ELISA

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Definition:

- The enzyme-linked immunosorbent assay (**ELISA**) is a common laboratory technique which is used to measure the concentration of an analyte (usually antibodies or antigens) in solution.

Why known as .....?

Enzyme Linked Immunosorbent Assay

1. Antigen/antibody of interest is absorbed on to plastic surface ('*sorbent*').

2. Antigen is recognised by specific antibody ('*immuno*').

3. This antibody is recognised by second antibody ('*immuno*') which has enzyme attached ('*enzyme-linked*').

4. Substrate reacts with enzyme to produce product, usually coloured.

History of Elisa

Radioimmunoassay was first described in a scientific paper by Rosalyn Sussman Yalow and Solomon Berson published in 1960.

In 1971, Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands independently published papers that synthesized this knowledge into methods to perform EIA/ELISA.

History of Elisa

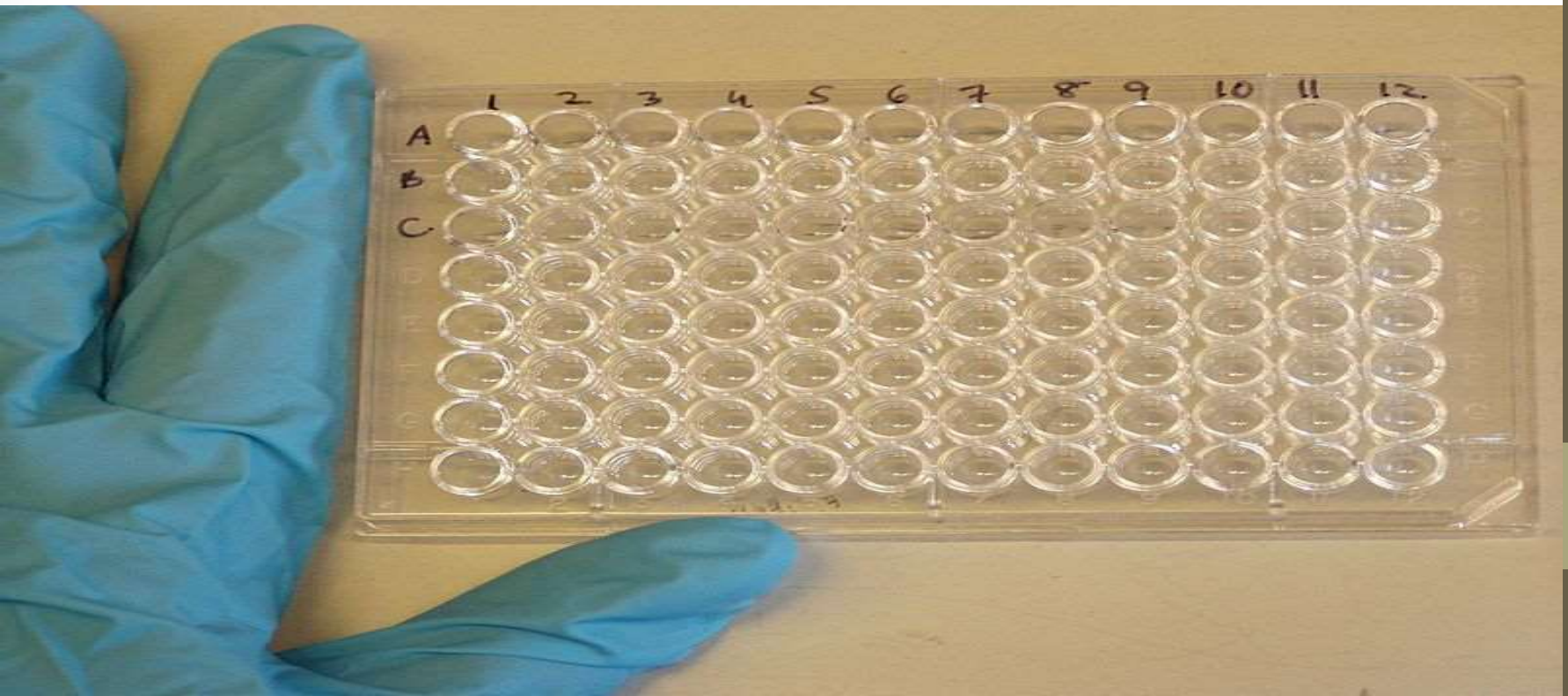
Rosalyn Sussman 1960

Eva Engvall 1971

Basic Terms:

- **Solid Phase:**

  Usually a microtiter plate well, having 8 ✖ 12 well format.



Basic Terms:

- **Adsorption:**

  The process of adding an antigen/antibody, diluted in buffer, so it attaches to the solid phase on incubation.

- **Washing:**

  The simple flooding & emptying of wells with a buffered solution to separate bound from un-bound reagents in ELISA.

Basic Terms:

- **Antigen:**

  Any molecule that elicits the production of antibodies when introduced into body.

- **Antibodies:**

  Proteins produced in response to antigenic stimuli.

- **Enzyme conjugate:**

  An enzyme that is attached irreversibly to an antibody.

  e.g: Horse-redish peroxidase (HRPO).

Basic Terms:

- **Chromogen:**

  A chemical alters color as a result of an enzyme interaction with substrate (color reaction used as signal) e.g Trimethyl benzidine (TMB).

- **Stopping:**

  The process of stopping the action of an enzyme on a substrate.

- **Reading:**

  Spectrophotometric measurement of color developed in ELISA.

Principle of ELISA:

- **Based on Basic Immunology Response**
- Lock and Key Concept:

1) Antigen (key) 2) Antibody (lock):

–Key fits into the lock

- Enzyme conjugate substrates
- Bound to a secondary antibody that binds with the antibody-antigen complex.

Equipments:

1) Microwell Plate:

Flat bottom polystyrene plate, contains 8 x 12 wells holding 350 µL each.

Equipments:

2) Multipipette :

An 8-channel 100 µL pipette is a good help for even small-scale work.

Equipments:

3) Washing Device:

- manually operated washing devices.
- may be of use particularly when there is a risk that the samples tested in ELISA contain infectious material, so must be collected for subsequent disinfection.

Equipments:

4) Microplate washer:

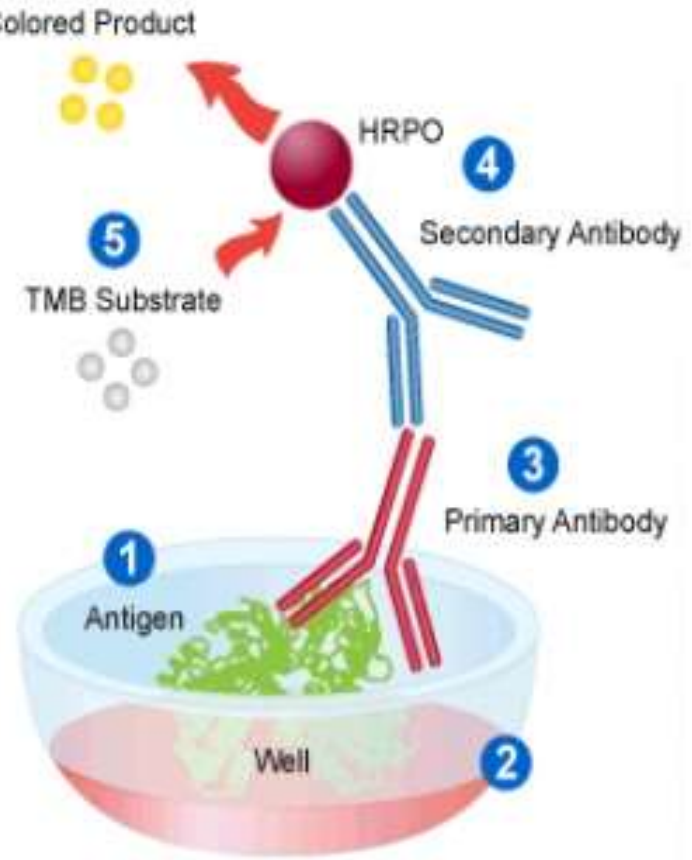
- These are very efficient with unusually low carry-over contamination.

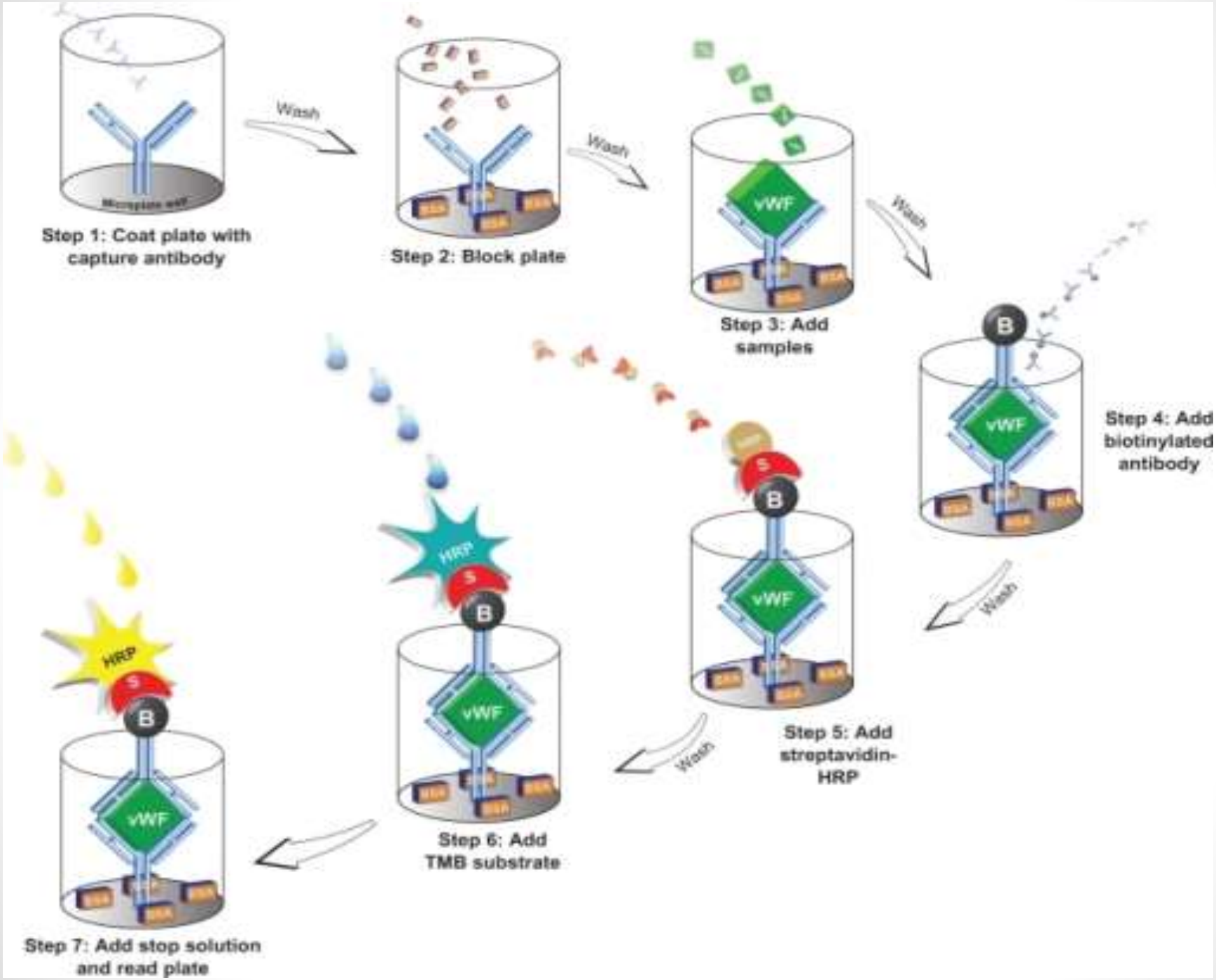
Reagents Used:

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

General Procedure:

1. Antigen/sample is added to plate.
2. Blocking buffer is added to block remaining protein-binding sites.
3. Next a suitable ***primary antibody*** is added.
4. A suitable ***secondary antibody – HRPO conjugate*** is then added which recognizes and binds to the primary antibody.
5. TMB substrate *(Leinco Prod. No. T118)* is added and is converted by HRPO to detectable form.



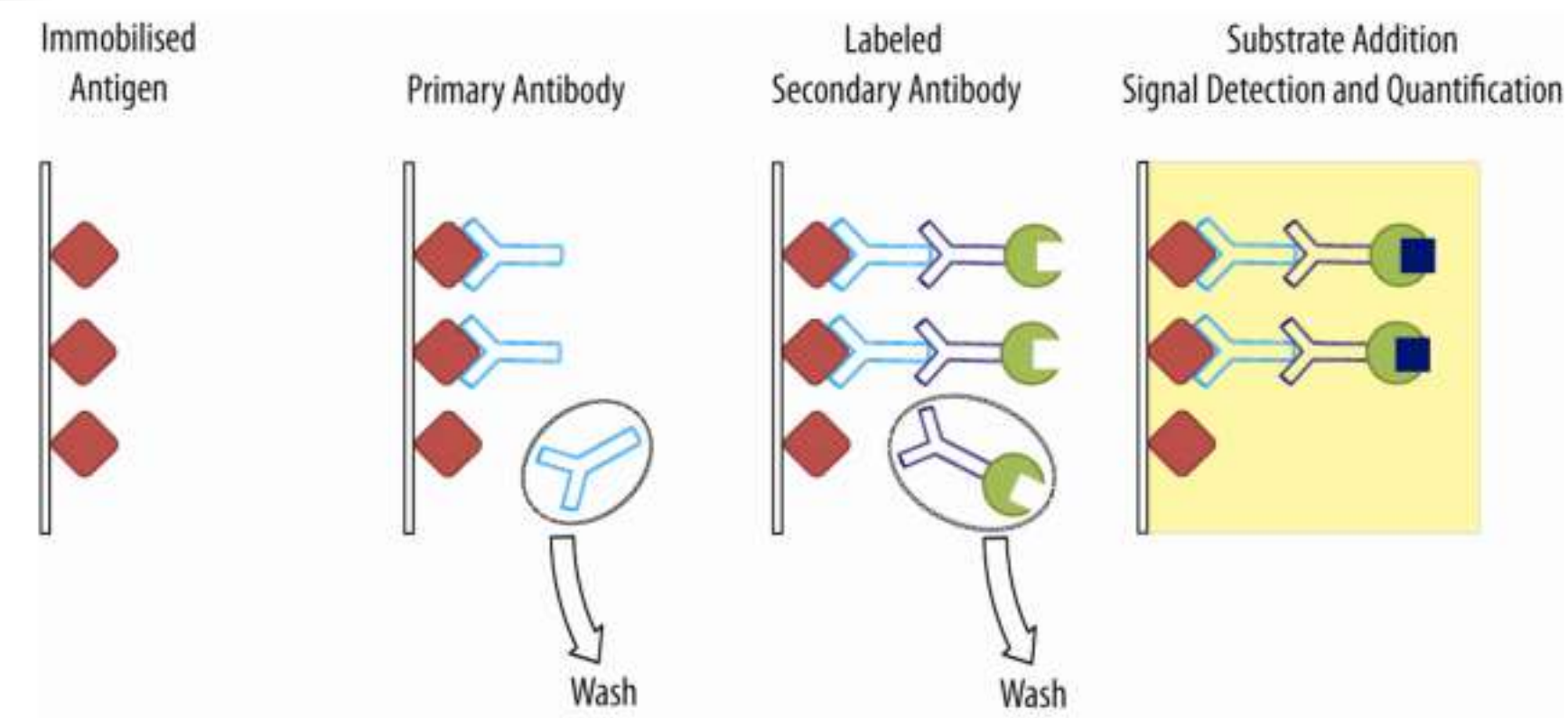

Step 1: Coat plate with capture antibody
Wash
Step 2: Block plate
Wash
vWF
Step 3: Add samples
Wash
B
vWF
Step 4: Add biotinylated antibody
Wash
S
B
vWF
Step 5: Add streptavidin-HRP
Wash
HRP
S
B
vWF
Step 6: Add TMB substrate
HRP
S
B
vWF
Step 7: Add stop solution and read plate

Types of ELISA:

- **On the Basis of Detection:**

1) Colorimetric ELISA:

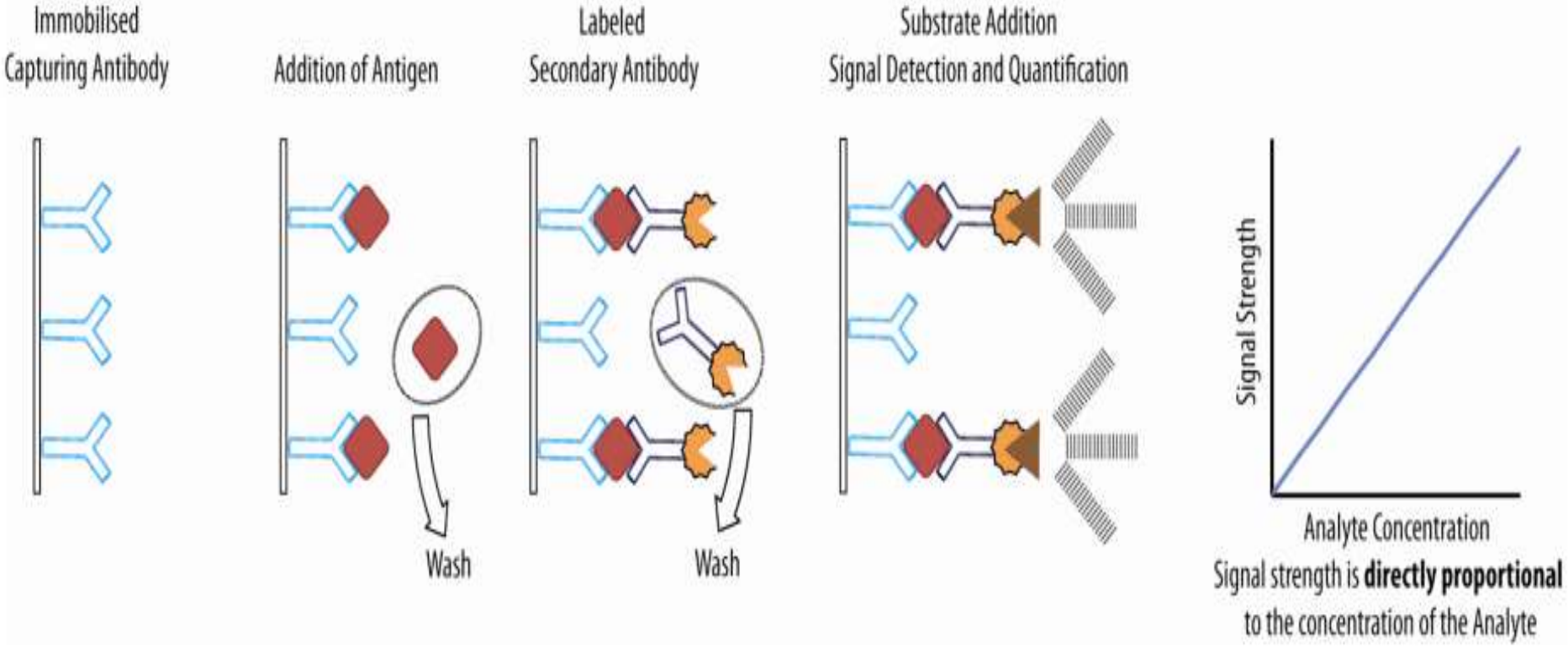
Assay to Determine the Antibody Concentration.



Types of ELISA:

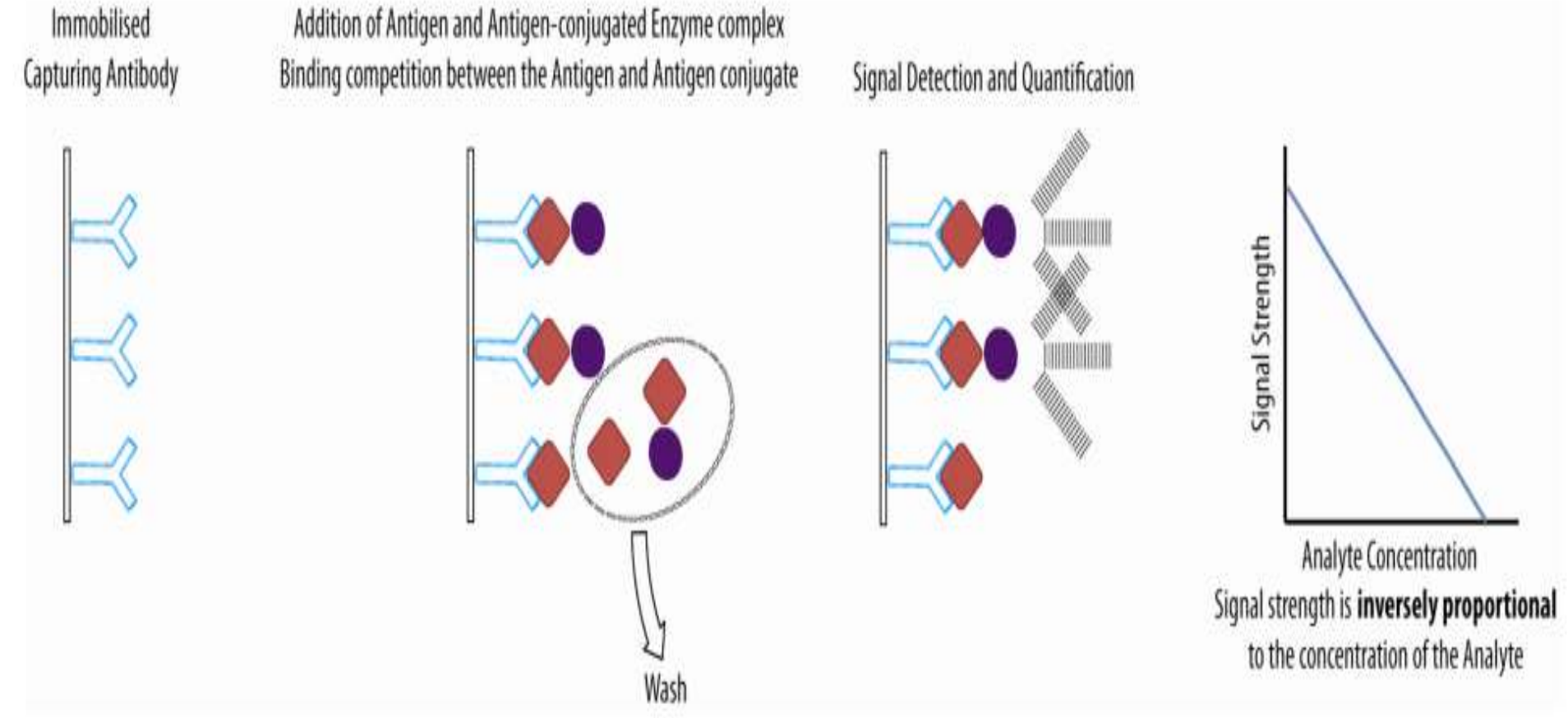
2) Chemiluminescent ELISA:

Assay for the Quantitation of an Antigen in a Biological Sample.



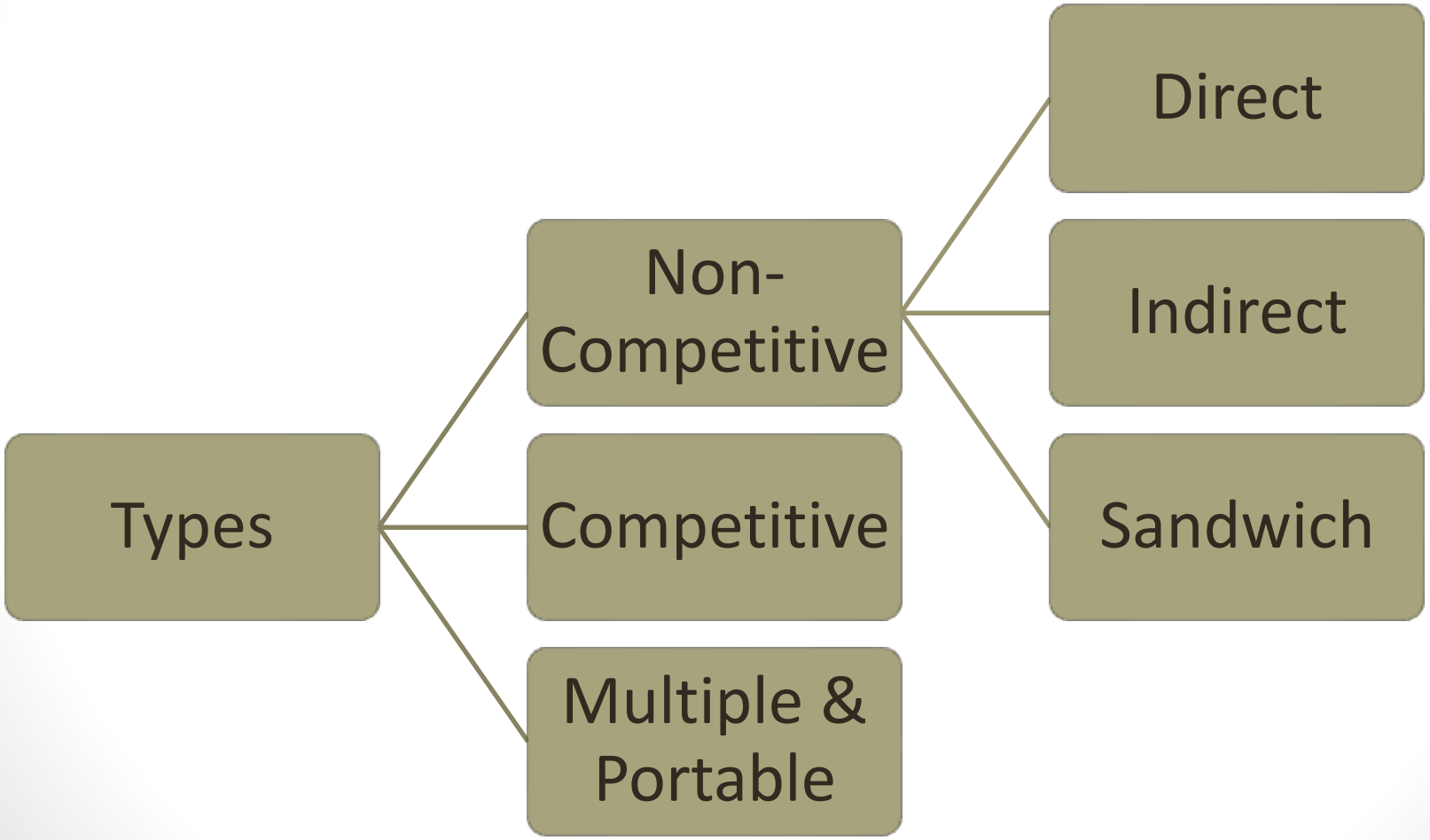
Types of ELISA:

3) Competitive Fluorescence ELISA:



Types of ELISA:

(on the basis of procedure)



Non-Competitive:

1) Direct ELISA:

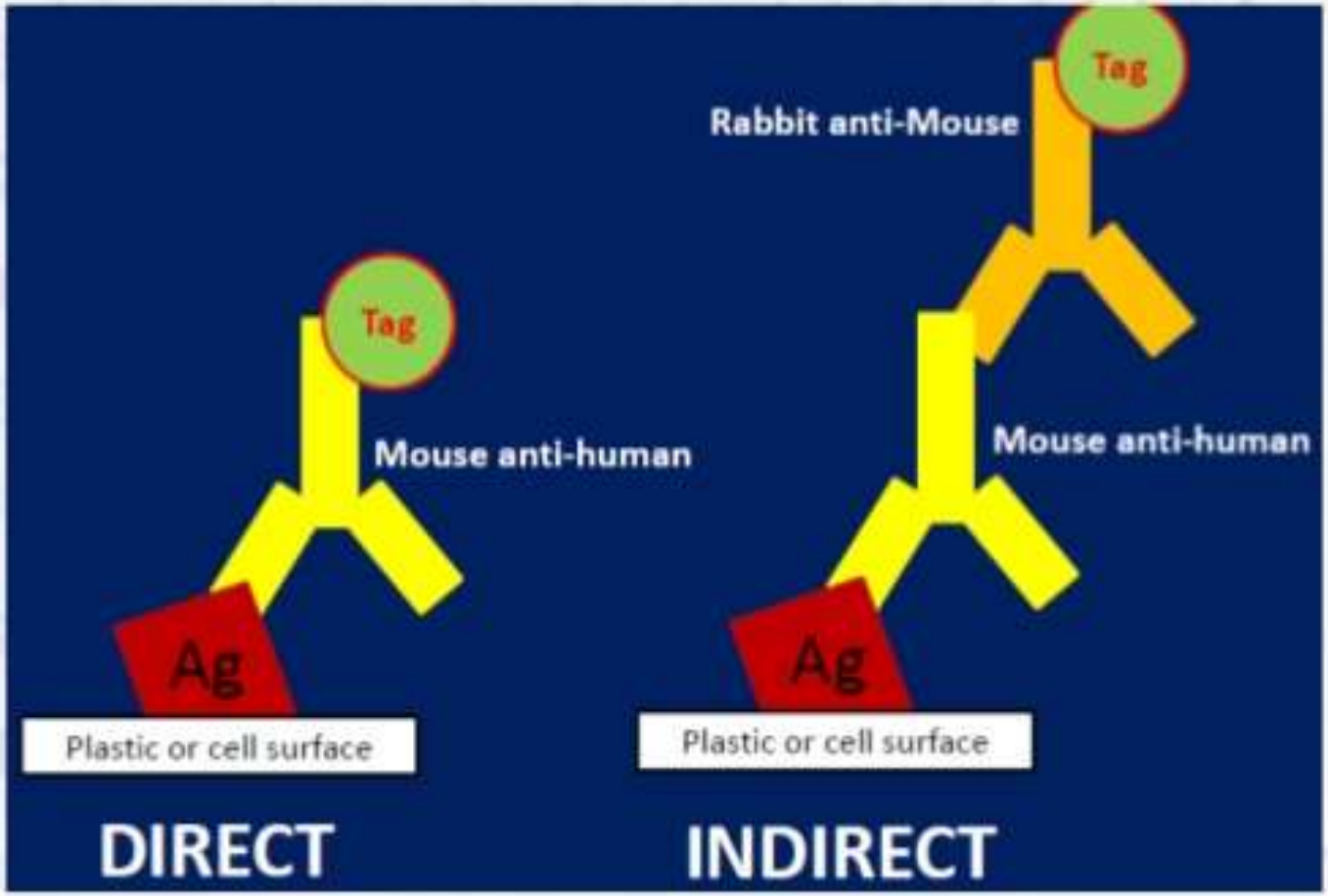
- It uses a primary labeled anti-body that react directly with the antigen.
- It can be performed with the antigen that is directly immobilized on assay plate.
- Not widely used but common for immuno-histochemical staining of cells & tissues.

Non-Competitive:

2) Indirect ELISA:

- It utilizes a primary un-labeled antibody in conjunction with a labeled secondary antibody.
- Secondary antibody has specificity for primary antibody.

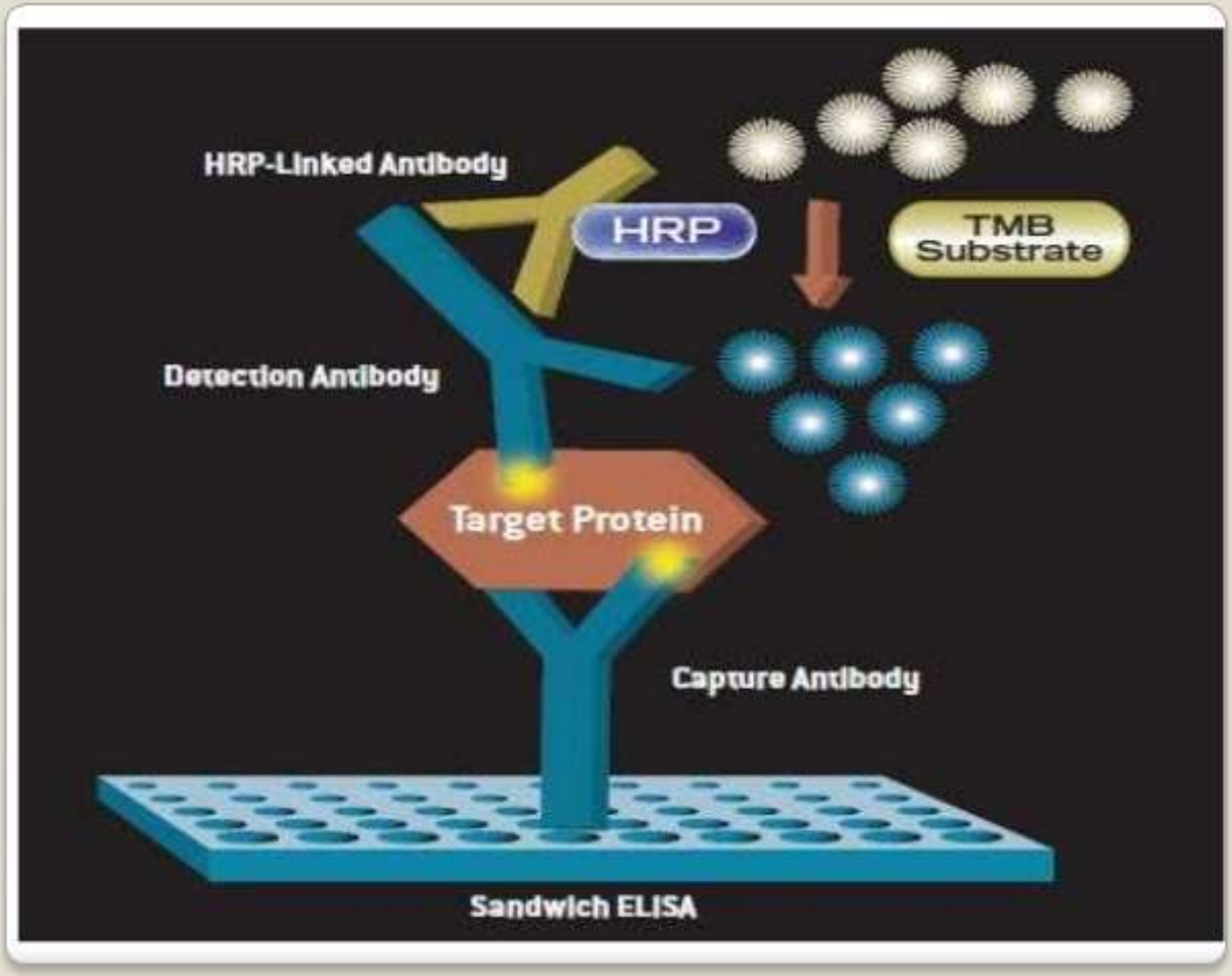
Direct and Indirect ELISA



Non-Competitive:

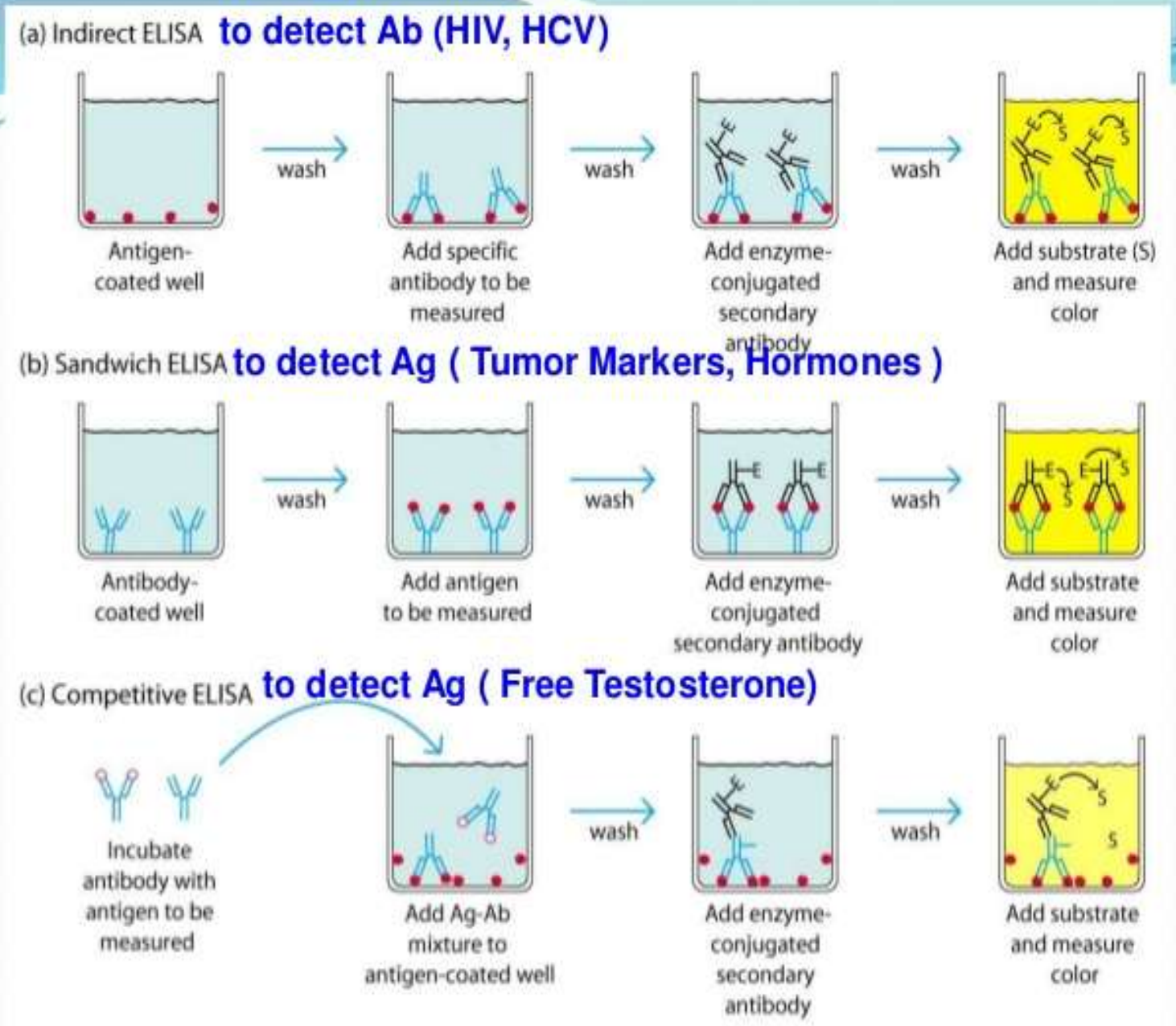
3) Sandwich ELISA:

- Antigens like Tumor markers, hormones, serum proteins may be determined.
- Antigens in the sample bind with the capture antibody & become immobilized.
- The antibody of the enzyme conjugate bind with the immobilized antigen to form a sandwich of Ab-Ag-Ab/ enzyme bound to microwell.


HRP-Linked Antibody
HRP
TMB Substrate
Detection Antibody
Target Protein
Capture Antibody
Sandwich ELISA

Competitive:

- Antibody coated microwell.
- Serum antigen & labeled antigen added together .... Competition
- Ab-Ag enzyme complex bound is inversely related to the conc. of antigen present in sample.
- Increased serum antigen results in reduced binding of Ag-enzyme conjugate with the antibody producing less enzyme activity & (yellow) color formation.
- Used to determine small molecules like T_3 , T_4 & Progesterone.

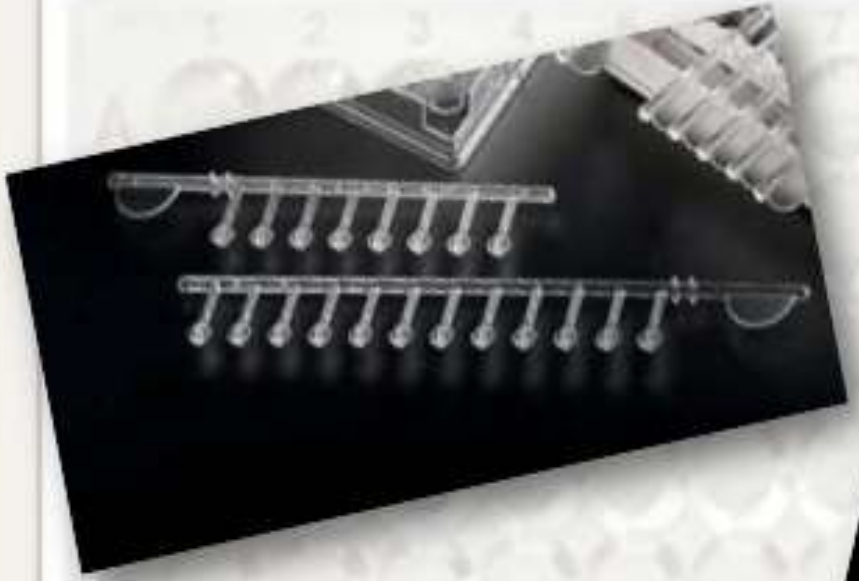


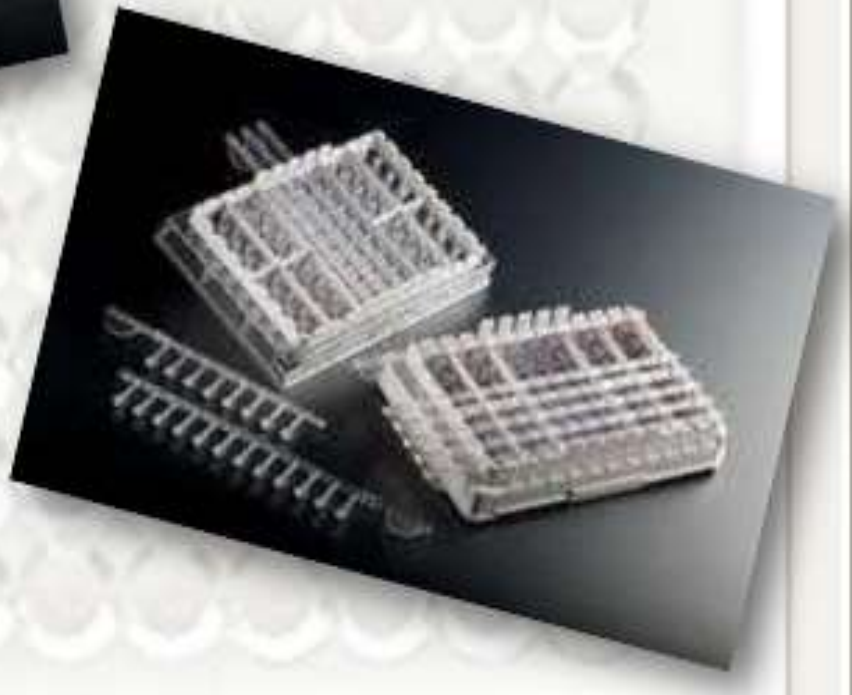
Comparison between Indirect Sandwich & Competitive ELISA

5-multiple and portable ELISA

A newer technique uses an solid phase made up of an immuno-sorbent polystyrene rod with 8-12 protruding ogives.

The entire device is immersed in a test tube containing the collected sample and the following steps (washing, incubation in conjugate and incubation in chromogenous) are carried out by dipping the ogives in microwells of standard microplates pre-filled with reagents



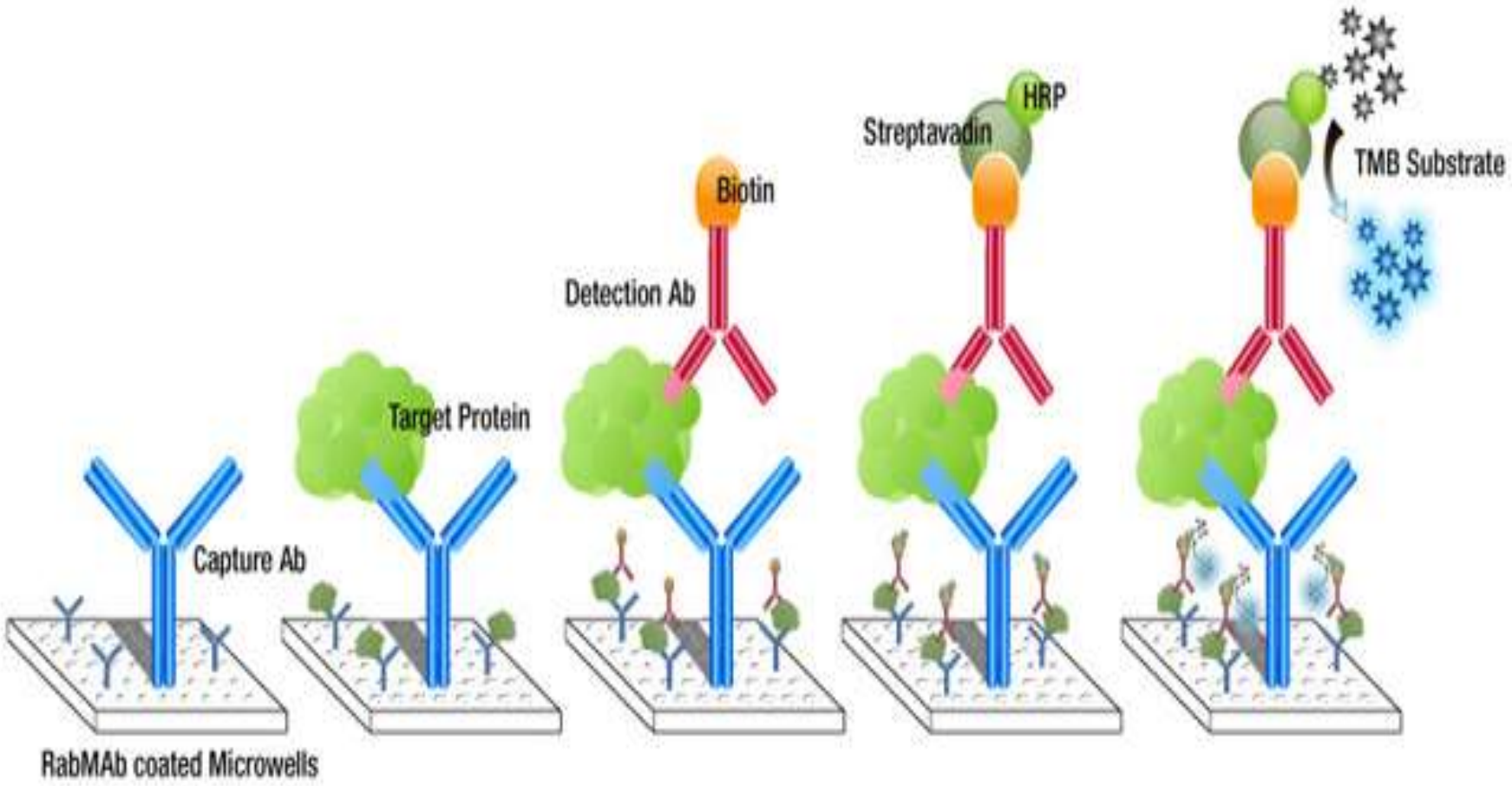


Ogives pins and rack

Modified ELISA:

- Enzyme  interfere with Ag-Ab interaction.
- Second antibody is often labeled with a very small molecular substance, **biotin** (MW=244.31), and a specific binding protein for biotin, **avidin** is conjugated with enzyme such as HRP.

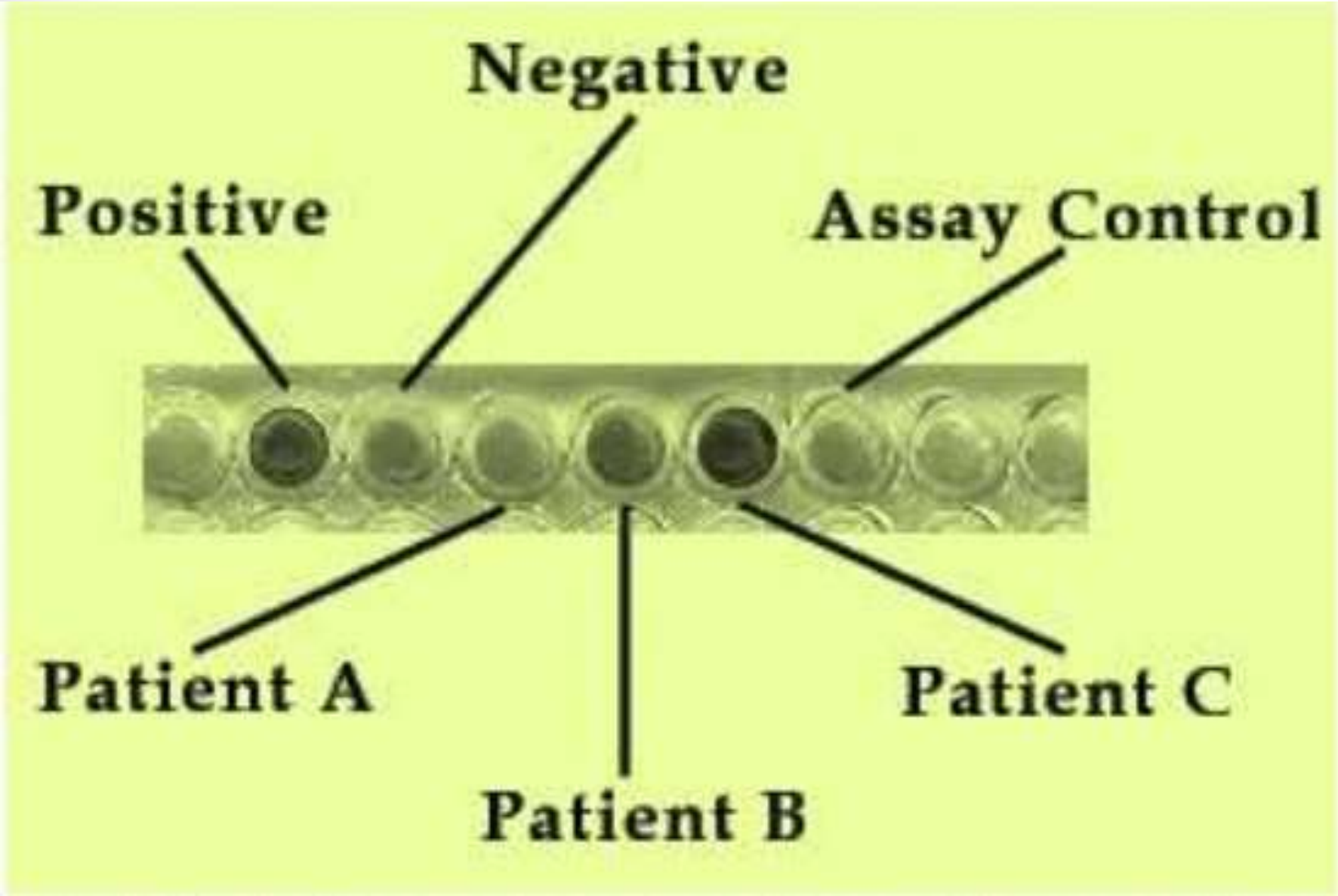
Modified ELISA:



Reading:

- Measure the absorbance at 450nm with the help of ELISA reader.
- Calculate the absorbance for each sample and reference.
- Ascent software for the calculation of results can be used.

Results:


Negative
Positive
Assay Control
Patient A
Patient C
Patient B

Troubleshooting in ELISA

*If the **negative controls** are giving positive results:*

- Contamination of the substrate solution, enzyme-labelled antibody, control themselves.
- Inadequate rinsing of plates.
- Inadequate blocking of plates.

- *If no colour has developed for the **positive controls** or for the samples:*

a. Check all reagents for dating and storage conditions.

b. Microwell plates not coated properly.

c. Reagents applied in wrong order or step omitted.

d. Enzyme conjugate defective or inhibited by contaminant.

- *If **very little colour** has developed for **positive controls** and the test samples:*

a. Check the dilution of the enzyme labelled antibody.
b. The concentration of the substrate.
c. Wash buffer not adequately drained after every wash step.
d. Inadequate incubation times.
e. Enzyme conjugate defective or inhibited by contaminant, Substrate defective or contaminated,
f. Micro well plates poorly coated.

- ***If colour has developed for the test samples <u>but not</u> the positive controls***:

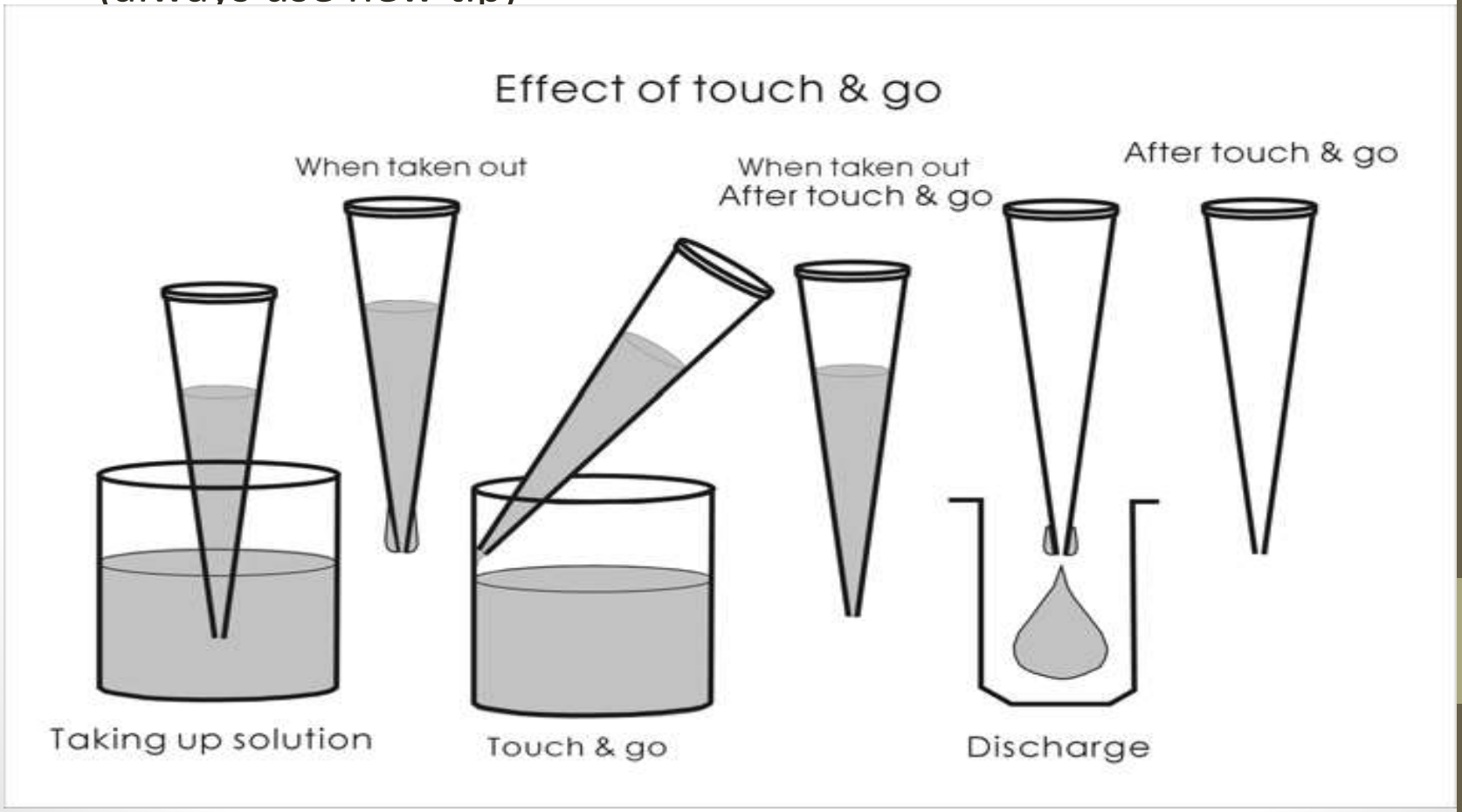
- Check the source of positive controls, their expiry date and storage.

- ***If the colour can be seen, but the absorbance is not high as expected,*** check the wave length.

Precautions:

1) Use of Exchange type pipette:

(always use new tip)

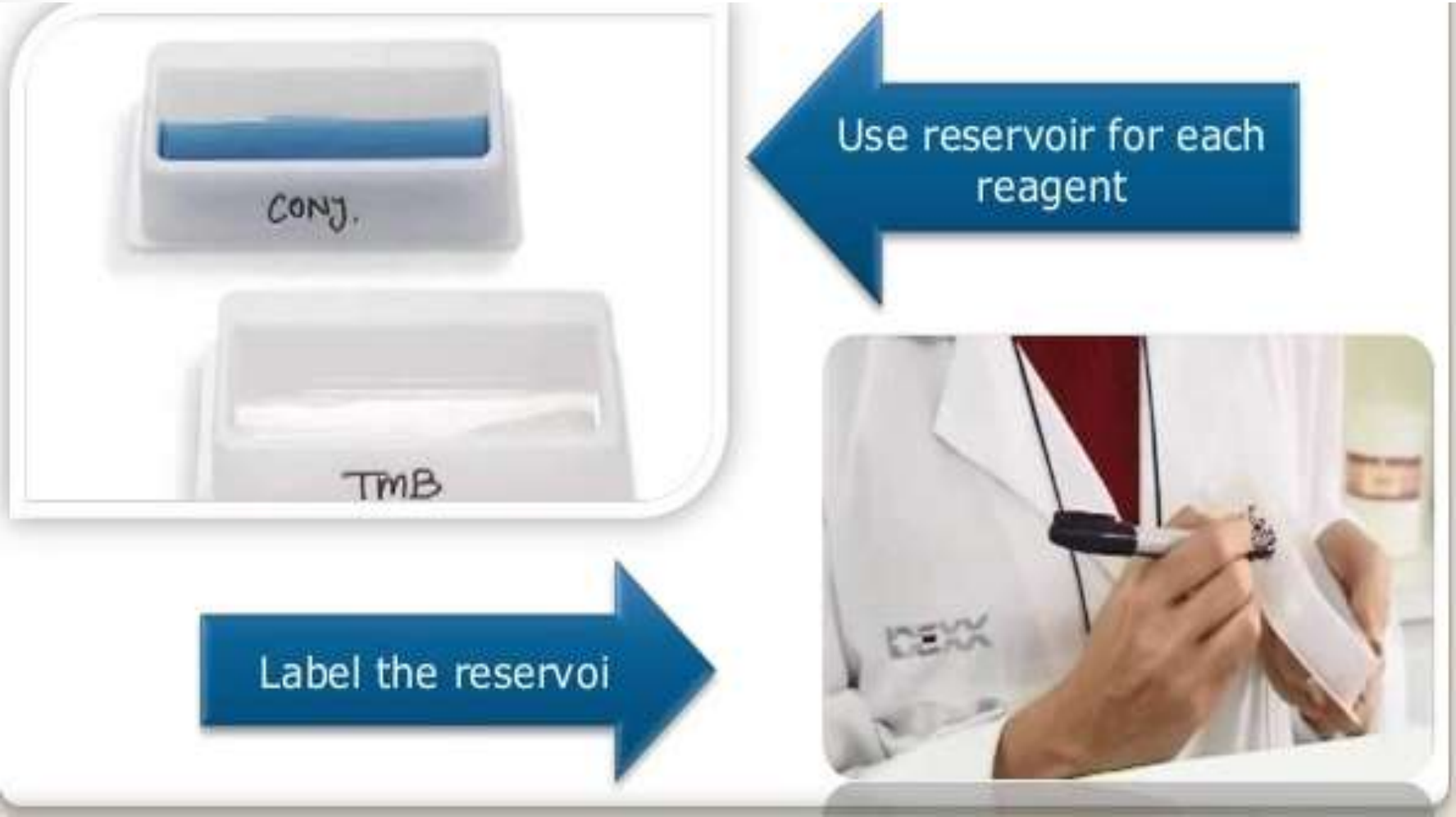


Precautions:

2) Washing:

Precautions:

3) Reagents:



Precautions:

3) Reagents:

Don’t use the same reservoir for multiple regents

Don’t return the reagents to the stock

Precautions:

4) Plate cover:

- During incubation, well plate should be covered using the plate cover
- Plate cover is effective only under suitable conditions i.e room temp. humidity > 50%, air steam <0.2 m/sec.

Precautions:

5) Coating of wells:

- Coating of wells should be proper with the addition of **Blocking solution.**

- Improper coating  False positive results

Advantages of ELISA:

- Reagents are relatively cheap & ‘ve long shelf life.
- It is highly specific & sensitive (<1pg/ml).
- No radiation hazards occur during labeling or disposal of waste.
- Easy to perform & quick procedures.
- Equipment is widely available.
- It can be used to a variety of infections.
- It can be used on most type of biological samples like plasma, serum, urine, cell extracts.

Disadvantages of ELISA:

- Measurement of enzyme activity can be more complex than the measurement of activity of some type of radioisotopes.
- Enzyme activity may be affected by plasma constituents.
- Kits are not cheap.
- Very specific to particular antigen but won’t recognize other antigens.
- False positive/ negative possible, especially with mutated/ altered antigen.

Limitations:

- Results may not be absolute.
- Antibody must be available(poor producer, interference).
- Concentration may be unclear.
- False positive possible (Ab already present).
- False negative possible.

APPLICATIONS

- 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)
- Detecting infections
  - Sexually-transmitted agents like HIV, syphilis and chlamydia
  - Hepatitis B and C
  - Toxoplasma gondii
- Detecting illicit drugs.
- Detecting allergens in food and house dust

References:

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- **ENDOCRINE MANUAL FOR REPRODUCTIVE ASSESSMENT OF DOMESTIC AND NON-DOMESTIC SPECIES** by Janine Brown, Ph.D , Sue Walker, M.S.
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Thank You