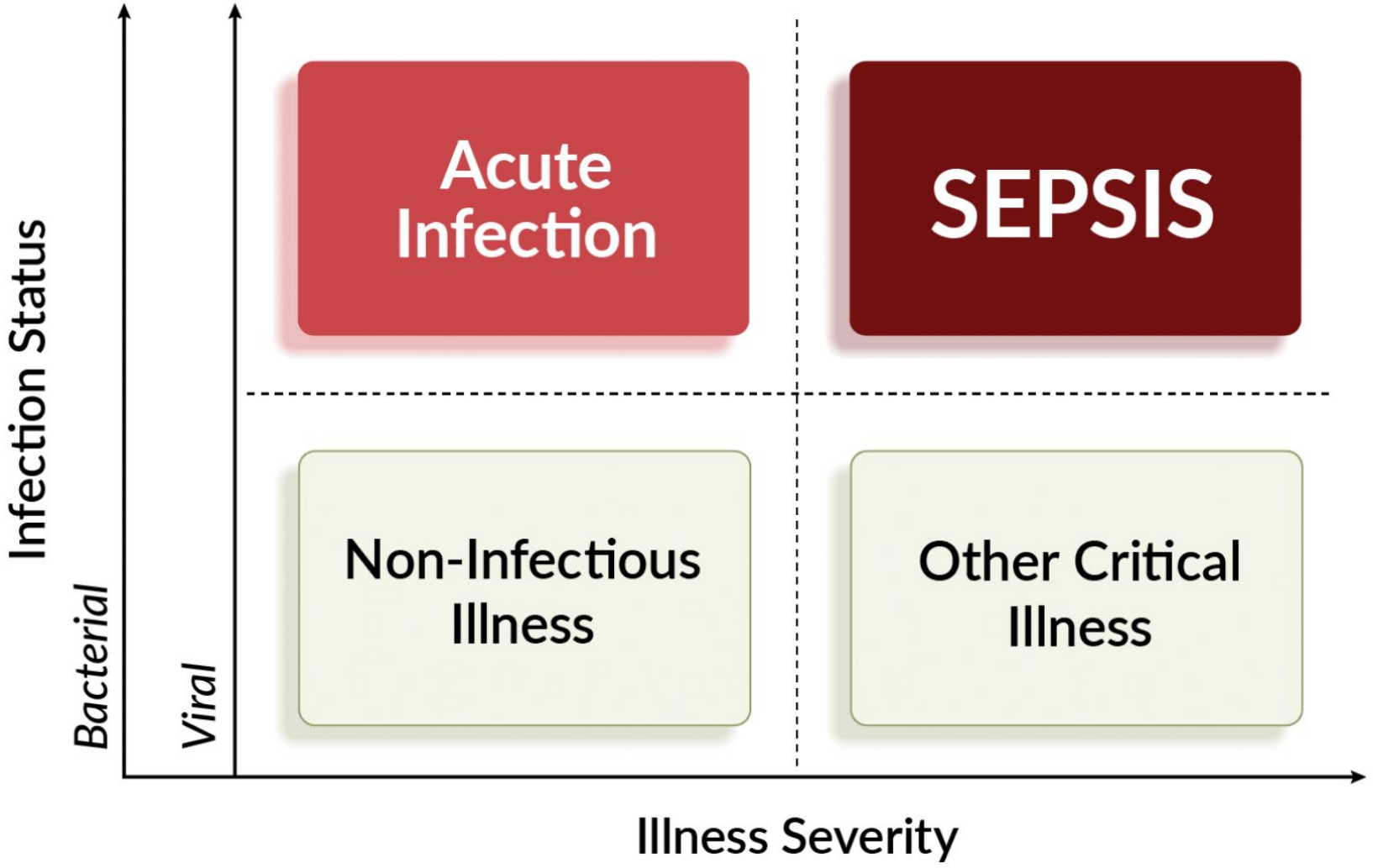


Western Blot / Immunoblotting

- Often used to confirm ELISA positive result
- Unlike ELISA, Western blot assays both **size of protein antigen** and **specific reaction with antibody**
- Size confirmation proves/disproves possible cross reaction (false positive)
- In Western blotting (WB), target **proteins are transferred** to a **hydrophobic membrane** after **SDS-PAGE** and **detected using specific antibodies.**
- After SDS-PAGE, a membrane is placed on the gel, to which the separated proteins in the gel are electrophoretically transferred.

Western Blot / Immunoblotting: Method

- Proteins collected from patient sample and separated by size on gel electrophoresis
- electric field drives proteins through gel:
- large stay near top, small move toward bottom

- separated proteins are transferred (blotted) to a nylon Membrane
- membrane is exposed to antiserum for suspect pathogen
  - antibodies in antiserum specifically bind to their epitope
- a colored substrate is added: reacts with the Fc region of bound antibodies thus coloring location of antigen with bound antibody
- pathogen is confirmed by:
  - binding of specific antiserum
  - size of the epitope

Electrophoresis for Protein Analysis

- uses cross-linked polymer polyacrylamide gels
- proteins migrate based on charge-to-mass ratio
- visualization = Coomassie blue dye binds to proteins

Migration of Proteins during Electrophoresis

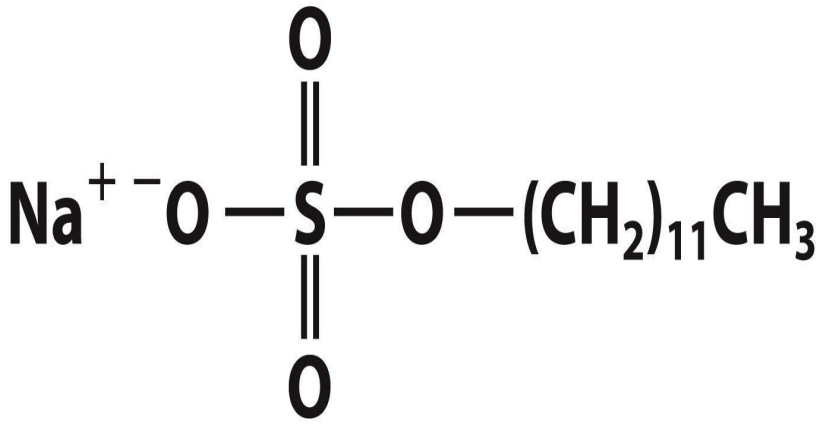
$$\mu = \frac{V}{E} = \frac{Z}{f}$$

μ = electrophoretic mobility
V = velocity
E = electrical potential
Z = net charge
f = frictional coefficient

migration of a protein in a gel during electrophoresis = function of size and shape

Sodium Dodecyl Sulfate (SDS)

- **sodium dodecyl sulfate (SDS)** =
  a detergent
  - binds and partially unfolds proteins
  - gives all proteins a similar charge-to-mass ratio
  - electrophoresis in the presence of SDS separates proteins by molecular weight
    - smaller proteins migrate more rapidly

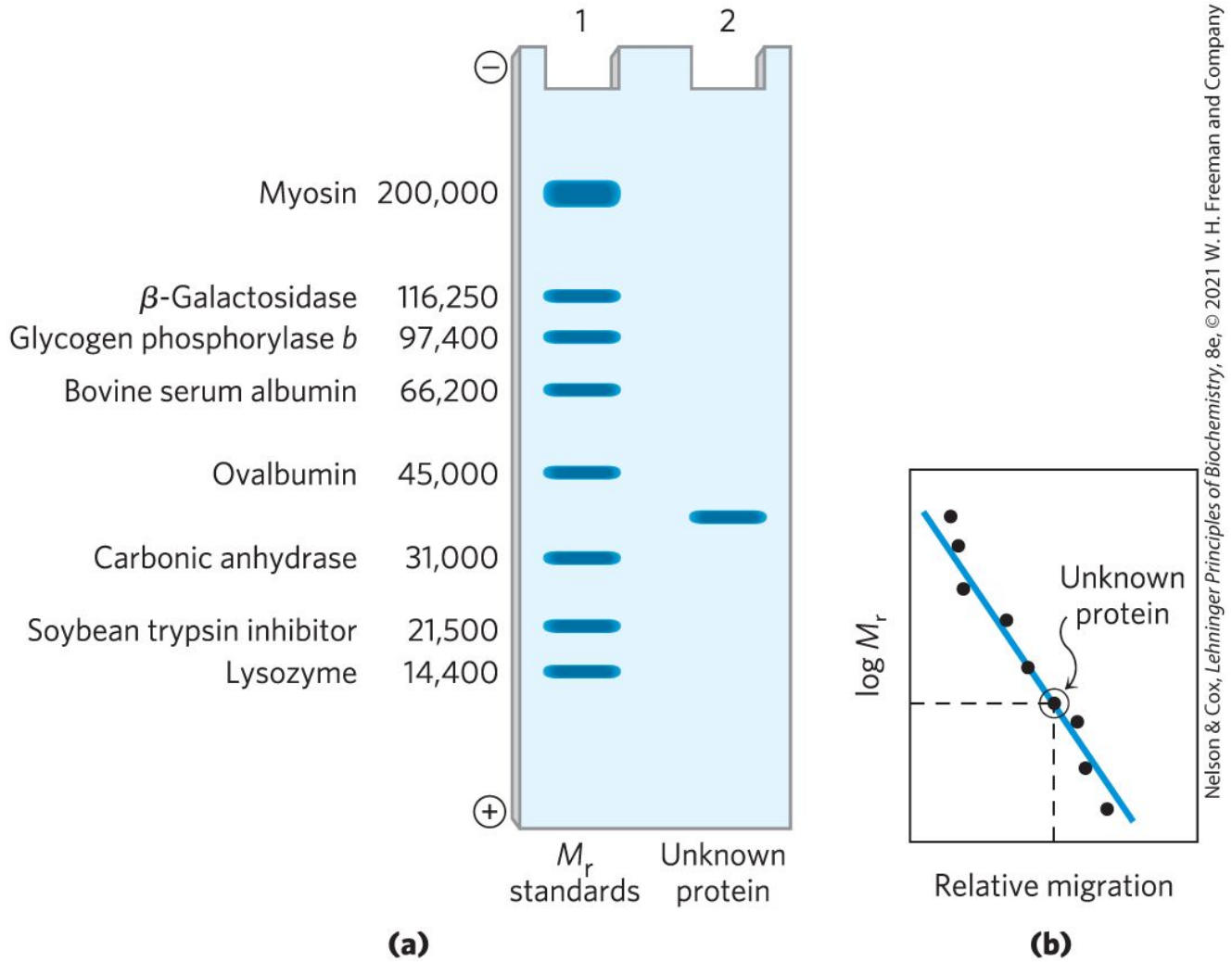


Sodium dodecyl sulfate (SDS)

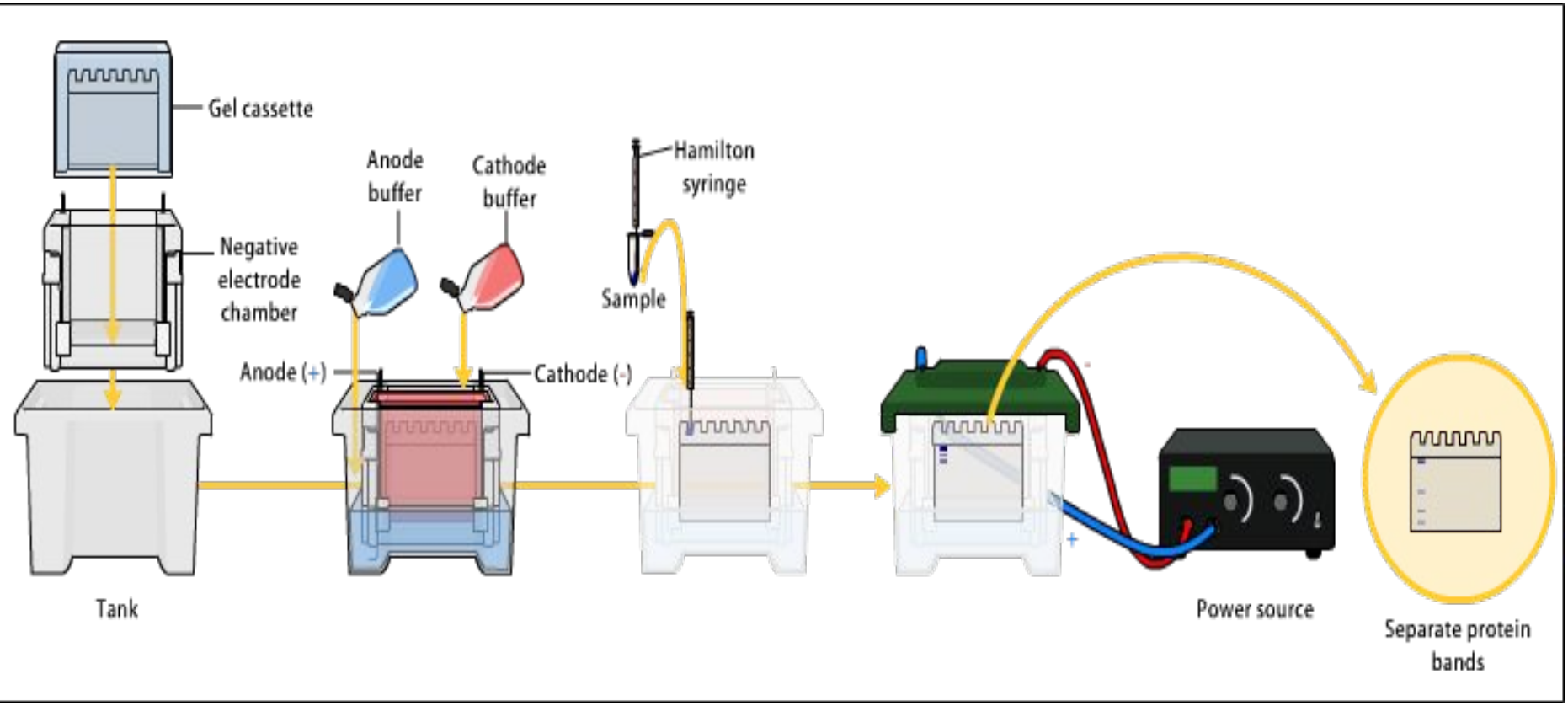
Unnumbered 3 p94
Lehninger Principles of Biochemistry, Seventh Edition

Estimating the Molecular Weight of a Protein

Plot of log M_r of marker proteins vs. relative migration during electrophoresis = linear



Protein Separation



APPLICATIONS OF IMMUNOLOGY

Monoclonal Antibodies in Diagnostics: Malaria RDT for Practicals

BCH 4047 TD/TP. #3

By

Dr Palmer Masumbe Netongo (PhD)

Malaria RDT
OUTLINE

- Introduction
- Learning Objectives
- Definition of TDR
- Principles of rapid diagnostic tests
- Types of antigens detected by the TDR

PREMISE OF MALARIA RDTs

- WHO recommends that malaria case management be based on parasite-based diagnosis in all cases.
- The use of antigen-detecting rapid diagnostic tests (RDTs) forms a vital part of this strategy,
- Provides the possibility of parasite based diagnosis in areas where good quality microscopy cannot be maintained.

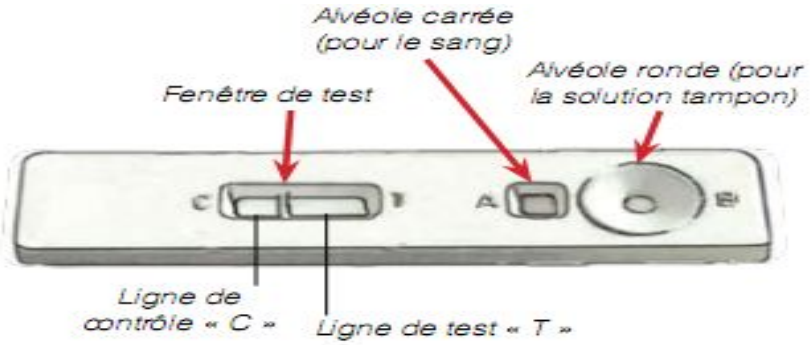
OBJECTIVES

- At the end of the presentation, Students should be able to:

- Define a rapid diagnostic test (RDT)
- Give the general principle of RDT
- Give different antigens detected by RDT currently

DEFINITION OF RDT

- A Rapid Diagnostic Test or RDT is a kit consisting of a set of materials and reagents to confirm the presence of the malaria parasite (Plasmodium) in the blood of a patient within a short time frame.
- The kit includes: a cassette tape or a buffer or lysis buffer, a lancet, a capillary tube, the alcohol swab and a pair of gloves.



PRINCIPLES OF RDT (1)

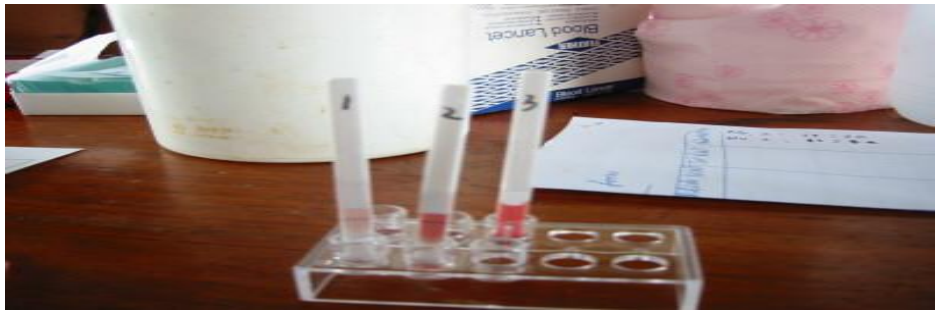
Malaria is caused by a parasite that infects red blood cells and produces chemicals (proteins) called antigens.

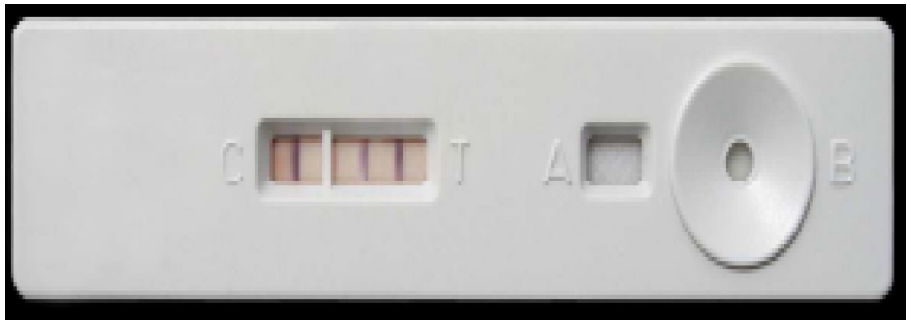
The malaria RDTs detect specific antigens (proteins) produced by malaria parasites. These antigens are present in the blood of infected individuals, the infection is recent or not.

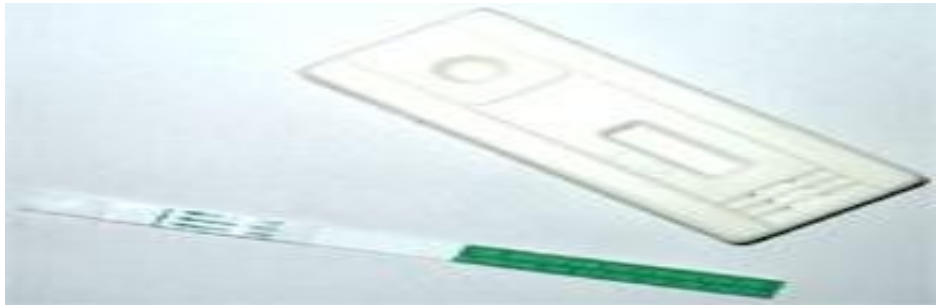
PRINCIPLES OF RDT (2)

- When we are infected by malaria parasite, our body defends itself naturally while producing the antibodies that recognize and specifically destroy these parasite
- Some of these antibodies have been purified and have been imbedded on paper filters and are currently used artificially to search for the malaria antigens (parasite) in a patient's blood, by production of a visible reaction.

DIFFERENTS FORMS OF RDTs







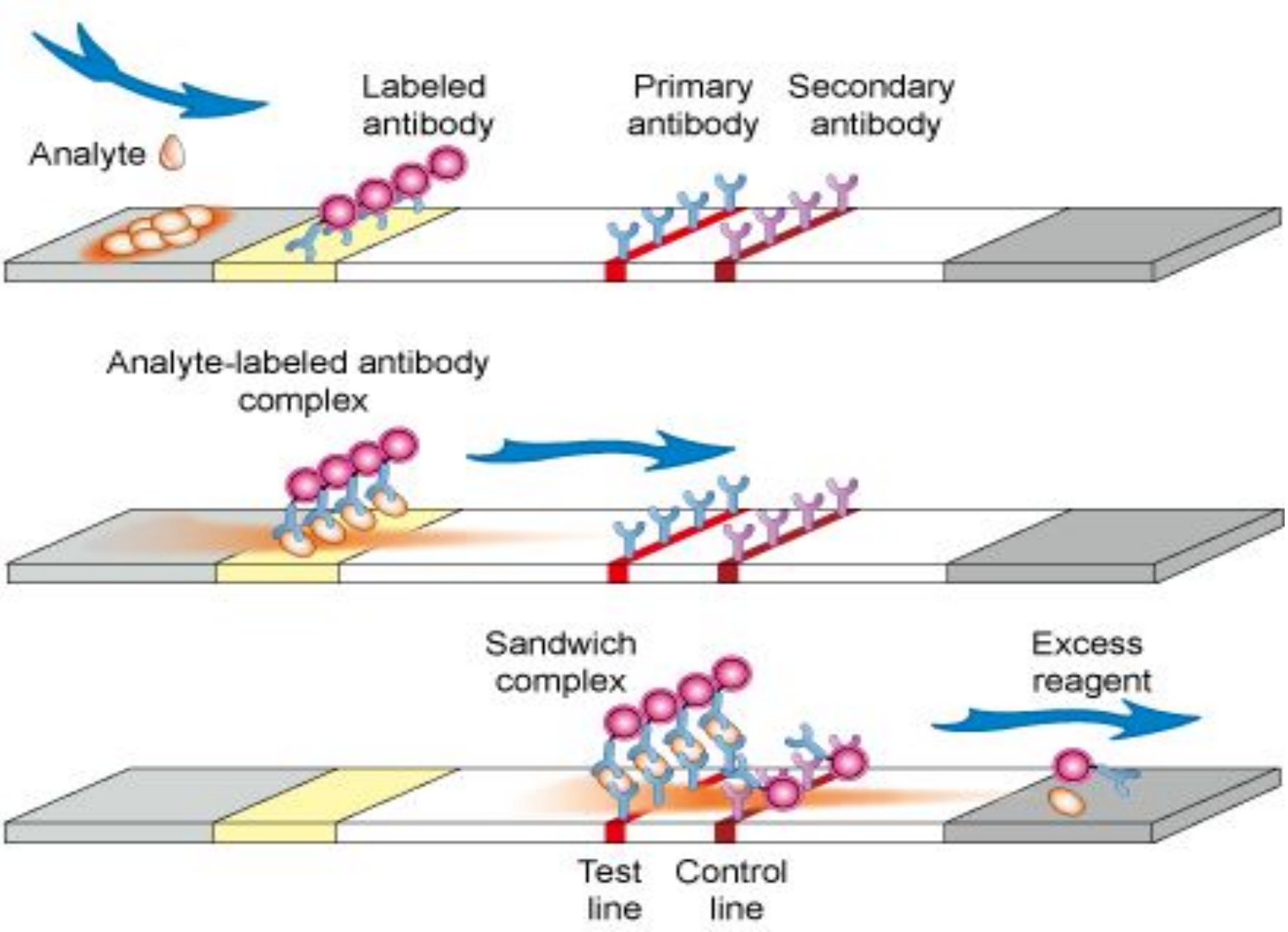
PRINCIPLES OF RDT (3)

- The principle is basically the same for the different tests and based on the immunochromatographic membrane or strip.
- A few microliters of whole blood obtained from capillary blood or venous blood is introduced to one end of a nitrocellulose membrane mounted on a plastic or cardboard. If desired antigen is present, it will bind with a labeled antibody most often with colloidal gold.
- Few drops of buffer or lysis buffer are deposited thereby facilitating lysis of red blood cells and the migration of the sample on the strip.

PRINCIPLES OF RDT (4)

- The complex antigen - antibody will then migrate by capillary action and the antigen will be captured by a sandwich capture antibody bound to the membrane. This screenshot will then result in the appearance of a colored line.
- A positive result is shown by the appearance of a colored line and an internal control to validate the test.
- Excess conjugate will continue to migrate and will be secured by a second antibody. The accumulation of colored complexes will again lead to the appearance of a second colored line. This second line or control line validates the proper functioning of the reaction.

Principle: Lateral Flow Technology of Immunochromatograghy



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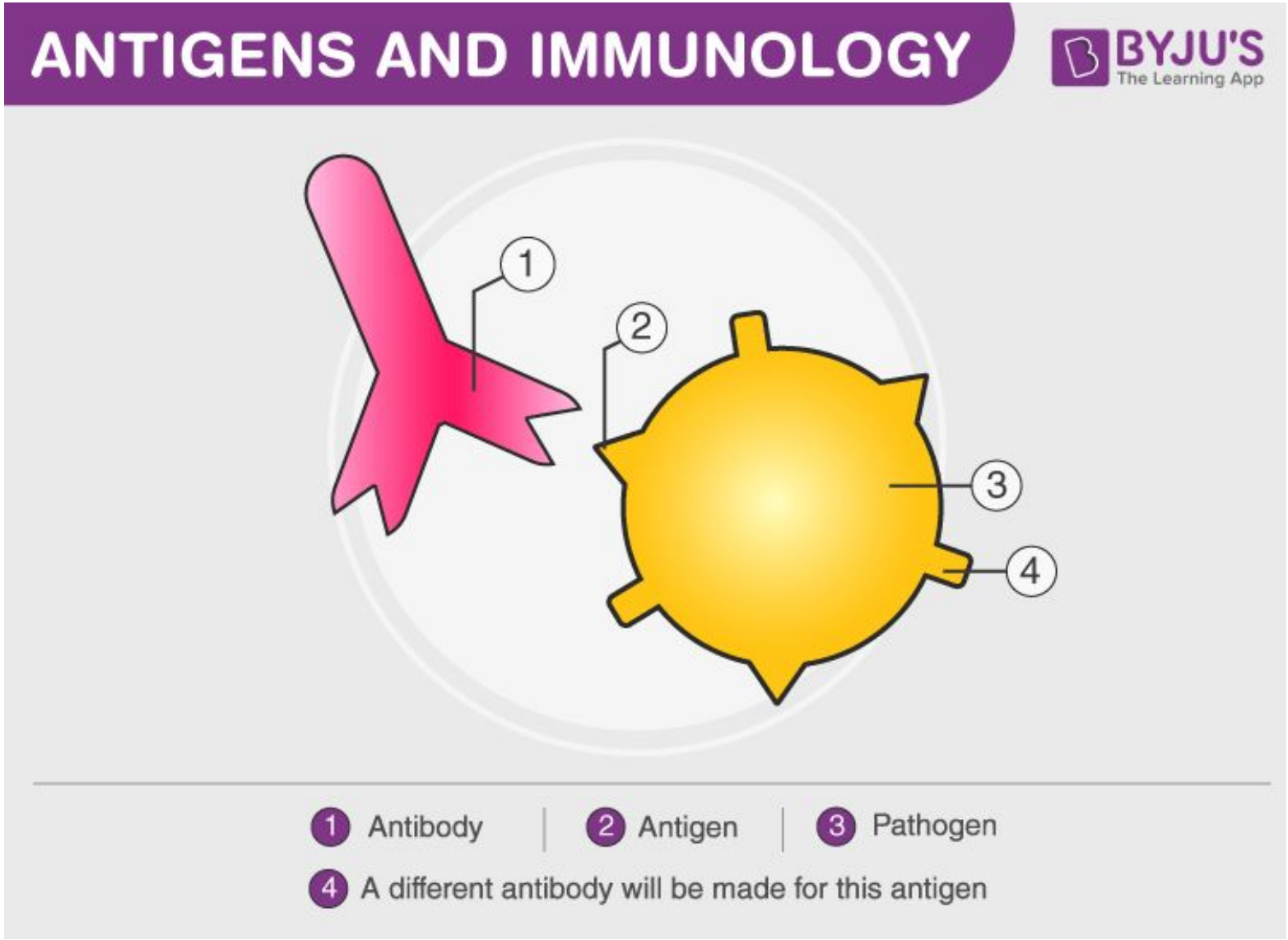
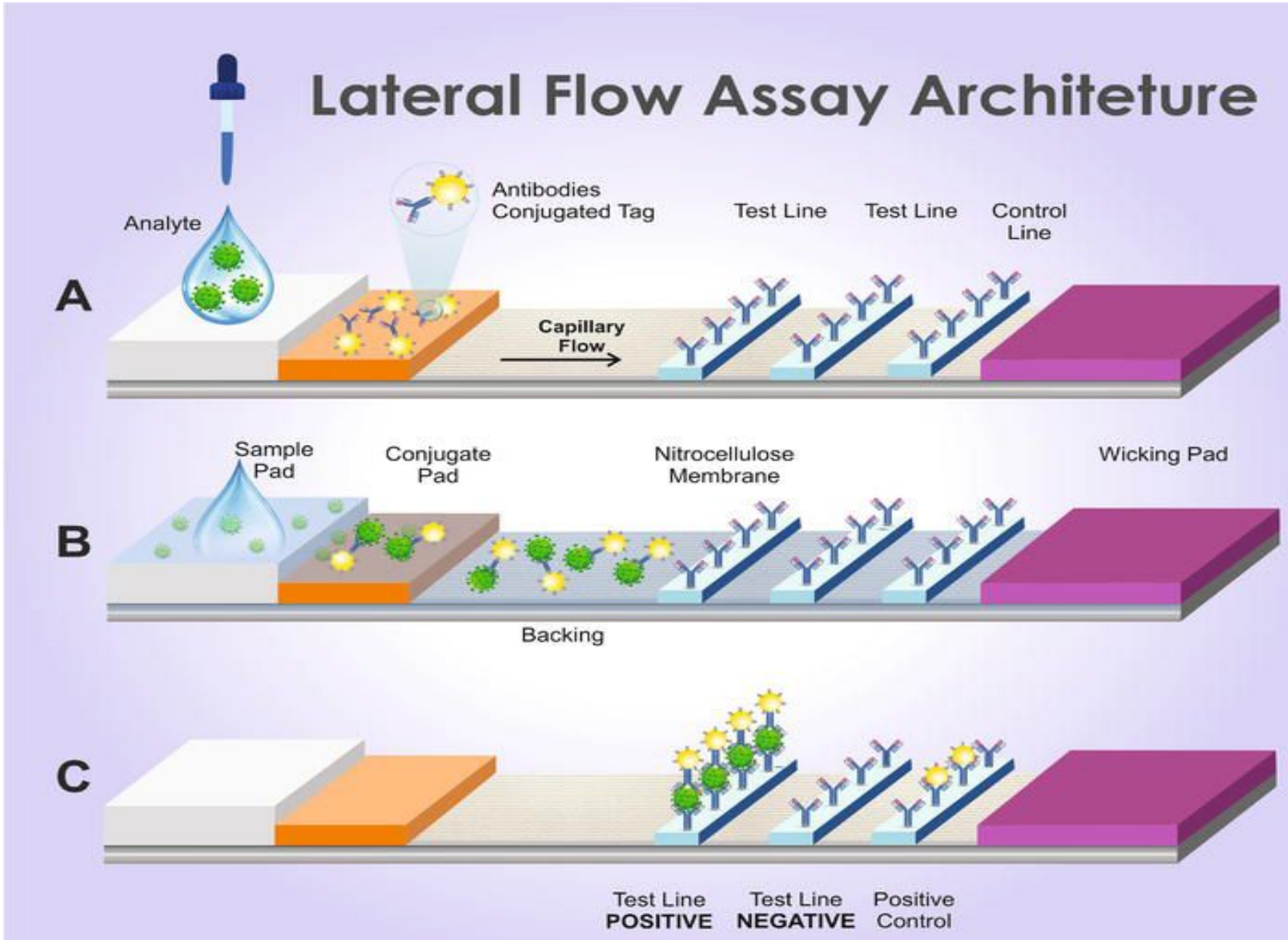


PRINCIPLE OF RDT



PRINCIPLE OF RDT (a)

- Dye-labelled antibody (Ab), specific for the target Ag, is present on the lower end of the nitrocellulose strip, or in a well provided by a casing covering the strip.
- Ab, specific for another epitope on the target Ag, is bound to the strip in a thin (test) line, and
- Ab specific for the labelled Ab is bound at the control line.

PRINCIPLE OF RDT (b & c)

- **b** | Blood and buffer, which have been placed on the strip or in the well, are mixed with labelled Ab and are drawn up the strip across the lines of bound Ab.

- **c** | If Ag is present, labelled Ab will be trapped on the test line.
  - Other labelled Ab is trapped on the control line.
  - If sufficient labelled Ab accumulates, the dye labels will become visible to the naked eye as a narrow line.

TYPES OF ANTIGENES DETECTED BY RDT (1)

- Different types of RDTs detect different antigens. Some antigens are produced by a single species of the parasite responsible for malaria (eg, *Plasmodium falciparum*), some are produced by all species of malaria parasite (including *P. falciparum, P. vivax, P. ovale and P. malariae*).

- Some RDTs are specific for a single species of parasite and are usually based on the detection of histidine-rich protein (HPR2) of *Plasmodium falciparum* or lactate dehydrogenase-(pLDH) or parasite aldolase.

- Other tests detect in addition to *P. falciparum*, one or more of the other three species of malaria parasites that infect humans, *P. malariae, P. ovale and P. vivax.*

TYPES OF ANTIGENES DETECTED BY RDT (2)

- Histidine-Rich Protein (HPR2) or may remain in the blood for at least another two weeks after the elimination of parasites by medication. Thus, RDT **can not** be used to verify the effectiveness of treatment for a person who has been on an antimalarial drug during the previous two weeks . He will have a positive test result, even if that person is not carrying parasites.

- Plasmodium lactate dehydrogenase (pLDH) and aldolase antigens disappear from blood quickly (about 5 days). Therefore, tests based on the detection of these antigens are negative a few days after effective treatment.

CONCLUSION

- TDR is a good method for malaria diagnosis that does not require expensive equipment or highly qualified personnel.

- TDR allows the rapid response of the patient.
- http://fr.scribd.com/doc/55548302/Immunology-Exam-Q-s-with-answers