# Antigen and Antibody Interactions (contd)

# Radioimmunoassay

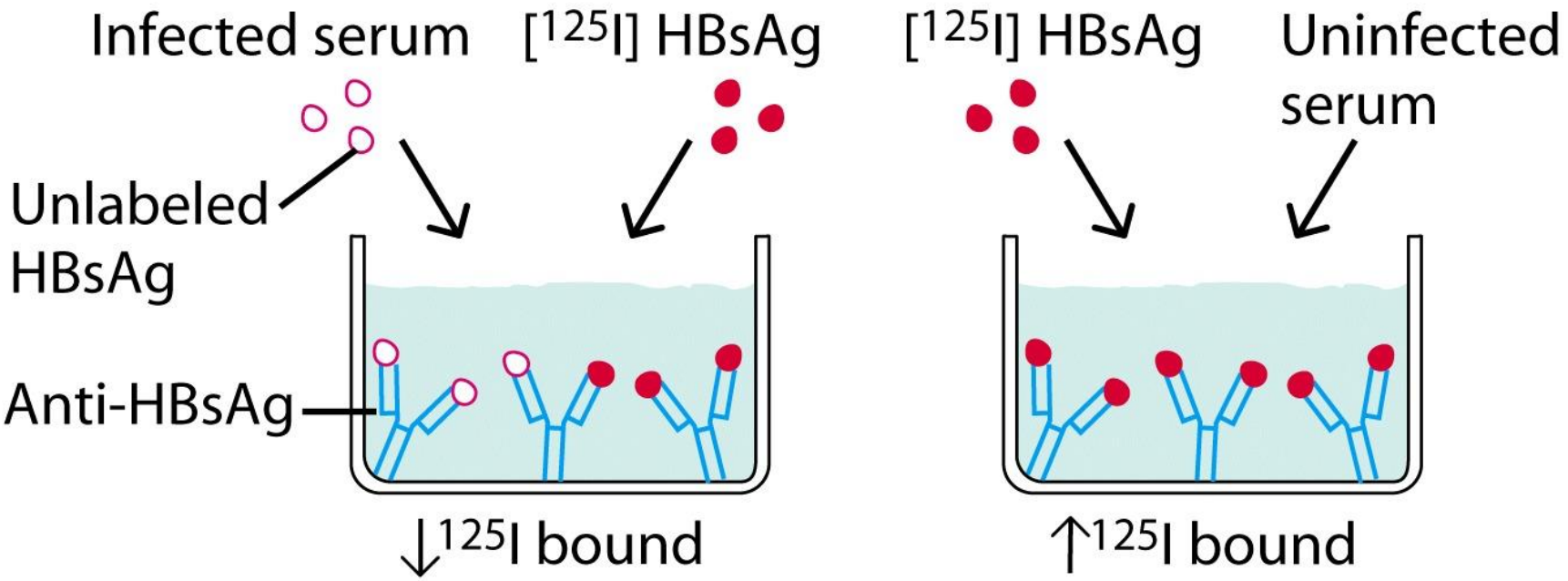
- It is a **highly sensitive** method for detecting antigen or antibody.

- It is based on competitive binding of **radiolabeled** (labeled with **radioactive isotopes**) **antigen** to a **high-affinity antibody.**

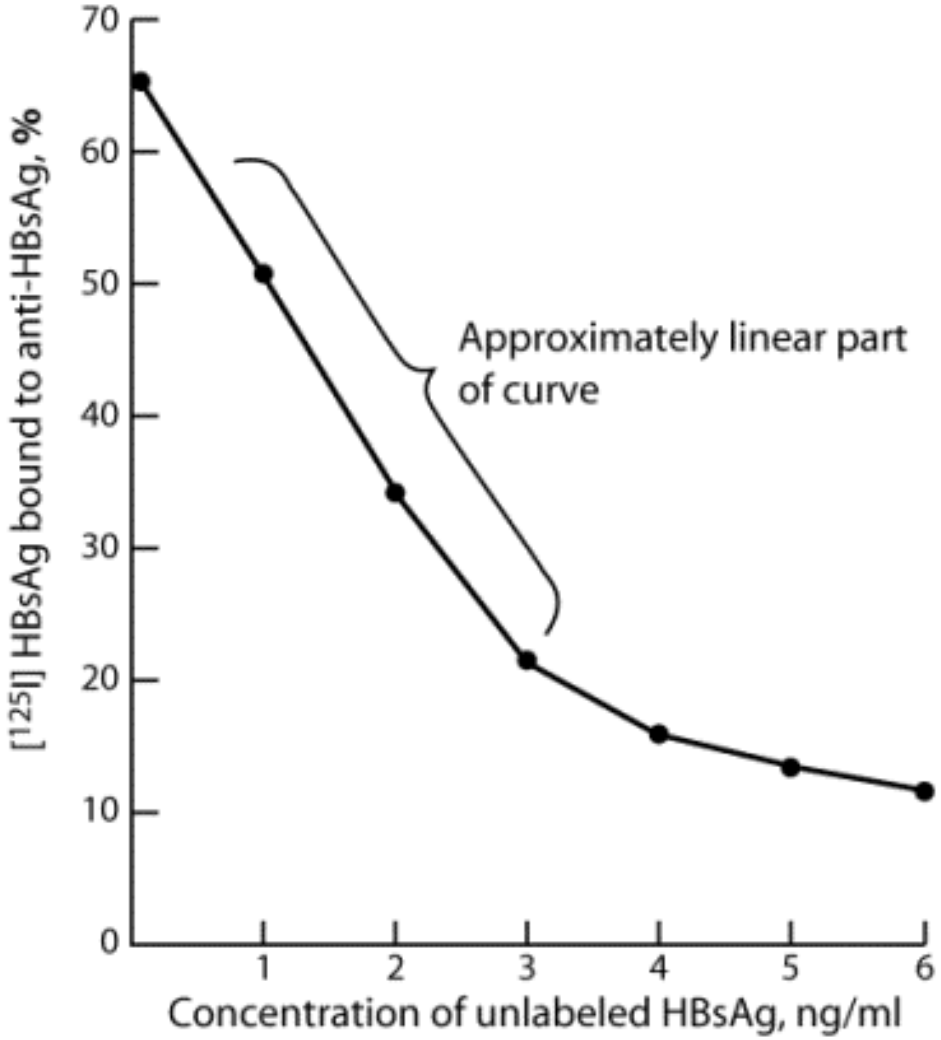
**Isotopes:** members of a family of an element that all have the same number of protons but different numbers of neutrons.

**Radioactive isotope:** An unstable form of an isotope that releases radiation as it breaks down and becomes more stable, e.g., C 14, I 131, I 125.

- A **specific antibody** is used to **coat** the wells of a plate.
- Then an **unknown sample** containing the antigen is applied.
- The **antigen** attaches to the **antibody.**
- In next step, **radiolabeled known antigen is added.**
- It **attaches only** to the **remaining antibody molecules.**
- Thus, **greater** is the amount of unknown antigen**, lesser** is the number of radiolabeled antigen attached to antibody**.**
- So signal gets **weaker** with **increasing** amount of unknown antigen **(inversely proportional).**


Infected serum
[125I] HBsAg
Unlabeled HBsAg
Anti-HBsAg
↓125I bound
[125I] HBsAg
Uninfected serum
↑125I bound

Using the **linear part** of the RIA curve one can determine the **concentration of Ag** in unknown serum samples



# ELISA

- **Enzyme-Linked ImmunoSorbent Assay**, also called **ELISA, Enzyme ImmunoAssay** or **EIA**, is a biochemical technique used to detect the presence of an antibody or an antigen in a sample.

There are various types of ELISA:

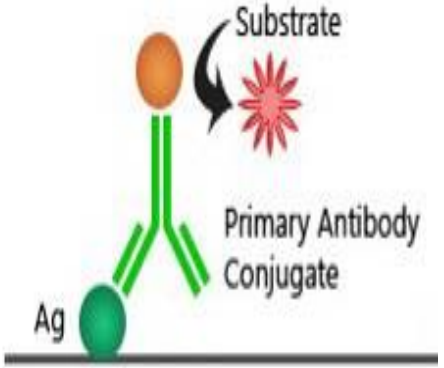
- **Direct ELISA**
- **Indirect ELISA**
- **Sandwich ELISA**
- **Competitive ELISA**

# Direct ELISA

- In simple ELISA (direct ELISA), an unknown amount of antigen is **affixed** to a surface, and then a **specific antibody** is applied to the surface so that it can bind to the antigen (or vice versa).

- This antibody is **linked** to an enzyme.

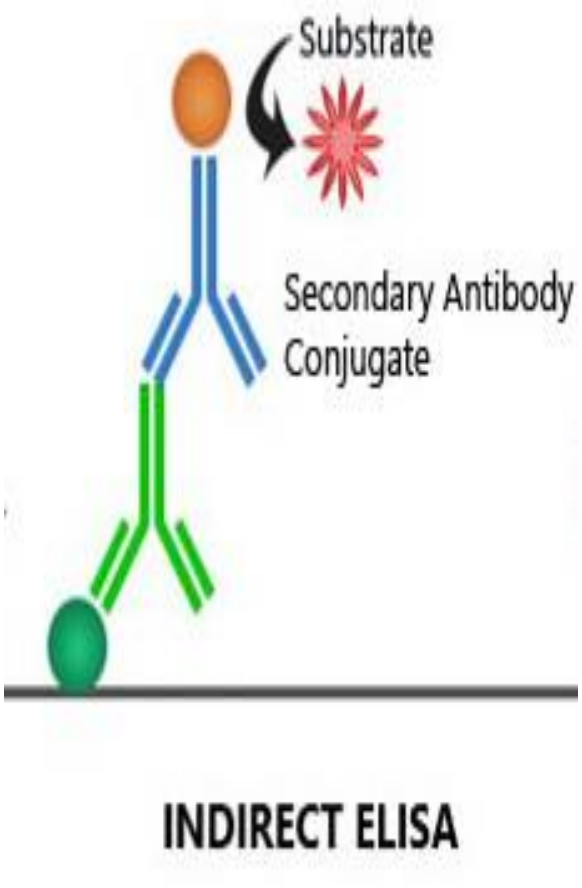
- In the final step a substance is added that the enzyme can convert to some **detectable signal**.




DIRECT ELISA

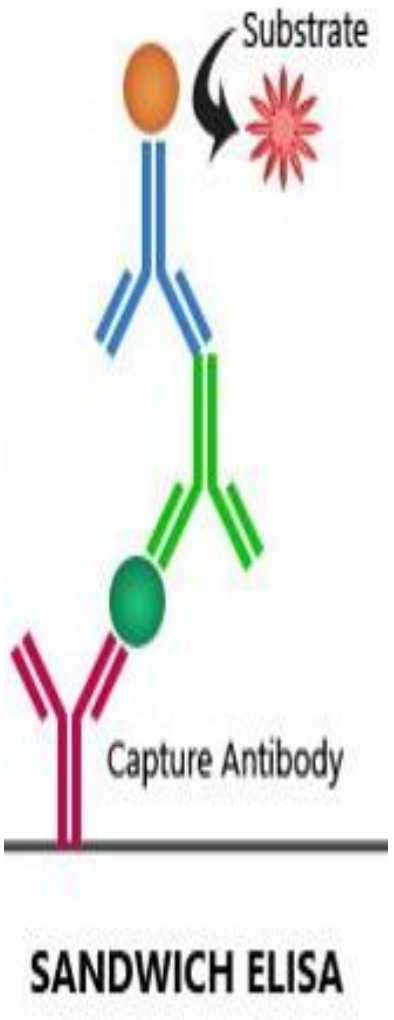
# Indirect ELISA

- In indirect ELISA, the antibody that reacts with bound antigen is **not labelled**. It is called **primary antibody**.
- A **secondary antibody** is used for which **primary antibody acts as an antigen** and it binds to it.
- This secondary antibody is **labeled with enzyme** which acts on substrate and a signal is generated..



# Sandwich ELISA

- In this, the well is **coated with a known antibody** which will attach to unknown antigen. This is called **capture antibody**.
- The **unknown sample** expected to contain the unknown antigen is added to the plate. If antigen is present it will bind to capture antibody.
- Unlabeled primary and labeled secondary antibodies are used as in indirect ELISA to get the results.



# Competitive ELISA

- The **well is coated with unknown sample** expected to contain antigen.
- The antigen will **occupy** binding spots, say 4 out of 10.
- In next step **labeled antigen** is added. It will occupy **remaining** six spots and a signal will be generated.

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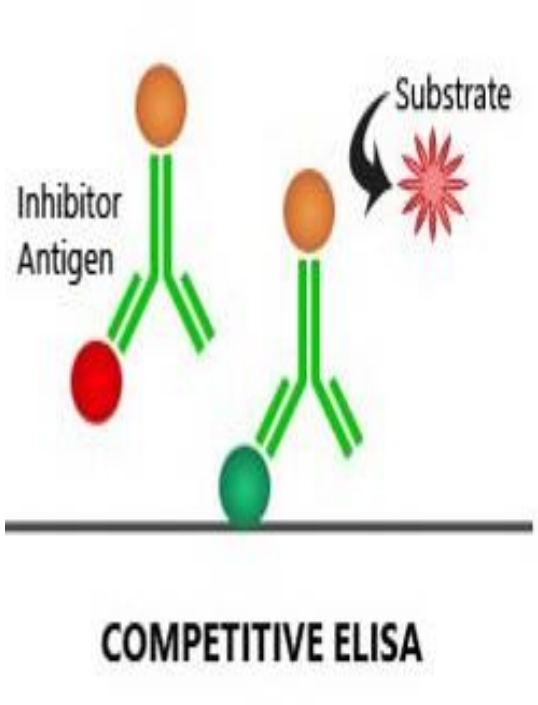
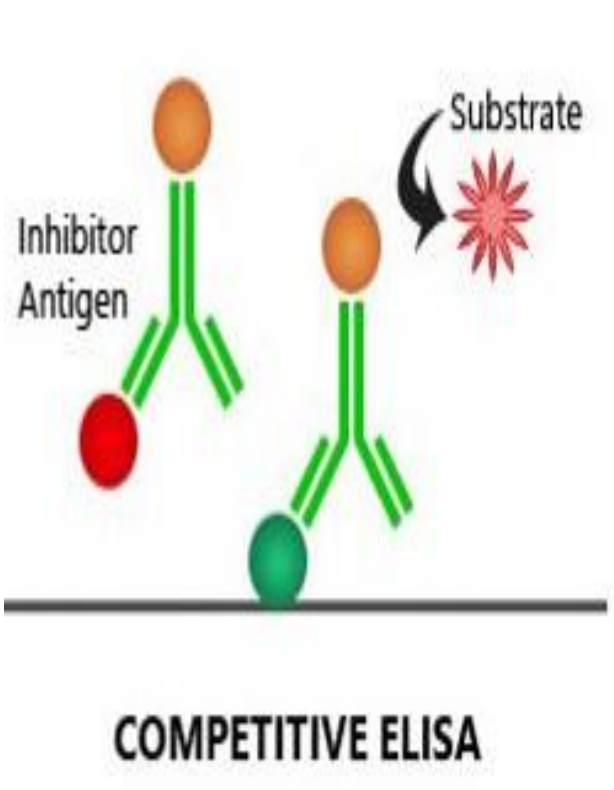
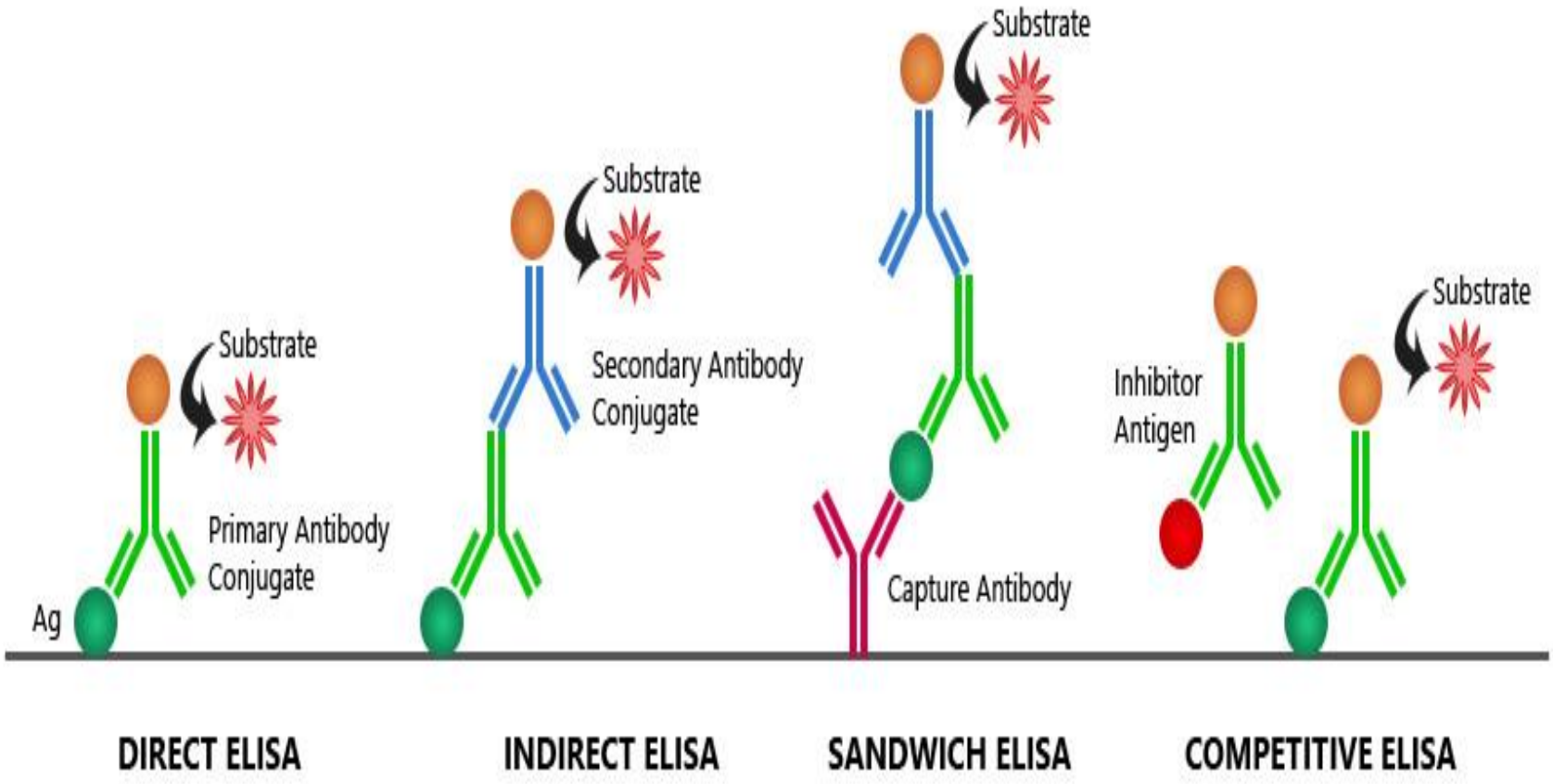


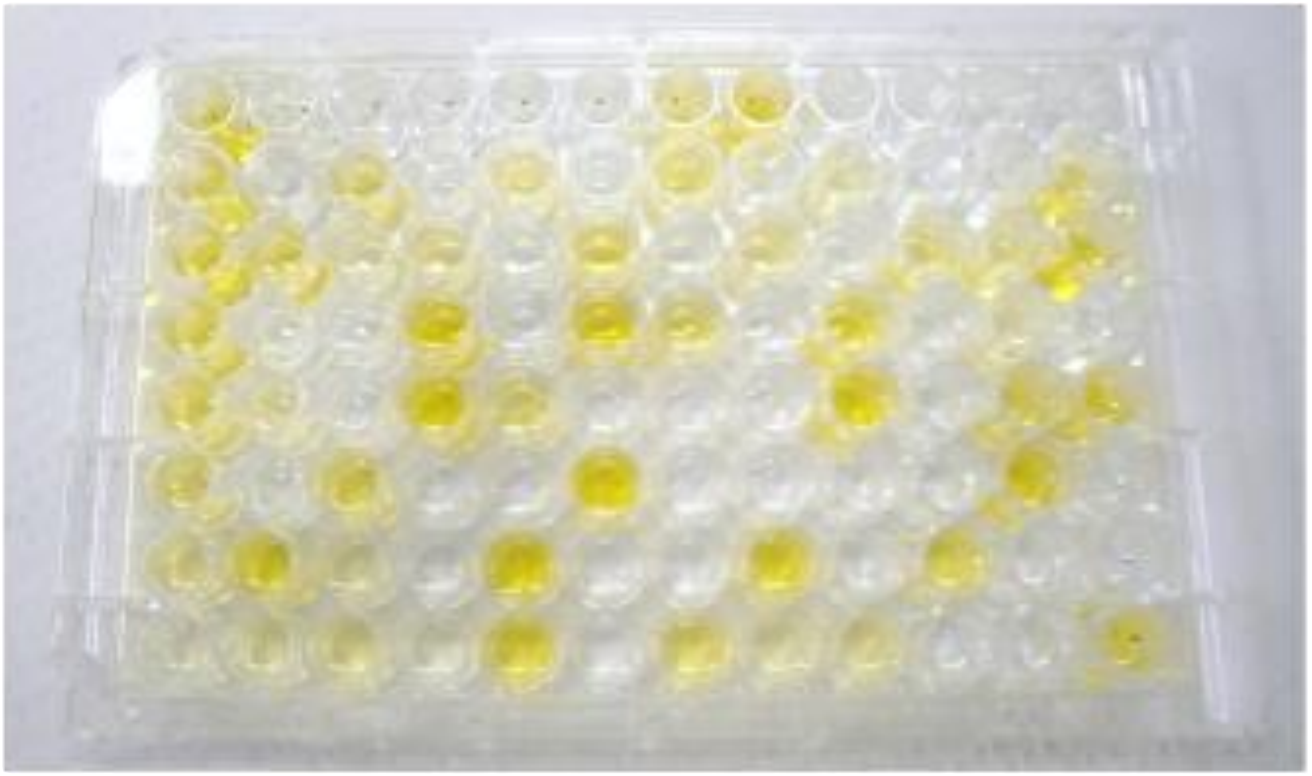
- If in another unknown sample, the amount of antigen was **larger** and it occupied say **8 of 10** available spots, there will be **only two spots left for labeled antigen** to bind. So in this case, **signal will be weaker.**

- This way we can **calculate** the amount of unknown antigen.

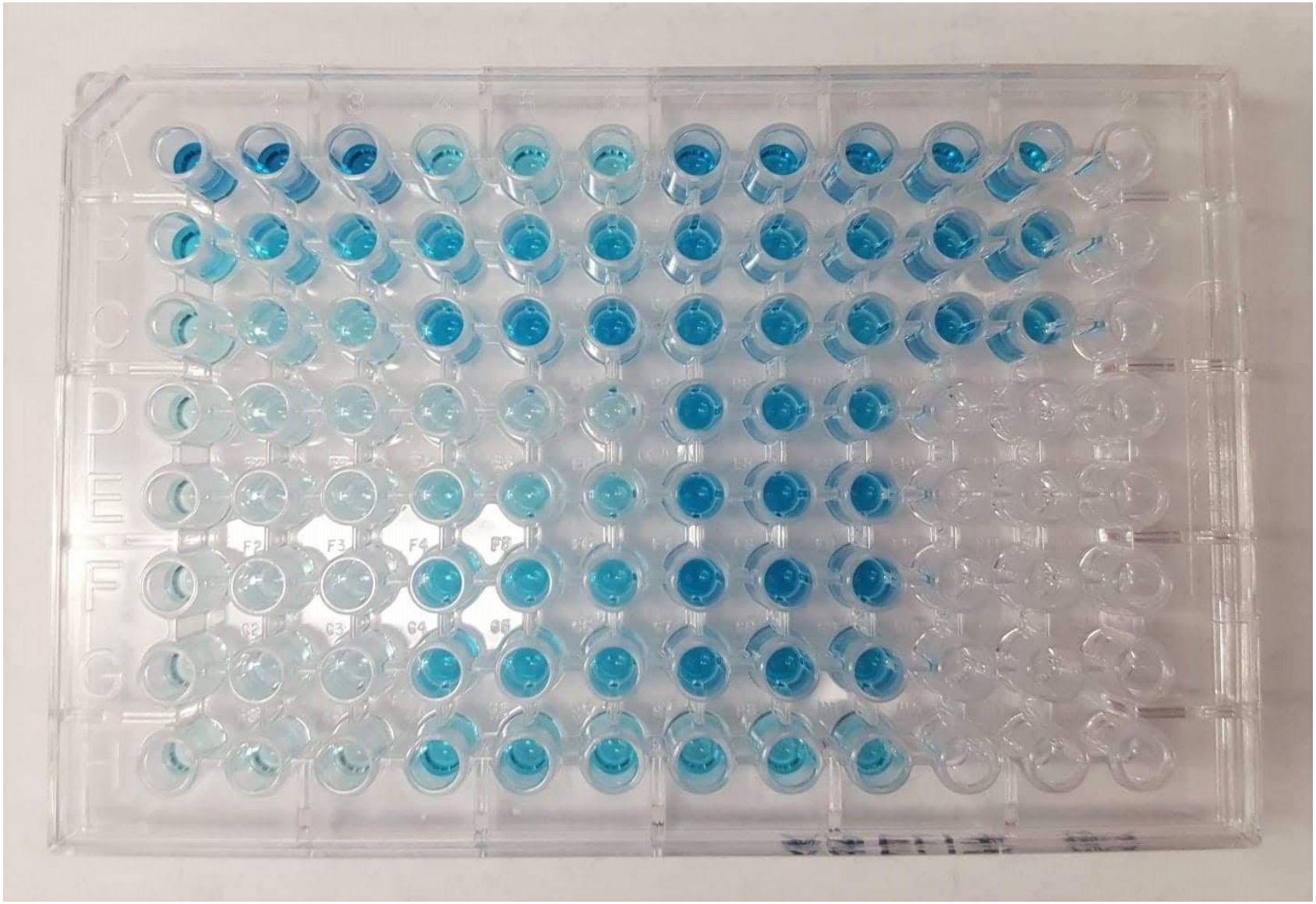



Substrate
Primary Antibody
Conjugate
Ag
Substrate
Secondary Antibody
Conjugate
Substrate
Capture Antibody
Inhibitor
Antigen
Substrate
DIRECT ELISA
INDIRECT ELISA
SANDWICH ELISA
COMPETITIVE ELISA

**ELISA: An example of an assay using a 96-well plate.**

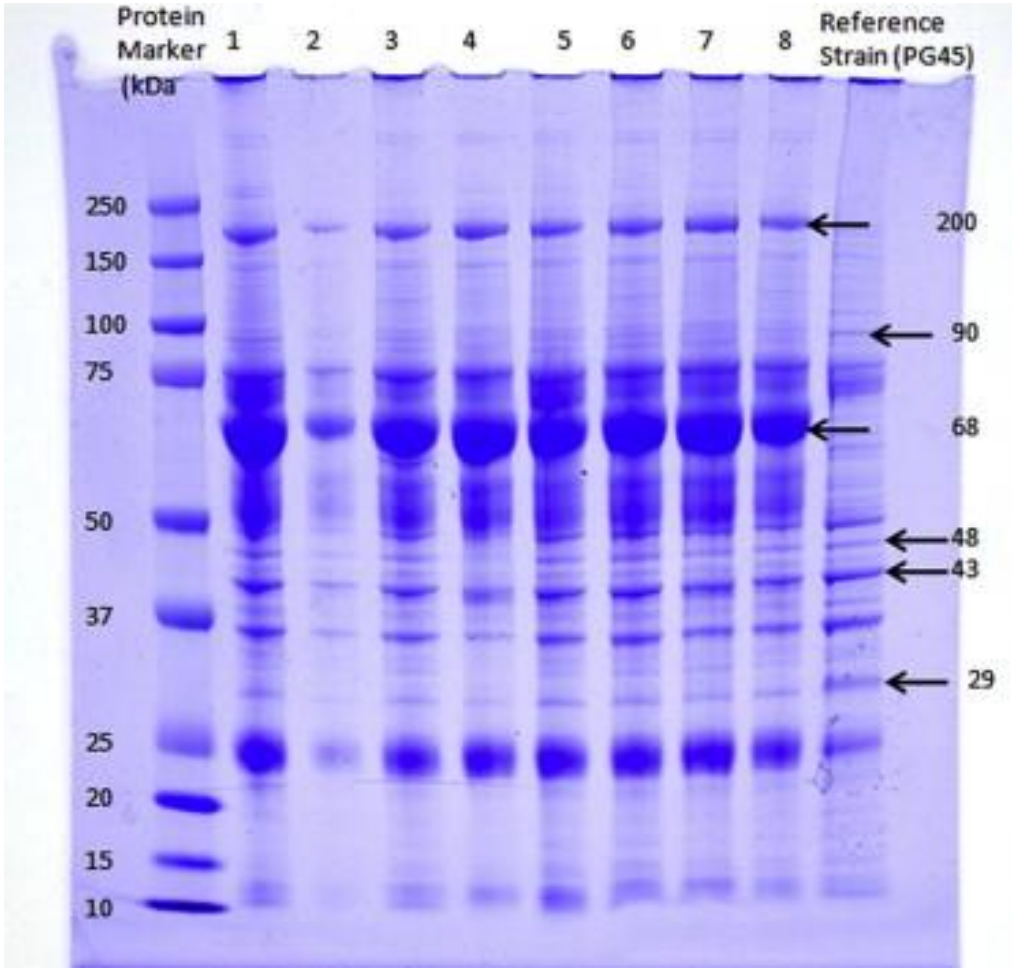


The yellow color indicates that the target protein is present. The higher degree of the color, the higher concentration of the target protein.



# Western Blotting

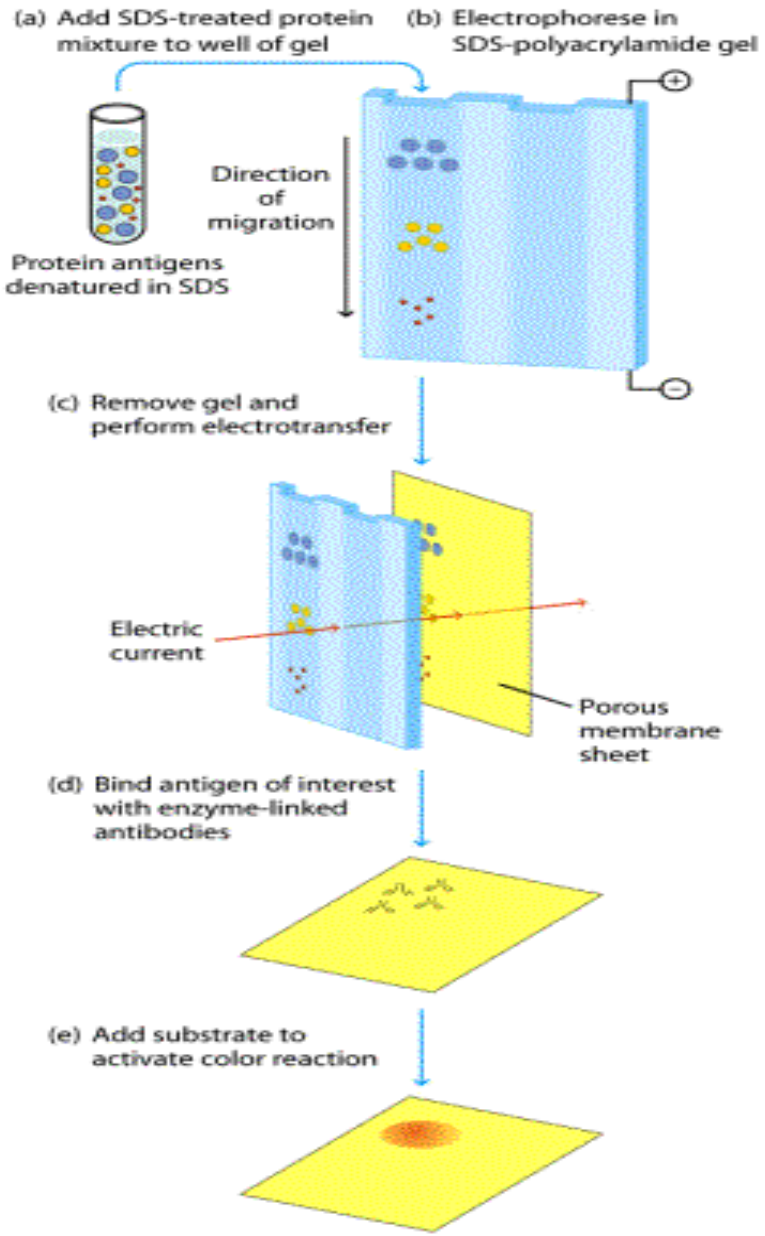
- In the first step, the proteins are **separated** on an SDS-PAGE gel.

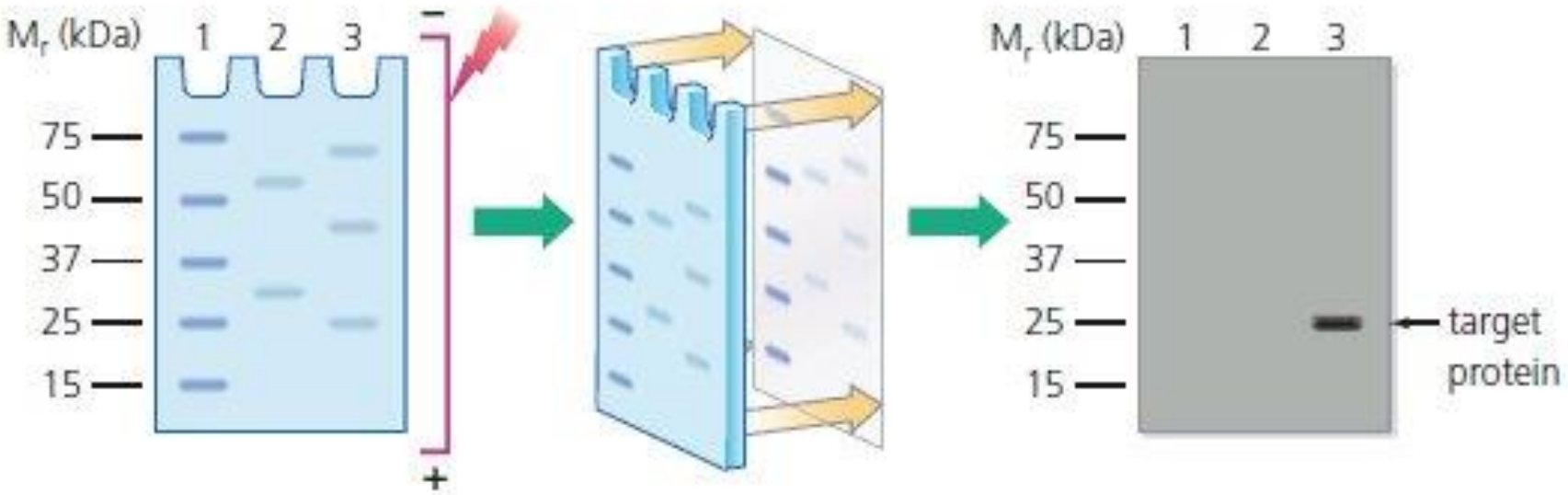


- The separated proteins on agarose gel are then **transferred to a nitrocellulose membrane** by **blotting** by bringing the gel and membrane in close contact, and applying electric current.

- After transfer, **enzyme-linked antibody** is used to detect **specific protein** (antigen).

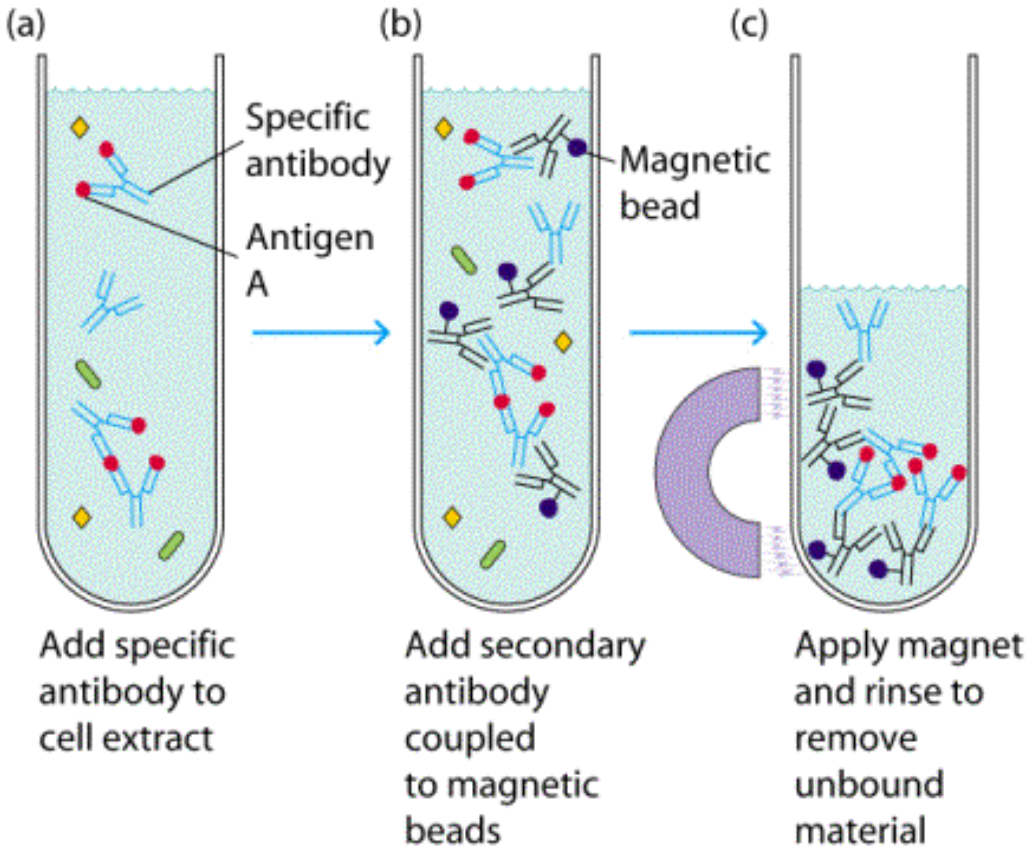
- This technique allows identification of **specific protein** in a complex mixture of proteins.


(a) Add SDS-treated protein mixture to well of gel
(b) Electrophorese in SDS-polyacrylamide gel
Direction of migration
Protein antigens denatured in SDS
(c) Remove gel and perform electrotransfer
Electric current
Porous membrane sheet
(d) Bind antigen of interest with enzyme-linked antibodies
(e) Add substrate to activate color reaction


$M_r$ (kDa)
1
2
3
75
50
37
25
15
−
+
$M_r$ (kDa)
1
2
3
75
50
37
25
15
target protein

# Immunoprecipitation

It is a **s**ensitive method for detecting Ag in a **cell or tissue extract.**



# Immunofluorescence

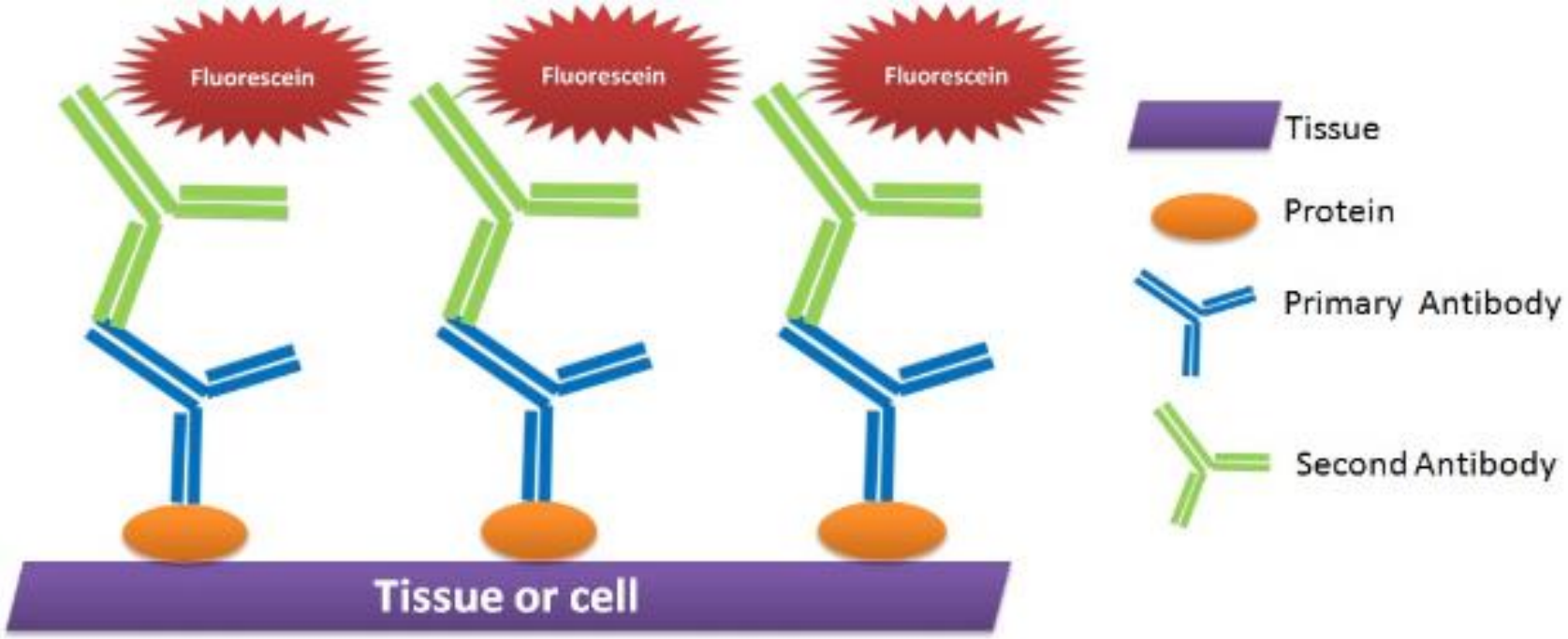
- **Fluorochrome labeled antibodies** are used to detect unknown antigens **in situ**.

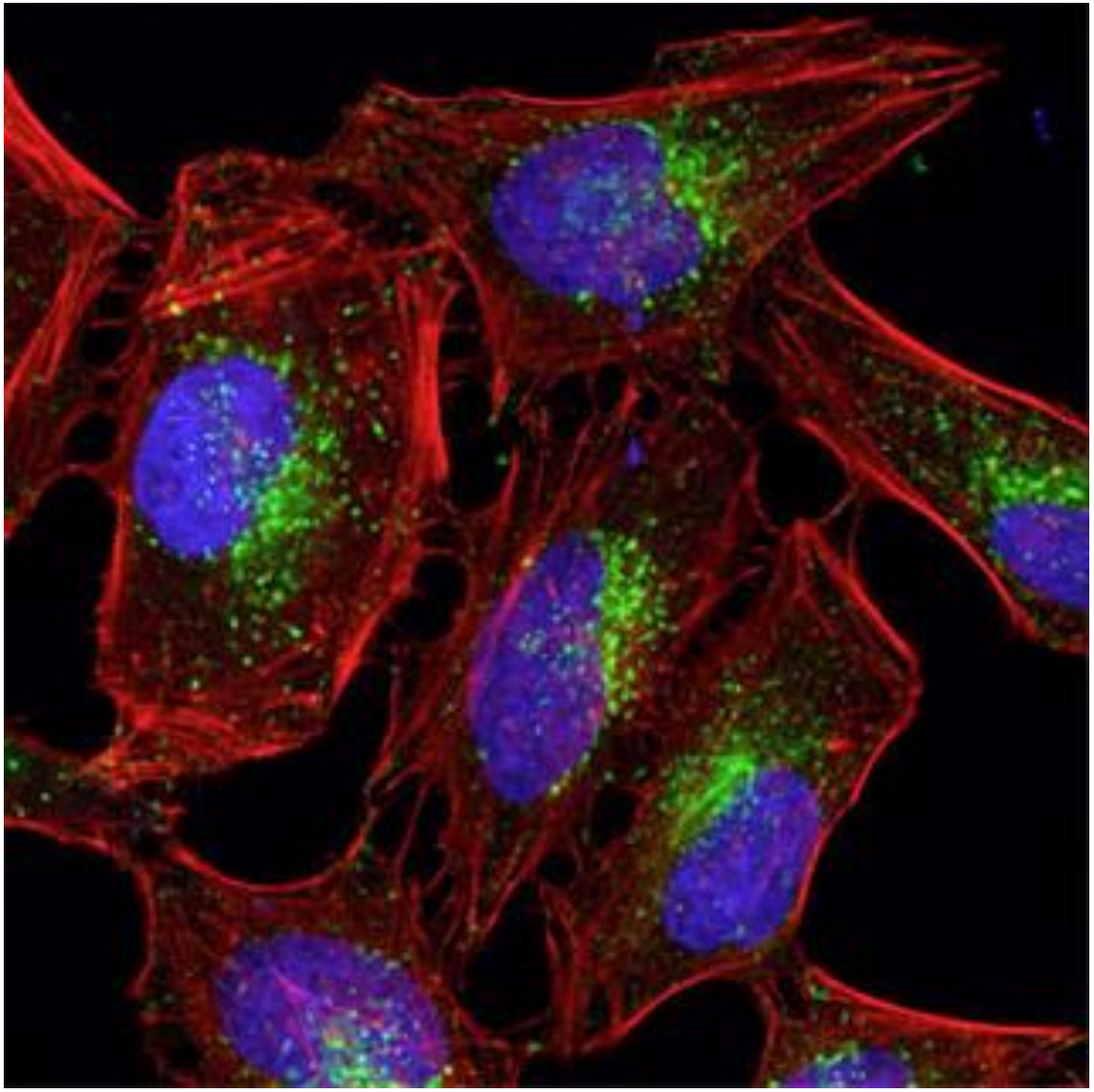
- After the reaction, the emitted light from a fluorochrome is viewed with a **fluorescent microscope.**

**Fluorochromes:** These are substances that absorb light energy of a specific wavelength and re-emit it at a longer wavelength.

## Common fluorochromes:

- **Fluorescein** (490nm absorption and 517nm emission-**green**)
- **Rhodamine** (515nm absorption and 546nm emission-**red**)
- **Pacific Blue** (401 nm absorption and 452 nm emission-**blue**)


Fluorescein
Fluorescein
Fluorescein
Tissue or cell
Tissue
Protein
Primary Antibody
Second Antibody



## Flow cytometry and fluorescence

- **Fluorochromes** are applied to a cell population
- **Fluorochrome labeled cells are counted** to yield quantitative data

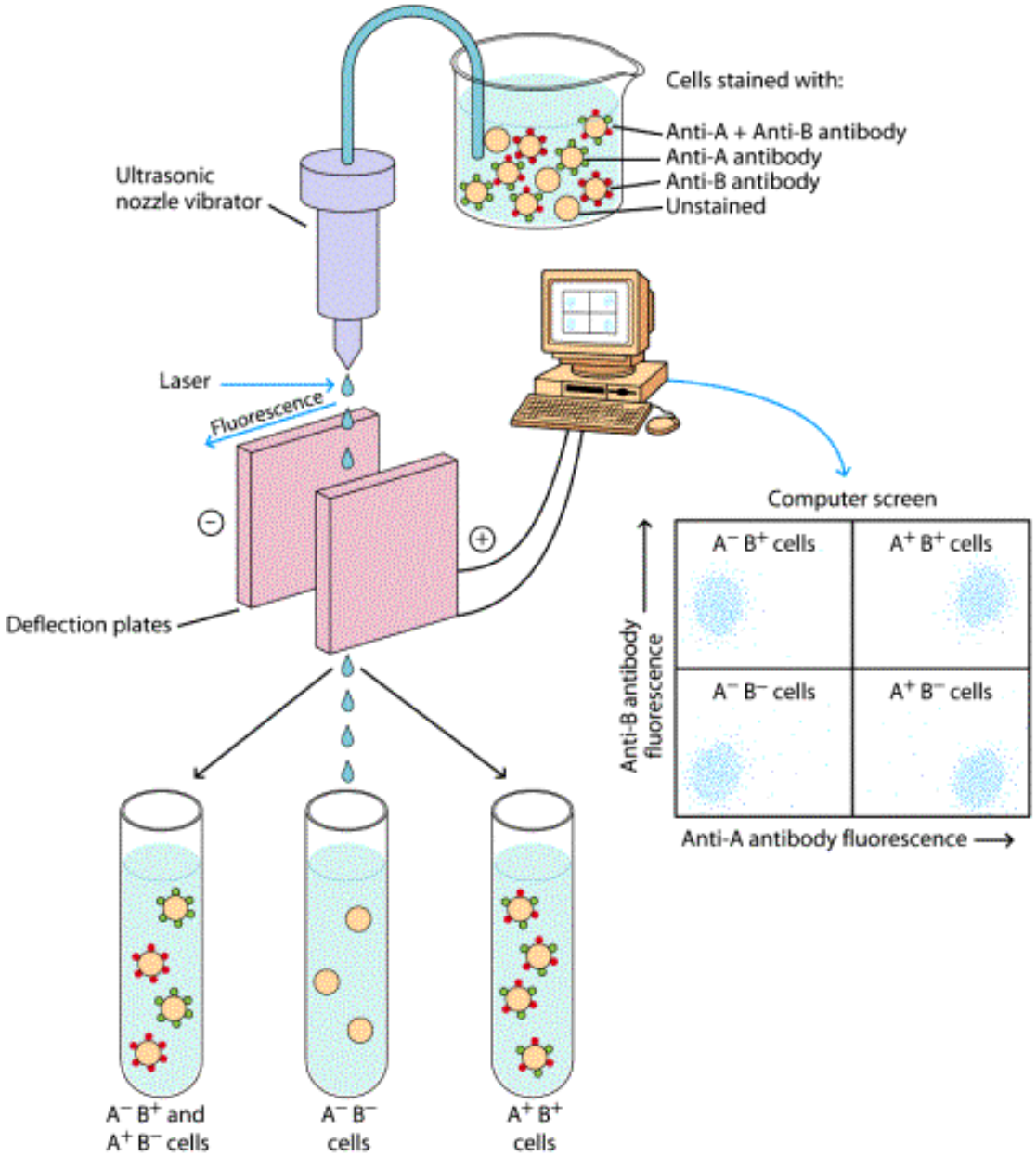

Cells stained with:
Anti-A + Anti-B antibody
Anti-A antibody
Anti-B antibody
Unstained
Ultrasonic nozzle vibrator
Laser
Fluorescence
Deflection plates
Computer screen
A⁻ B⁺ cells
A⁺ B⁺ cells
A⁻ B⁻ cells
A⁺ B⁻ cells
Anti-B antibody fluorescence
Anti-A antibody fluorescence
A⁻ B⁺ and A⁺ B⁻ cells
A⁻ B⁻ cells
A⁺ B⁺ cells

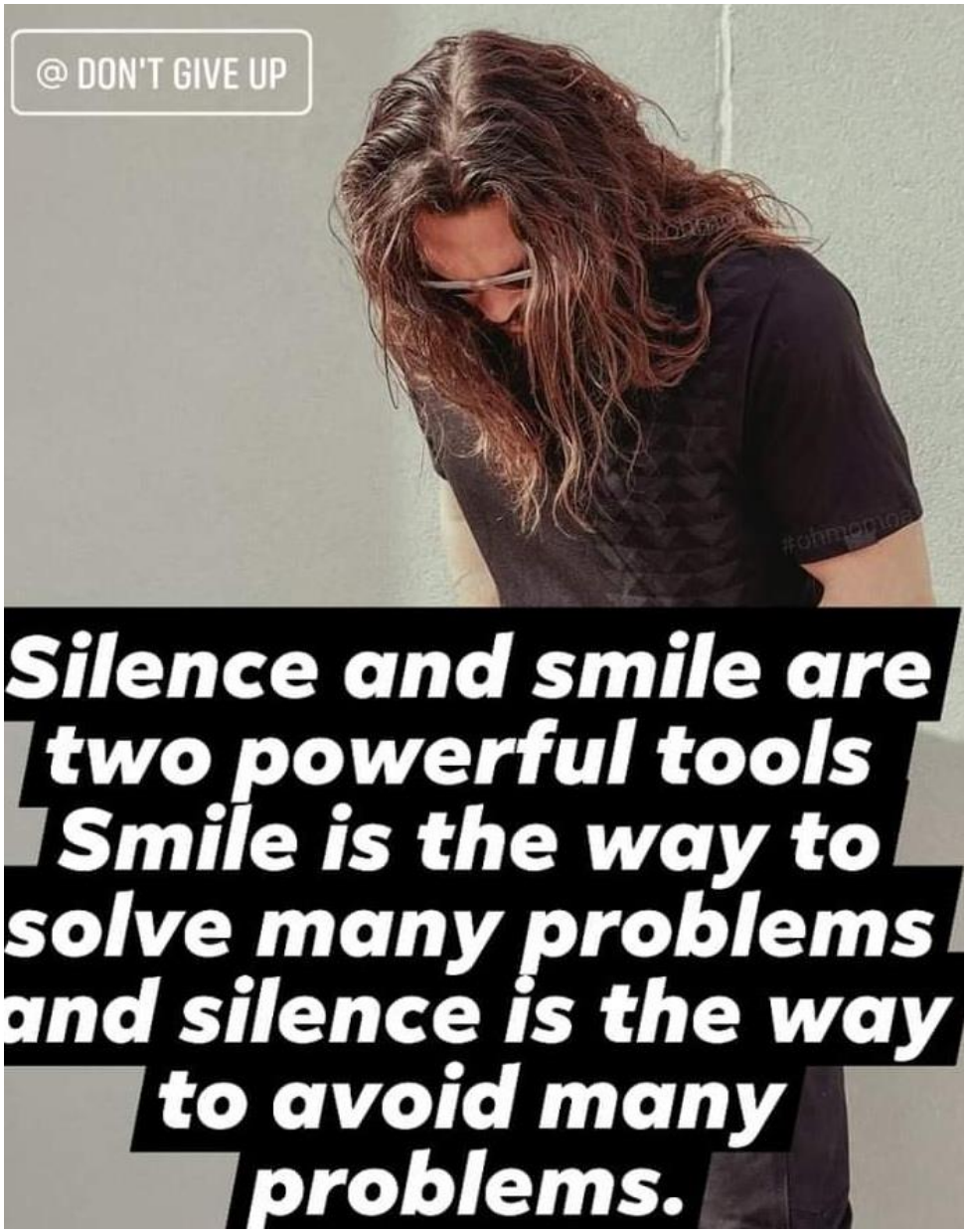
**TABLE 6-3** Sensitivity of various immunoassays

| Assay | Sensitivity* (μg antibody/ml) |
|---|---|
| Precipitation reaction in fluids | 20–200 |
| Precipitation reactions in gels | |
| Mancini radial immunodiffusion | 10–50 |
| Ouchterlony double immunodiffusion | 20–200 |
| Immunoelectrophoresis | 20–200 |
| Rocket electrophoresis | 2 |
| Agglutination reactions | |
| Direct | 0.3 |
| Passive agglutination | 0.006–0.06 |
| Agglutination inhibition | 0.006–0.06 |
| Radioimmunoassay | 0.0006–0.006 |
| Enzyme-linked immunosorbent assay (ELISA) | <0.0001–0.01 |
| ELISA using chemiluminescence | <0.0001–0.01† |
| Immunofluorescence | 1.0 |
| Flow cytometry | 0.06–0.006 |

*The sensitivity depends upon the affinity of the antibody as well as the epitope density and distribution.

†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology*, 5th ed., American Society for Microbiology, Washington, D.C.


@ DON'T GIVE UP
Silence and smile are two powerful tools Smile is the way to solve many problems and silence is the way to avoid many problems.

Great Photography: An ant caught in a raindrop


Thank You!