SEROLOGICAL TECHNIQUES – II

(Primary Binding Tests)

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Antigen antibody reactions

Ag-Ab reaction occurs in three stages:

- Primary Stage
  - Formation of Ag-Ab complex **combined by weaker intermolecular forces**
- Secondary stage
  - leads precipitation
  - agglutination
  - lysis of cells etc.
- Tertiary stage (reaction):
  - Leads to tissue damage
  - Destruction of Ag or its Neutralization

CLASSIFICATION

- **Primary binding test -** directly measure the binding of antigen to antibody e.g. RIA, IF, ELISA.
- **Secondary binding test -** measure the results of antigen – antibody interaction *in vitro*, e.g. precipitation, complement fixation.
- ***In vivo* test -** measures the actual protective effect of antibodies in a host, e.g. passive cutaneous anaphylaxis.

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 (RIA)	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	~0.0001–0.01
ELISA using chemiluminescence	~0.00001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.006–0.06

*The sensitivity depends on the affinity of the antibody used for the assay as well as the epitope density and distribution on the antigen.
†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Updated and adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology,* 5th ed., American Society for Microbiology, Washington, DC.

Table 6-3
Kuby IMMUNOLOGY, Sixth Edition

RADIO IMMUNO ASSAY (RIA)

RADIO IMMUNO ASSAY

- RIA was developed by Barson and Yalow in 1960
- The test use radioactive isotopes for detecting antigen or antibody. The commonly used isotopes are H^3, C^{14}, or I^{125}
- RIA is one of the most sensitive immunological technique (Using antibodies of high affinity - $K_0 = 10^8$–10^{11} M^{-1}, it is possible to detect a few picograms (10^{-12} g) of antigen in the tube.
- **Uses of Radioimmunoassay**
  - The test can be used to determine very small quantities of antigens and antibodies in the serum (anti DNA Abs in SLE).
  - The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.
  - Analyze nanomolar and picomolar concentrations of hormones in biological fluids.
- **Limitations of Radioimmunoassay** – expensive (gamma counters), hazards of radioactivity, limited shelf-life of labeled reagents, availability of radioisotopes and environmental concerns with safe disposal of radioactive material

Principle of Radioimmunoassay

- It involves a combination of three principles.
  - An immune reaction i.e. antigen, antibody binding.
  - A competitive binding or competitive displacement reaction. (It gives specificity)
  - Measurement of radio emission. (It gives sensitivity)

- The classical RIA methods are based on the principle of competitive binding:
  - an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity
  - the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.

Technique of Radioimmunoassay

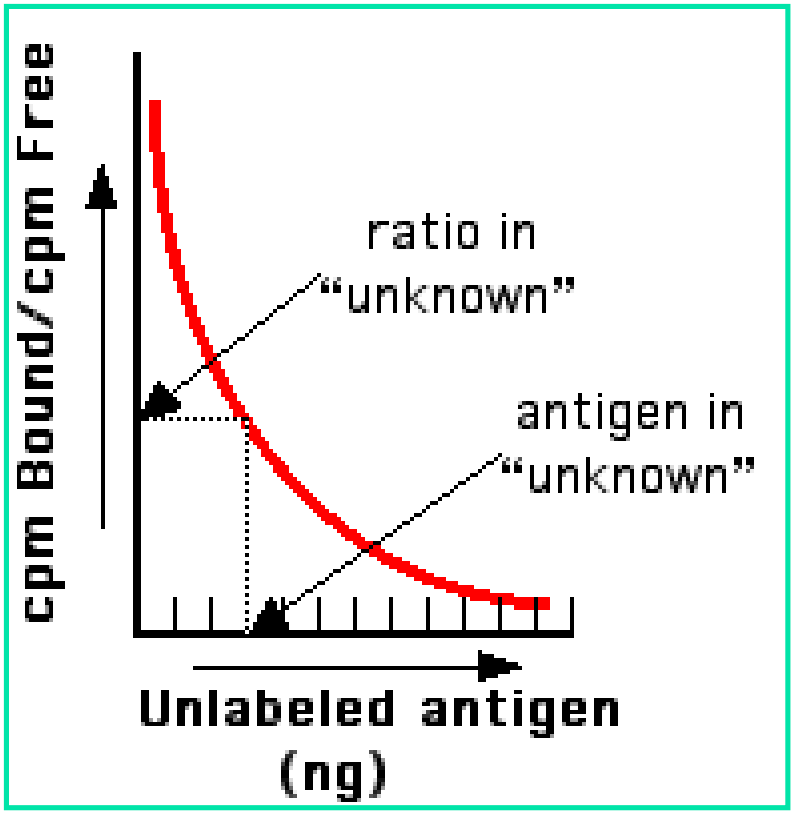
- A mixture is prepared of
  - radioactive antigen (“hot")
  - antibodies ("First" antibody) against that antigen.
- Known amounts of unlabeled ("cold") antigen are added to samples of the mixture. These compete for the binding sites of the antibodies.
- At increasing concentrations of unlabeled antigen, 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, and the radioactivity of each is measured.
- From these data, a standard binding curve can be drawn
- The samples to be assayed (the unknowns) are run in parallel.
- After determining the ratio of bound to free antigen ("cpm Bound/cpm Free") in each unknown, the antigen concentrations can be read directly from the standard curve

Technique of Radioimmunoassay

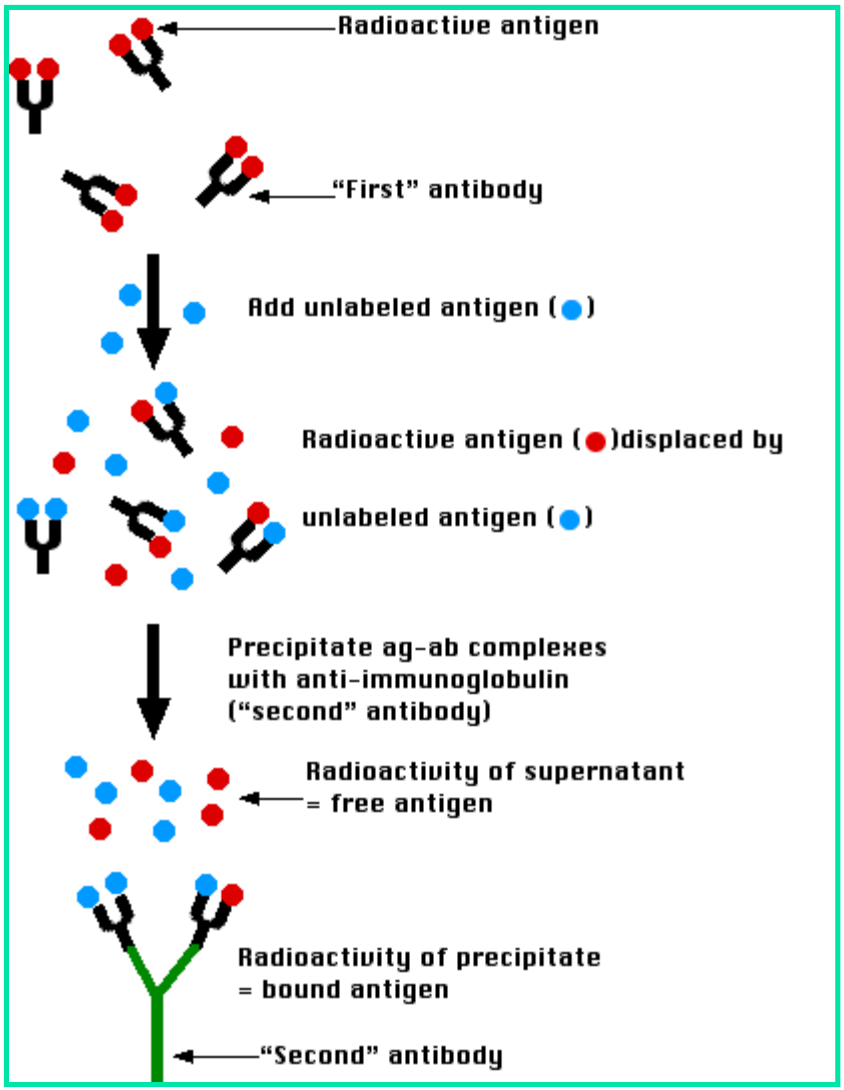
Separating Bound from Free Antigen

- Precipitate the antigen-antibody complexes by adding a "second" antibody directed against the first. For example, rabbit IgG and anti rabbit-IgG
- The antigen-specific antibodies can be coupled to the inner walls of a test tube and after incubation:
  - the contents ("free") are removed;
  - the tube is washed ("bound"), and
  - the radioactive of both is measured.
- The antigen-specific antibodies can be coupled to particles, like Sephadex. Centrifugation of the reaction mixture separates
  - the bound counts (in the pellet) from
  - the free counts in the supernatant fluid.

Technique of RIA



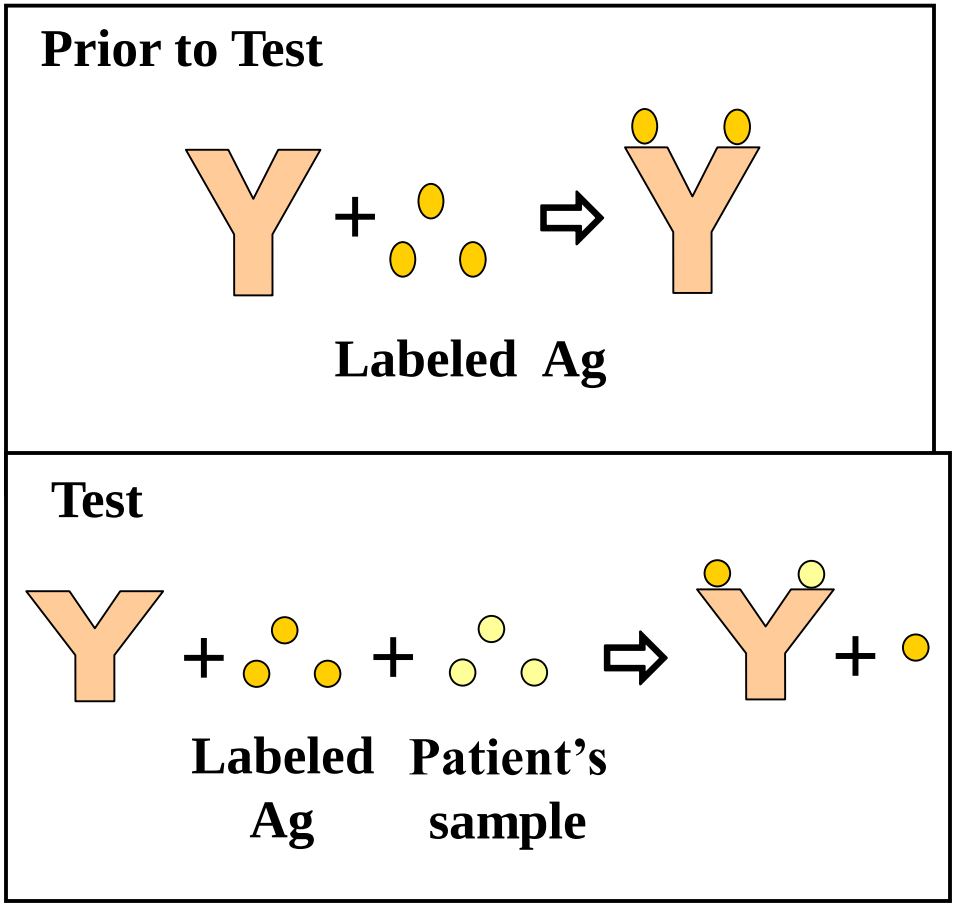
Standard curve



Separating antigens by precipitation

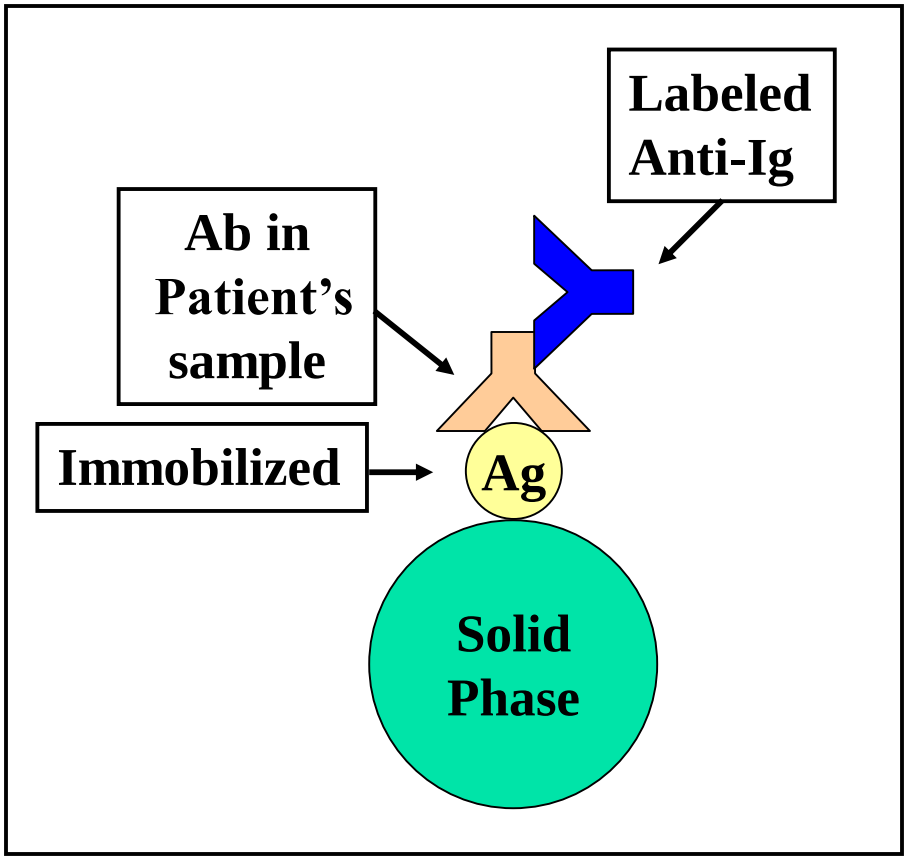
Competitive RIA for Ag detection

- Determine amount of Ab needed to bind to a known amount of labeled Ag
- Use predetermined amounts of labeled Ag and Ab and add a sample containing unlabeled Ag as a competitor
- Determine amount of labeled Ag bound to Ab
- Concentration of Ag in sample is determined from a standard curve using known amounts of unlabeled Ag



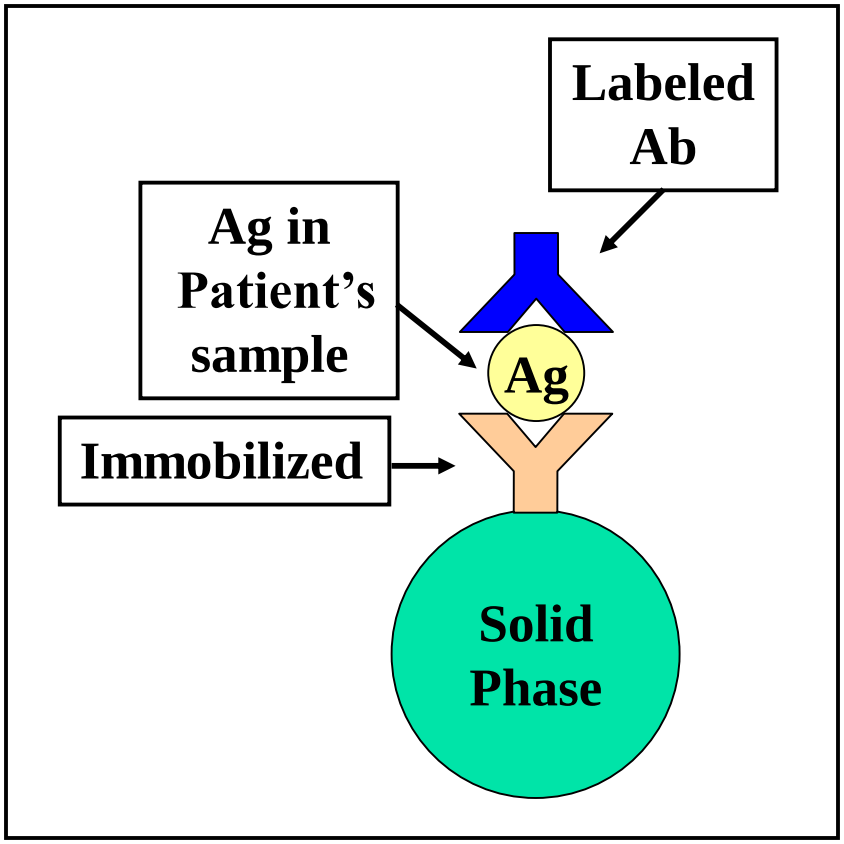
Non- Competitive RIA for Ab detection

- Immobilize Ag
- Incubate with patient's serum sample
- Add labeled anti-Ig
- Amount of labeled Ab bound is proportional to amount of Ab in the sample



Non- Competitive RIA for Ag detection

- Immobilize Ab
- Incubate with patient's sample containing antigen
- Add labeled antibody
- Amount of labeled Ab bound is proportional to the amount of Ag in the sample



IMMUNO FLUORESCENCE ASSAY (IFA)

INTRODUCTION

- **Immunofluorescence assay** (IFA) is a standard serological technique to identify the presence of antigens in infected cells by their ability to react with specific antibodies, which conjugated with are visualized by incubation with Fluorescent dyes.

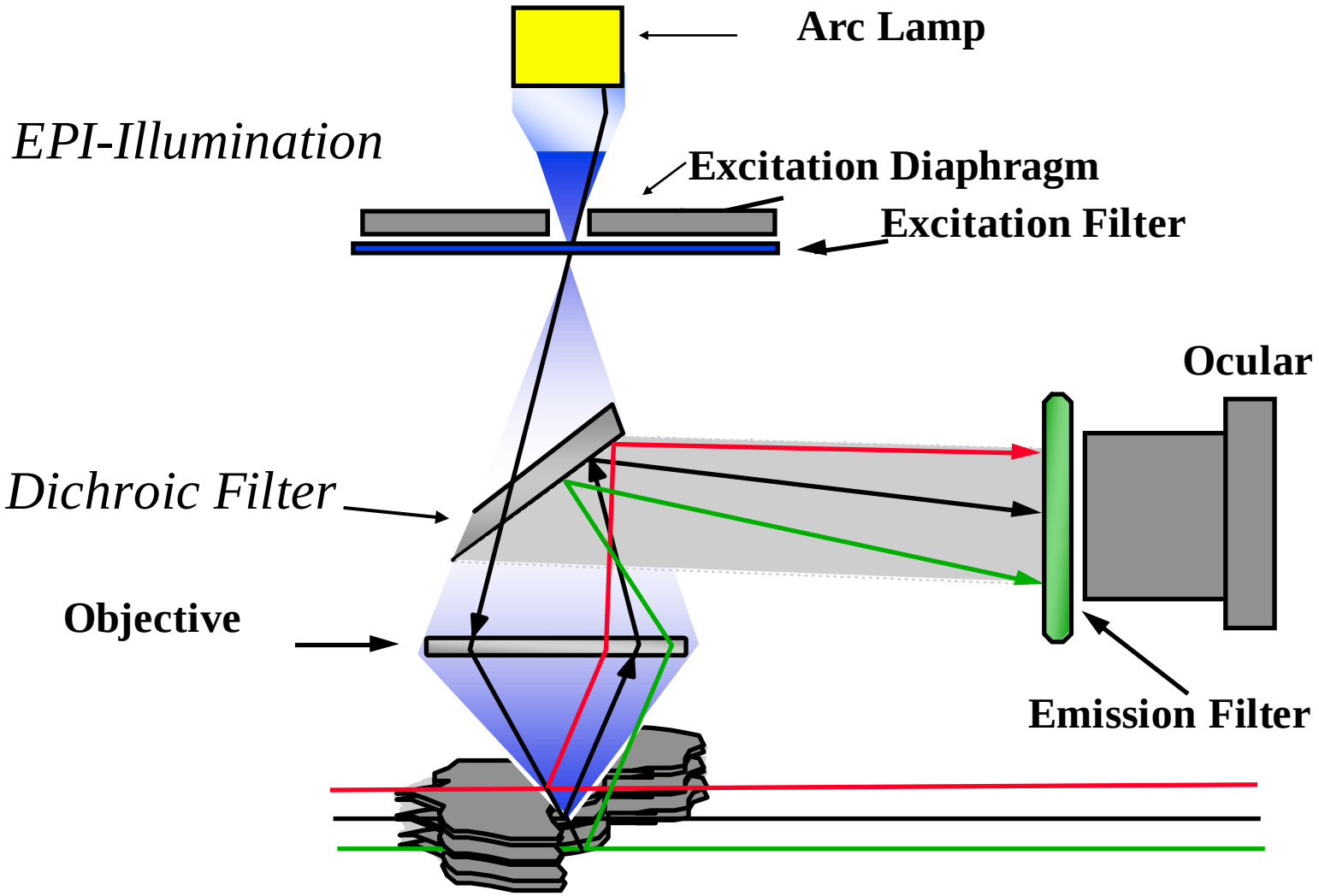
- Fluorescent molecules are organic dyes that absorb light of one (shorter) wavelength (excitation) and emit light of another (larger) wavelength.

- The emitted light can be viewed with a fluorescent microscope, which is equipped with a UV light source.

- The most commonly used fluorescent dyes are fluorescein isothiocyanate (FITC) and lissamine rhodamine

- FA tests are performed on frozen sections of tissues, blood smears, tissue imprints and scrapings or in cell cultures.

Fluorescent Microscope



Flurochrome labelled antibodies

- When possible use fluorochrome-labeled, affinity purified antibodies

»	Excitation (nm)	Emission (nm)
• Fluorescein	495	524(green/yellow)
• Texas Red	595	620(red)
• R-phycoerythrin	565	574(orange/red)

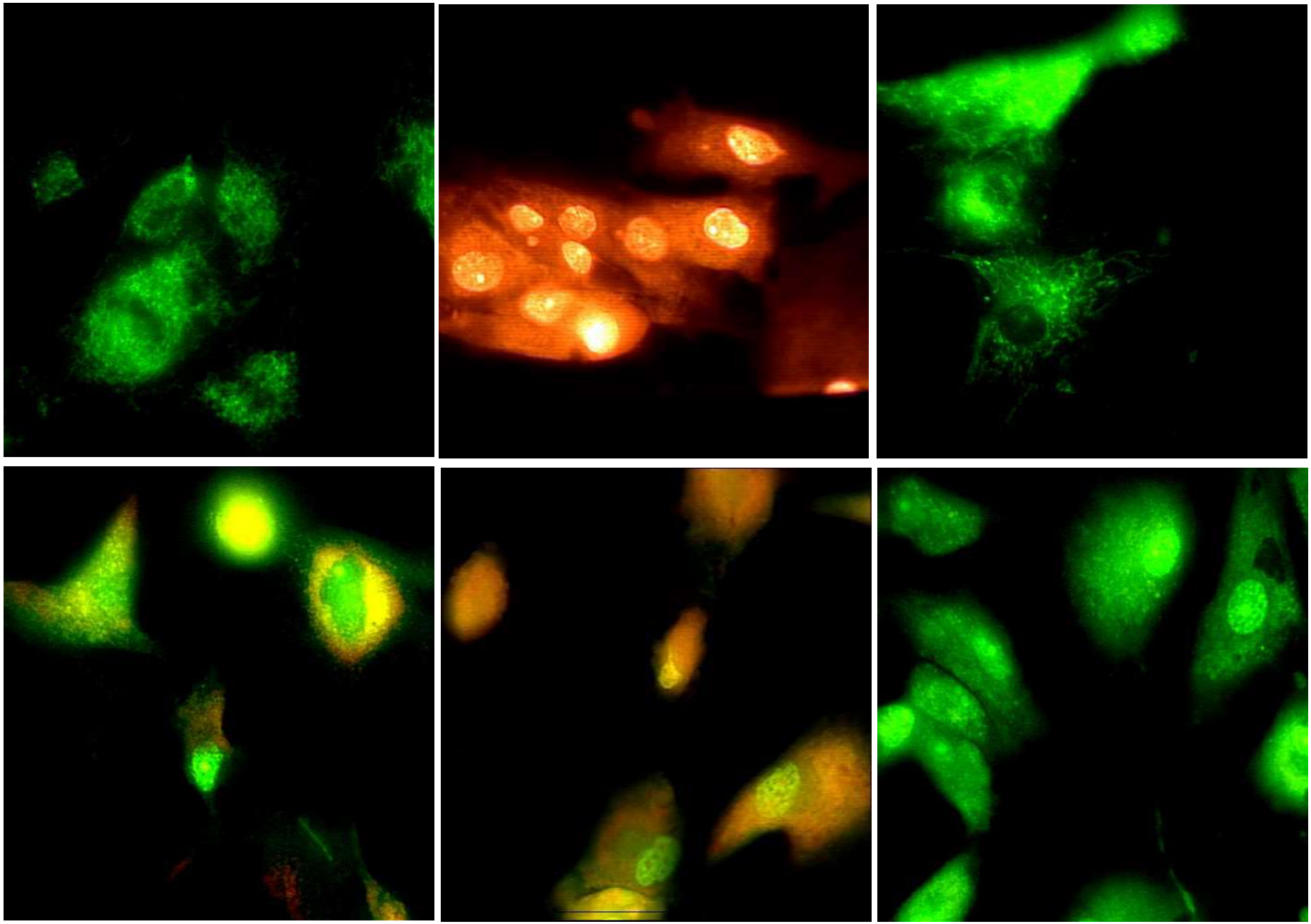
INTRODUCTION

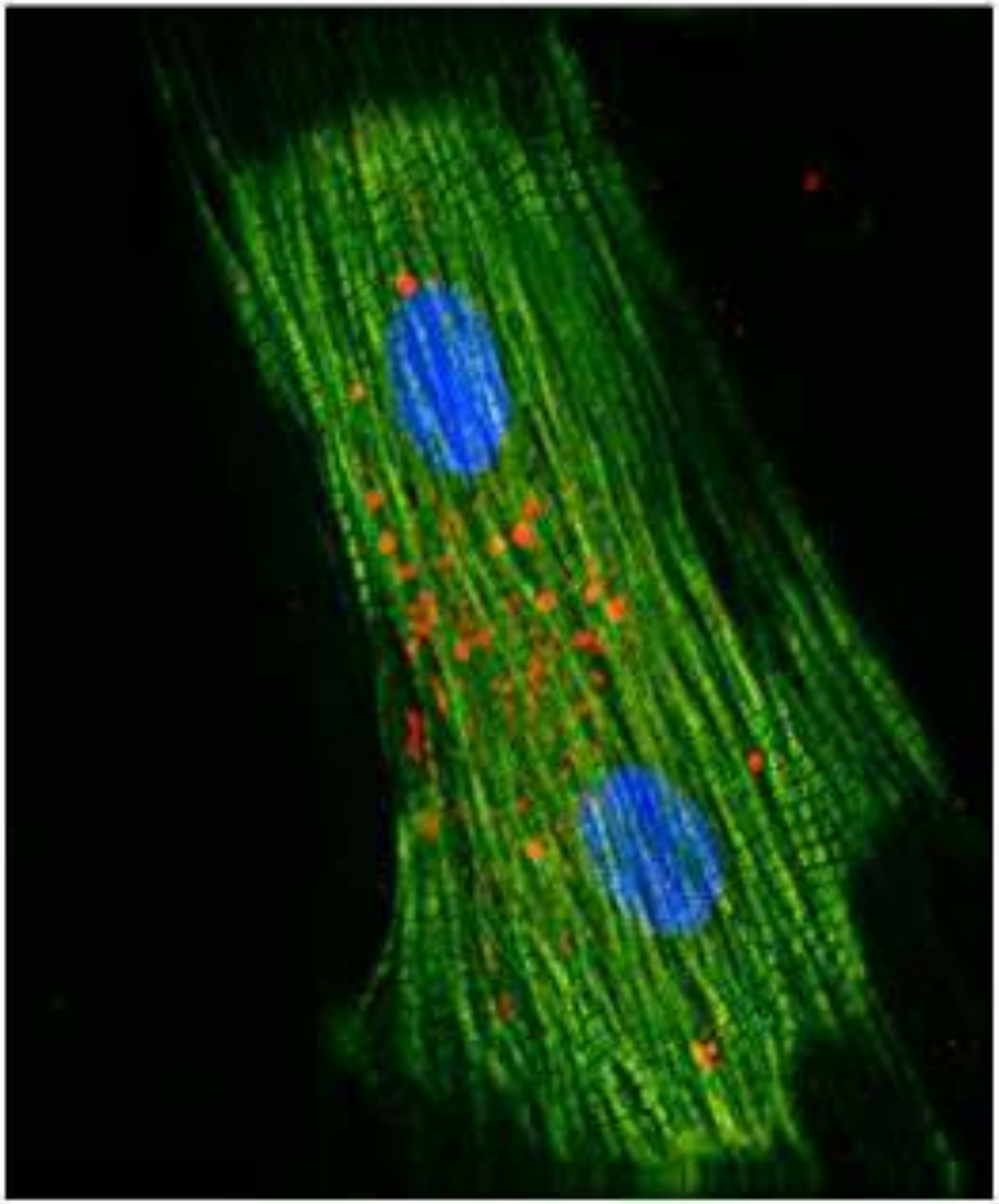
Advantages:

- The method is rapid and specific.
- IF assays can also be used to detect co-localisation of two or more different antigens within same specimen
- The method can also be used for visualization of intracellular localization of antigens, such as viruses.

Limitations:

- expensive fluorescence microscope and reagents,
- trained personnel
- have a factor of subjectivity that may result in erroneous results





Fluorescence Deconvolution Microscopy Image of a differentiating adult cardiomyocyte.

Blue = DAPI staining of nucleic acids to show nuclei

Green = BODIPY staining of F-actin

Red = Texas RED staining of calcitonin gene related peptide (CGRP) receptor

DAPI, Bodipy and Texas Red are fluorescent probes of different wavelengths (INVITROGEN)

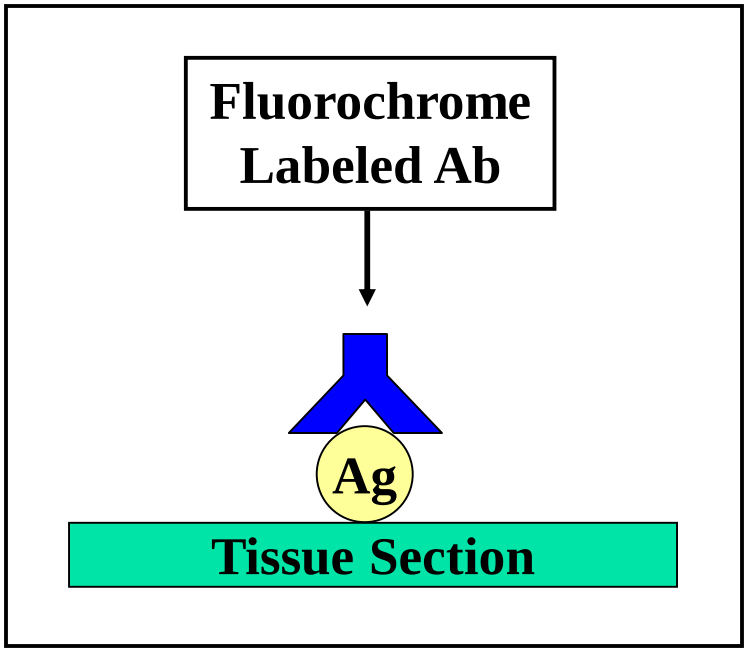
Mag. x 600

TYPES OF IFA

- There are two basic types of IF techniques:
  - direct (DFT) - is used to detect unknown antigen in a cell or tissue by employing a known labeled antibody that interacts directly with unknown antigen.
  - indirect (IFT) - is a double antibody technique and has the advantage of using a single labeled antiglobulin (antibody to IgG) as a “universal reagent” to detect many different specific antigen–antibody reactions.
- Indirect immunofluorescence test is used widely to:
  - detect specific antibodies for serodiagnosis of syphilis, amoebiasis leptospirosis,, toxoplasmosis, and many other infectious diseases;
  - Identify the class of a given antibody by using fluorescent antibodies specific for different immunoglobulin isotypes;
  - Identify and enumerate lymphocyte subpopulations by employing monoclonal antibodies and cytofluorographs (FACS); and
  - Detect autoantibodies, such as antinuclear antibodies in autoimmune diseases.
  - Diagnosis of diseases such as rabies
  - Demonstration of non-cytopathic viruses in cell cultures

DIRECT IMMUNOFLUORESCENCE

– Ab to tissue Ag is labeled with fluorochrome
– quicker to perform



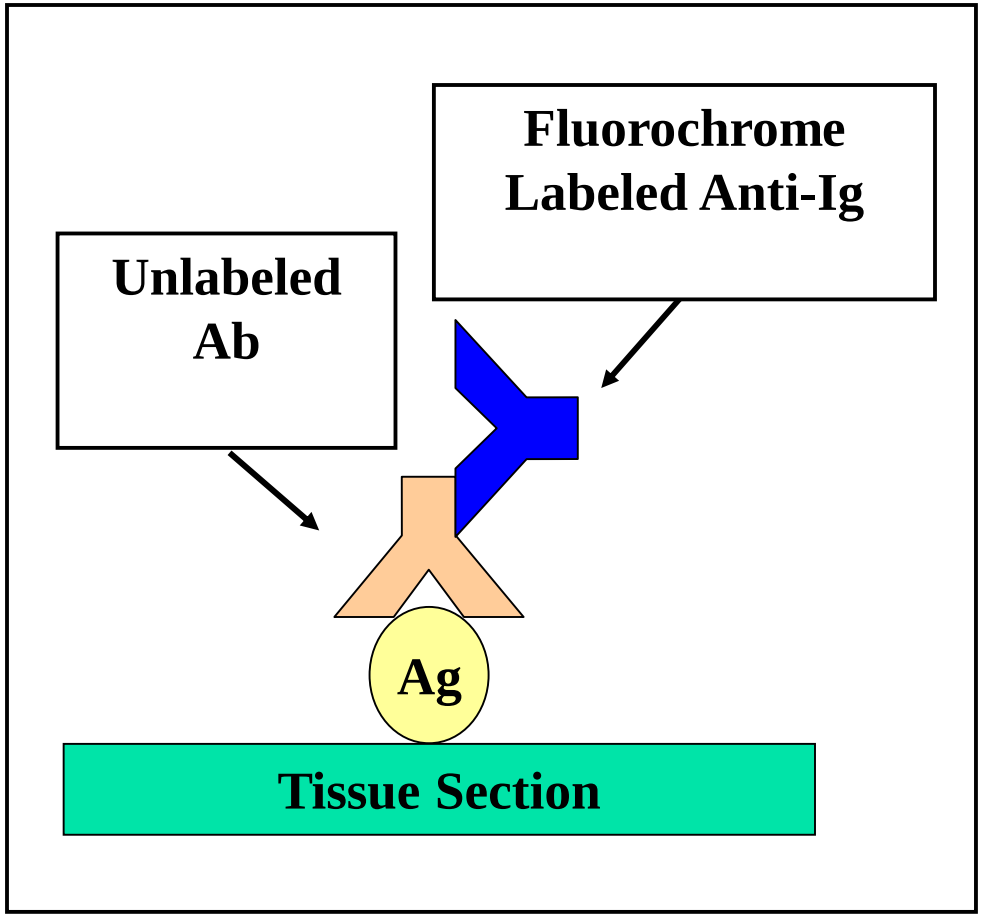
Procedure of DFA

- The specimen with suspected antigen is placed on a slide and fixed by acetone
- Fluorescent labeled specific antibody is then added to it and incubated
- The preparation is then washed which will allow the removal of other components except the complex of antigen and fluorescent labeled antibody.
- On microscopy (Fluorescence Microscopy), antigen- antibody complex are observed fluorescing due to the dye attached to antibody.
- The need for preparation of separate labeled antibody for each pathogen is the major disadvantage of the direct immunofluorescence test.

INDIRECT IMMUNOFLUORESCENCE

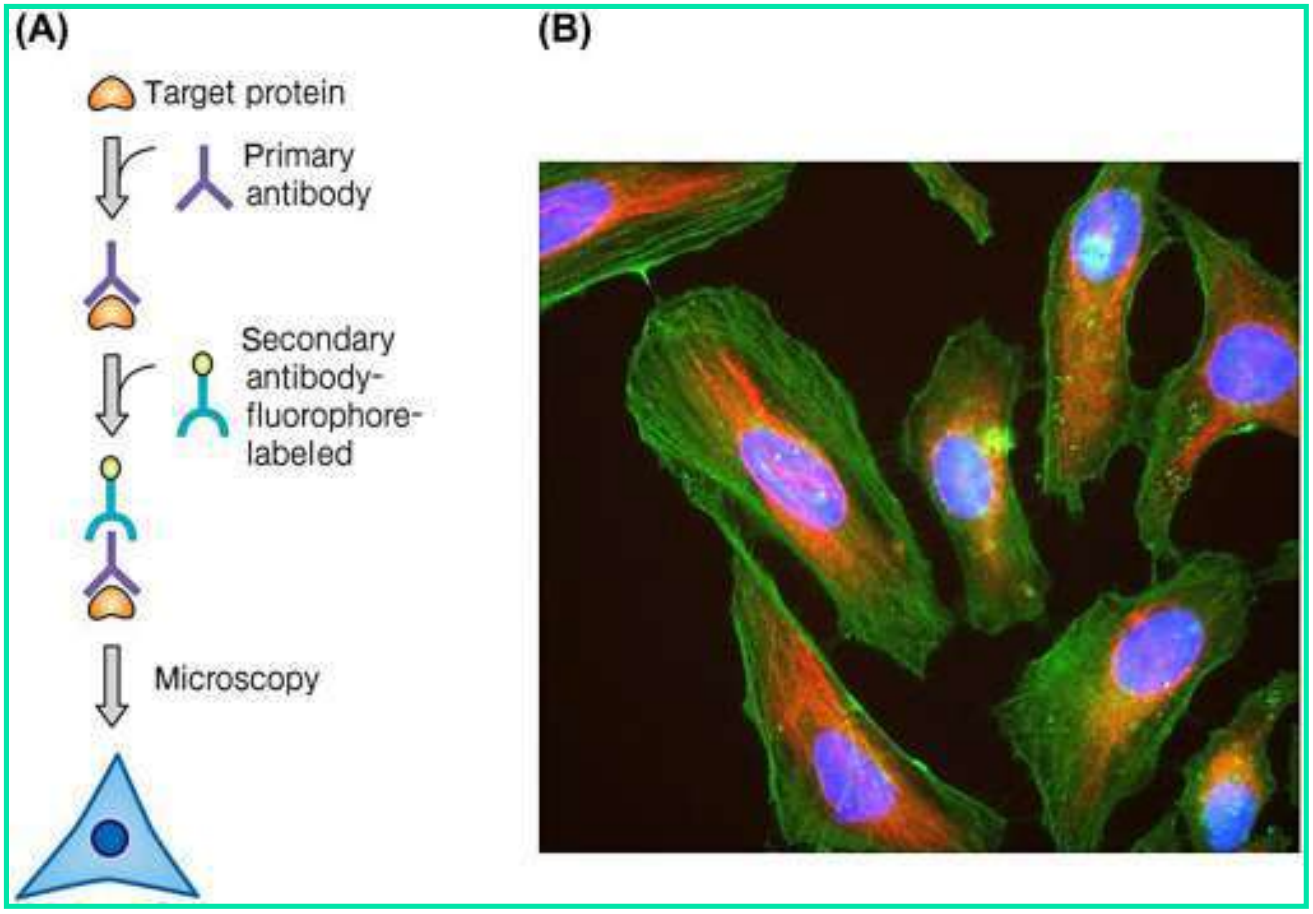
- Primary Ab to tissue Ag is unlabeled
- Fluorochrome-labeled anti-Ig (Secondary or detecting antibody) is used to detect binding of the first Ab.
- More sensitive (since several molecules of fluorochrome-labeled detecting reagent bind to each primary Ab molecule)
- Specific (if monoclonal antibodies are used)
- More time consuming
- Economical as it requires only one anti-species IgG labeled antibody for detecting all pathogens
- **IFT is choice of test for diagnosis of Rabies as per WHO and OIE**

Indirect Immunofluoroscence Test



Procedure of IFT

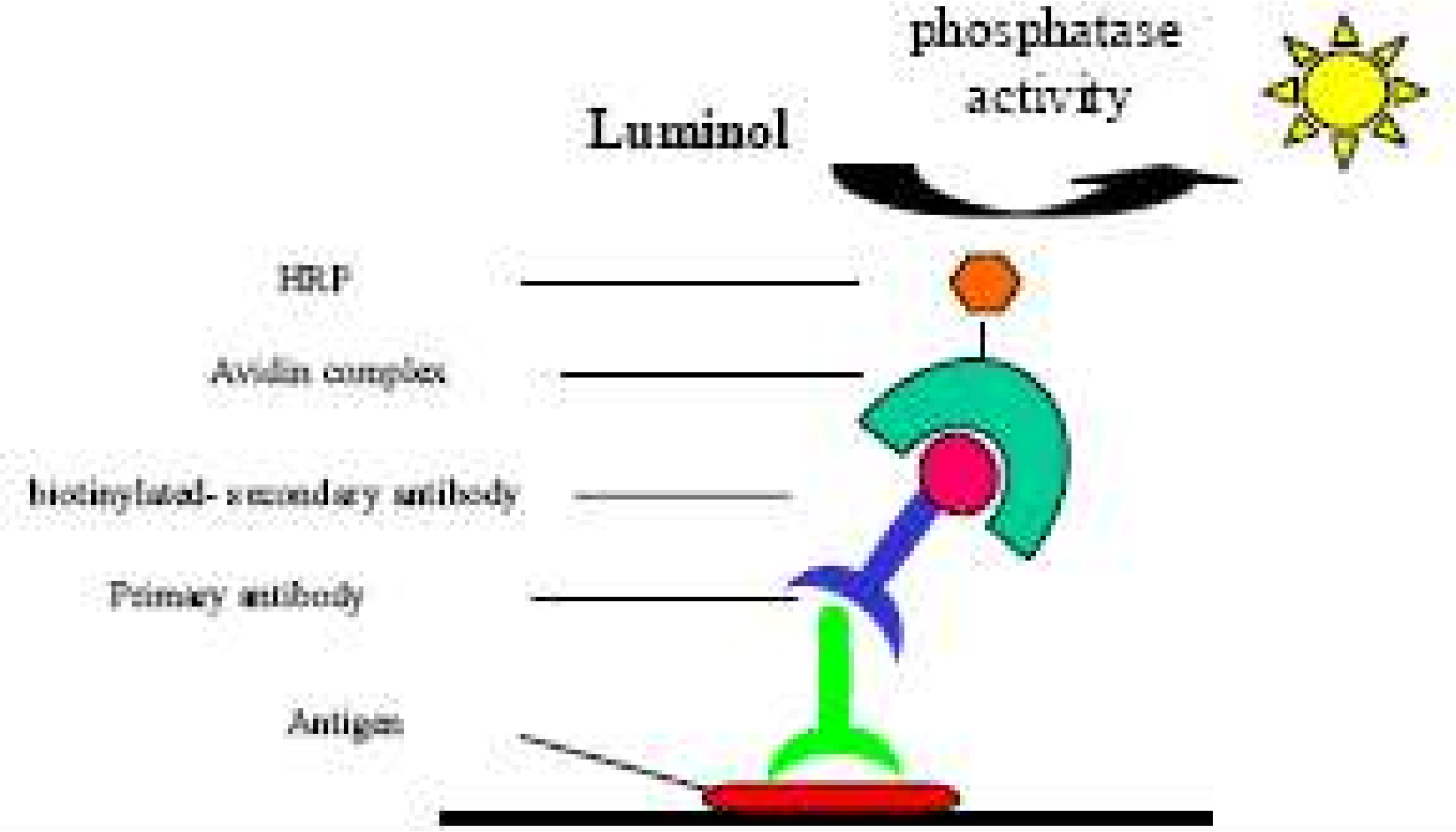
- First of all, the virus infected cells are grown on cover glass.
- The cells are then fixed by paraformaldehyde so that the proteins in the cells are poised to antibody binding.
- Cells are then permeabilized by a proper detergent such as triton X-100.
- A specific antibody is applied on the surface so that the viral antigen is recognized by the antibody.
- Then, the secondary antibody, which is specific to Fc fragment of IgG molecule of the primary antibody, is applied. Since a fluorescence dye is linked to the secondary antibody, the antigen can be visualized under a fluorescence microscope.


(A)
Target protein
Primary antibody
Secondary antibody-fluorophore-labeled
Microscopy
(B)

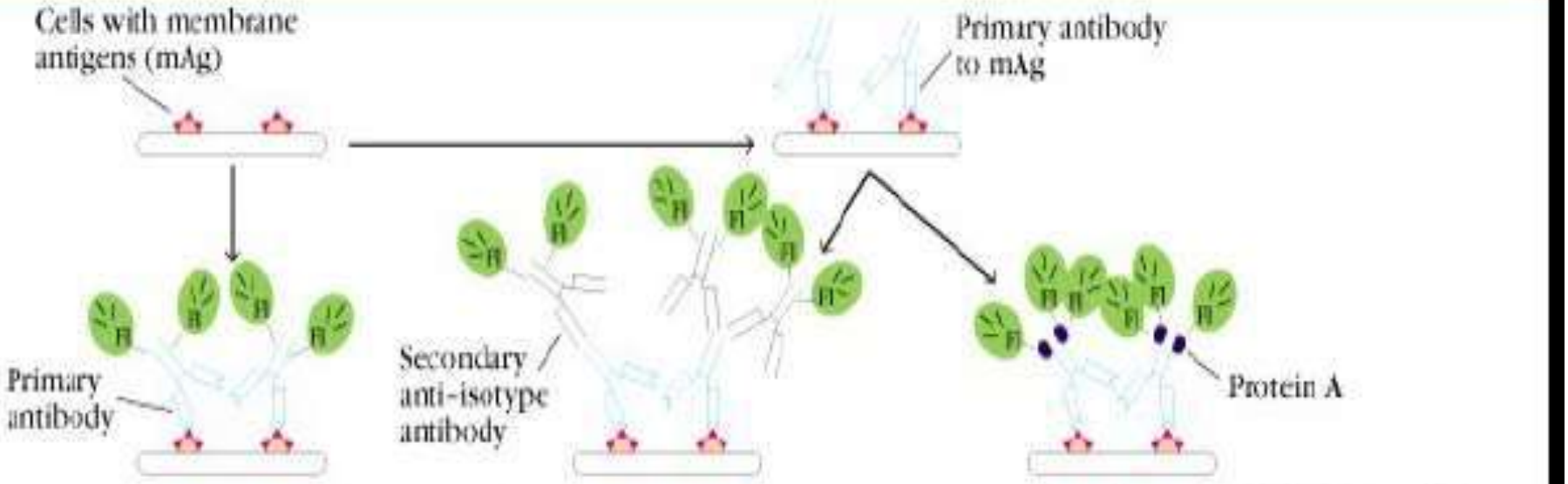
New flurochrome reagents

- Fluorochrome-labeled protein A, which binds to the Fc region of primary IgG antibody molecules
- Fluorochrome-conjugated avidin, which binds to biotin molecule of secondary biotinylated anti-species antibody
- Fluorochrome conjugated anti-c3 antibody, which binds to multiple c3b molecules that binds to Ag-Ab complex due to activation of classical complement pathway following interaction between antigen and antibody.

Biotinylated antibodies



Immunofluorescence



IMMUNOPRECIPITATION

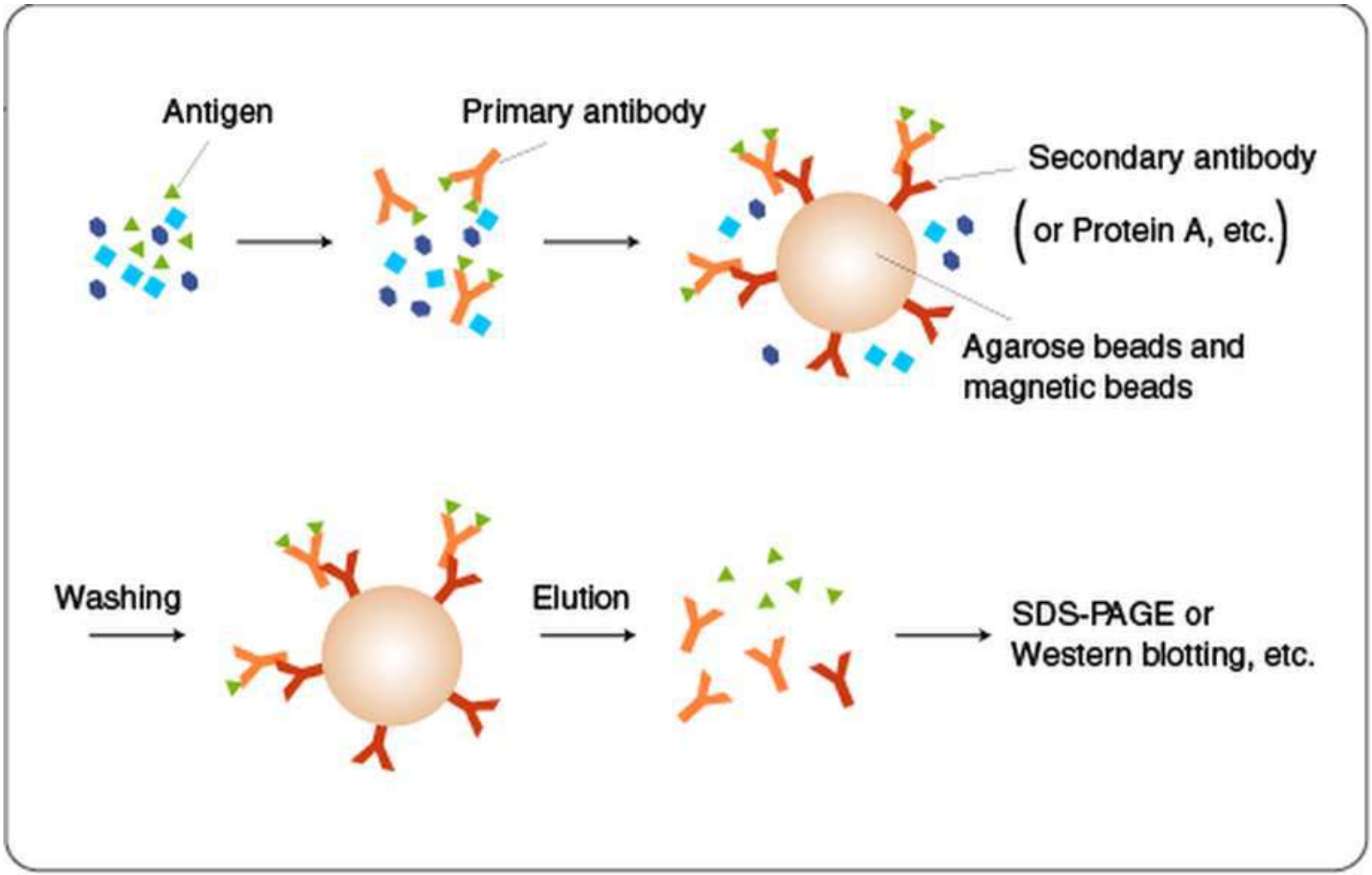
Immunoprecipitation

- Immunoprecipitation (IP) is a method to isolate a specific antigen from a mixture, using the antigen-antibody interaction. Antigens isolated by IP are analyzed by SDS-PAGE or Western blotting.
- It is a modification of traditional precipitation technique and is useful to determine the presence and quantity of the viral antigen, particularly when its concentration is very low in infected tissues or cell cultures.

The principle

- In IP, an antibody is added first to a mixture containing an antigen, and incubated to allow antigen-antibody complexes to form. Subsequently, the antigen-antibody complexes are incubated with an immobilized antibody against the primary antibody (secondary antibody) or with protein A/G-coated beads to allow them to absorb the complexes. The beads are then thoroughly washed, and the antigen is eluted from the beads by an acidic solution or SDS.
- The use of an antibody with high binding specificity and affinity for the antigen is critical for successful IP.

Principle of Immunoprecipitation



Procedure of Immunoprecipitation

The immunoprecipitation procedure can be divided into following steps:

- labeling the antigen (optional)(with a radioactive precursor or avidin);
- lyses of the cells to release the antigen;
- addition of primary antibody and formation of Ag-Ab complexes;
- purification of the immune complex by the addition of co-precipitating agents, such as biotinylated anti-immunoglobulin antibodies, synthetic (agarose) beads coupled to protein A or protein G or supermagnetic beads
- the unbound antigen is removed by washing and the immune complex are then collected by centrifugation.
- SDS-PAGE or Western Blotting

Cell protein extract containing the two interacting proteins of interest in protein complex

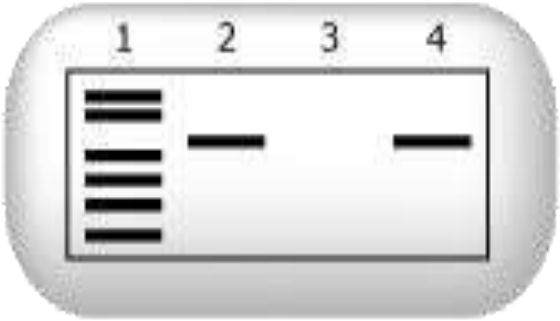
Protein Extraction

Add antibody against one of the protein of interest

Add antibody binding beads

Immunoprecipitate the proteins of interest

Wash and collect the immunoprecititated proteins

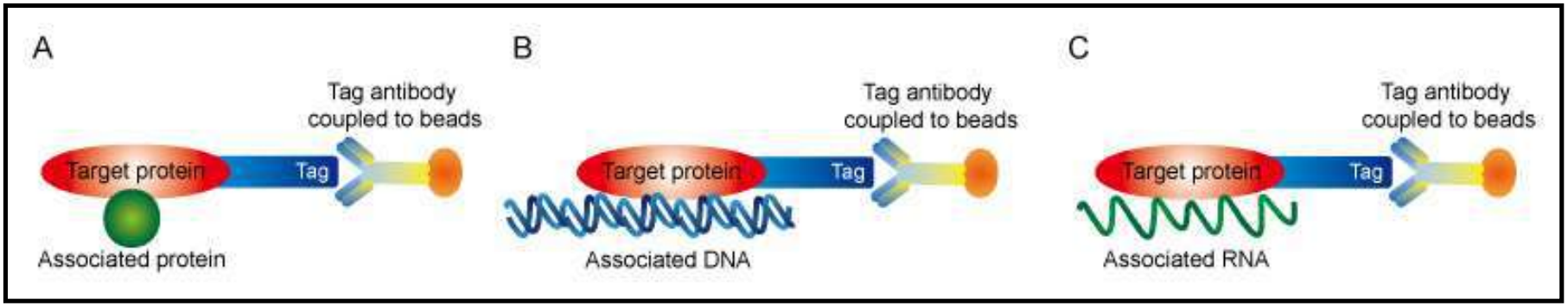


1) Mol. weight marker
2) Extract
3) Supernatant
4) Co-IP

Western blot analysis of the immunoprecipitated proteins using an antibody directed against the second protein of interest

Types of IP

- **Individual protein immunoprecipitation (IP) -** Involves using an antibody that is specific for a known protein to isolate that particular protein out of a solution containing many different proteins.
- **Complex immunoprecipitation (Co-IP) -** Immunoprecipitation of intact protein complexes, i.e. antigen along with any proteins or ligands that are bound to it.
- **Chromatin immunoprecipitation (ChIP) -** an antibody that is specific to a putative DNA binding protein is used to immunoprecipitate the protein–DNA complex out of cellular lysates.
- **RNP Immunoprecipitation (RIP) -** ribonucleoproteins are immunoprecipitated using an antibody targeting the ribonucleoprotein of interest.
- **Tagged-protein IP –** proteins of interest are tagged on either the C- or N- terminal end with an epitope to which a high-affinity antibody is available. The most commonly used tags include FLAG, c-Myc, Hemagglutinin (HA), V5 and Green fluorescent protein (GFP).



Tag strategies for co-IP (A), ChIP (B) and RNP-IP (C)

Advantages and Limitations of IP

Advantages

- IP is ideal for small-scale enrichment of proteins; protein at a very low concentration can be concentrated from the relatively large volume of 1–2 mL.
- It is fast and relatively easy
- antigens are allowed to react with the antibodies in their native conformation prior to their subsequent separation and quantification.
- Co-IP is considered as the golden standard assay for protein-protein interaction.

Limitations

- might not be able to capture low affinity and transient protein interactions.
- limited by the availability of antibodies that recognize the bait protein

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

What is ELISA?

- An immunological technique using an enzyme as a label to detect (assay) presence of a target protein (antigen or antibody).
- A conjugate in ELISA is an enzyme (label) bound to an antibody which bind to target protein
- Can be qualitative or quantitative.
- Very sensitive.
- Commonly used in medicine and scientific research.

Principle of ELISA

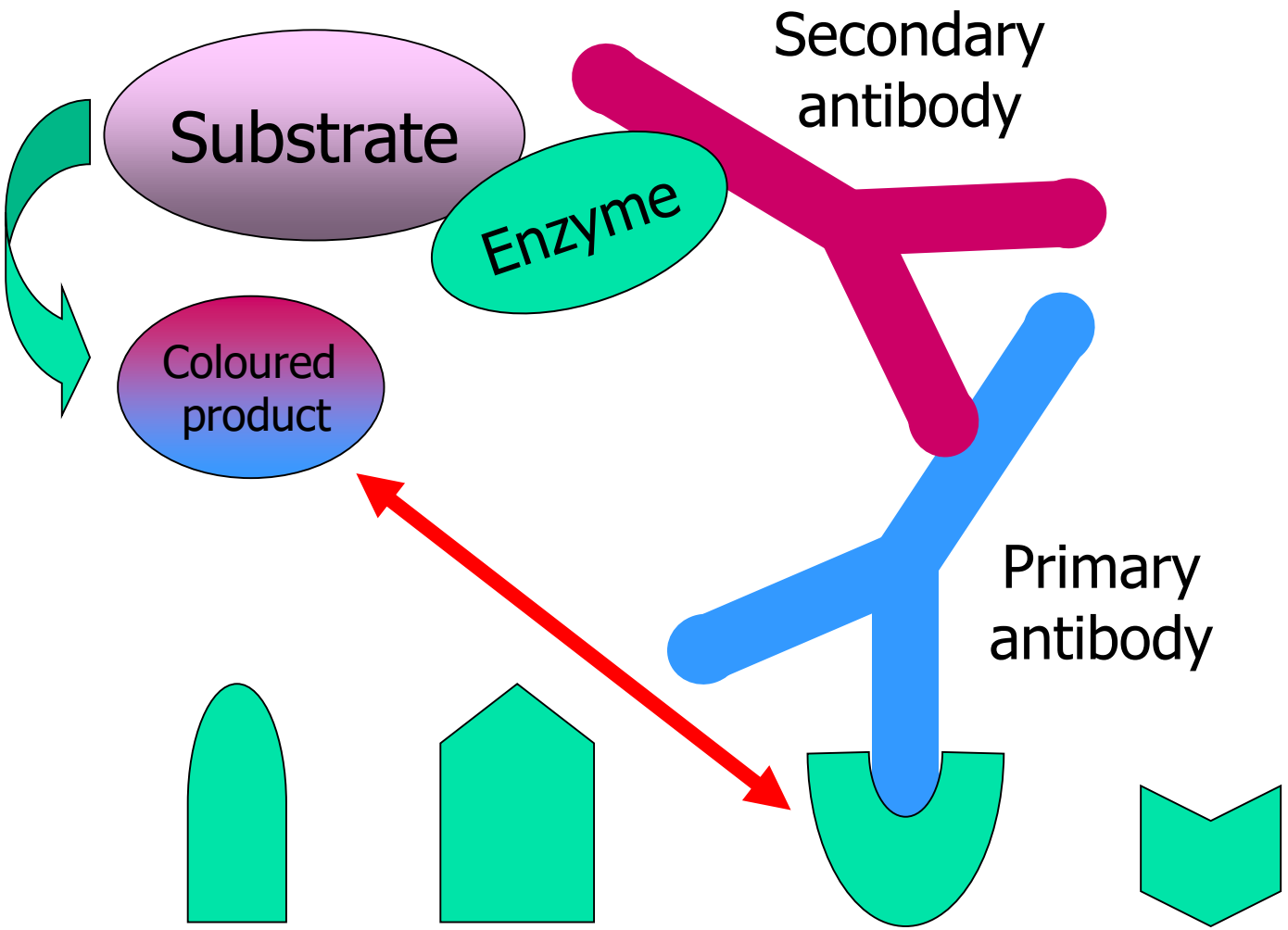
***E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay*

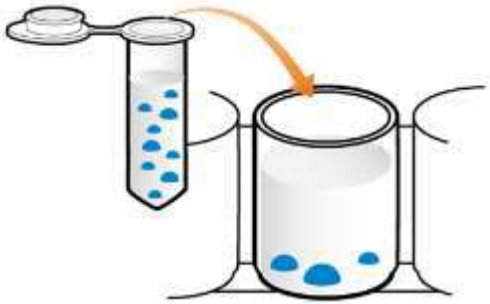
1. Antigen of interest is adsorbed on to plastic surface (‘*sorbent*’).
2. Antigen is recognised by specific antibody (‘*immuno*’).
3. This antibody is recognised by second antibody (‘*immuno*’) which has enzyme attached (‘*enzyme-linked*’).
4. Substrate reacts with enzyme to produce product, usually coloured.

Irrespective of type and format, washing is mandatory after each step of ELISA except after adding substrate

Coloured product
=
measure (*assay*) of antigen present

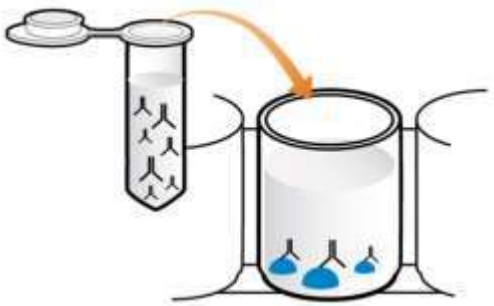
Principle of ELISA





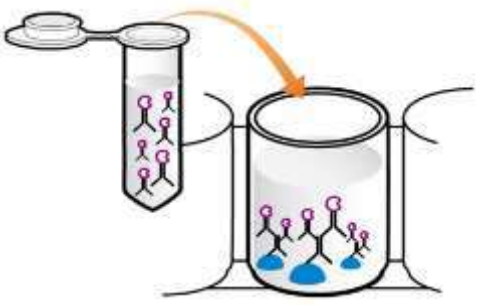
1. Add antigen

2. Wash with PBST



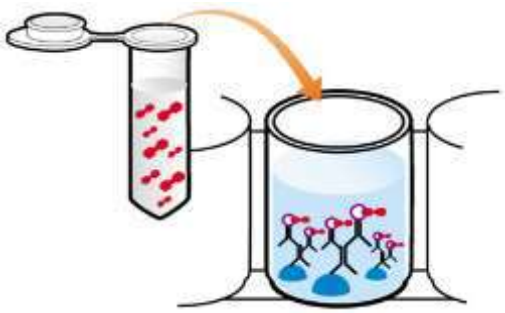
3. Add primary antibody

4. Wash with PBST



5. Add secondary antibody

6. Wash with PBST



7. Add substrate for enzyme

8. Observe colour development

Incubation for a predetermined time after each step is required

Washing after each step is mandatory to remove unbound reactants


Substrate
100 ml
Sample Diluent
250 ml
MicroGeneSys, Inc.
Stopping
CAUTION
100 ml

Enzymes used in ELISA

- React with a colourless substrate to produce a coloured product.
- Must work fast at room temperature so the colour develops quickly.
- Have minimal interference from factors in sample.

Peroxidase from horseradish

Alkaline phosphatase from *E. coli*

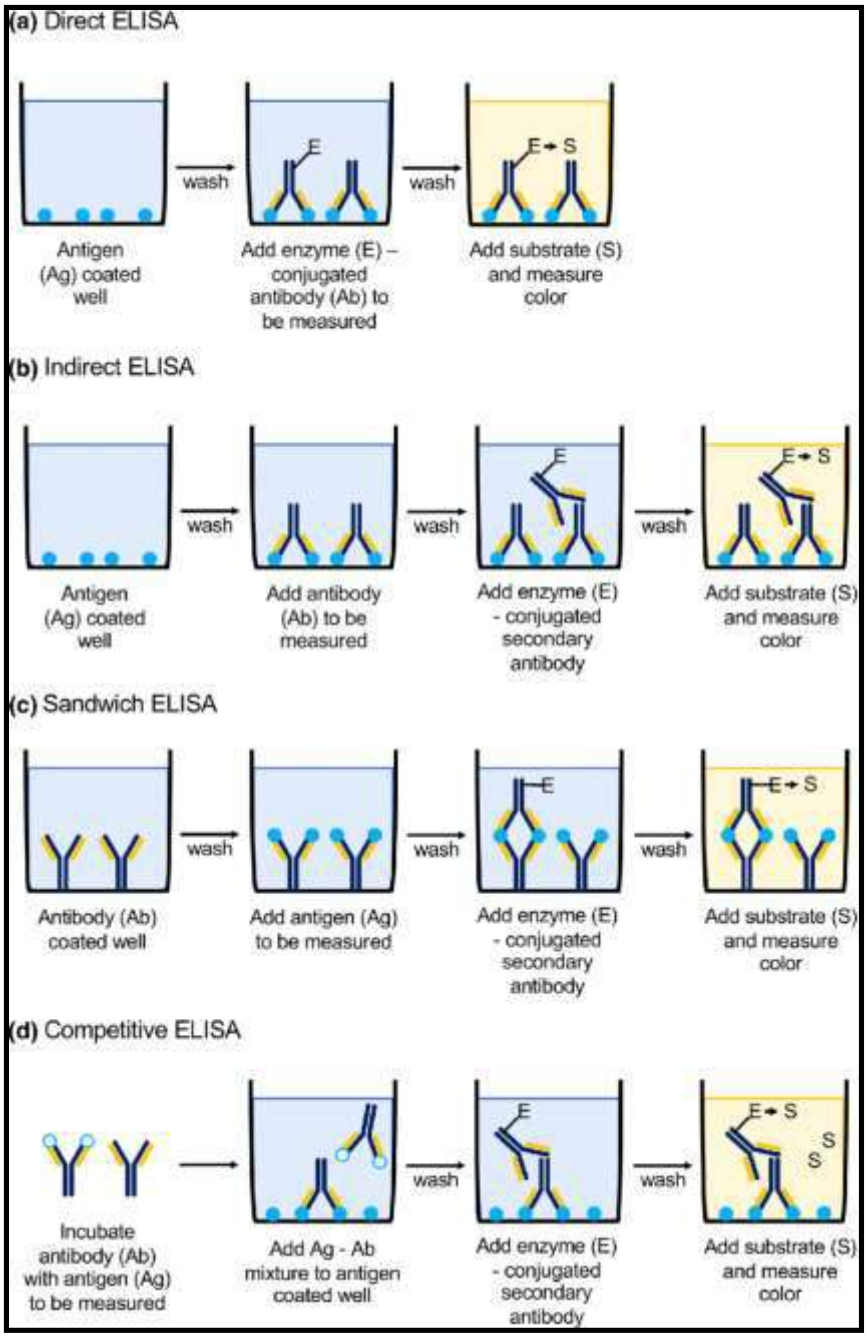
β-galactosidase from *E. coli*

Colorimetric ELISA substrates

Enzyme	Product	Absorbance and Color
Alkaline Phosphatase (AP)	**PNPP** (*p*-Nitrophenyl Phosphate)	405nm Yellow
Horse Radish Peroxidase (HRPO)	**ABTS** (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid])	410nm (650nm) Green
HRPO	**OPD** (*o*-phenylenediamine dihydrochloride)	492 nm Yellow orange
HRPO	**TMB** (3,3',5,5'-tetramethylbenzidine)	450nm (652nm) Yellow
B-galactosidase	**ONPG**	410nm Yellow

Types of ELISA

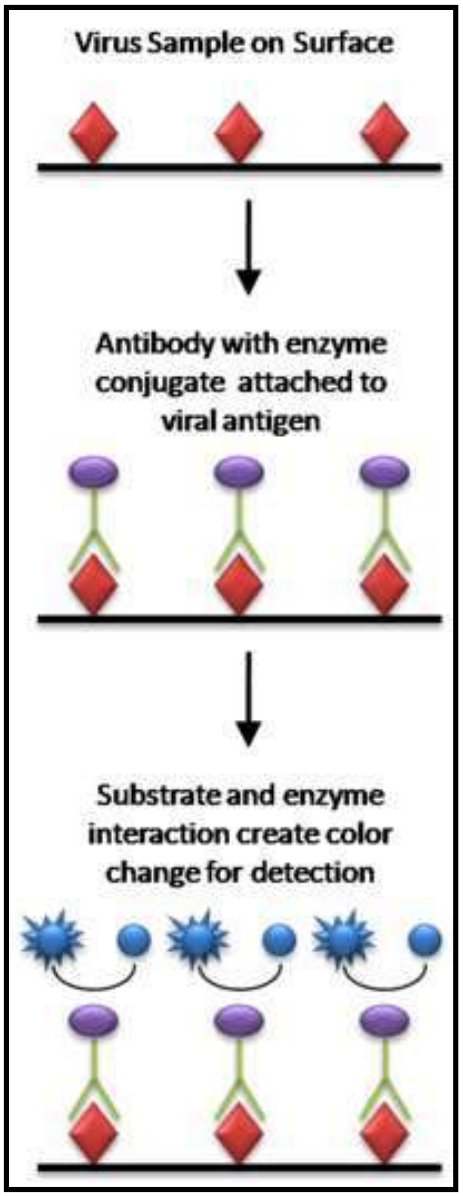
- Direct ELISA
- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA


(a) Direct ELISA
wash
wash
E
E → S
Antigen (Ag) coated well
Add enzyme (E) – conjugated antibody (Ab) to be measured
Add substrate (S) and measure color
(b) Indirect ELISA
wash
wash
wash
E
E → S
Antigen (Ag) coated well
Add antibody (Ab) to be measured
Add enzyme (E) - conjugated secondary antibody
Add substrate (S) and measure color
(c) Sandwich ELISA
wash
wash
wash
E
E → S
Antibody (Ab) coated well
Add antigen (Ag) to be measured
Add enzyme (E) - conjugated secondary antibody
Add substrate (S) and measure color
(d) Competitive ELISA
wash
wash
E
E → S
S
S
Incubate antibody (Ab) with antigen (Ag) to be measured
Add Ag - Ab mixture to antigen coated well
Add enzyme (E) - conjugated secondary antibody
Add substrate (S) and measure color

Direct ELISA

- A direct ELISA is one which the antigen is bound directly to the well of a microplate and the detecting (or binding) antibody is directly conjugated with enzyme.
- The steps are:
  - Coating of ELISA plate with antigen in a buffer, incubated and washed
  - Blocking of non-specific binding sites, incubated and washed
  - Addition of the enzyme conjugated detecting antibody, which binds specifically to the test antigen coating the well, incubated and washed
  - A substrate for this enzyme is then added which changes color upon reaction with the enzyme.
  - After short incubation stop solution is added and the signal is measured on a plate reader.

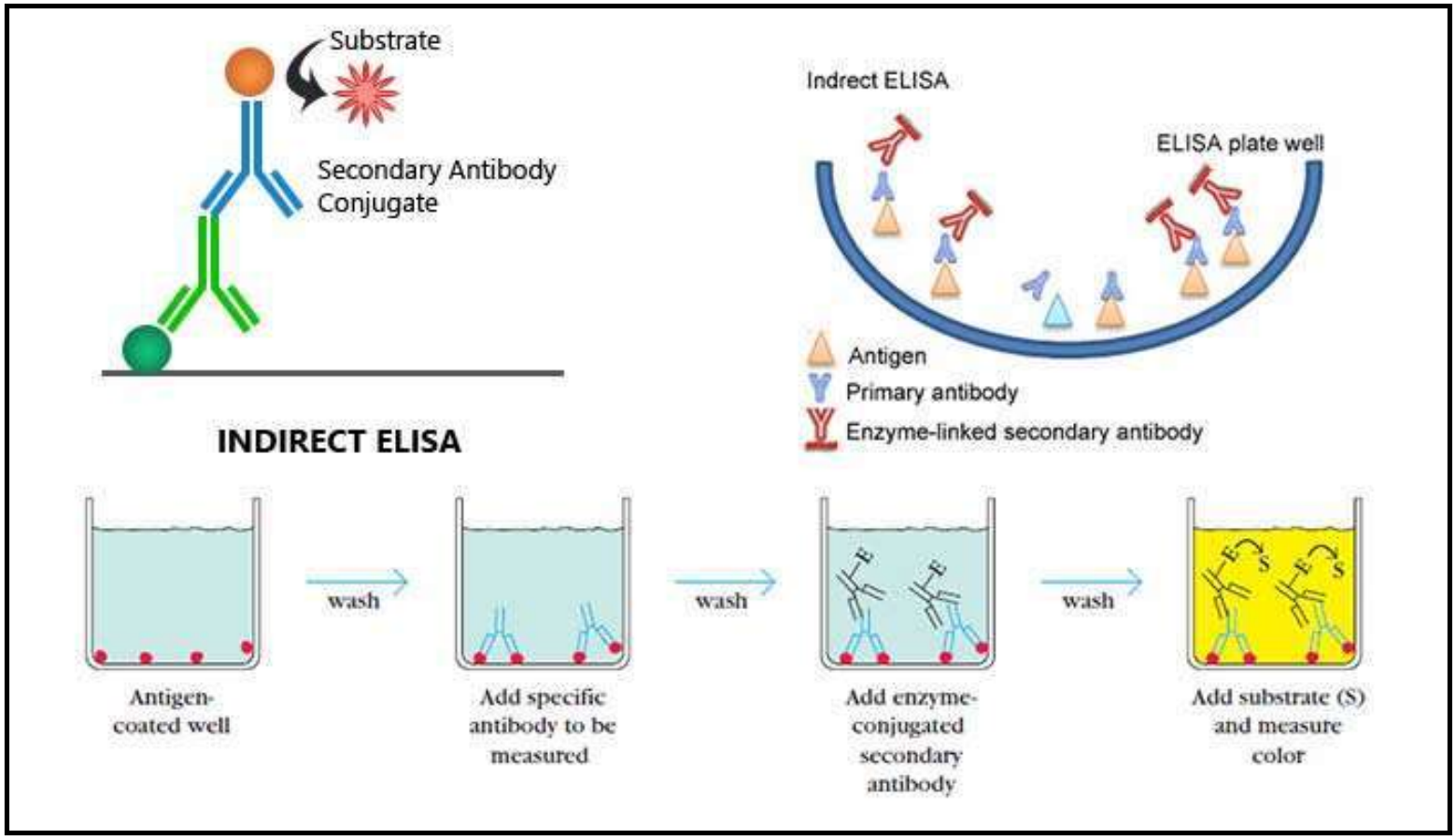
Direct ELISA



Indirect ELISA

- Indirect ELISA is a two-step ELISA which involves two antibodies - primary unlabeled antibody (test sample) and a labeled, secondary antibody. The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody, which is enzyme conjugated anti-species Ig .
- The steps are:
  - Coating of ELISA plate with antigen in a buffer, incubated and washed
  - Blocking of non-specific binding sites , incubated and washed
  - Addition of serum sample containing primary antibody, which binds specifically to the antigen coating the well, incubated and washed
  - Enzyme linked secondary antibody are added, incubated and washed.
  - A substrate for this enzyme is then added which changes color upon reaction with the enzyme.
  - After short incubation stop solution is added and the signal is measured on a plate reader.

Indirect ELISA



Indirect ELISA

- The indirect ELISA is used for the quantitative estimation of antibodies in the serum and other body fluids.

Advantages

- High sensitivity: More than one labeled antibody is bound per antigen molecule;
- Flexible: Different primary antibodies can be used with a single labeled secondary antibody;
- Cost-saving: Fewer labeled antibodies are required.

Disadvantages

- Cross-reactivity might occur with the secondary antibody, resulting in non-specific signal (noise).
- An extra incubation step is required in the procedure.

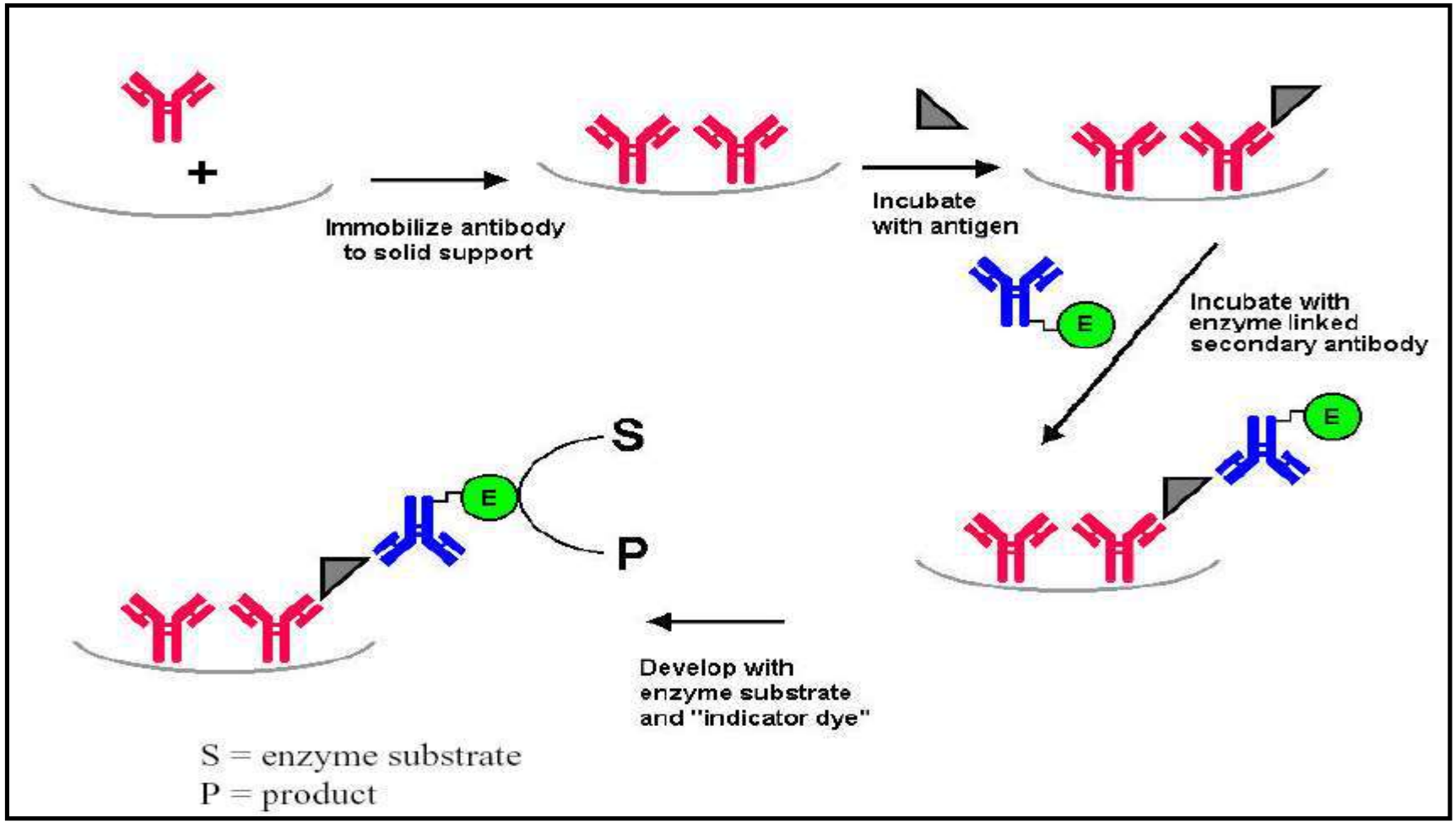
Sandwich ELISA

- The sandwich assay uses two different antibodies (capture and detection) that are reactive with different epitopes on the antigen; it is named so as **antigen is sandwiched between two antibodies.**
- The antigen to be measured must contain at least two different antigenic epitopes.
- Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems; mAbs improves sensitivity and specificity of test
- A polyclonal is often used as the capture antibody and mAb as detection antibody.
- Sandwich ELISA is used as a qualitative and quantitative test for antigen detection.
- A standard curve is drawn with absorbance readings against known concentrations of antigen and the test antigen is quantified by extrapolation.

Sandwich ELISA

- Sandwich ELISA is a two-step test which involves two antibodies – capturing and detection. The capturing antibody is incubated with the antigen followed by the incubation with the detecting antibody, which either itself may be labeled or an anti-speices Ig labeled secondary antibody can be used.
- The steps are:
  - Coating of ELISA plate with capture antibody in a buffer, incubated and washed
  - Blocking of non-specific binding sites by blocking agents, incubated and washed
  - Addition of test antigen sample, which binds specifically to the capture antibodies coating the well, incubated and washed
  - Addition of detecting antibody, incubated and washed
  - A substrate for enzyme is then added which changes color upon reaction with the enzyme.
  - After short incubation stop solution is added and the signal is measured on a plate reader.

Sandwich ELISA



Sandwich ELISA

- The sandwich ELISA is most preferred ELISA format used for the qualitative and quantitative detection of viral pathogens, such as FMDV, BTV, PPRV, etc.

Advantages

- High specificity since two antibodies are used and antigen is specifically captured and detected.
- High sensitivity since two antibodies are used
- Purification of antigen from a complex mixture is not required
- Flexible, since both direct and indirect detection methods can be used.

Disadvantages

- An extra incubation step is required in the procedure.

Comparison of Direct, Indirect and Sandwich ELISA

Direct ELISA detection	
Advantages	• Quick because only one antibody and fewer steps are used. • Cross-reactivity of secondary antibody is eliminated.
Disadvantages	• Immunoreactivity of the primary antibody might be adversely affected by labeling with reporter enzymes or tags. • Labeling primary antibodies for each specific ELISA system is time-consuming and expensive. • Limited number of conjugated primary antibodies available commercially. • No flexibility in choice of primary antibody label from one experiment to another. • Minimal signal amplification.
Indirect ELISA detection	
Advantages	• A wide variety of labeled secondary antibodies are available commercially. • Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection. • Maximum immunoreactivity of the primary antibody is retained because it is not labeled. • Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. • Different detection methods can be used with the same primary antibody (colorimetric, chemiluminescent, etc.).
Disadvantages	• Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal. • An extra incubation step is required in the procedure.
Sandwich ELISA	
Advantages	• Highly sensitive and highly specific for target antigen as two antibodies are used for capture and detection. • Different detection methods can be used with the same capture antibody.
Disadvantages	• Requires more optimization to identify antibody pairs and to ensure there is limited cross-reactivity between the capture and detection antibodies.

Competitive ELISA (c-ELISA)

- A competitive ELISA, also known as an **inhibition or blocking** ELISA, measures the amount of analyte in a sample by **quantification of its interference with an expected signal.**
- The signal inversely correlates with the amount of analyte such that, if the concentration of the target analyte is high, then the reference signal is diminished through its competitive binding to a limited amount of labeled antibody.
- Less is more - more target analyte in your sample will mean more antibody competed away, which will lead to less signal and vice-versa.
- The test can be used to measure the concentration of an antigen or antibody in a sample.
- The ELISA currently being used for assay of vaccine-induced seroconversion in FMD control programme is an example of c-ELISA.

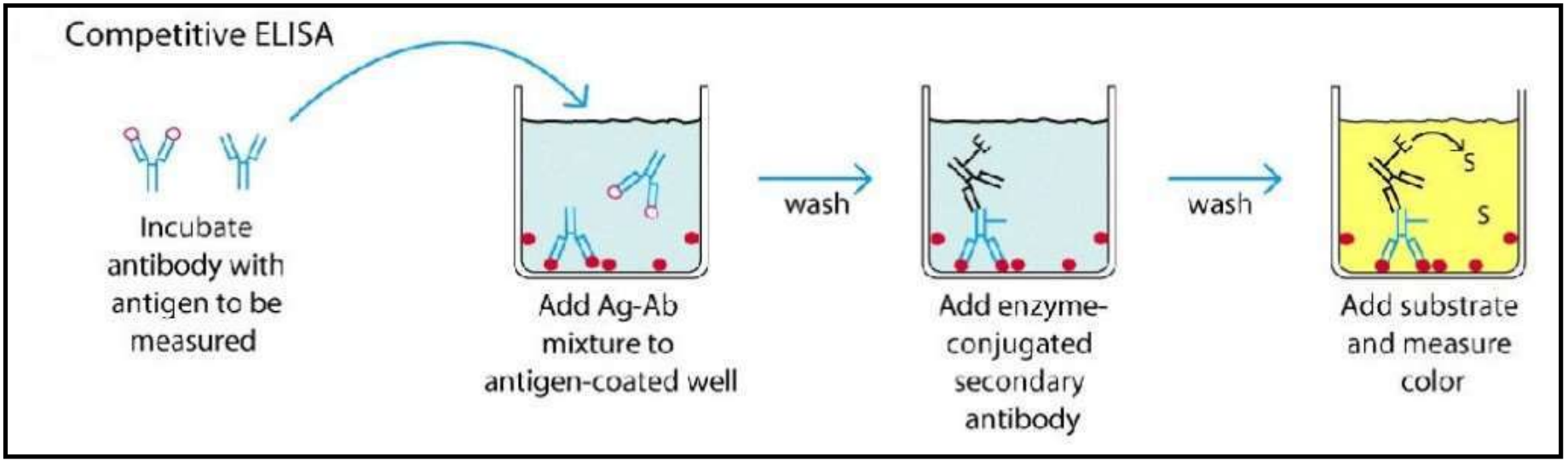
Competitive ELISA for Antigen - Principle

- A sample containing unknown antigen (test antigen) is first incubated with antibodies specific for reference antigen in a solution (liquid phase); the two combine to form immune complexes, if specific for each other.
- The antigen-antibody mixture is then added to the ELISA plate which has wells pre-coated with known antigen (reference antigen).
- After washing, enzyme conjugated secondary antibody specific for isotype of the primary antibody is added followed by color development
- If both antigens are homologous then less free antibody will be available to bind to the reference antigen coated on well (hence **competition**)
- Thus the higher the concentration of antigen in the sample, the lower the absorbance.

Competitive ELISA for Antibody - Principle

- Principle of the test is that two specific antibodies for same antigen, one conjugated with enzyme and the other present in test serum (unlabeled), are used.
- A sample containing unknown antibodies (test serum) is first incubated with a known antigen in a solution (liquid phase); the two, if specific, combine to form immune complexes.
- The antigen-antibody mixture is then added to the ELISA plate which has wells pre-coated with same antigen.
- After washing, enzyme conjugated antibody specific for antigen is added followed by color development.
- Competition occurs between the two antibodies for the same antigen.
- Appearance of color indicates a negative test (absence of antibodies), while the absence of color indicates a positive test (presence of antibodies); the color being **blocked**

Competitive/Blocking ELISA



Competitive ELISA

Advantages

- High specificity, since two antibodies are used and the antigen/analyte is specifically captured and detected
- Suitable for complex samples, since the antigen does not require purification prior to measurement
- High sensitivity since two antibodies are used
- Flexible, since both direct and indirect detection methods can be used.

Disadvantages

- An extra incubation step is required in the procedure.

Modifications of ELISA

- Dot ELISA
- Avidin-Biotin ELISA (AB-ELISA)
- Multiplex ELISA
- ELISPOT/FLUOROSPOT Assay
- Chemiluminescence Immunoassay

Dot ELISA

- Dot ELISA is a type of solid phase micro enzyme-linked immunosorbent assay utilizing antigen dotted onto nitrocellulose filter discs.
- It is a rapid immunochemical test which is extensively used as an immunological tool in research as well as analytical/diagnostic laboratories
- The most significant feature of dot-ELISA is the precipitation of the chromogenic substrate only into the area with the enzymatic activity, hence the name **Dot-ELISA**
- The enzyme activity is indicated by intensity of spot, which is directly proportional to the antigen concentration.
- Dot ELISA protocols usually are of sandwich ELISA

Dot ELISA

- In sandwich Dot-ELISA, the antigen is sandwiched directly between two antibodies which react with two different epitopes on the same antigen.
- Here the capturing antibody is immobilized onto a solid support, which is a nitrocellulose membrane and the second (detecting) antibody is linked to an enzyme.
- Antigen in the test sample first reacts with the immobilized antibody and then with the second enzyme-linked antibody.
- The amount of enzyme linked antibody bound is assayed by incubating the strip with an appropriate chromogenic substrate, which is converted to a coloured, insoluble product.

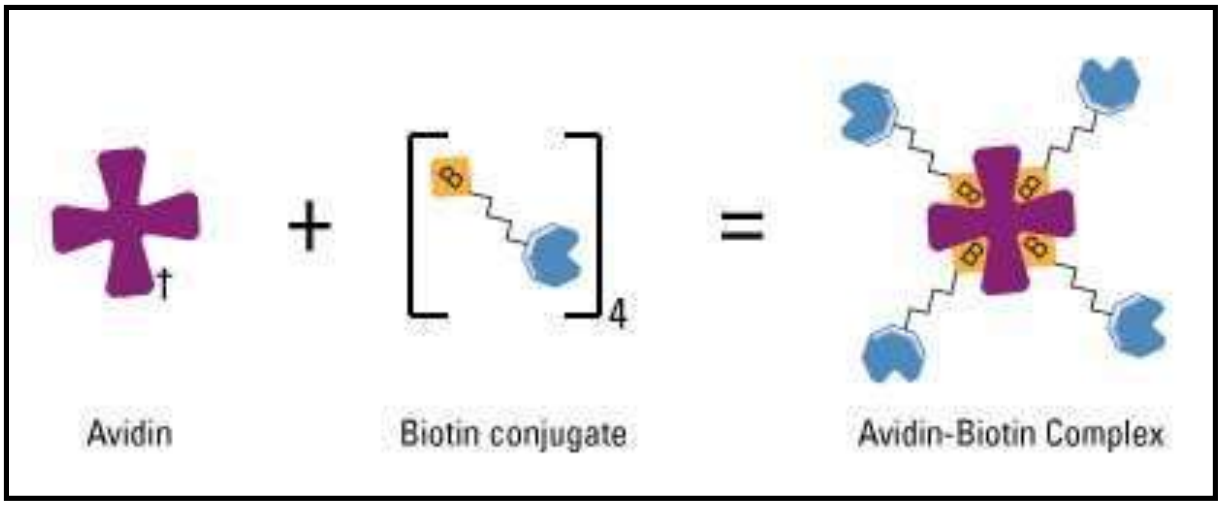
ELISA VERSUS DOT ELISA

ELISA	DOT ELISA
A rapid immunochemical test which involves an enzyme used for measuring a wide variety of tests of body fluids	A solid-phase immunoassay that can be used to detect either antigen or antibody
Three types are direct ELISA, indirect ELISA, and sandwich ELISA	A type of sandwich ELISA
Involves detecting a specific protein with the help of an antibody and detecting bound antibodies through an enzymatic reaction	Chromogenic substrate only binds to the area with enzymatic activity
Mainly uses microtiter plates as the solid phase	Uses nitrocellulose membranes as the solid phase

Avidin Biotin ELISA (AB-ELISA)

- Avidin is a protein derived from both avians (whites of eggs) and amphibians that shows a high affinity for biotin.
- Biotin is a small vitamin molecule (Vitamin H, Vitamin B7, Coenzyme R) that is easily modified so that it can be chemically attached to proteins, antibodies and other biomolecular probes of interest.
- Streptavidin is another proteins that originate from different source but bind very strongly and specifically to the biotin molecule
- The avidin-biotin affinity system is frequently employed in the design of tagging and detecting systems for ELISA
- In AB system the antibodies are biotinylated and avidin/streptavidin is conjugated with enzymes.
- Avidin/streptavidin proteins are tetrameric and have four biotin-binding sites per molecule.

Avidin-biotin interaction



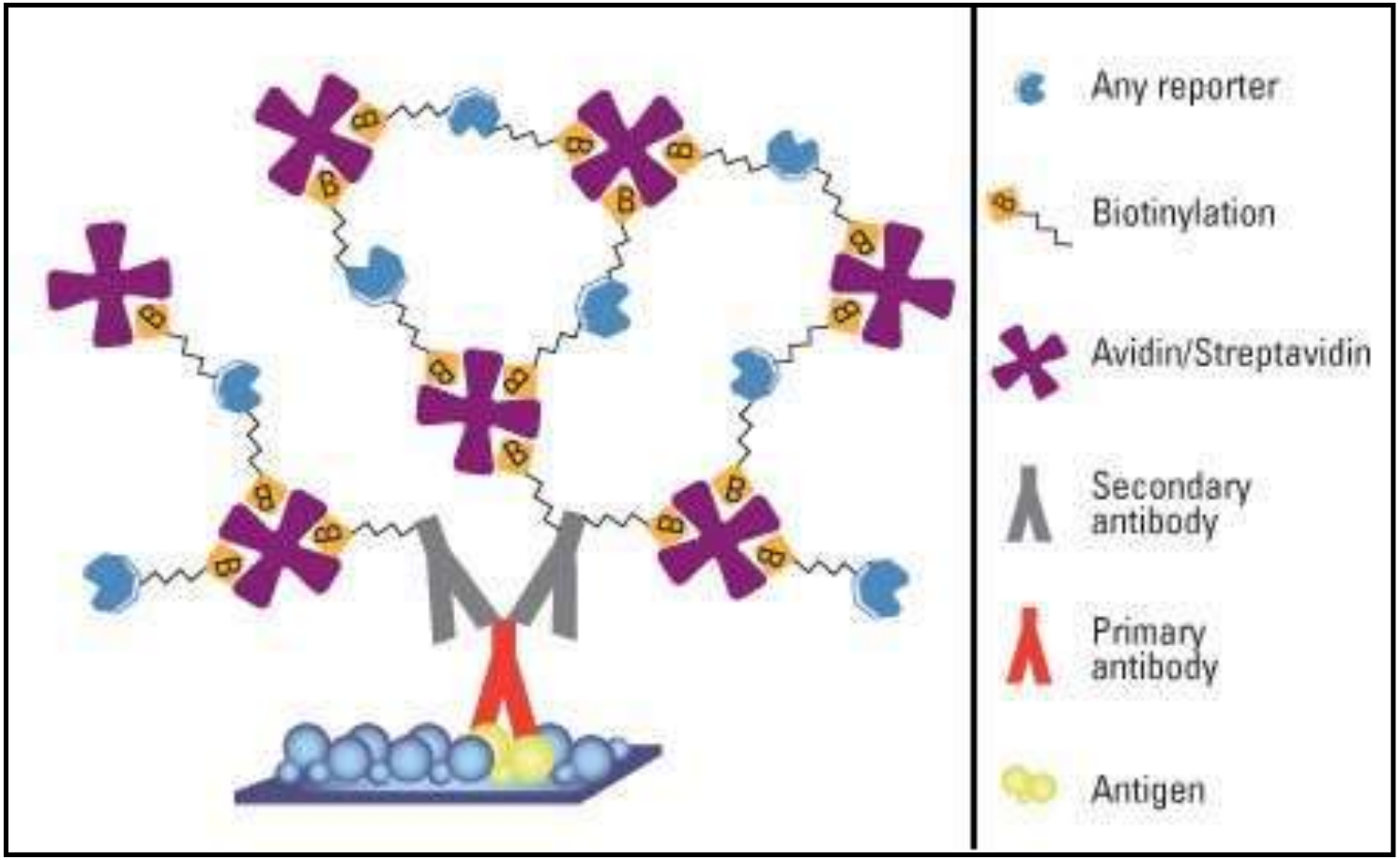
† denotes that Avidin is also often conjugated to an antibody, target protein or immobilized support

Avidin, Streptavidin or NeutrAvidin proteins can bind up to four biotin molecules, which are normally conjugated to an enzyme or antibody to form an Avidin-biotin complex.

Avidin Biotin ELISA (AB-ELISA)

- The avidin-biotin chemistry results in amplification of signal in ELISA in indirect detection. There are two approaches:
  - The first method involves using a biotinylated detection antibody, which is probed using avidin or streptavidin protein conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes.
  - The second approach also employs a biotinylated detection antibody, but it is probed with a pre-incubated mixture of avidin and biotinylated enzyme, a process known as “avidin-biotin complex” (ABC) signal amplification.
- Signal amplification occurs through two mechanisms:
  - First, biotinylation results in multiple biotin tags per antibody molecule, thus allowing more than one streptavidin molecule to bind to each antibody.
  - Second, the process of either labeling the streptavidin molecule with enzymes or using a pre-incubated mixture of streptavidin plus biotinylated enzyme results in conjugates having more than one enzyme.
- The combined effect of this multiple labeling is to increase the number of enzyme molecules in the final immune complex. This increases the catalysis of appropriate substrate and gives a stronger signal compared to a conventional enzyme-labeled secondary antibody.

Signal Amplification by AB-ELISA



Avidin Biotin ELISA (AB-ELISA)

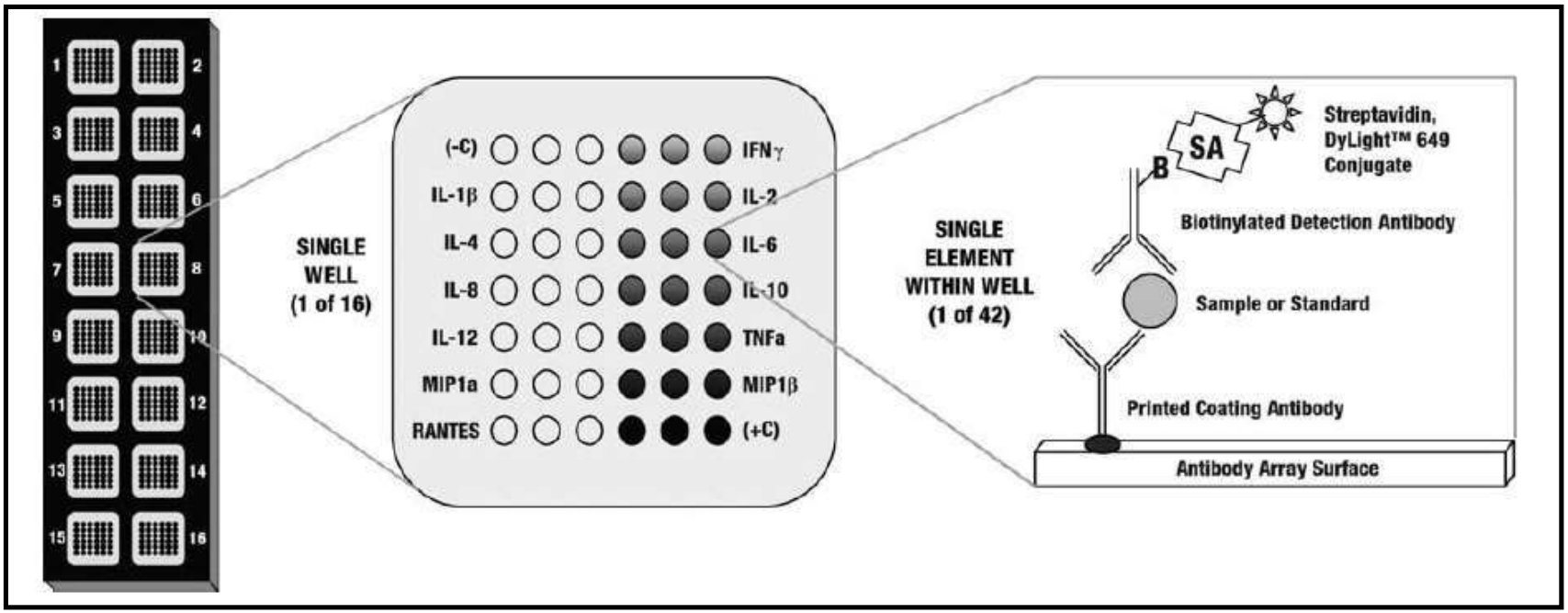
Advantages of using Avidin-biotin systems

- The Avidin-biotin complex is the strongest known non-covalent interaction ($K_d = 10^{-15}M$) between a protein and ligand.
- The bond formation between Avidin and biotin is very rapid, and once formed, is unaffected by extremes of pH, temperature, organic solvents and other denaturing agents.
- These features of biotin and Avidin are useful for detecting proteins conjugated to either component of the interaction without affecting their structure
- The Avidin-biotin systems amplify the original protein signal to improve detection of proteins expressed at low levels by forming large Avidin-biotin complexes and thus are highly sensitive.

Multiplex ELISA

- A multiplex ELISA enables a researcher to assess the levels of more than one protein target at a time.
- It is commonly used to determine the expression of multiple cytokines simultaneously.
- The multiplex ELISA methods utilizes one or more of the following techniques: flow cytometry, fluorescence, chemiluminescence, or electrochemiluminescence.
- Flow cytometric analysis is the most commonly employed multiplex ELISA method and is performed using bead-conjugated antibodies.
- Typically, a greater number of cytokines can be detected and/or measured at a time by using fluorophores, flow cytometry and bead-conjugated antibodies, compared to other methods.
- In fluorescence assays, the detection antibody is either labeled directly or the secondary antibody (or occasionally avidin) is labeled for indirect detection

A multiplex array ELISA using fluorescence



ELISPOT

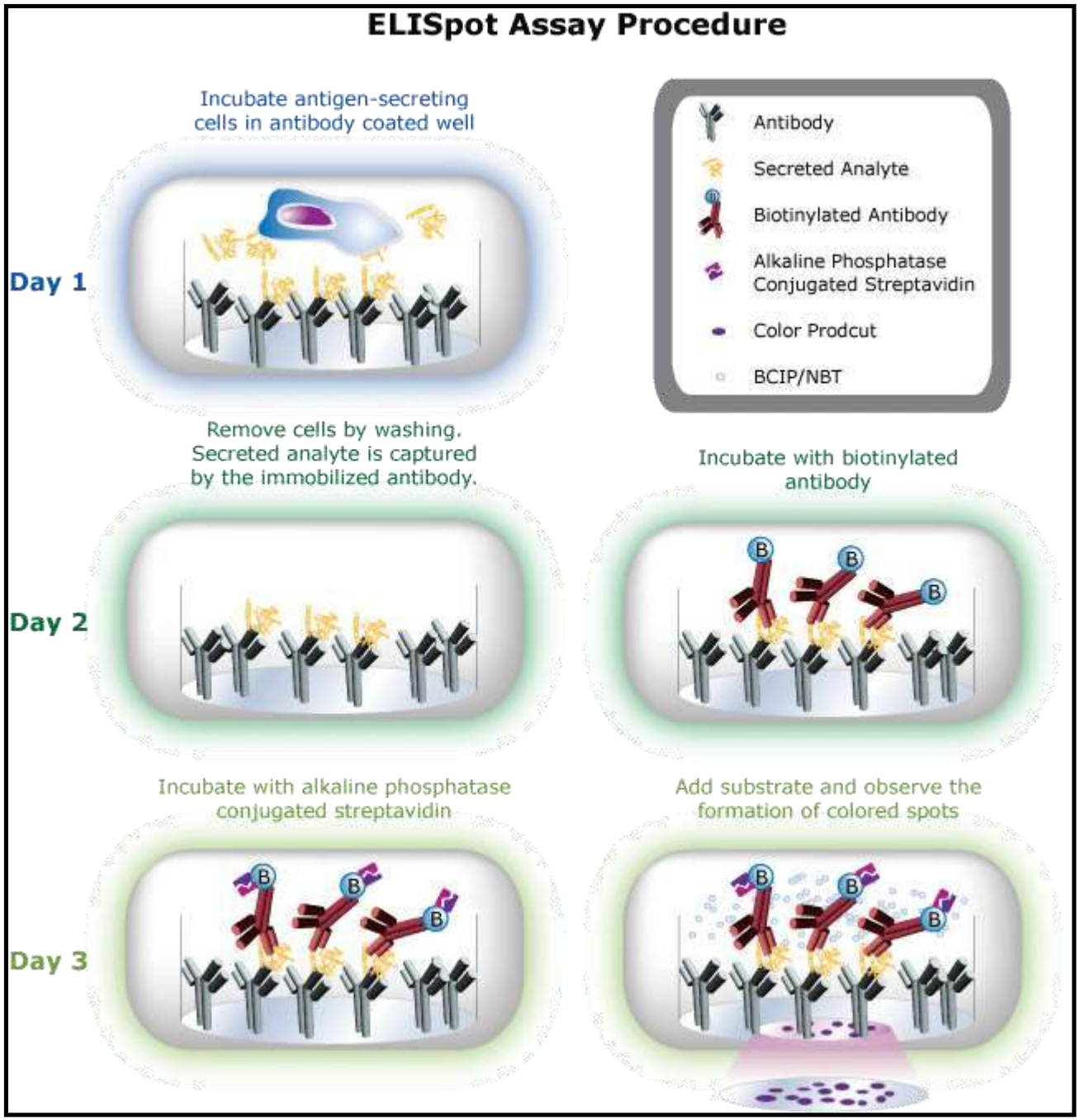
- ELISPOT assay is based on the enzyme-linked immunosorbent technique designed to enumerate cytokine-secreting cells
- It is both a quantitative and qualitative assay, is extremely sensitive (can detect 1/300 000 cytokine-secreting cells -).
- The cytokine released in response to antigen can be mapped to a single cell cells hence T cell responder frequencies can be calculated.
- Cells can be stimulated either in the anti-cytokine antibody coated plate (direct assay) or pre-stimulated and then transferred to the pre-coated plate (indirect assay);
- Once the assay is complete, the plates can be analyzed on a plate reader.
- The main application of the ELISPOT assay is in monitoring of immune responses in both humans and animals

ELISPOT

The steps are:

- cells are cultured on a surface coated with a specific capture antibody in the presence or absence of stimuli.
- Proteins, such as cytokines, that are secreted by the cells are captured by the specific antibodies on the surface.
- After an appropriate incubation time, cells are removed and the secreted molecule is detected using a detection antibody in a similar procedure to that employed by the ELISA.
- The detection antibody is either biotinylated and followed by a streptavidin-enzyme conjugate or the antibody is directly conjugated to an enzyme.
- By using a substrate with a precipitating rather than a soluble product, the end result is visible spots on the surface. Each spot corresponds to an individual cytokine-secreting cell.

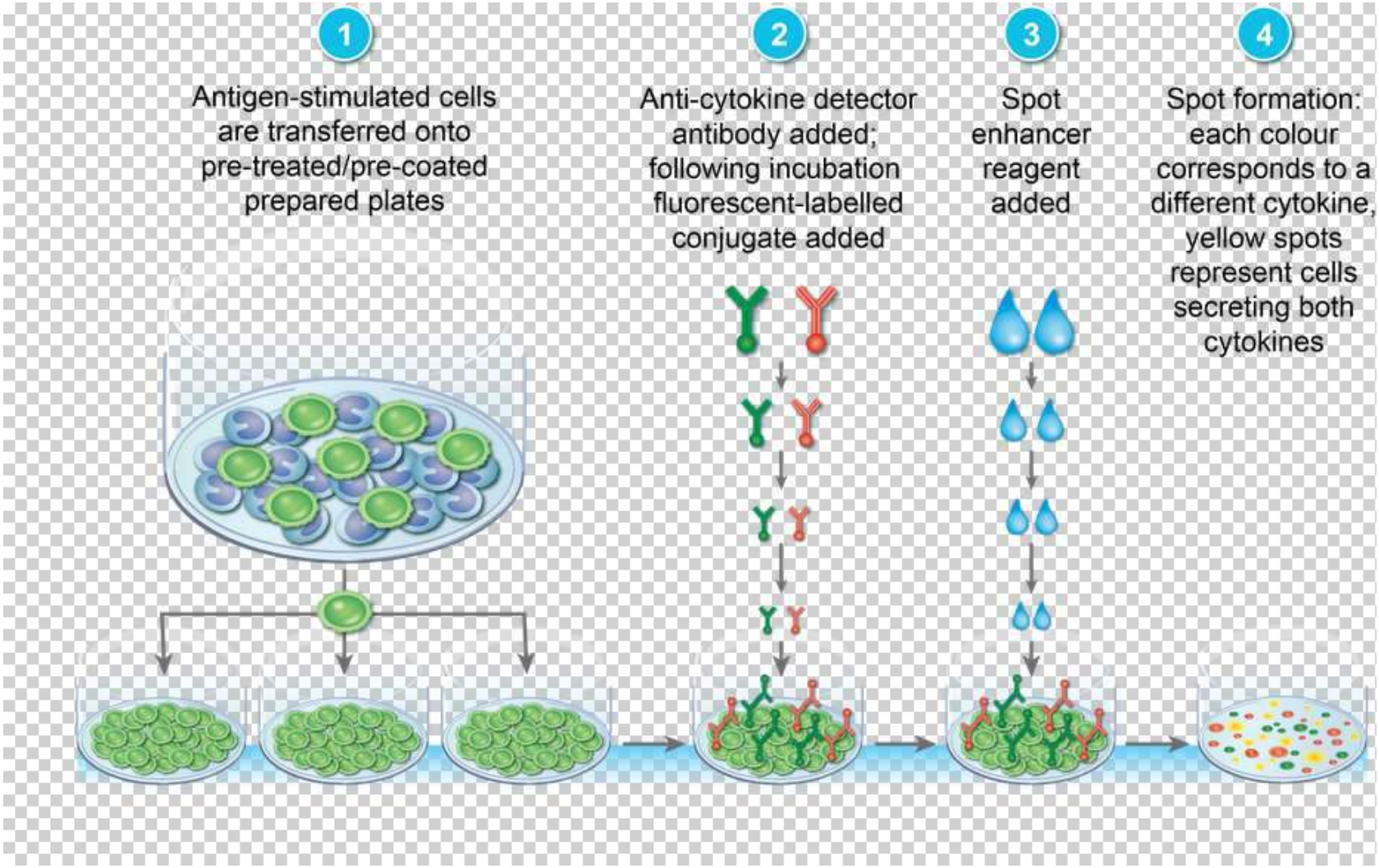
ELISpot Assay Procedure



ELISPOT

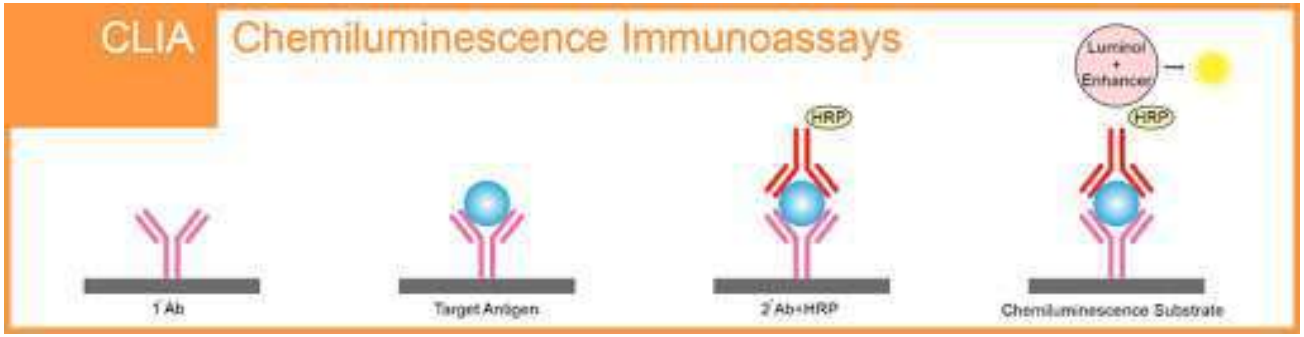
- More recently, the assay has been adapted to a FluoroSpot assay which utilizes fluorochrome-conjugated detection antibodies thereby allowing the simultaneous detection of multiple distinct cytokines and subsequent T cell sub-population analysis .
- The cytokine ELISPOT has been successfully used across several disciplines of Immunology, including organ-transplantation, cancer research, infectious diseases, vaccine development and autoimmune diseases.
- Both ELISPOT and FluoroSpot assays are widely used in immuno-monitoring of clinical trials where both quantitative information and T cell phenotype identification at a single cell level is highly informative, e.g in SARS CoV-2 immunology studies

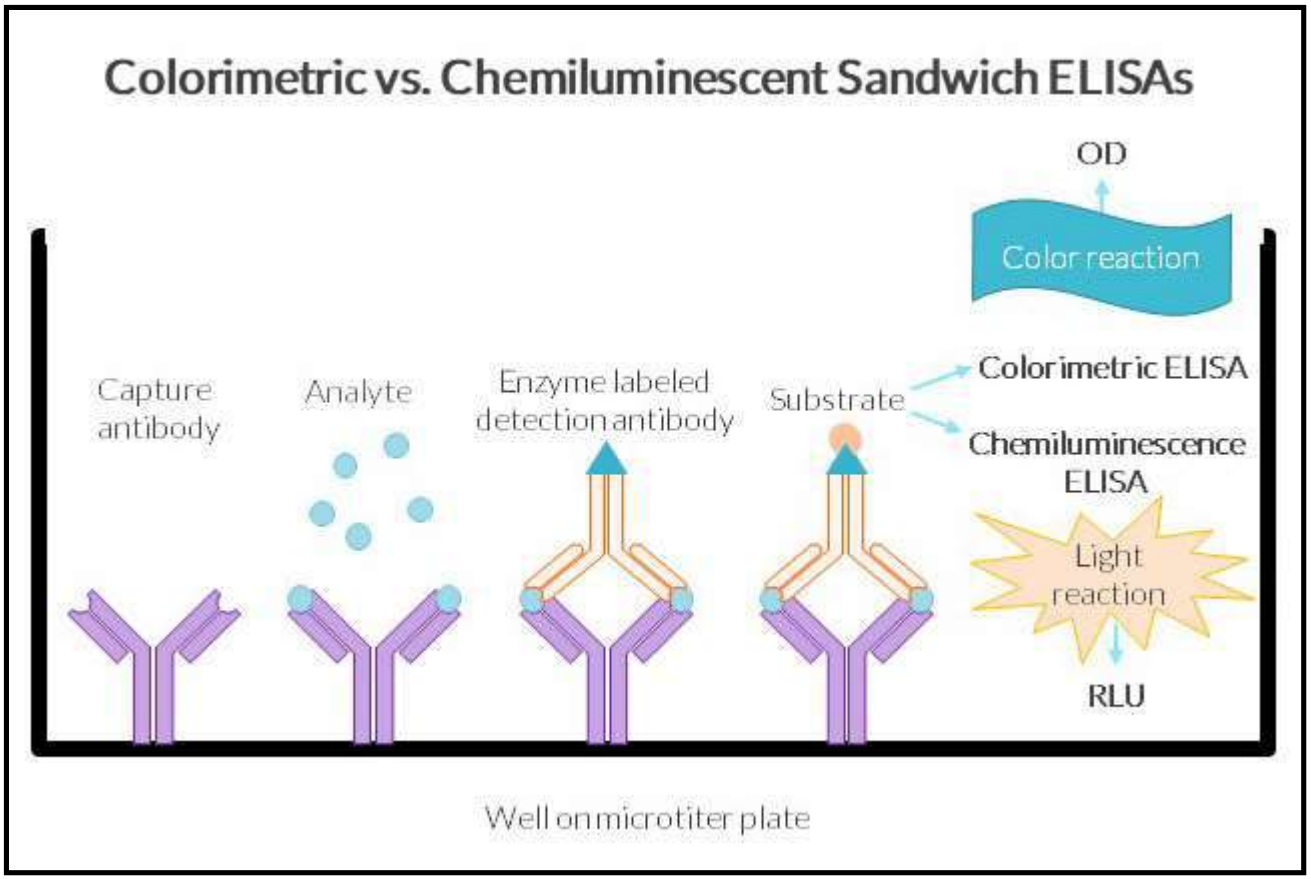
FLUOROSPOT ASSAY



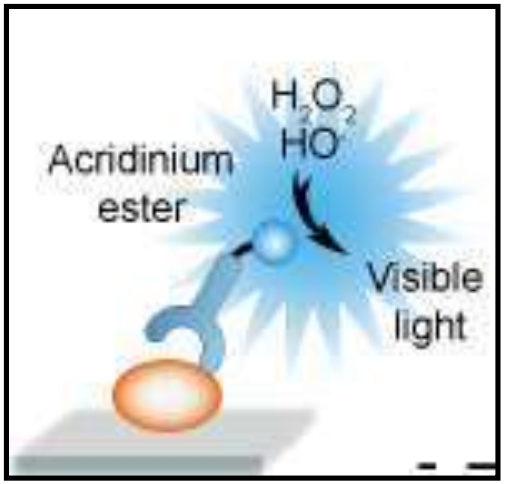
Chemiluminescence Immunoassay (CLIA)

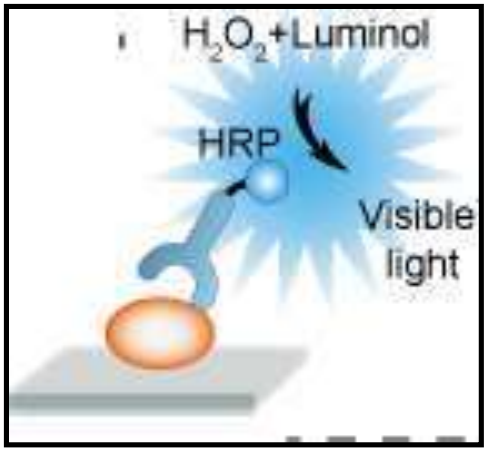
- CL is defined as the emission of electromagnetic radiation caused by a chemical reaction to produce light.
- CLIA is an assay that combine chemiluminescence technique with immunochemical reactions. It utilize chemical probes which could generate light emission through chemical reaction to label the Ab
- CLIA has high sensitivity, good specificity, wide range of applications, simple equipment and wide linear range.
- CLIA have three different label systems according to the difference of physical chemistry mechanism of the light emission:
  - **Label chemical directly involved in the light emission reaction -** chemical that can transfer to an excited state through chemical reaction, e.g. acridinium ester
  - **Enzyme catalyzed light emission reaction -** utilizes enzymes to label antibody, but the substrate is a luminescent chemical instead of chromogen, e.g. Luminol instead of OPD for HRPO enzyme
  - **Redox Reaction Mediated Light Emission Reaction -** utilizes ruthenium tris-bipyridine (bpy) as label which by electrochemical reaction generates an excited state of $Ru(bpy)_3^{2+}$

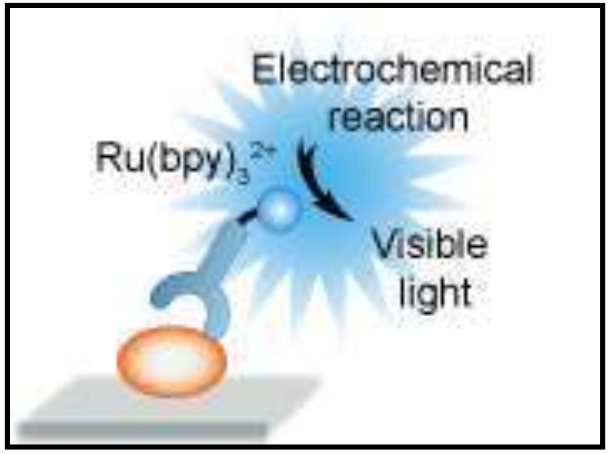

CLIA
Chemiluminescence Immunoassays
Luminol + Enhancer
HRP
HRP
1 Ab
Target Antigen
2 Ab+HRP
Chemiluminescence Substrate


Colorimetric vs. Chemiluminescent Sandwich ELISAs
OD
Color reaction
Capture antibody
Analyte
Enzyme labeled detection antibody
Substrate
Colorimetric ELISA
Chemiluminescence ELISA
Light reaction
RLU
Well on microtiter plate

CLIA Labels


H_2O_2
HO
Acridinium ester
Visible light


H_2O_2+Luminol
HRP
Visible light

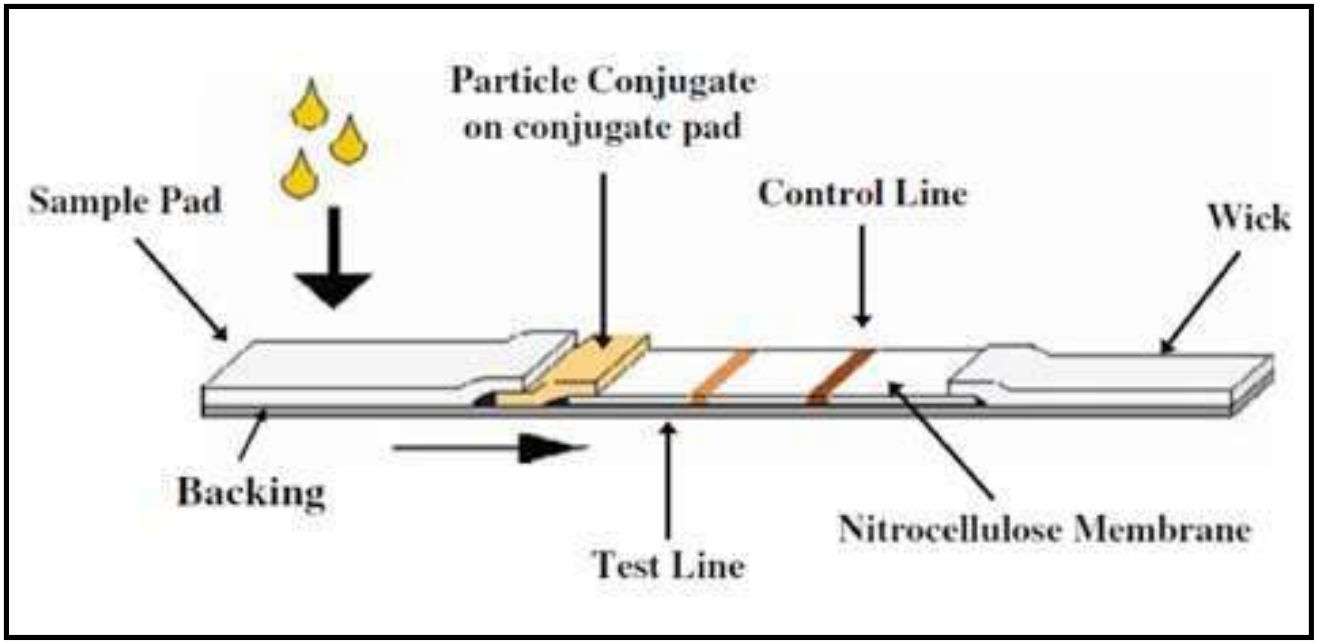

Electrochemical reaction
$Ru(bpy)_3^{2+}$
Visible light

IMMUNOCHROMATOGRAPHY ASSAY (ICA)
OR
LATERAL FLOW ASSAYS (LFA)

IMMUNOCHROMATOGRAPHY ASSAY

- Immunochromatography assay (ICA), namely lateral flow test, is a simple device intended to detect the presence or absence of the target analyte (antigen or antibody)
- The concept of immune-chromatography is a combination of chromatography (separation of components of a sample based on differences in their movement through a sorbent) and immunochemical reactions, which can be recognized according to the change of colors.
- Assays using this format are rapid, taking approximately 15 min to run and are also simple to use, requiring only the dilution of the test agent in a sample buffer and applying several drops (~200µl) to the test strip.
- Strips used for ICA contain four main components - sample application pad (cellulose and/or glass fiber), conjugate pad (where labeled antibodies are dispensed), substrate(Nitrocellulose) membrane, and adsorbent pad (works as sink at the end of the strip)

Typical layout of a lateral flow test strip.



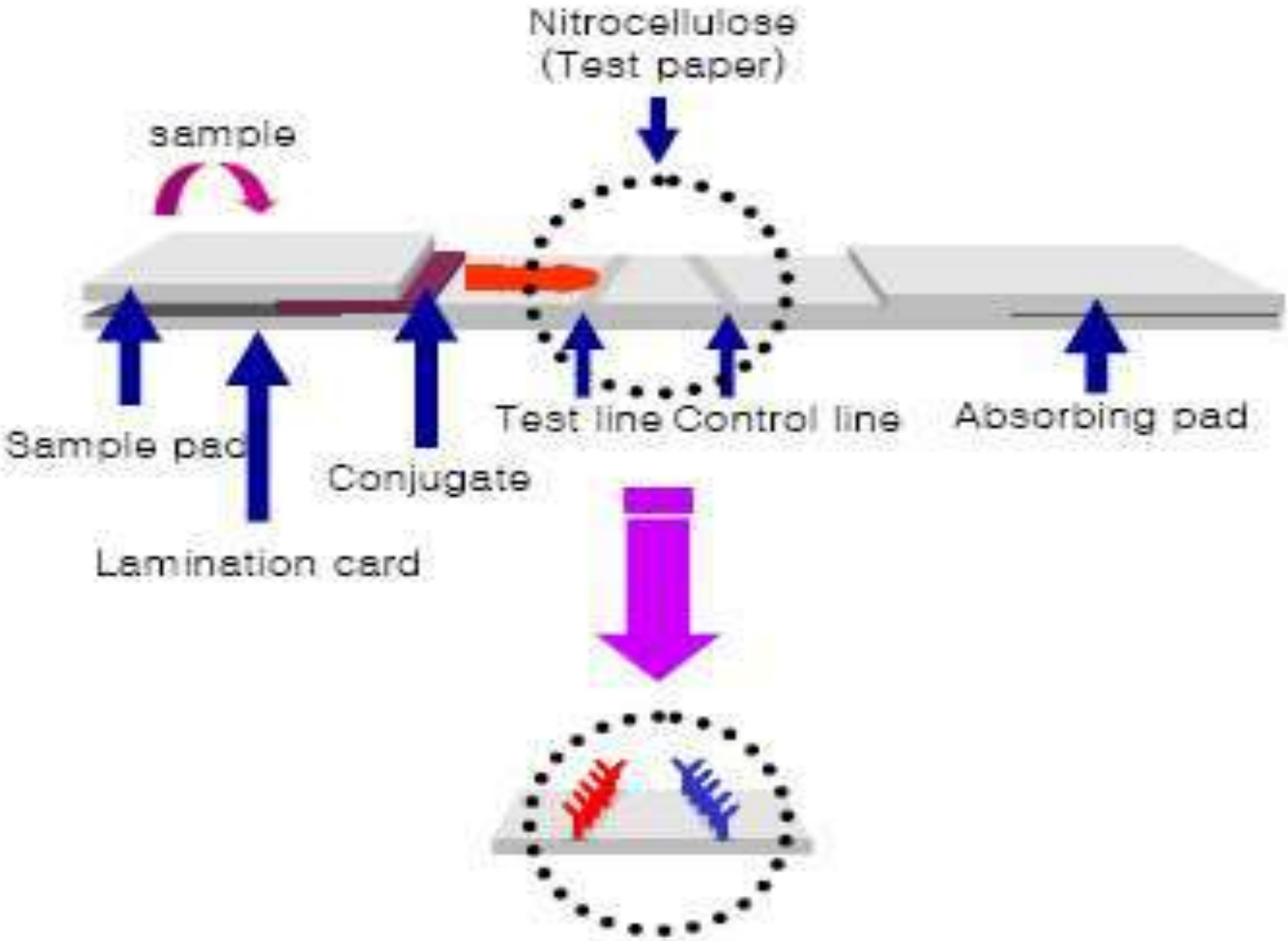
Principle of ICA

- Typical handheld assay devices contain a colloidal gold (or other) labeled antibody dried onto a filter pad affixed to a nitrocellulose strip.
- A capture antibody is applied in a line on the strip and dried.
- To perform the test, a specimen is suspended in buffer and added to the pad containing the colloidal gold labeled antibody.
- The antibody specifically binds to antigen present in the specimen, and the resulting complex wicks down the membrane where it binds to the capture antibody.
- A positive reaction is visualized as a red line created by the bound colloidal gold
- Lateral flow immunoassays are choice of test for point-of-care tests (POCT) or field use applications.

Steps in ICA

Major steps in ICA are:

- Preparation of labeled antibody and capture antibody against target analyte
- Immobilizing the labeled antibody onto conjugate pad, and the capture antibody onto the strip membrane to form the test / control line.
- Assembling of all components onto a backing card after dispensing of reagents at their proper pads.
- Add samples and buffer onto sample pad.
- Wait the sample flow through the test and control line for 5-10 minutes.
- Read the result when the color reveal.

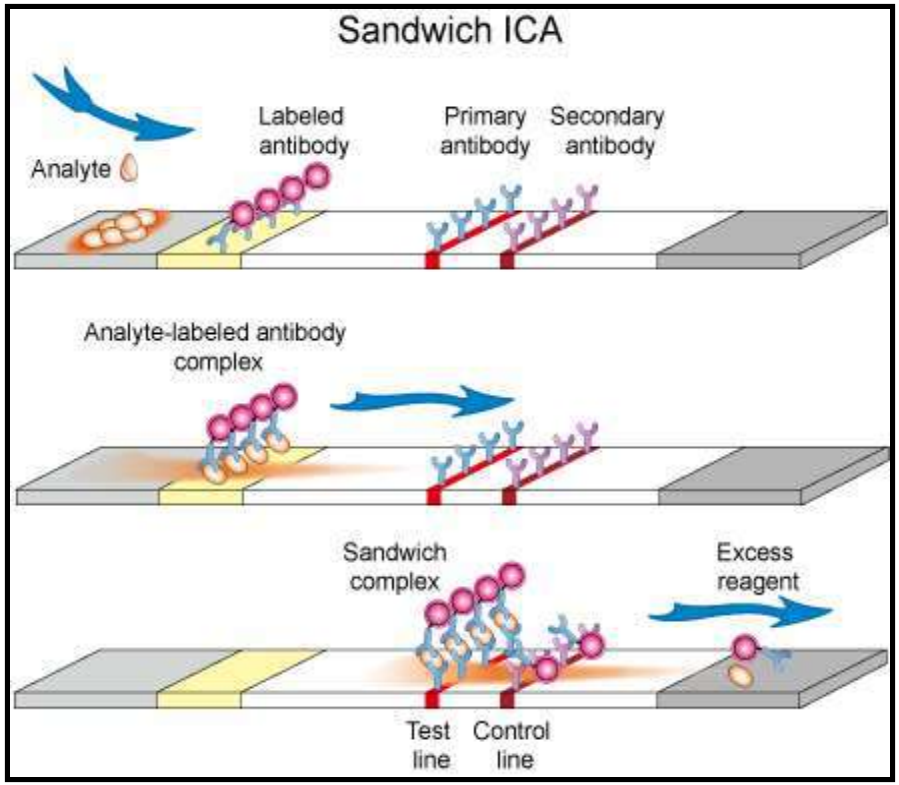

Nitrocellulose
(Test paper)
sample
Sample pad
Lamination card
Conjugate
Test line
Control line
Absorbing pad

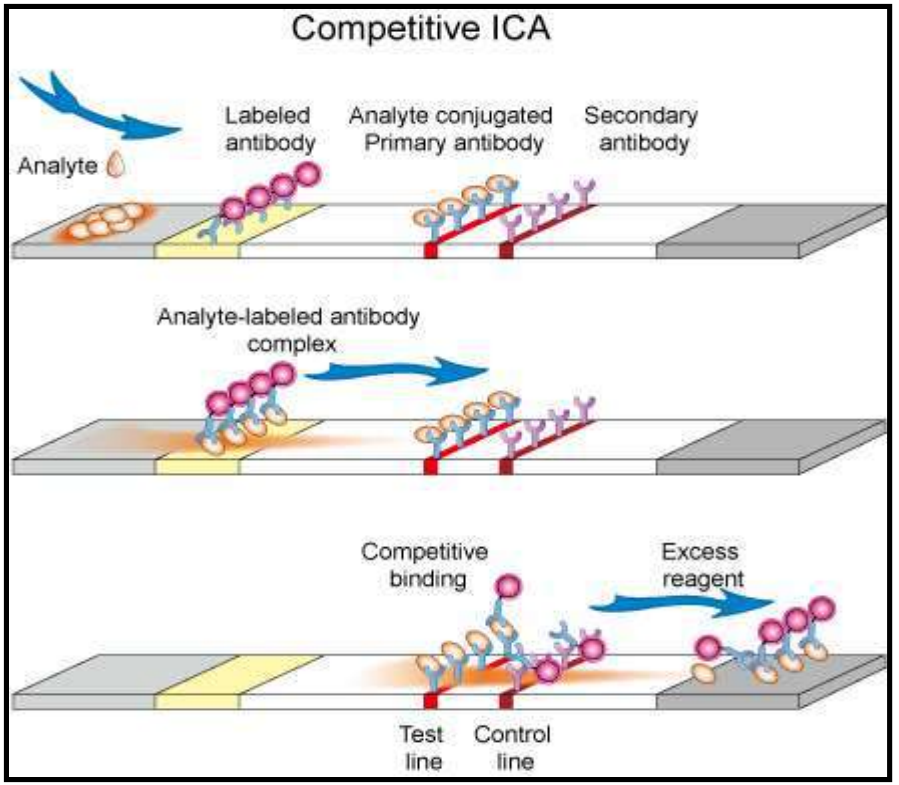
Types of ICA

Three formats are in common use in ICA. These are:

1. **Sandwich Assay** - In this assay, label coated antibody is immobilized at conjugate pad. A capture antibody against target antigen is immobilized over test line. A secondary antibody against labeled antibody is immobilized at control zone.
2. **Competitive Assay** – In this assay, solution containing target antigen is applied onto the sample application pad, which also has prefixed labeled antibody. Test line contains pre-immobilized antigen which binds specifically to label conjugate. Control line contains pre-immobilized secondary antibody which has the ability to bind with labeled primary antibody. Antigen in the sample solution and the one which is immobilized at test line of strip compete to bind with labeled conjugate .
3. **Multiplex Detection Assay** – This assay is used for detection of more than one target species. It is performed over the strip containing test lines equal to number of target antigens to be analyzed.

Types of ICA


Sandwich ICA
Analyte
Labeled antibody
Primary antibody
Secondary antibody
Analyte-labeled antibody complex
Sandwich complex
Excess reagent
Test line
Control line


Competitive ICA
Analyte
Labeled antibody
Analyte conjugated Primary antibody
Secondary antibody
Analyte-labeled antibody complex
Competitive binding
Excess reagent
Test line
Control line

Advantages and Limitations of ICA

ADVANTAGES

- Relative ease of manufacture
- Easily scalable to high-volume production.
- Stable: shelf-lives of 12-24 months often without refrigeration.
- Ease of use: minimal operator-dependent steps and interpretation
- Can handle small volumes of multiple sample types
- Can be integrated with onboard electronics, reader systems, and information systems (POCT)
- Relatively low cost and short timeline for development and approval
- Market presence and acceptance - minimal education required for users and regulators

LIMITATIONS

- Sensitivity issues in some systems
- Simultaneous analysis of multiple markers may difficult
- Test-to-test reproducibility challenging

Applications of ICA