

Ag-Ab reactions

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What is serology

- **Is a branch of immunology dealing with study of Ag –Ab interactions in Vitro by different serological tests.**
- **Ag/Ab**
- **Importance of Lab diagnosis:**
 - 1- Save patient's life
 - 2- Prevent spread of disease
 - 3- Treatment therapy
 - 4- Confirm clinical diagnosis

Lab diagnosis of infectious diseases

1. Isolation and identification of causative agent by:

- a. Morphological tests (microscopy)
- b. Biochemical reactions
- c. Cultural identification
- d. Biotechnology: PCR-DNA probe- DNA finger printing

2. Detection of specific Ab in sera of infected patients using serological techniques

3. Diagnosis of other diseases or change of condition

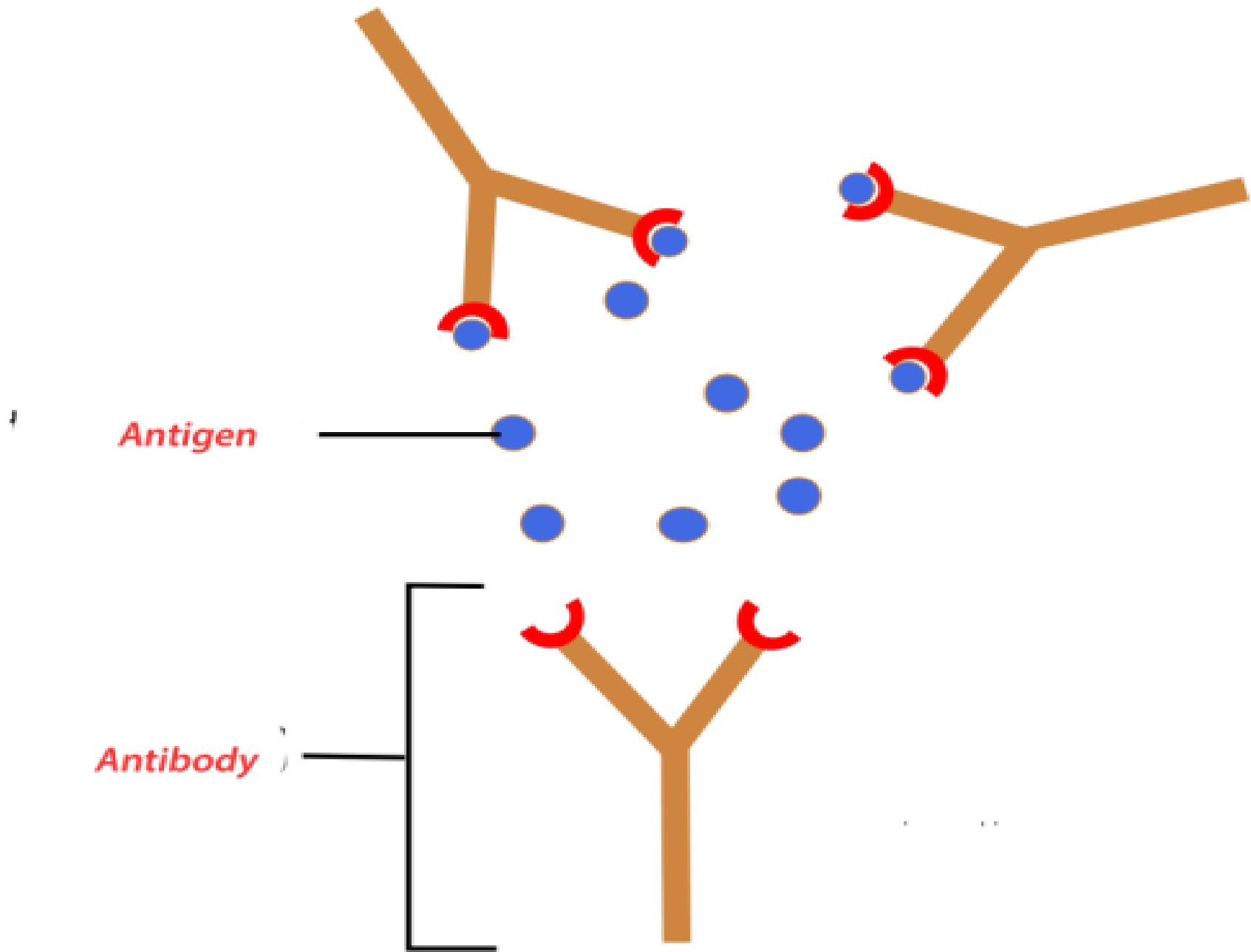
Ag & Ab

- **Ag**
- Antigen (antibody generation)
- **Antigen** – substance capable of binding specifically to components of the immune response; ie BCR, TCR, antibody
- **Immunogen** - A substance that induces a specific immune response. Therefore all immunogens are antigens but not all antigens are immunogens
- **Epitope (antigenic determinant)**
- **Ab**
 - Igs are **Glycoprotein** molecules that are produced by **plasma cells** in response to an immunogen and which function as antibodies
 - Plasma proteins electrophoresis
 - Immunoglobulins (Igs) belongs to **Gamma** and partially beta regions
 - **Paratope (single combining site)**

Principles of Ag-Ab reactions

Maximal binding of an antigens by an antibody will occur in the presence of such conditions :

- Complementarity
- Close proximity
- High **affinity** and **avidity**
- Presence of equal no of multivalent sites
- **Appropriate concentration of Ag and Ab**
- Appropriate environmental factors: ionic strength, (PH=6.5-7.5), temperature (37) , time , stirring ,shaking ,centrifuge



Antigen-antibody interactions:

❖ Are *reversible* specific non-covalent biochemical reactions:

- Hydrogen bonds (*A chemical bond in which a hydrogen atom of one molecule is attracted to an electronegative atom of another molecule*)
- Electrostatic bonds (*A valence bond in which two atoms, attracted by electrostatic forces, transfer one or more electrons between atoms*)
- Van der Waal forces (*forces acting between nonbonded atoms or molecules*)
- Hydrophobic bonds (*The attractive force between molecules due to the close positioning of non-hydrophilic portions of the two molecules*)

❖ Can be represented by the formula:

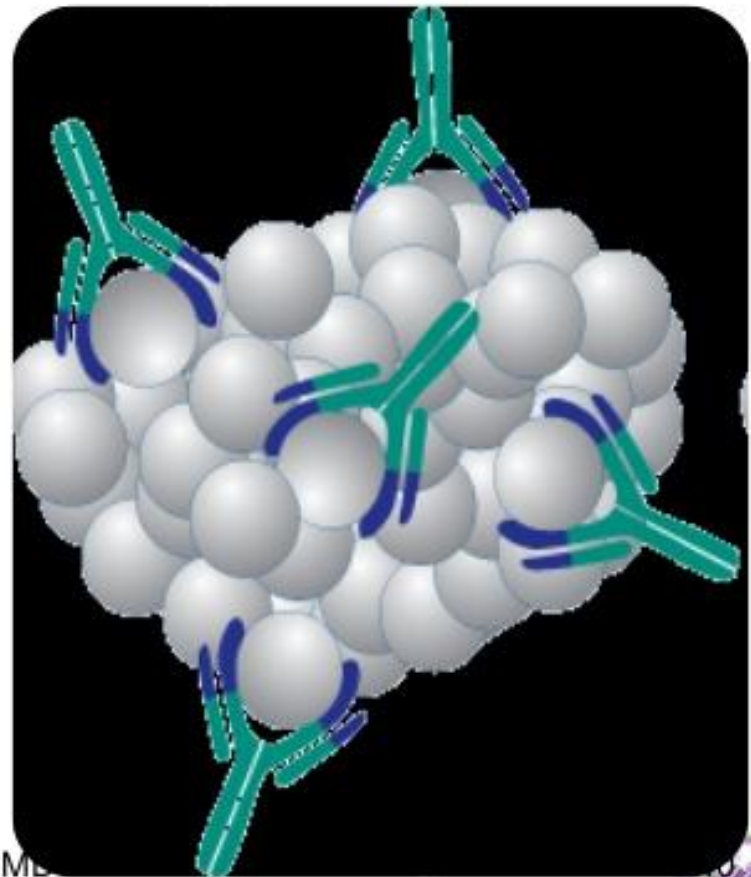


K1=constant of association

K2=constant of dissociation

Affinity

- Antibody affinity is the strength of the reaction between a single antigenic determinant and a single combining site on the antibody.
- It is the sum of the attractive and repulsive forces operating between the antigenic determinant and the combining site .



Dr.T.V.Rao MB



Avidity

		
$K_{eq} = 10^4$	10^6	10^{10}
Affinity	Avidity	Avidity

Dr.T.V.Rao MD

Avidity is a measure of the overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies

Avidity is influenced by both the valence of the antibody and the valence of the antigen.

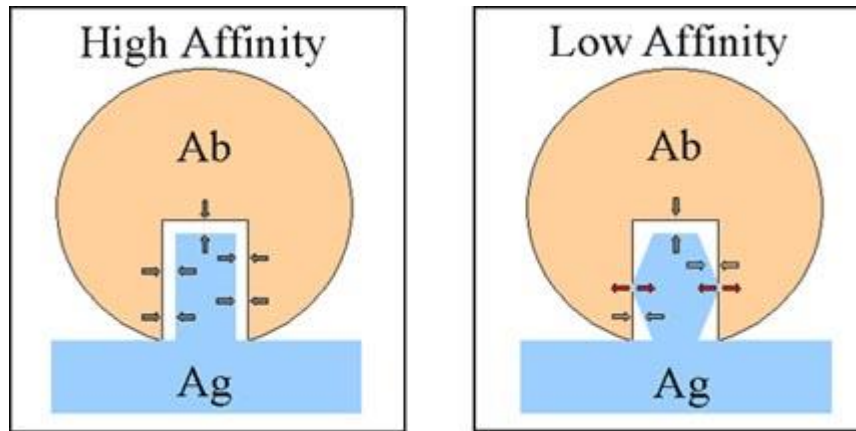
Avidity is more than the sum of the individual affinities.

The affinity:

is the strength of the reaction between a single antigenic determinant and a single combining site on the antibody

or it is the association constant for binding (K_A)

$$K_A = k_1/k_2$$



Valence: the number of epitopes

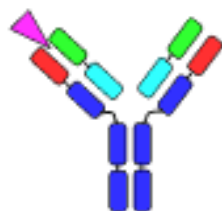
Avidity: is the collective affinity of multiple binding sites (*affinity + Valence*)

Affinity and avidity

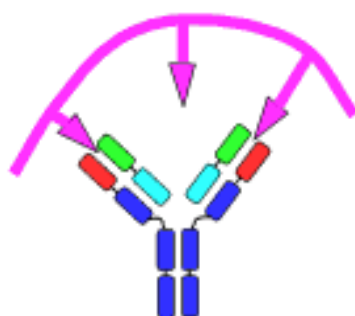
Fab



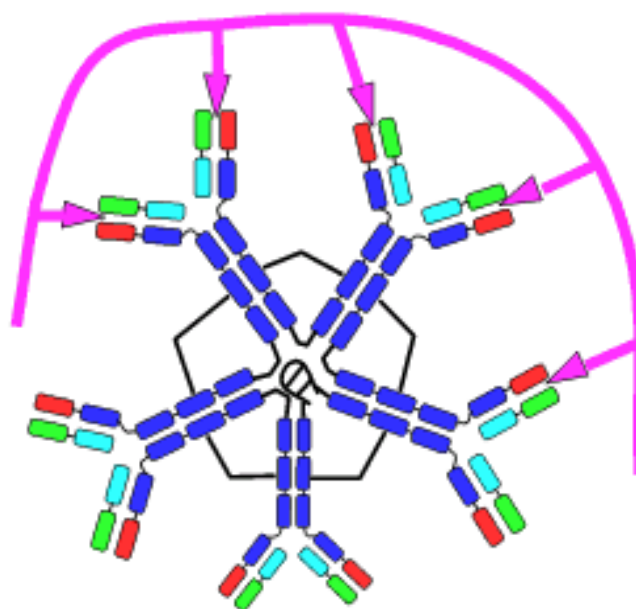
IgG



IgG



IgM



1

1

2

5

effective valence

1

1

100

$10^4 - 10^5$
relative equilibrium
constant

monovalent affinity

multivalent avidity

Concentration of Ag and Abs

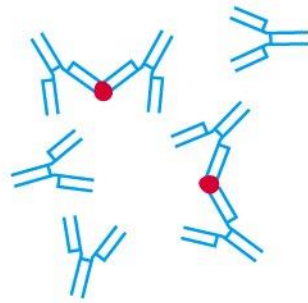
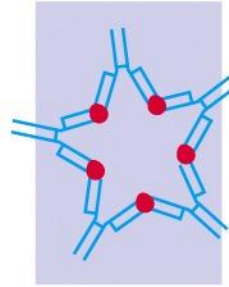
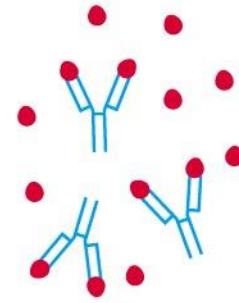
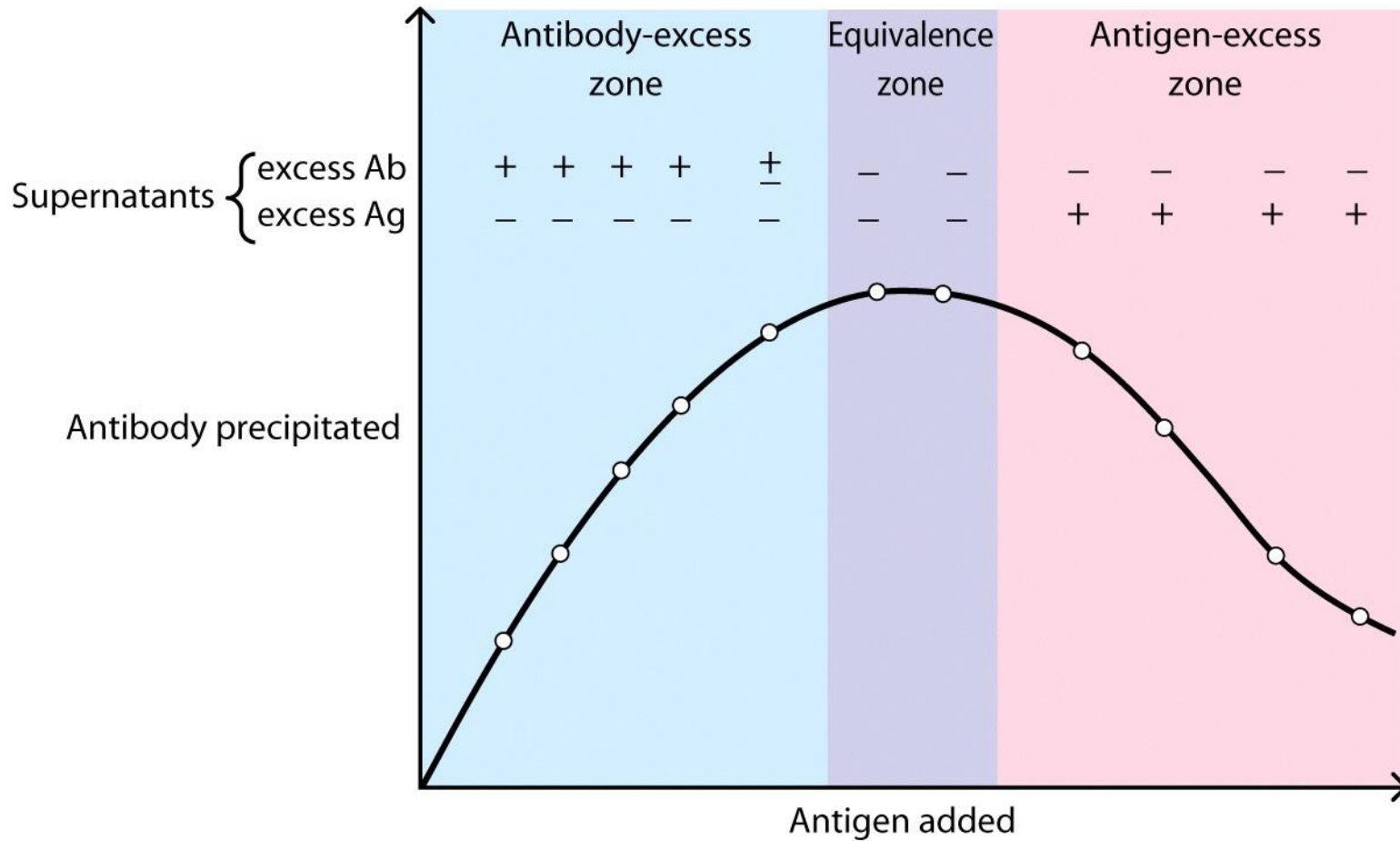
Relative molar concentration, the no of epitopes and the no of Ag binding sites

Precipitin zones :

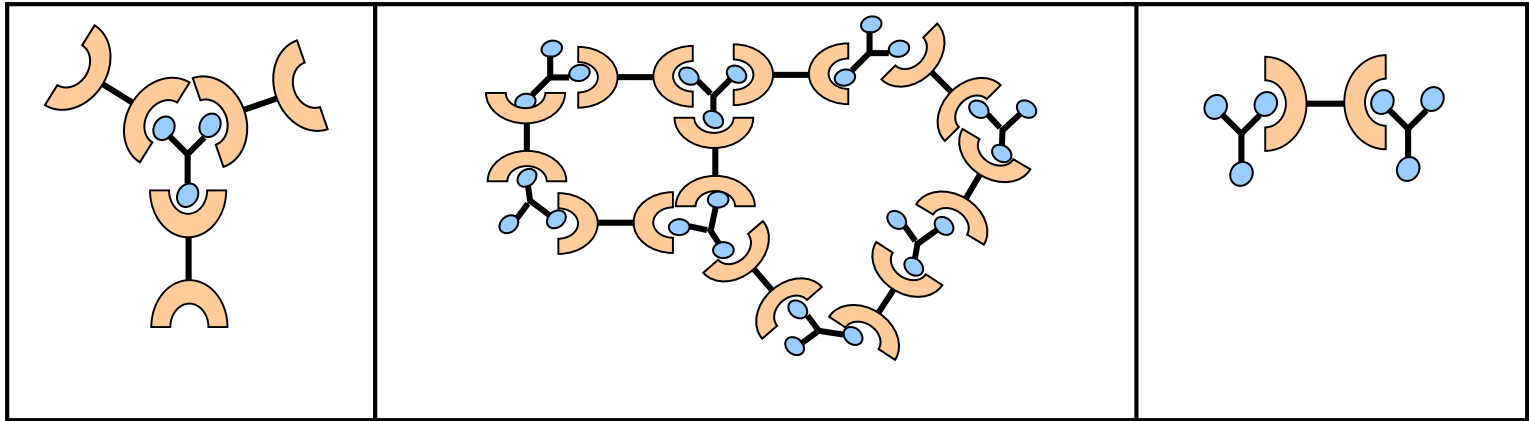
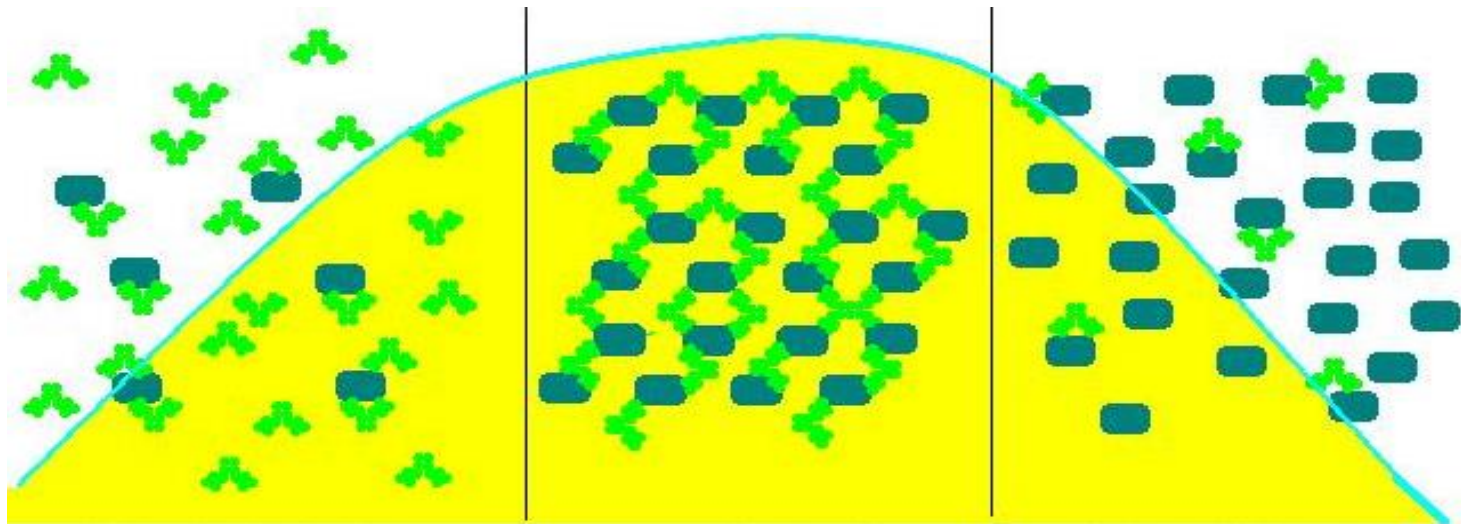
- Zone of equivalence
- Prozone
- Postzone

- Heidelberger
- Ag : polysaccharide : variable level in tube
- Anti Ag (Ab) : constant level add to tube

- **Hook effect** in immunoassay and nephelometry



Precipitation Curve

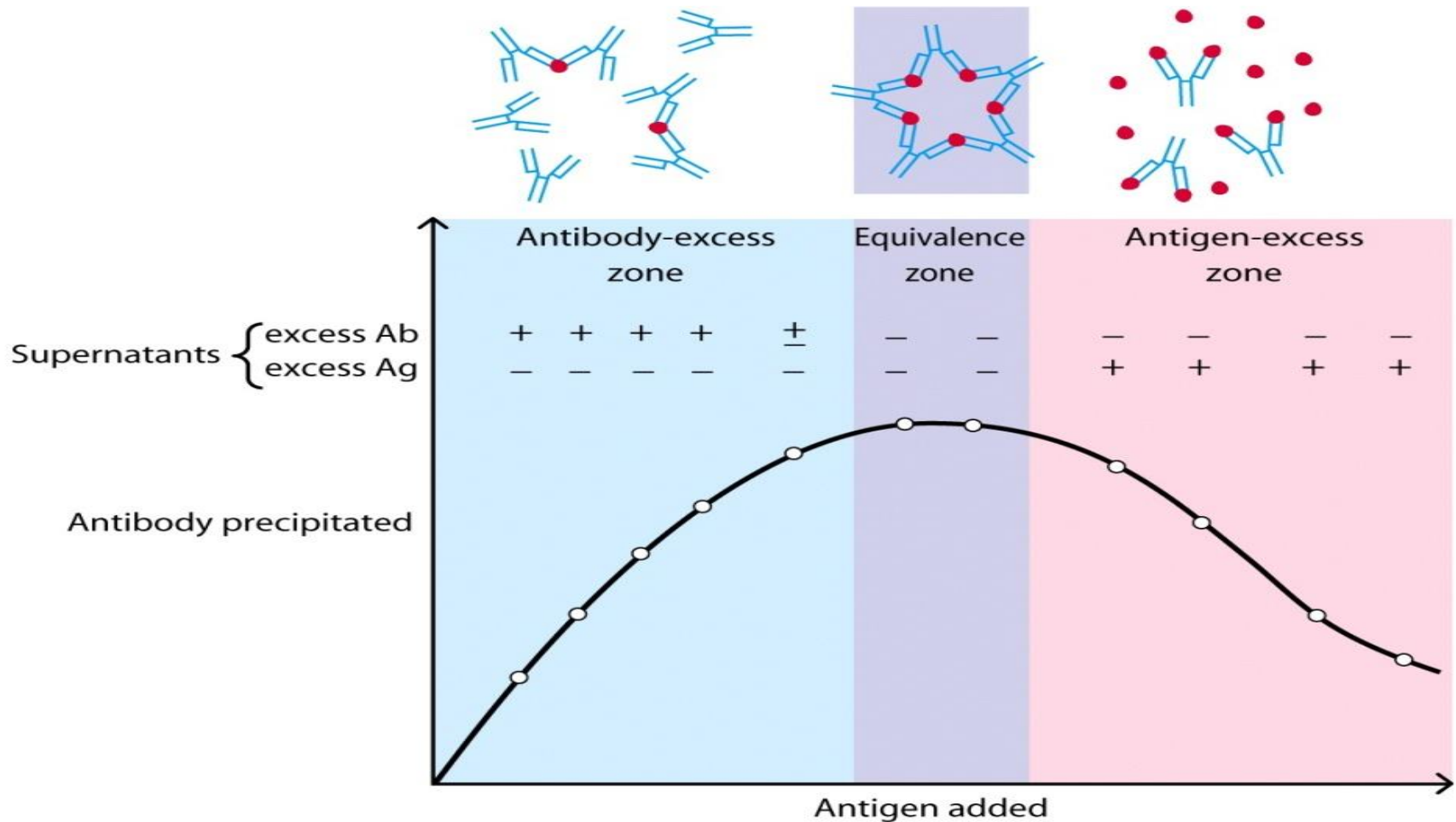


Ab excess

**Equivalence -
Lattice formation**

Ag excess

Precipitation Curve



Postzone / postzone

The **prozone** and **postzone** phenomena must be considered in the clinical setting, because **negative reactions** occur in both.

A false-negative reaction may take place in the **prozone** due to **high antibody concentration**. If it is suspected that the reaction is a false negative, **diluting out** antibody and performing the test again may produce a positive result.

In the **postzone**, excess antigen may obscure the presence of a small amount of antibody. Typically, such a test is repeated with an additional patient specimen taken about a week later. This would give time for the further production of antibody.

Terms

- **Validity** : A serological test should provide an indication of which individuals actually have the disease and which do not.
- **Specificity**: ability of a test to identify correctly those who do not have the disease.(have least cross reactivity)
- **Sensitivity**: Ability of a test to identify correctly those who have the disease(can detect v. small amounts)

Sensitivity / specificity

- The **Sensitivity** of a test is defined as the proportion of diseased individuals in whom the test is positive. Low sensitivity False neg
- The **Specificity** of a test is the proportion of individuals without a given disease in whom the test is negative. Low specificity False pos

Example

Sensitivity : True positive rate of the test(no false –ve)

Specificity: True negative rate of the test(no false +ve)

	Test result	Test result	Total No
	positive	negative	of people
Really have AIDS	99	1	100
Do not have AIDS	199	9701	9900
Totals	298	9802	10,000
Sensitivity = $99/100 \times 100 = 99\%$			
Specificity = $9701/9900 \times 100 = 98\%$			

PPV/NPV

- **Positive predictive value** is the probability that subjects with a **positive** screening test truly have the disease.
- **Negative predictive value** is the probability that subjects with a negative screening test truly don't have the disease

		True class		Measures
		Positive	Negative	
Predicted class	Positive	True positive <i>TP</i>	False positive <i>FP</i>	Positive predictive value (PPV) $\frac{TP}{TP+FP}$
	Negative	False negative <i>FN</i>	True negative <i>TN</i>	Negative predictive value (NPV) $\frac{TN}{FN+TN}$
Measures		Sensitivity $\frac{TP}{TP+FN}$	Specificity $\frac{TN}{FP+TN}$	Accuracy $\frac{TP+TN}{TP+FP+FN+TN}$

Terms

- **Quantitative test:**
- It measures the amount of Ag or Ab.
- **Qualitative test :**
- It detects the presence or absence of Ag or Ab.

Binding forces :

- **Non covalent** and relatively weak
- **Ionic interaction** (charge complementarity)
- **Hydrogen bonds** : form between-negative charged and positive charged H^+
- **Hydrophobic bonds** : between nonpolar molecules eliminating H_2O during the process
- **Van der waals forces** : very weak and non specific
become stronger as the
epitope comes closer to the Ag
binding site (**paratope**)

Environmental factors affecting Ag/Ab interaction

- Hydrogen ion concentration : (pH)
Ionization of Abs changes its conformation and modify affinity
- Temperature :
Optimum 37°C , increasing temp decreases stability of the complex
- Salt concentration :
Low salt is optimum
- **Zeta potential** :
Cell agglutination, IgG is too short to overcome zeta potential, Anti-IgG added to form bridges between the cells (**Anti globulin test**)
Agitation, high or low pH or high salt concentration

Serological Reactions

- **Primary** : It measures the direct interaction between Ag and Ab in Vitro(test tube).
Example: Elisa, IFA, RIA tests.
- **Secondary** : It measures the consequences of interaction between Ag and Ab in Vitro.
Example: Agglutination, CFT, Precipitation, Neutralization tests.
- **Tertiary** : It measures Ag and Ab interactions in Vivo (in body)

Classification of Ag-Ab Reactions

Primary(immuno Assay)

Labeled immunoassays (Ag/Ab) , More sensitive,
Time consuming, Heterogenous

(Lattice formation not required)

Secondary:

(Lattice formation is required)

Primary Serologic Reactions and their labels

RIA : **Radio** Immuno Assay

FIA : **Fluorescent** Immuno Assay

CLIA : Chemi **Luminescent** Immuno Assay

ECL : Electro chemiluminescence

ELISA/EIA : **Enzyme** Linked Immuno Sorbent Assay

ELFA : Enzyme Linked Florescent Assay

IHC : Immuno **Histo** Chemistry

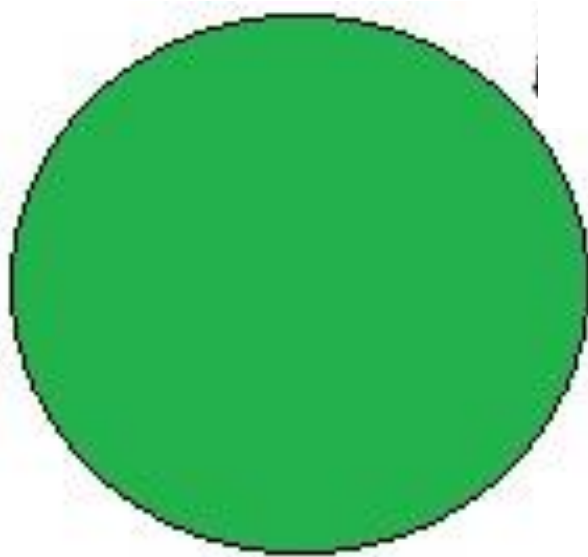
Relative sensitivities of tests (approximate)

Method	Usual operating range of Ab or Ag
Percipitation	10 -1000 µg/ml
IFA	0.1 – 10 µg/ml
ELISA	0.1 – 10 ng/ml
RIA	0.01 – 10 ng/ml
CLIA	0.01 – 10 ng/ml

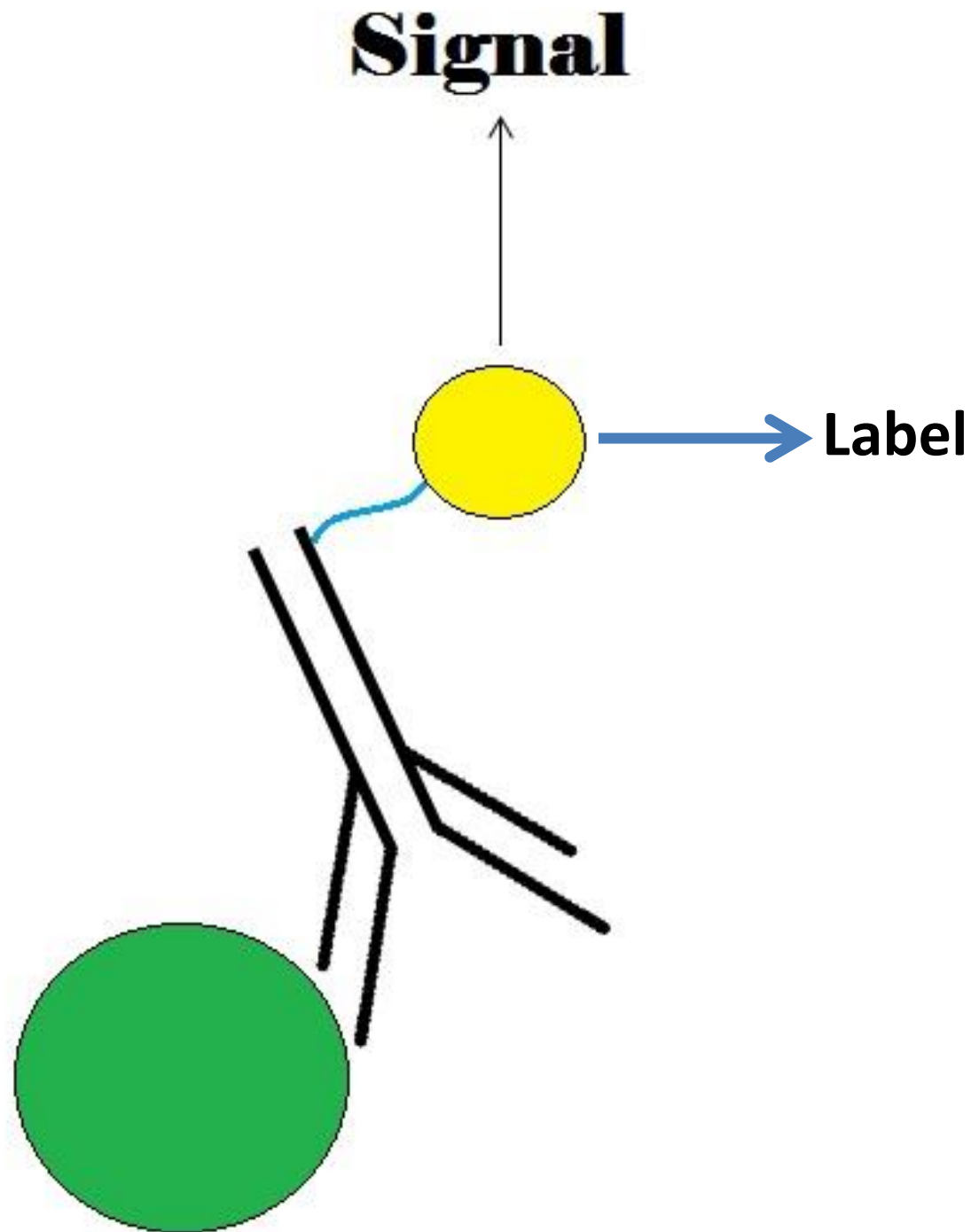
Why primary reactions are sensitive than others?

Immunoassay

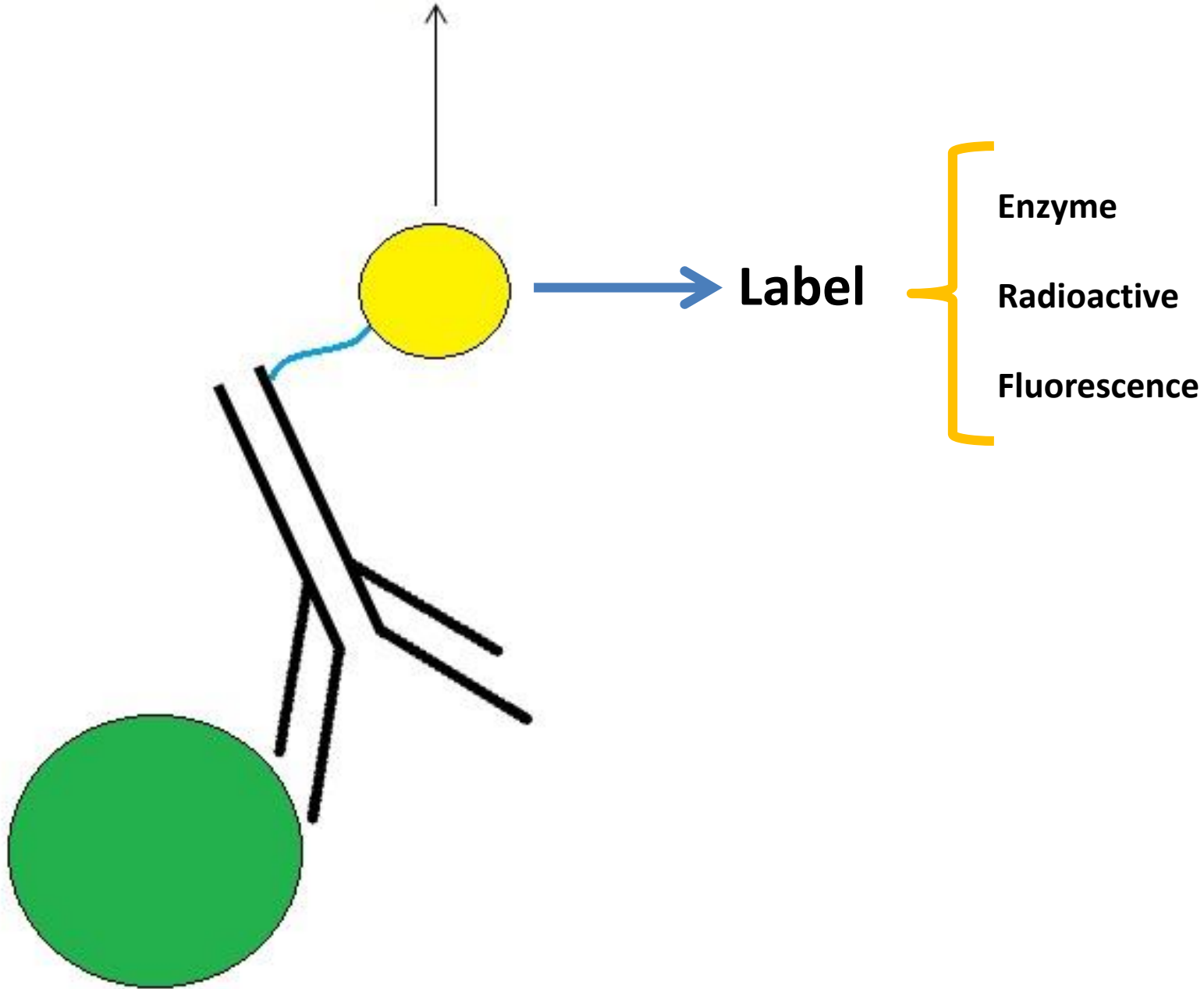
Determine concentration of a macromolecule in a solution through the use of an **antibody**



Macromolecule (Ag)



Signal

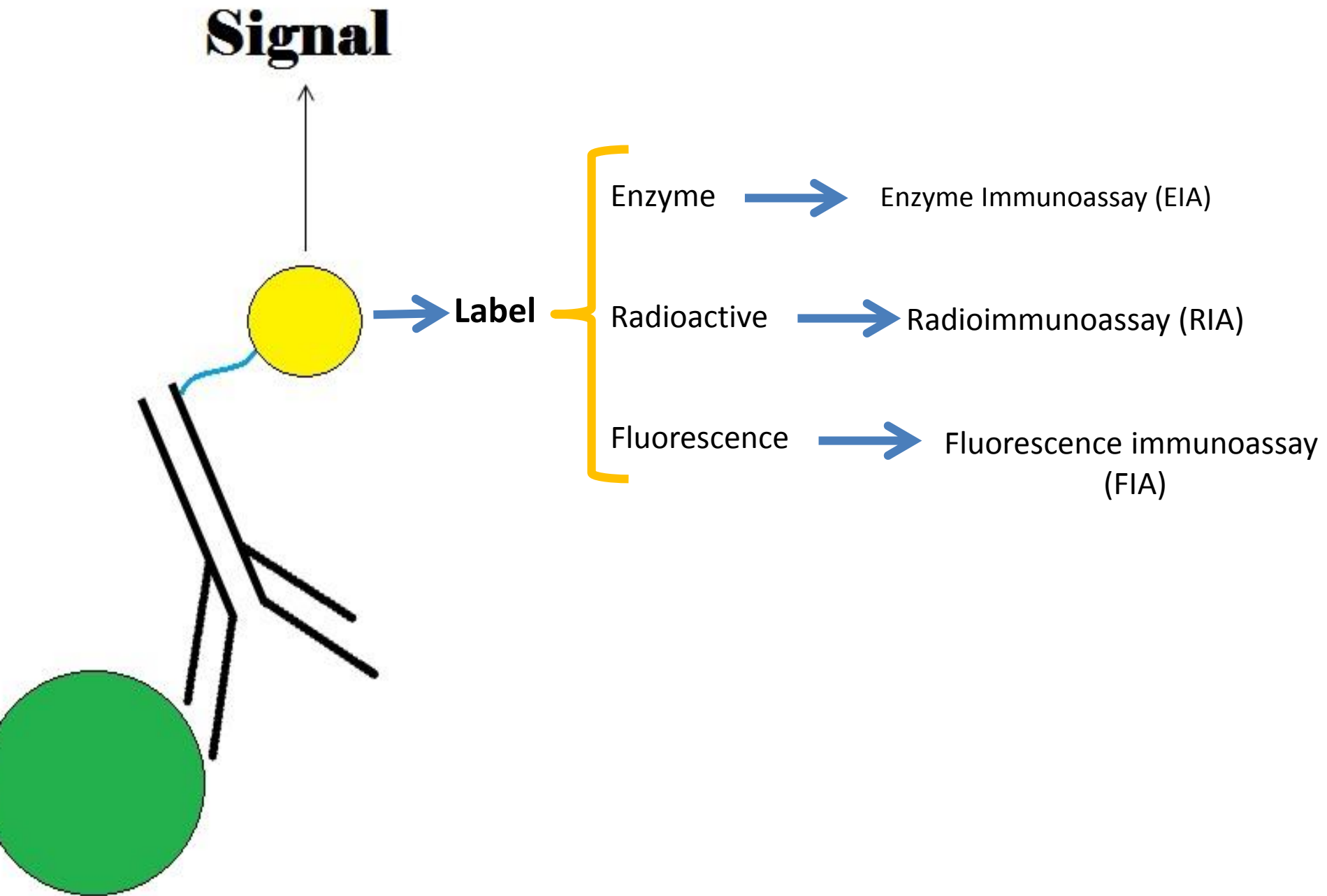


Label

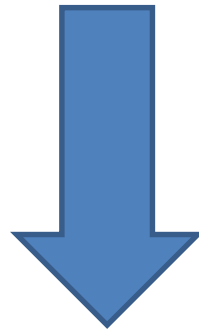
Enzyme

Radioactive

Fluorescence



EIA (Enzyme Immunoassay)



Enzyme Linked Immunosorbent Assay (ELISA)

ELISA

- ELISA combines the **specificity of antibodies** with the **sensitivity of enzyme assays**, by labeling the Abs or Ags with an easily assayable enzyme.

ELISA procedure in details

- 1. Coating the plate with Ag or Ab**
- 2. Blocking free sites**
- 3. Addition of standards & samples**
- 4. Conjugated Ab**
- 5. Substrate**
- 6. Stopping**
- 7. Reading optical density by ELISA reader**

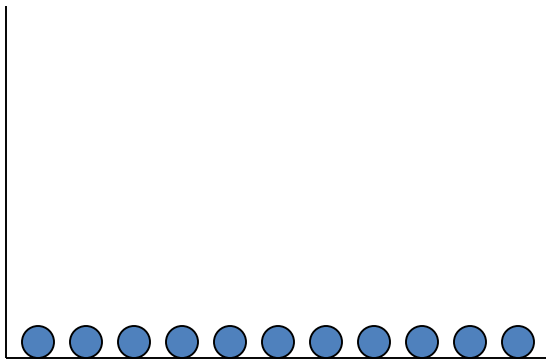
1. Coat solid phase with

antigen when analyzing **antibody**

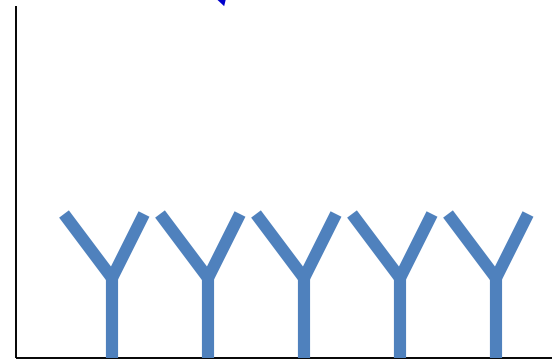
antibody when analyzing **antigen**

Coat Ag or Ab at 0.5 - 20 $\mu\text{g/ml}$

100 $\mu\text{l/well}$, 4°C overnight



Analyte = antibody



Analyte = antigen

Incubate, wash

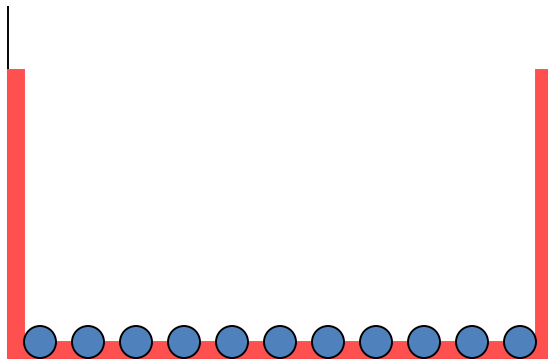
Washing

- **Buffer + 0.05% Tween 20, 200-300 μ l/well**
- **3 - 6 washes with 30-60 Second soaking time**
- **Washing methods**
 - **Automated washer**
 - **Flood and Flick (biohazard)**
 - **Multi-channel pipette for dispensing**

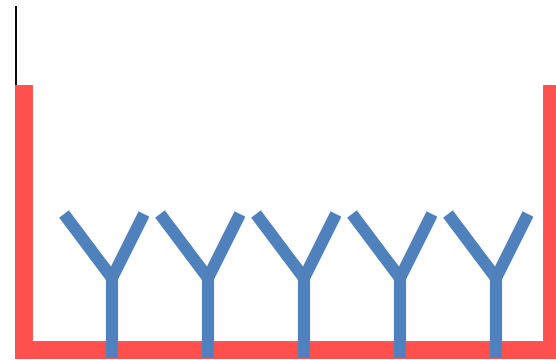
2. Block free binding sites

Inert Protein as Albumin , Casein
Or Tween 20

0.5 - 2% Bovine Serum Albumin
2% Non-fat Dried Milk (skimmed
milk)



Analyte = antibody



Analyte = antigen

Incubate, wash

We obtain the commercial ELISA kits while the coating and blocking steps was already done

3. Add sample

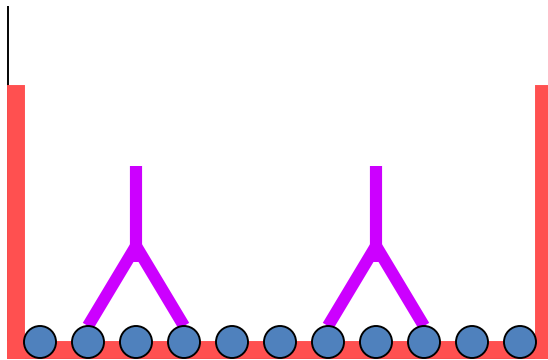
Include known Positive and Negative Controls
and Standards or Cutoff

Recombinant protein

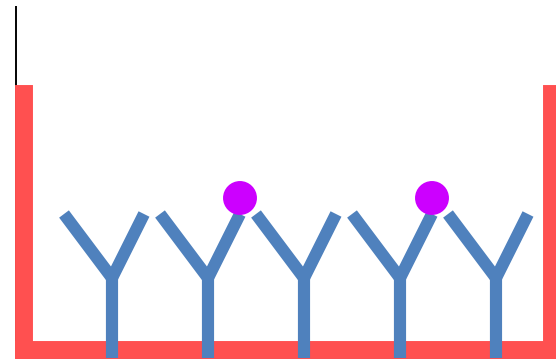
International standard antibody

Double-dilute from 10 pg/ml - 10 ng/ml

100 µl/well, duplicates



Analyte = antibody



Analyte = antigen

Incubate, wash

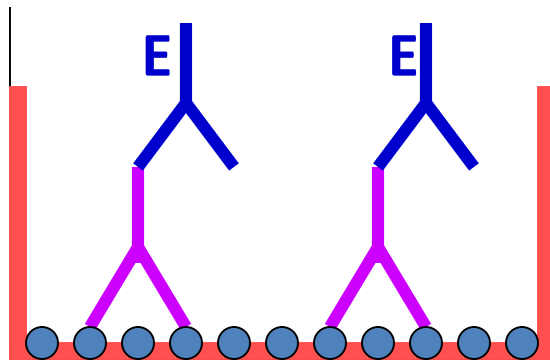
4. Add conjugate.

Monoclonal **capture** antibody

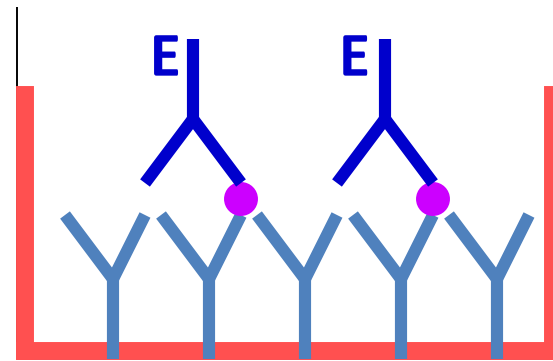
Polyclonal **detection** antibody

Anti-(human) Ig-enzyme

IgG / A / M / E / subclass-specific



Analyte = antibody



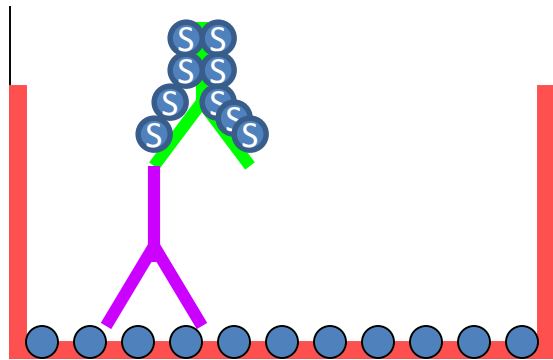
Analyte = antigen

Common Enzymes:

Incubate, wash **Horse Radish peroxidase (HRP)**
Alkaline Phosphatase (Alk.p)

Amplification of Signals

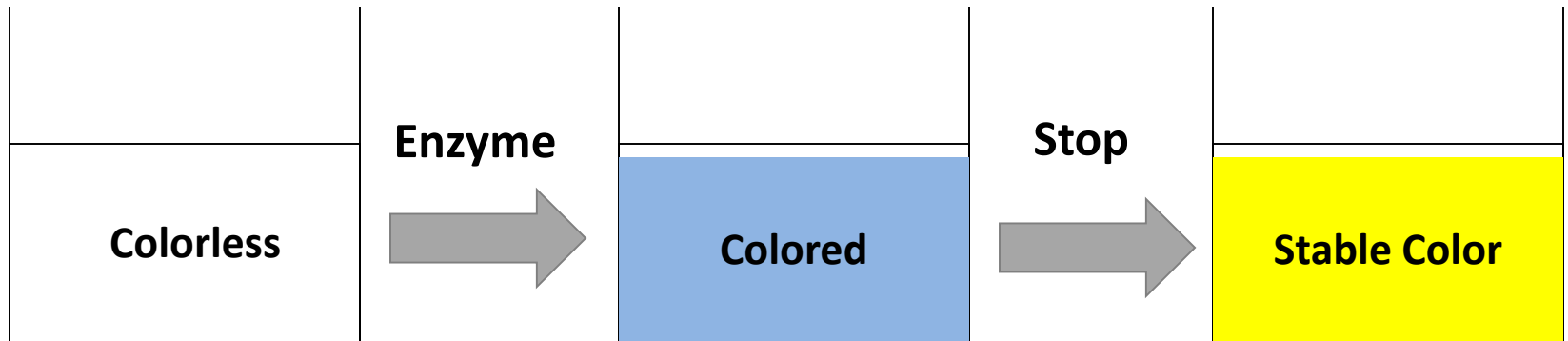
Directly enzyme conjugated antibody may give weak signals



Instead you may add
Biotin-labelled anti-Ig
followed by
Streptavidin - Enzyme

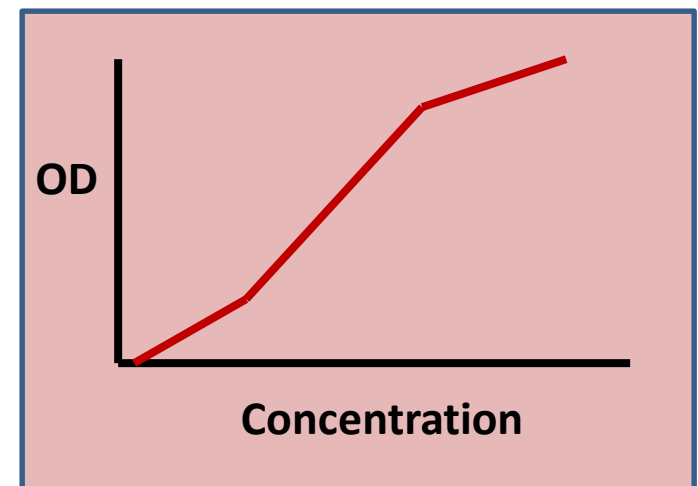
5. Add substrate/ Chromogen

6. Incubate, stop enzyme, measure color change

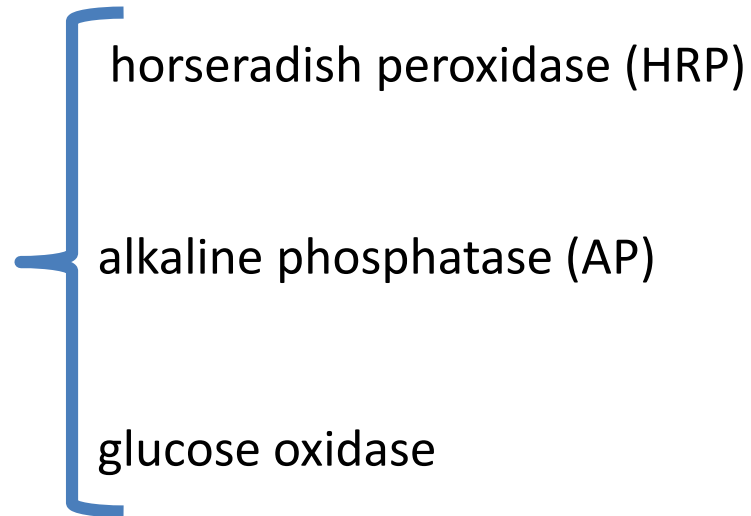


HRP:

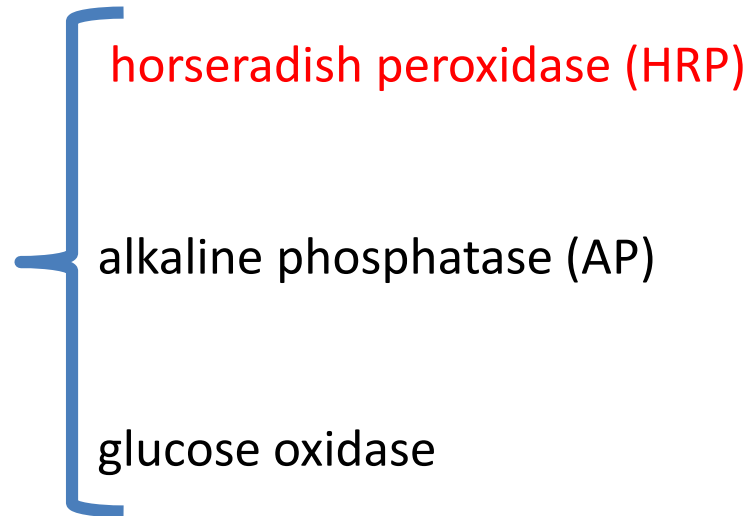
$\text{H}_2\text{O}_2 + \text{TMB (Tetra methyl Banzidin)} \rightarrow 450 \text{ nm}$



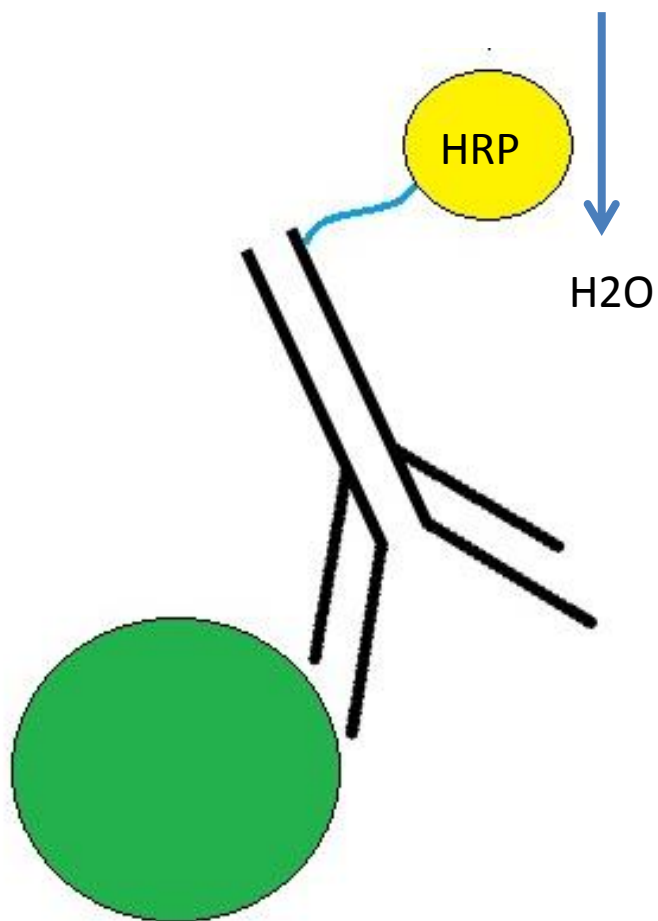
Enzymes



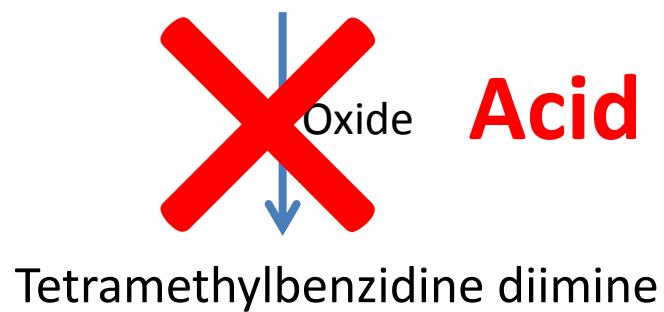
Enzymes



H₂O₂



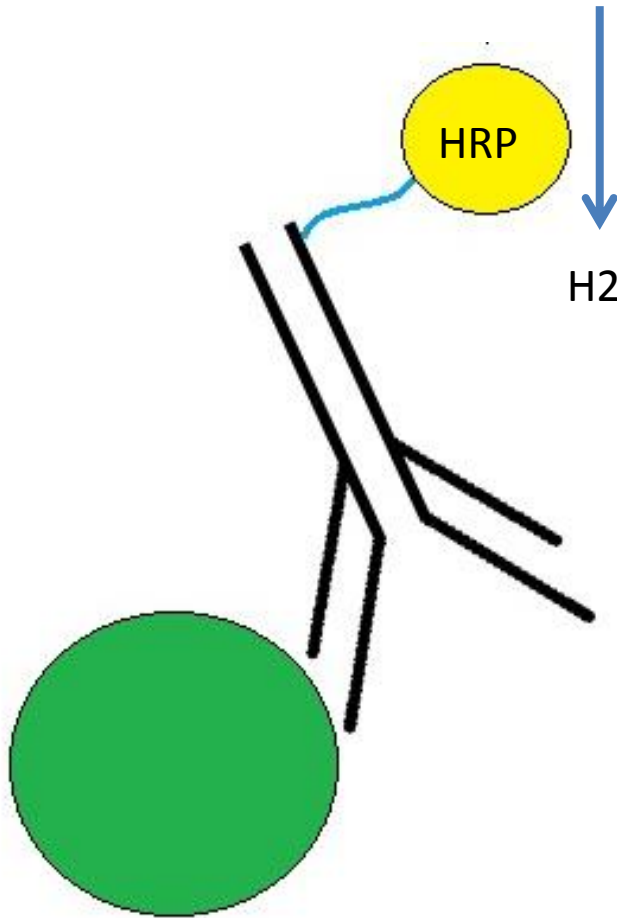
TMB (Tetramethylbenzidine)



H₂O₂

HRP

H₂O

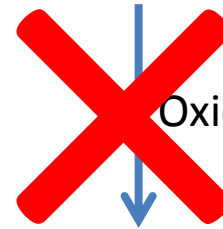


TMB (Tetramethylbenzidine)

Oxide

Acid

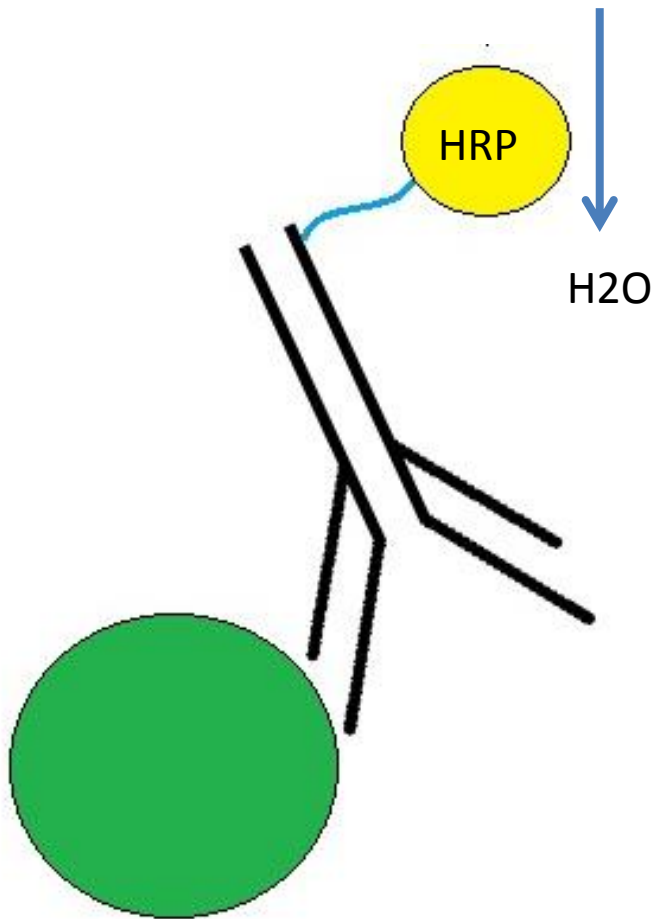
Tetramethylbenzidine diimine



H₂O₂

HRP

H₂O

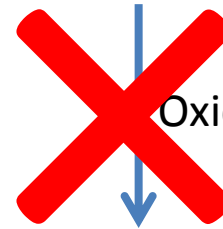


TMB (Tetramethylbenzidine)

Oxide

Acid

Tetramethylbenzidine diimine



H₂O₂

HRP

H₂O

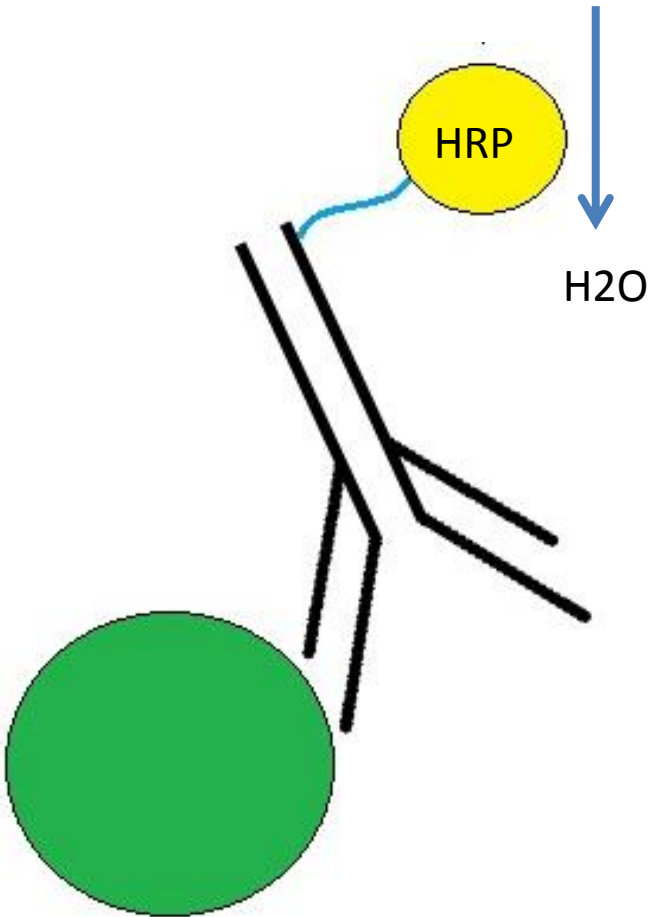
TMB (Tetramethylbenzidine)

Oxide

Acid

Tetramethylbenzidine diimine

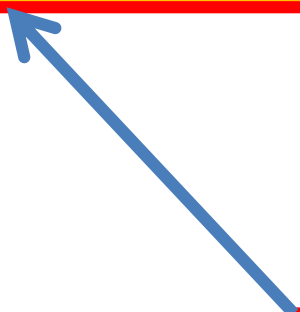
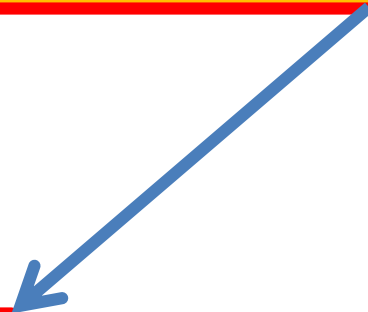
Measuring the absorbance
(450nm)



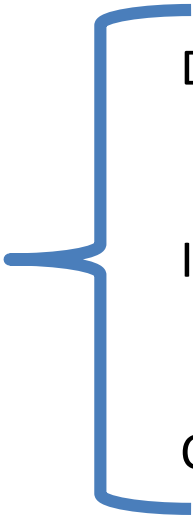
color intensity

levels of target

HRP activity



ELISA formats

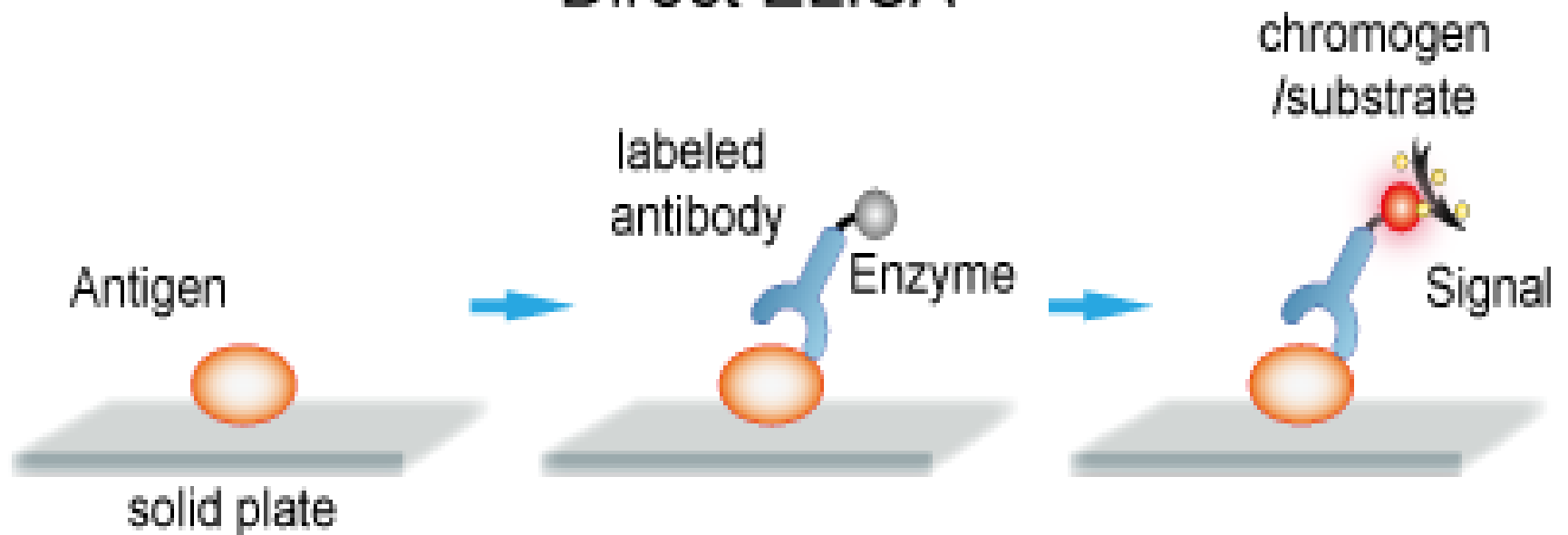


Direct Assay

Indirect Assay

Capture Assay (Sandwich)

Direct ELISA



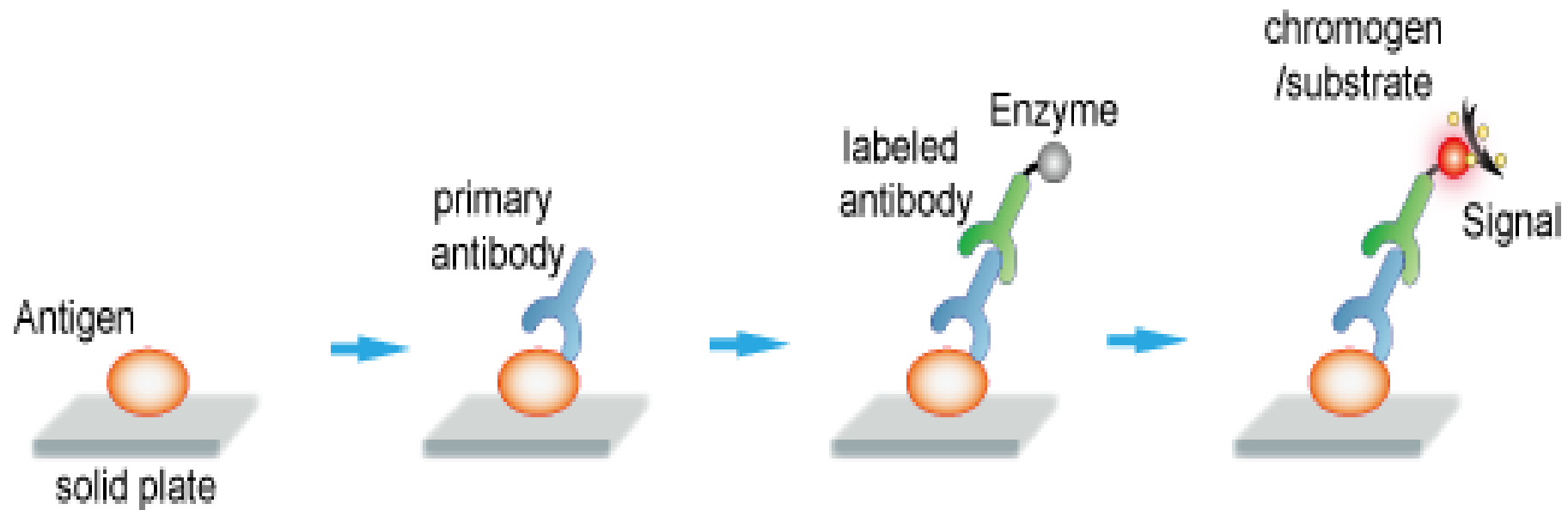
1. Antigen is coated onto wells by passive adsorption

2. Antibody conjugated with enzyme is added and incubated with antigen and incubation.

3. Substrate / chromophore is added and colour develops.

Research

Indirect ELISA



1. Antigen is coated onto wells by passive adsorption and incubation.

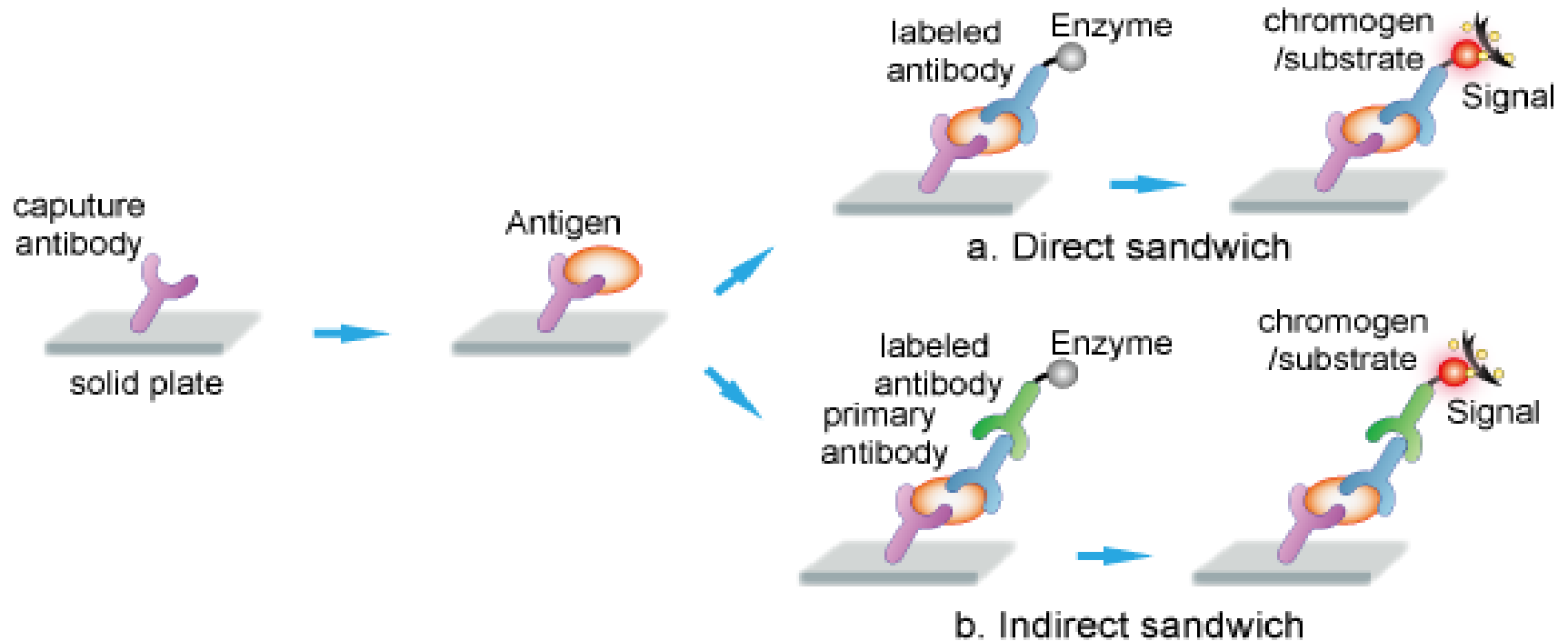
2. Primary antibody is added and incubated with

3. Anti-species antibody conjugated with enzyme is added and incubated.

4. Substrate / chromophore is added and colour develops.

Auto Ab, Toxo G , CMV G , H.Pylori G ,

Sandwich ELISA



1. Capture antibody is coated onto wells by passive adsorption and incubation.

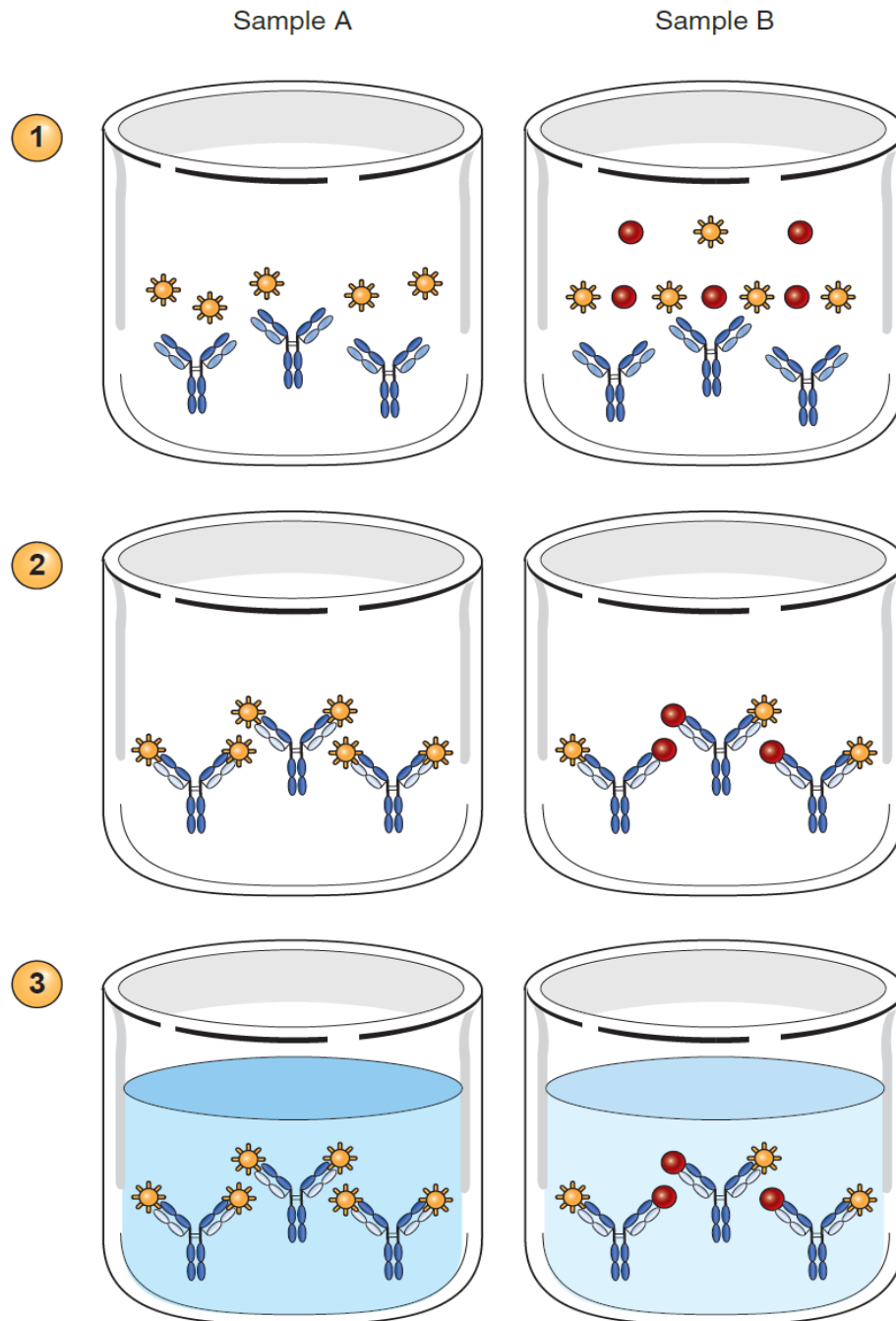
2. Antigen sample is added and incubated with capture antibody

3. labeled antibody (a)/primary antibody and labeled antibody (b) is added and incubated with antigen

4. Substrate / chromophore is added and colour develops.

Ag Capture : Tumor markers , PSA , FSH, LH, TSH, HCG , Cytokine,...

Ab Capture : HBS Ab , ...



1. Unknown concentration of analyte in patient sample (red dots) competes with labeled analyte (yellow stars) for binding sites on immobilized antibody. Sample A is a negative control.
2. Wash to remove unbound materials.
3. After substrate is added, a colored product (signal) is generated with an intensity proportional to the amount of enzyme-labeled analyte bound to the antibody.

The signal strength is usually **inversely** related to the analyte concentration.

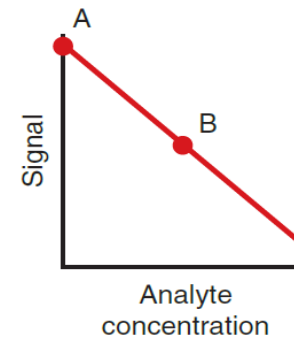
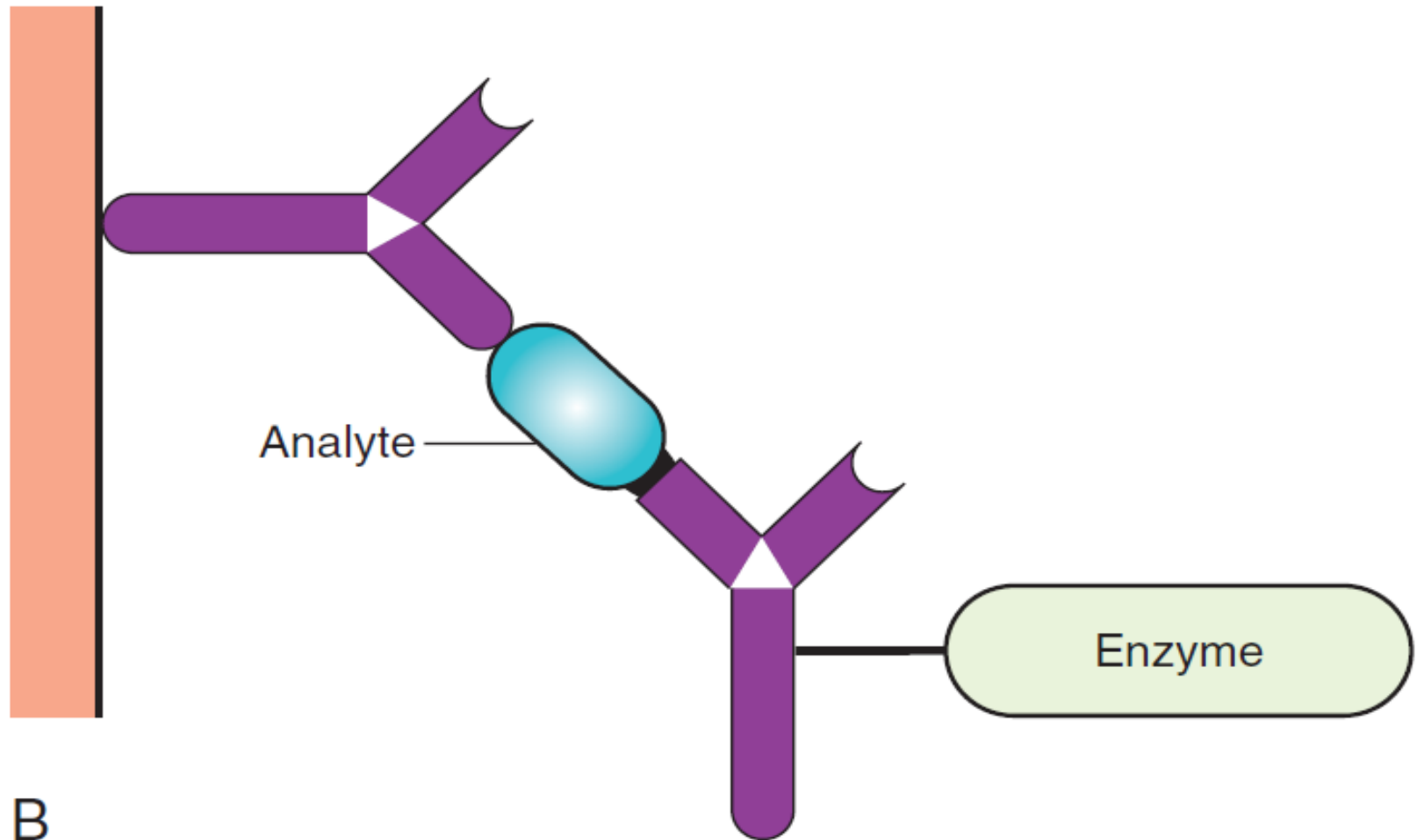
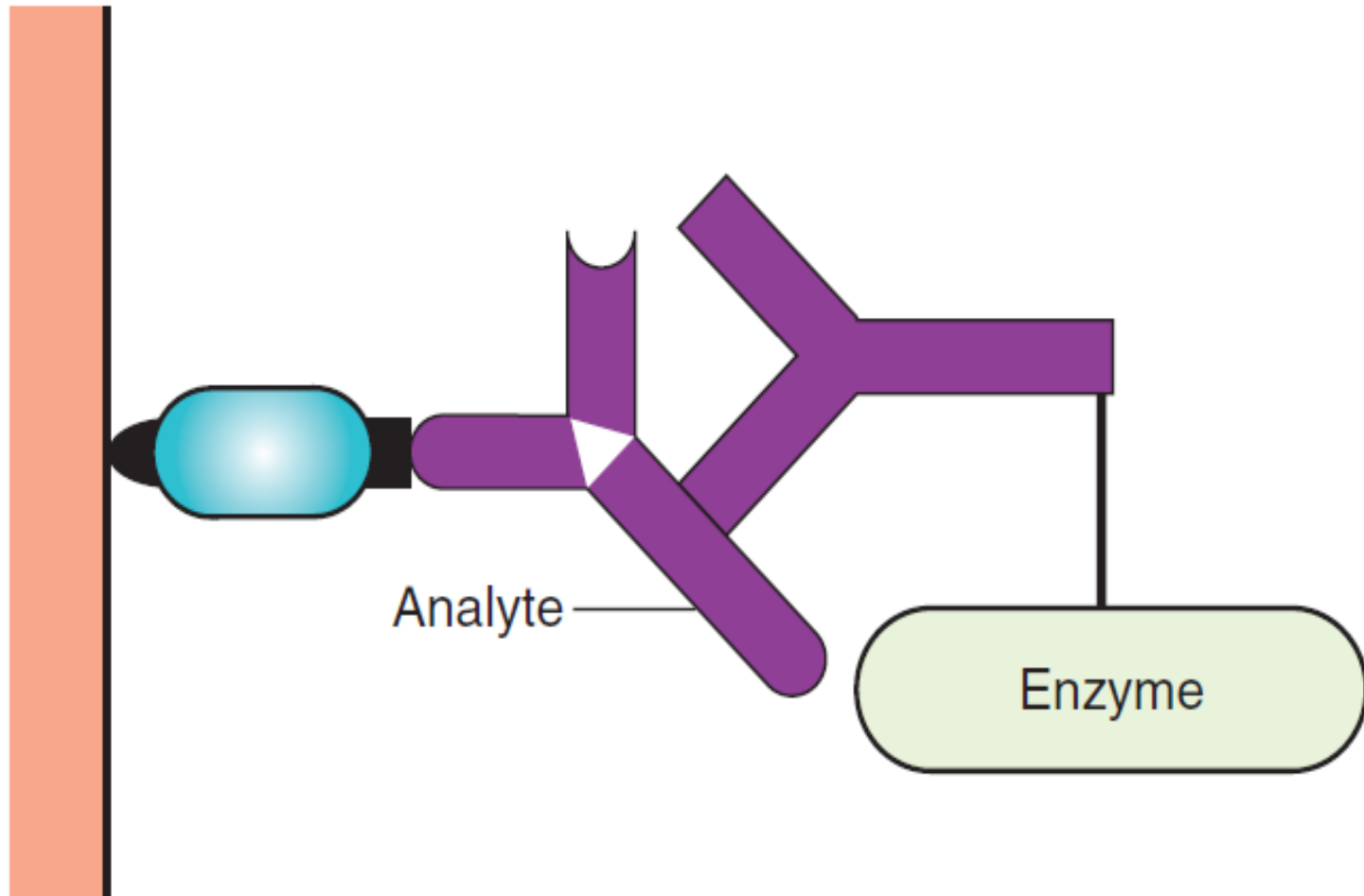


FIGURE 11-1 Principle of a competitive immunoassay.

Noncompetitive Sandwich Assay



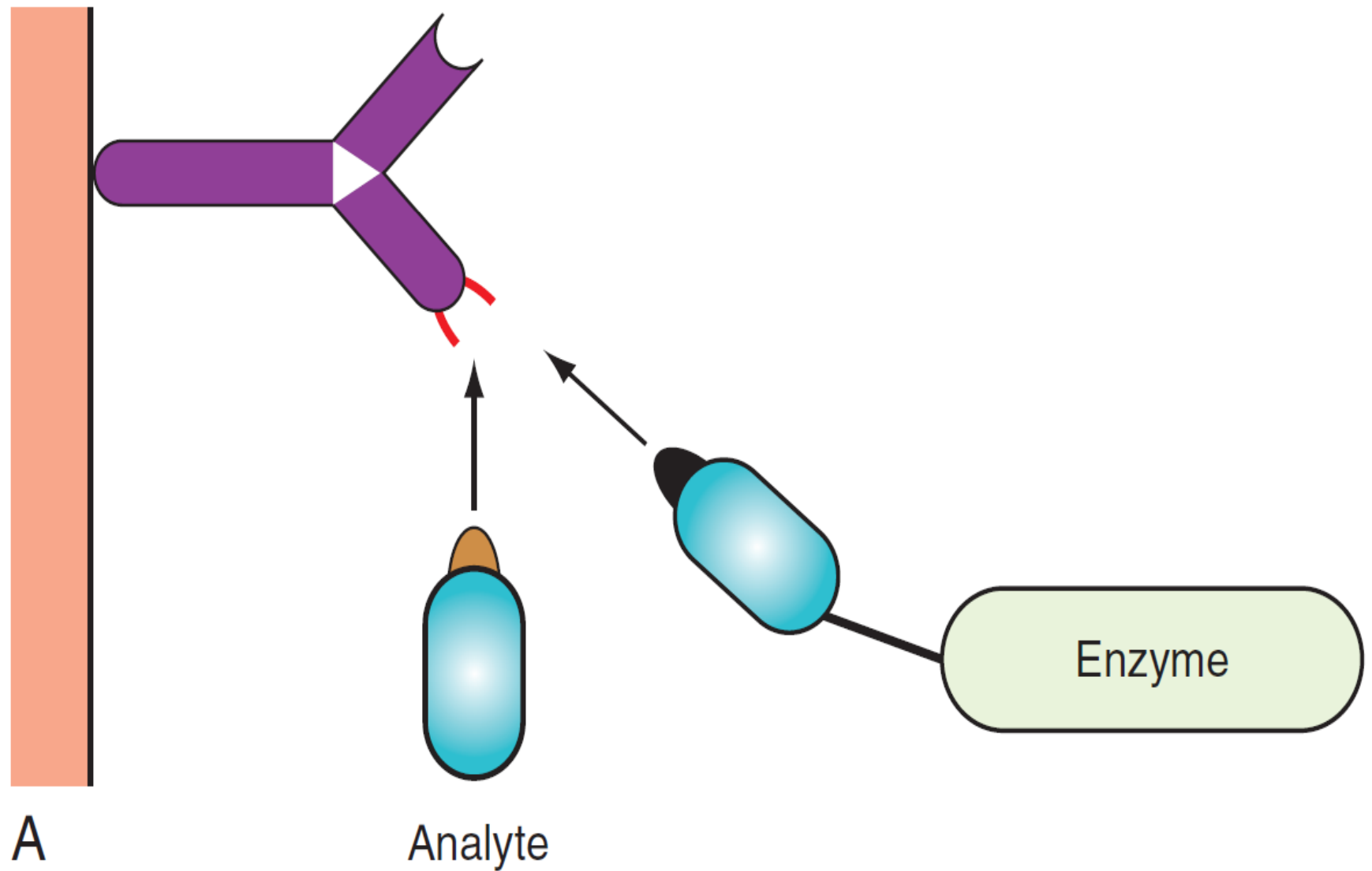
Antigen Assay



C

Antibody Assay

Competitive Assay



Advantages

- Sensitivity
- Quantitative
- Reproducible
- Kit format
- No Radiolabel

Relative sensitivities of tests (approx)

Usual operating range
[Ab] or [Ag]

precipitation
immunoelectrophoresis
double/radial diffusion }

10 µg/ml - 1 mg/ml

immunofluorescence

0.1 - 10 µg/ml

ELISA (colour)
(chemiluminescence)

0.1 - 10 ng/ml

0.01 - 10 ng/ml

radioimmunoassay

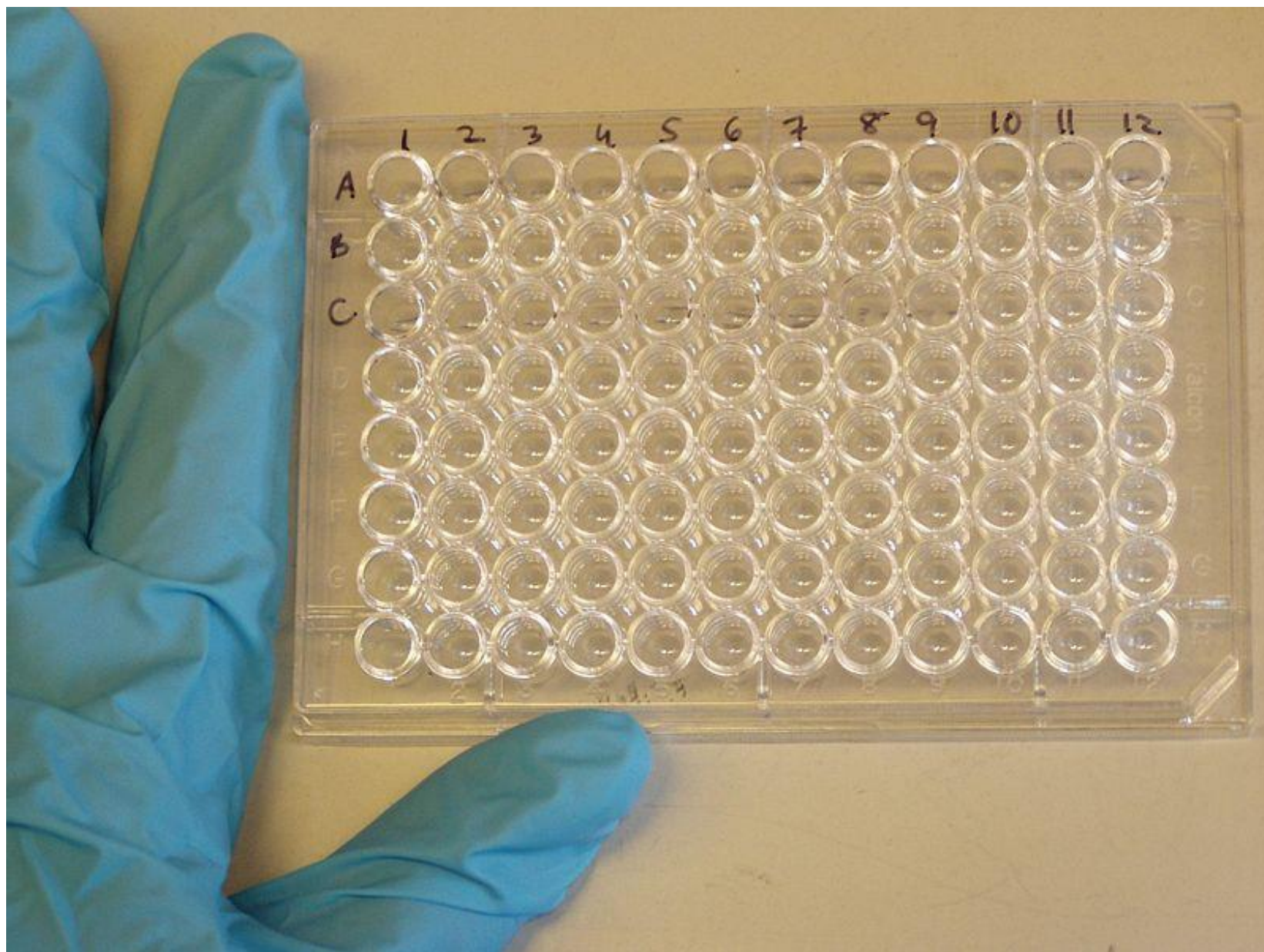
0.01 - 10 ng/ml

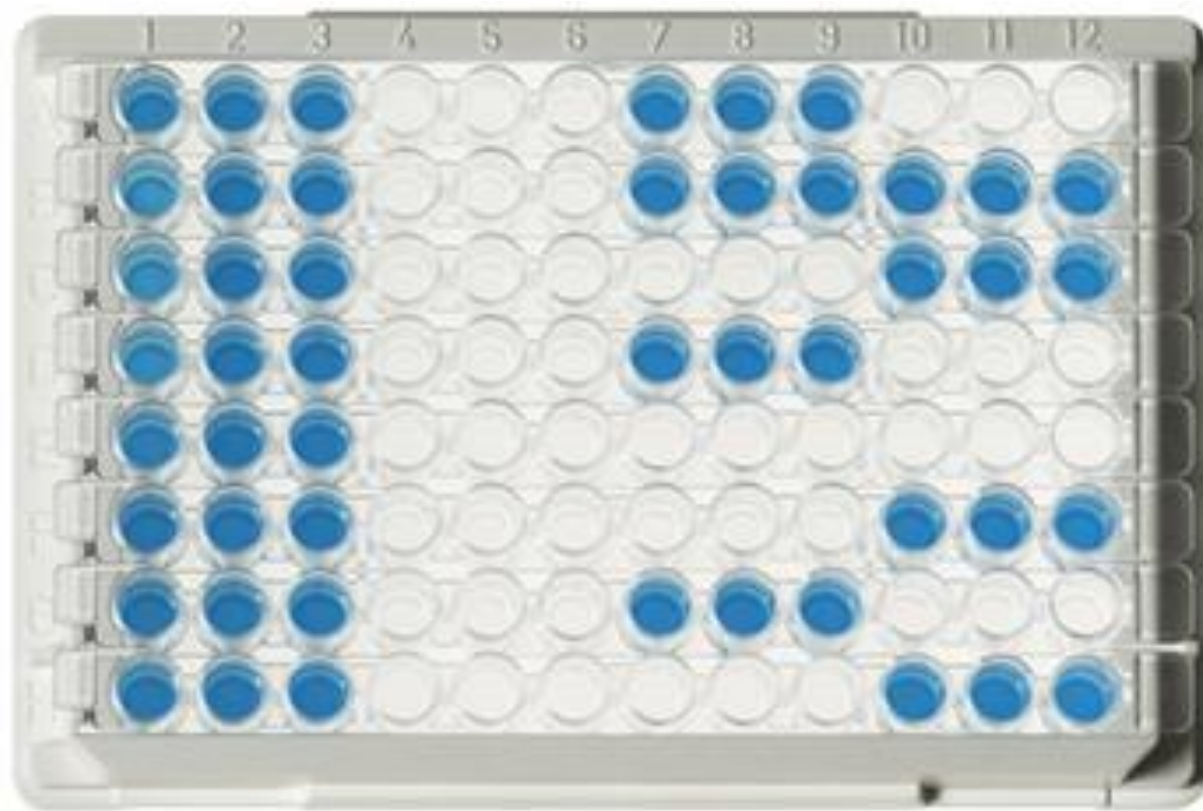
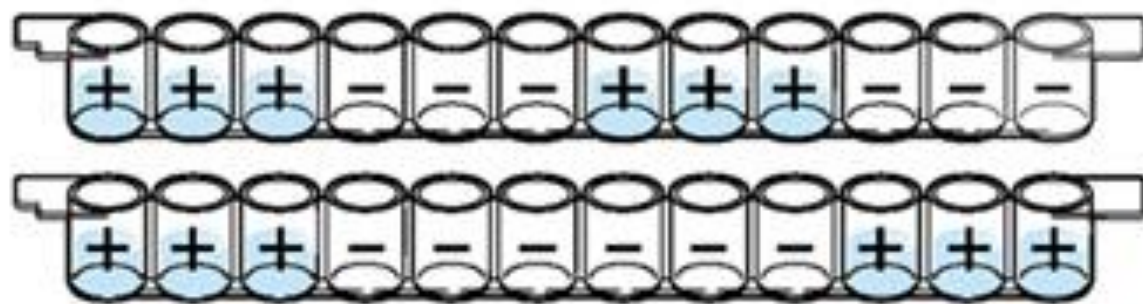
What is it used for?

- Measure antibody levels (allergies, vaccines)
- Detect viruses (hepatitis, HIV, venereal diseases)
- Detect circulatory inflammatory markers (cytokines)
- Detect hormonal changes (pregnancy)

ELISA Kits







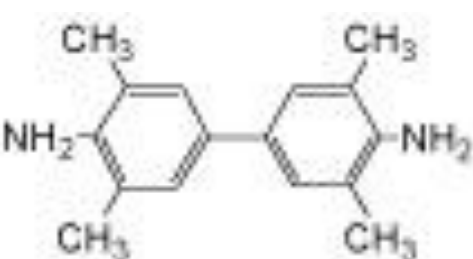
ELISA reader



OD (optical Density)

How covert OD to concentration?

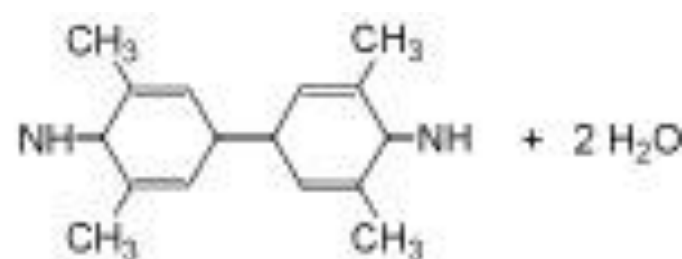
by standard Curve



**2,2',5,5'-Tetramethyl
-benzidine
(TMB)**

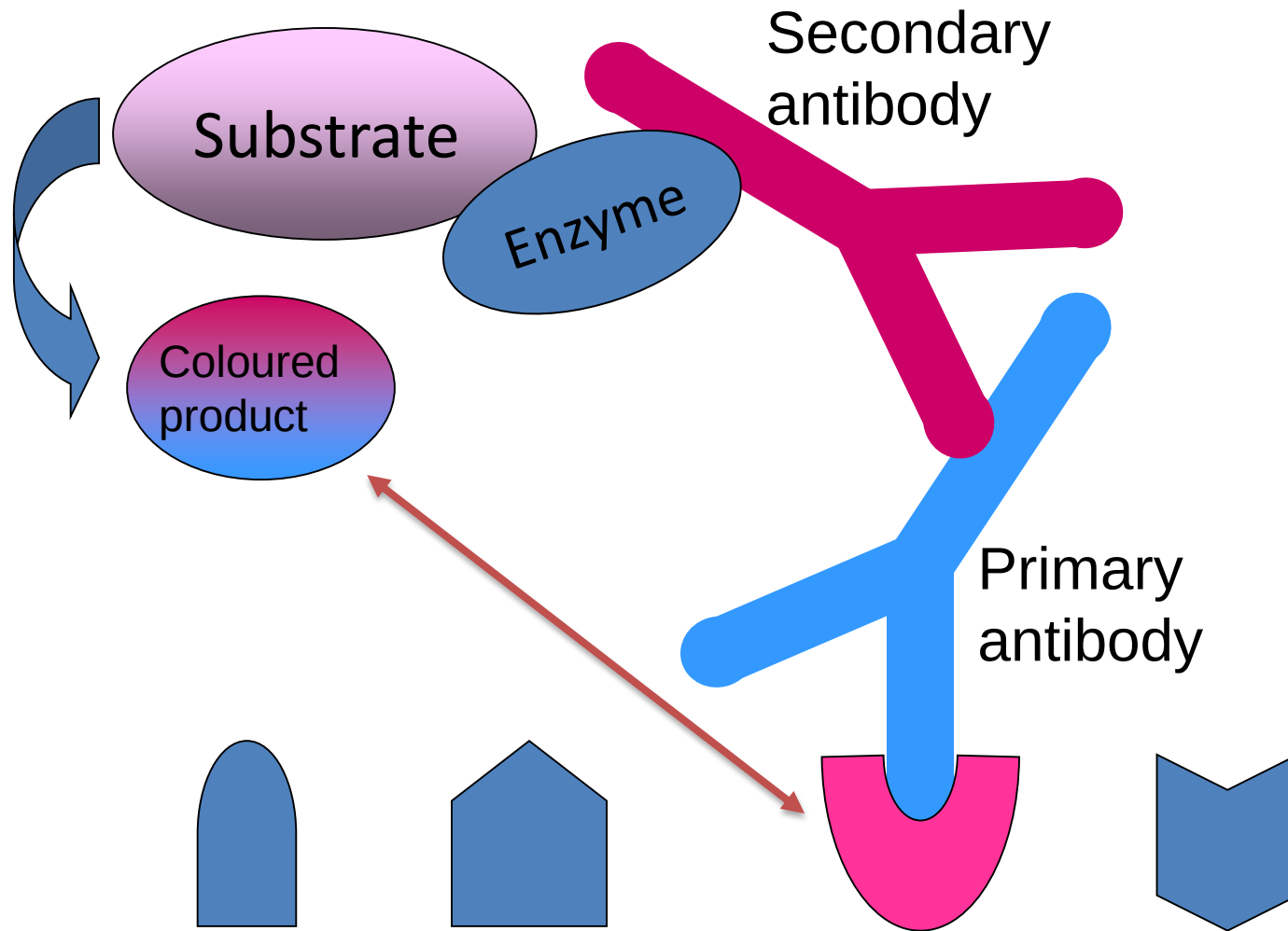


Horseradish-Peroxidase



absorbs at 285 nm

principle?



Different antigens in sample

What is an ELISA?

- Enzyme-linked immunosorbent assay
- Name suggests three components
 - Enzymatic amplification
 - Allows you to turn a little capture into a visible color change that can be quantified using an absorbance plate reader
 - Antibody (immuno)
 - Allows for specific detection of analyte of interest
 - Solid phase (sorbent)
 - Allows one to wash away all the material that is not specifically captured

Types of Primary Reactions

1. Noncompetitive binding method

- a) Ag measuring system (Ab coated wells)
- b) Ab measuring system (Ag coated wells)

2. Competitive binding method

Competition of labelled and unlabelled Ag to attach the coated Ab

Enzyme Linked Immunosorbent Assay

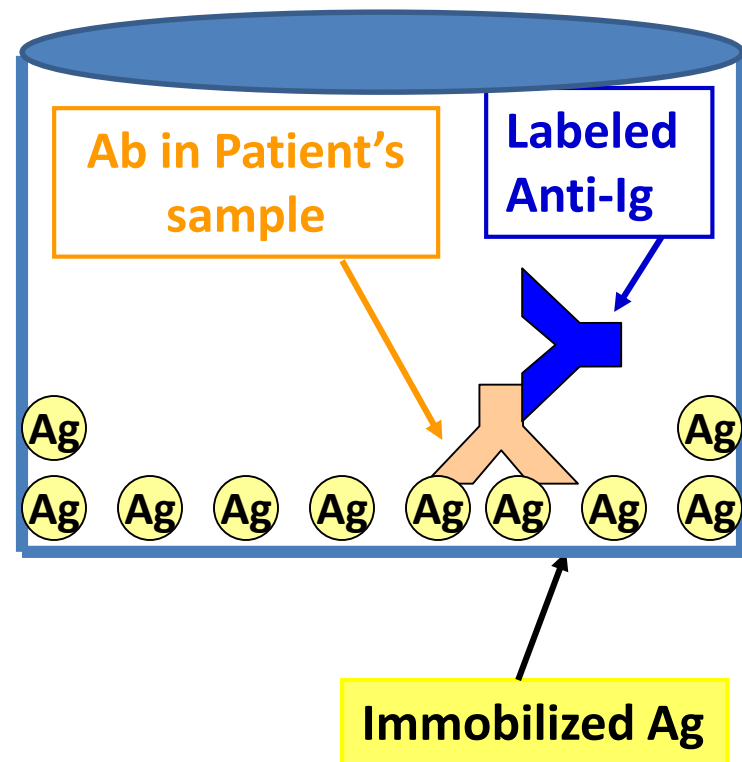
- In 1971 **Peter Perlmann** and **Eva Engvall** at Stockholm University invented ELISA which use an immune reaction similar to previously invented RIA method (1959), but differed in detection system.
- Detection was based on
 - Enzyme catalysed reaction
 - NOT radioactivity



Solid Phase Non-Competitive ELISA

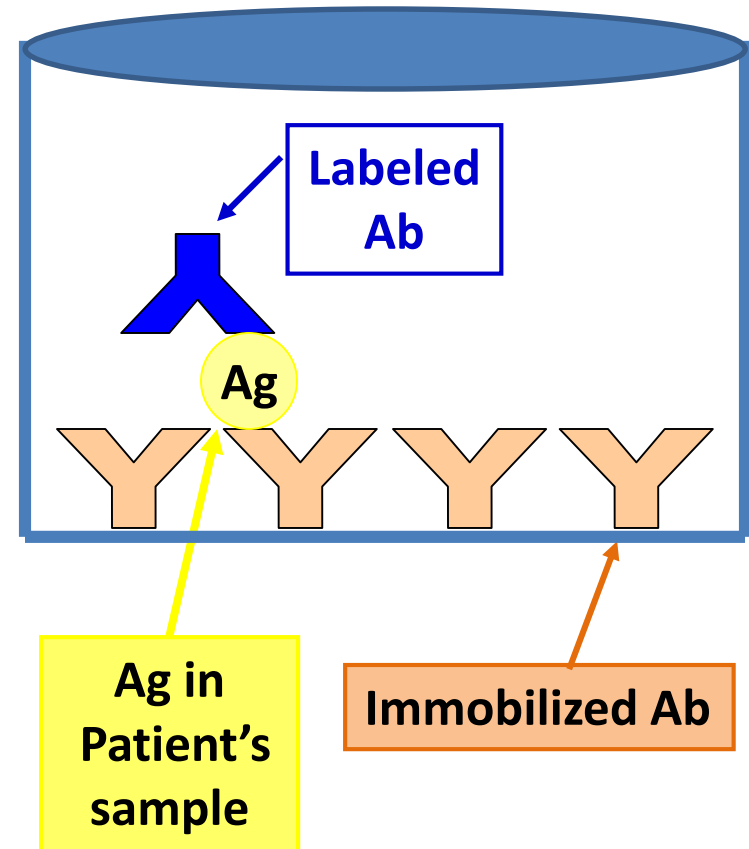
- **Ab detection**

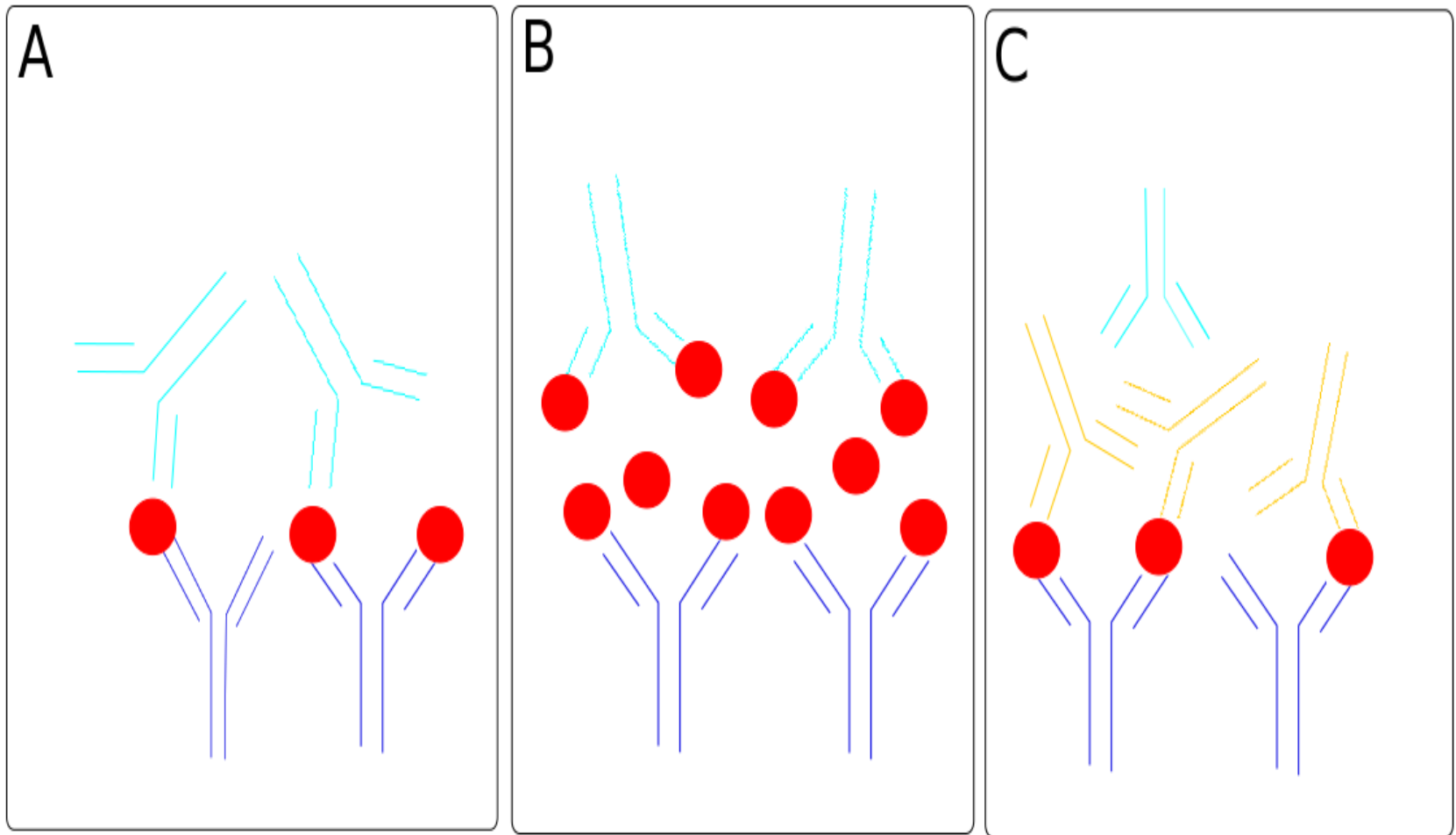
- Immobilize Ag
- Incubate with sample (containing Ab)
- Add labeled anti-Ig
- Amount of labeled Ab bound is proportional to amount of Ab in the sample



Solid Phase Non-Competitive ELISA

- **Ag detection**
 - Immobilize Ab
 - Incubate with sample (containing Ag)
 - Add labeled antibody
 - Amount of labeled Ab bound is proportional to the amount of Ag in the sample





Panel A: Normal sandwich elisa. The capturing antibody is shown in purple, antigen in red and detection antibody in turquoise.

Panel B: Excessive antigen binds up sites on both capturing and detection antibodies, causing reduced detection levels.

Panel C: If blocking antibodies are present (in orange), they compete with detection antibodies for antigen binding. Reduced detection levels results.

Toxoplasma IgG (ELISA) Kit contents

- **Microwells/Strips:** Toxoplasma Ag coated wells
- **Serum Controls:** Negative, Cut off, Positive
- **Diluent:** BSA 1% which is used for dilution of controls and patient sera
- **Secondary Ab:** Enzyme (HRP) conjugated anti-human Ab
- **Substrate:** HRP substrate+ chromogen
- **Stop solution:** 0.1 M acid
- Some of solutions contain **inert dyes** which help for better distinguishing.

Toxoplasma IgG (ELISA) Sampling

- **Serum Diluent:** Used as **Blank**, contains No Ab
- **Negative:** (1:21 diluted) Negative serum control
- **Calibrator:** (1:21 diluted) **Cut off** serum control
- **Positive:** (1:21 diluted) Positive serum control
- **Samples:** Sera from patients with unknown immune status to toxoplasma Gondii
- **How to load your strips**
 - Blank , Neg , Cal , Pos , Sample × 2
 - Incubate 30 min in RT

ELISA troubleshooting tips

No signal

Possible Causes	What can you do?
Assay set up incorrectly	Make sure that the instructions in the protocol is followed carefully.
Incorrect secondary antibody used	Check if the correct secondary antibody is used.
Insufficient antibodies used	Increase concentration of primary or secondary antibody.
Substrate reagents not fresh	Use fresh substrate reagents
Wrong settings of plate reader	Check the settings (wavelength, filters, gain etc) of plate reader.
Insufficient incubation	Follow the incubation time as indicated in the protocol booklet.
Sample concentration falls below detection limits of kit	Decrease dilution factor or concentrate samples.
Plate washing too vigorous	Check the setting of plate washer. Pipette wash buffer into wells gently.
Wells dried out	Cover plate with adhesive film or incubate in humidified chamber throughout experiment.
Enzyme inhibitor present in buffers or reagents	Inhibitors such as Sodium Azide can affect enzyme and assay performance. Ensure that there is no enzyme inhibitor in any buffers.

Weak signal

Possible Causes	What can you do?
Insufficient coating	Use more antigens or antibodies for coating.
Substrate reagents have expired or prepared at a wrong pH	Use fresh substrate reagents.

Variation among replicates

Possible Causes	What can you do?
Improper washing	Make sure that the washing is done as according to protocol.
Poor mixing of samples	Mix samples gently and evenly.
Dirty plate	Make sure that the bottom of plate is clean.
Reagents too old	Make sure that the reagents are not expired. Use freshly prepared reagents.
Bubbles in wells	Make sure that there is no bubble in wells before reading.
Inconsistent pipetting	Calibrate pipettes to make sure that the correct volume is dispensed.
Edge effects	Make sure that the plate and reagents are equilibrated to room temperature before starting assay.

High background

Possible Causes	What can you do?
Too much antibodies was used	Reduce the concentration of primary or secondary antibodies.
Antibodies bind nonspecifically	Use blocking buffer or choose another affinity-purified antibody.
Too much substrate reagent used	Use substrate with higher dilution.
Insufficient washing	Increase washing cycles.
Wrong concentration of blocking reagent	Check the recommended concentration of blocking buffer.
Reaction not stopped	Stop reactions with STOP buffer before reading.
Plate left too long before reading	Take measurements shortly after addition of substrate and STOP buffer.
Insufficient Tween in wash buffer	Use PBS+0.05% Tween as wash buffer.
Incubation temperature too high	Optimize incubation temperature for each experiment.
Plate stacking during incubation lead to uneven temperature throughout the plate	Avoid stacking plates together during incubation.
Pipetting error	Calibrate pipettes to make sure that the correct volume is dispensed.
Reagents not mixed properly	Make sure that all reagents are mixed properly and equilibrated to room temperature before assay
Salt concentration of incubation and wash buffer	Increase salt concentration to reduce nonspecific interaction.
Substrate incubation carried out in light	Perform substrate incubation in dark.
Dirty plate	Make sure that the bottom of plate is clean.

Poor standard Curve

Possible Causes	What can you do?
Improper standard dilution	Use appropriate diluent as blank. Make sure that the dilution is performed as according to protocol.
Standard improperly reconstituted	Briefly spin standard vial before opening. Make sure that there is no undissolved material after reconstituting.
Standard degraded	Store standards as according to protocol.
Curve doesn't fit the scale	Try plotting log-log or 5 parameter logistic curve fit.
Pipetting error	Calibrate pipettes to make sure that the correct volume is dispensed.
Incomplete washing	Increase washing cycles.

History of RIA

In 1959, Rosalyn Yalow & Soloman Berson invented RIA.

They applied radioisotopes for measurement of insulin.

RIA is the predecessor of modern immunoassays.



Dr. Rosalyn Yalow became the first female to win a Nobel Prize with her work on the radioimmunoassay

RIA

- RIA combines the **specificity of antibodies** (or any other ligands) with the **sensitivity and simplicity of radioactivity determination.**

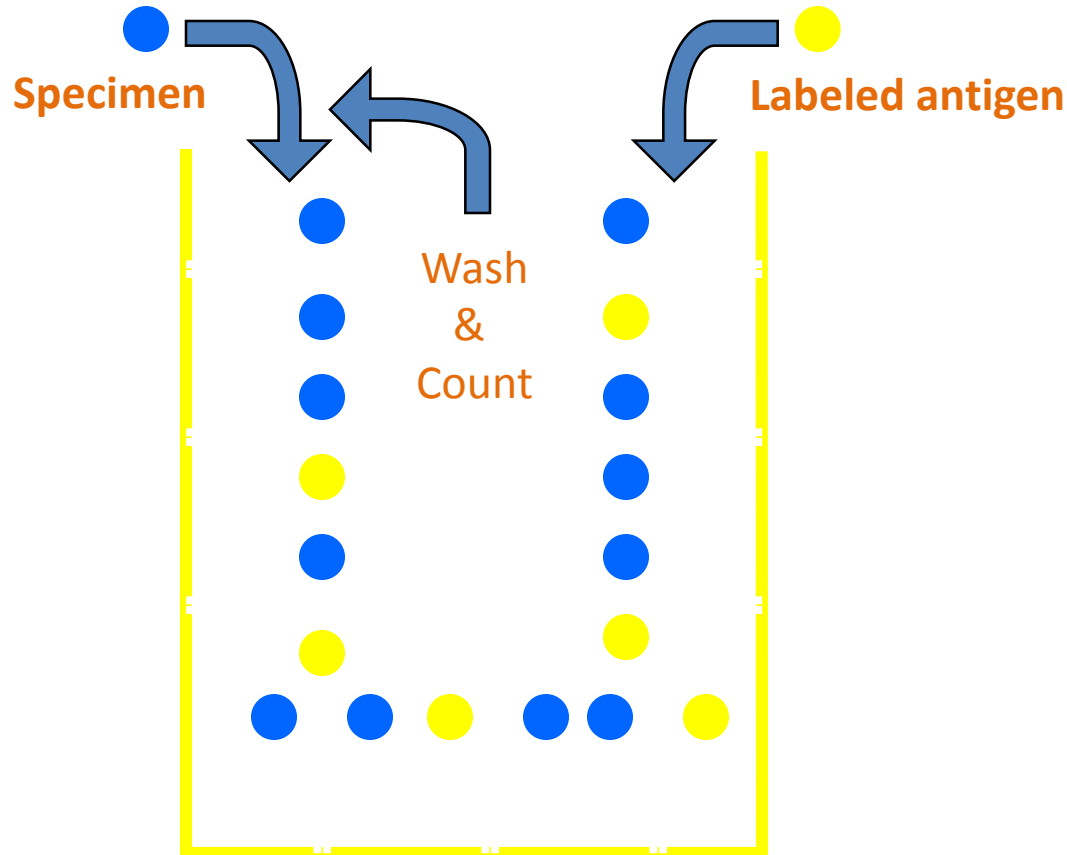
RIA (Radioimmunoassay)

- A highly sensitive and specific analytical assay which **uses the competition between radiolabelled and unlabelled substances in an Ag-Ab reaction** to determine the concentration of the unlabeled substance.
- Mostly applied for measurement of Hormones, Vitamins, Metabolites, Drugs and other small Ags.
 - (e.g. T3, T4, TSH, LH, FSH, ACTH, B12, Folic acid, Glucagon, Insulin, Testosterone, Barbiturates, Digoxin, Morphine, Prostaglandins, Glucocorticoids)

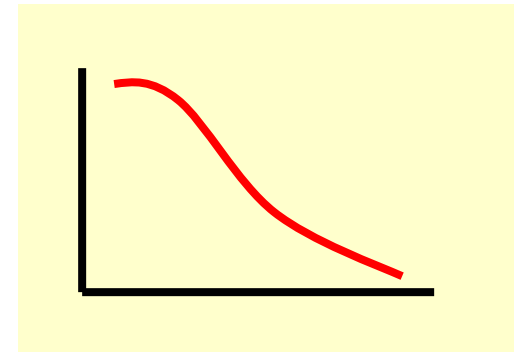
Competitive RIA in Coated tubes



Ag^* : RadAg : Ligand to be measured
labelled ligand



Radioactivity of bound residue is measured (CPM)
Ligand concentration is inversely related to radioactivity



Assay Procedure in RIA

- Sampling: Add known amounts of the sample / standards + labelled antigen into the tubes
- Incubate: Allow the reaction to reach completion
- Washing: Decant & wash contents of the well to remove all unbound antigens
- Measure radioactivity: The remaining radioactivity of the standard tubes is measured by a gamma counter
- Intensity of radioactivity is inversely correlated with the concentration of antigens in the test sample

Requirements for RIA

1. Preparation and Radiolabelling of the Antigen (^3H , ^{14}C , ^{125}I)
2. Binding of the Specific Antibody to tubes
3. Gamma counter

Disadvantages of RIA to ELISA

- **Radiation hazards**
- **Trained persons**
- **Special license for laboratory**
- **Shorter shelf life of reagents**
- **Gamma counter is needed**

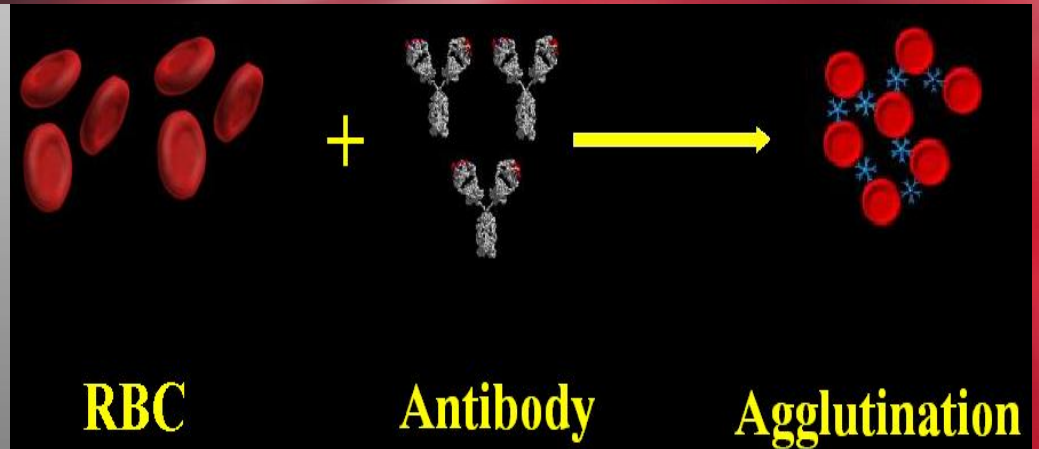
Secondary Ag / Ab Reaction

- **Agglutination**
- **Percipitation**
- **Fluccolation**

Agglutination Reactions

Principles and Practice

Immunology Department / TUMS



Outlines of Agglutination

- **Definition**
- **Classifications**
- **Applications**
- **Active Microbial Agglutination tests**
- **Passive Agglutination tests**

Serology Present & Past

- Serology as a science began in 1901 by the pioneering studies of **Karl Landsteiner** (1868-1943) on blood groups.
- Nowadays, complicated immunodiagnostic assays have become very popular in clinical laboratories.
- However, the initially described simple serology tests are still very useful and is routinely used too.



Agglutination

- Agglutination is a simple, sensitive test with different applications.
- Firstly observed by Gruber and Durham in 1896. They showed serum reaction with bacterial cells
- Overall, agglutination is based on **aggregation of particulate Ags by agglutinins (e.g. Abs).**

Approximate compression of sensitivity of common immunology tests

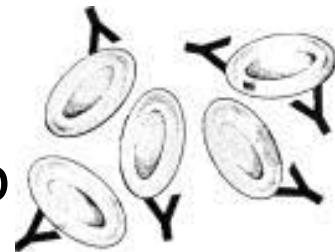
Method	Sensitivity ($\mu\text{g/ml}$)
Bacterial Agglutination	0.01
Heamagglutination	0.001-0.01
Passive agglutination	0.001
Precipitation	10
IFA	1
ELISA	0.001- 0.0001
RIA	0.001- 0.0001

Agglutination Steps

Agglutination takes place in two steps:

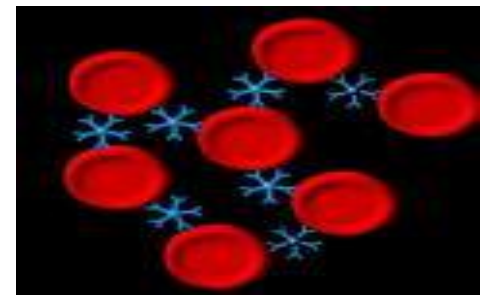
1. Sensitization or initial binding of molecules

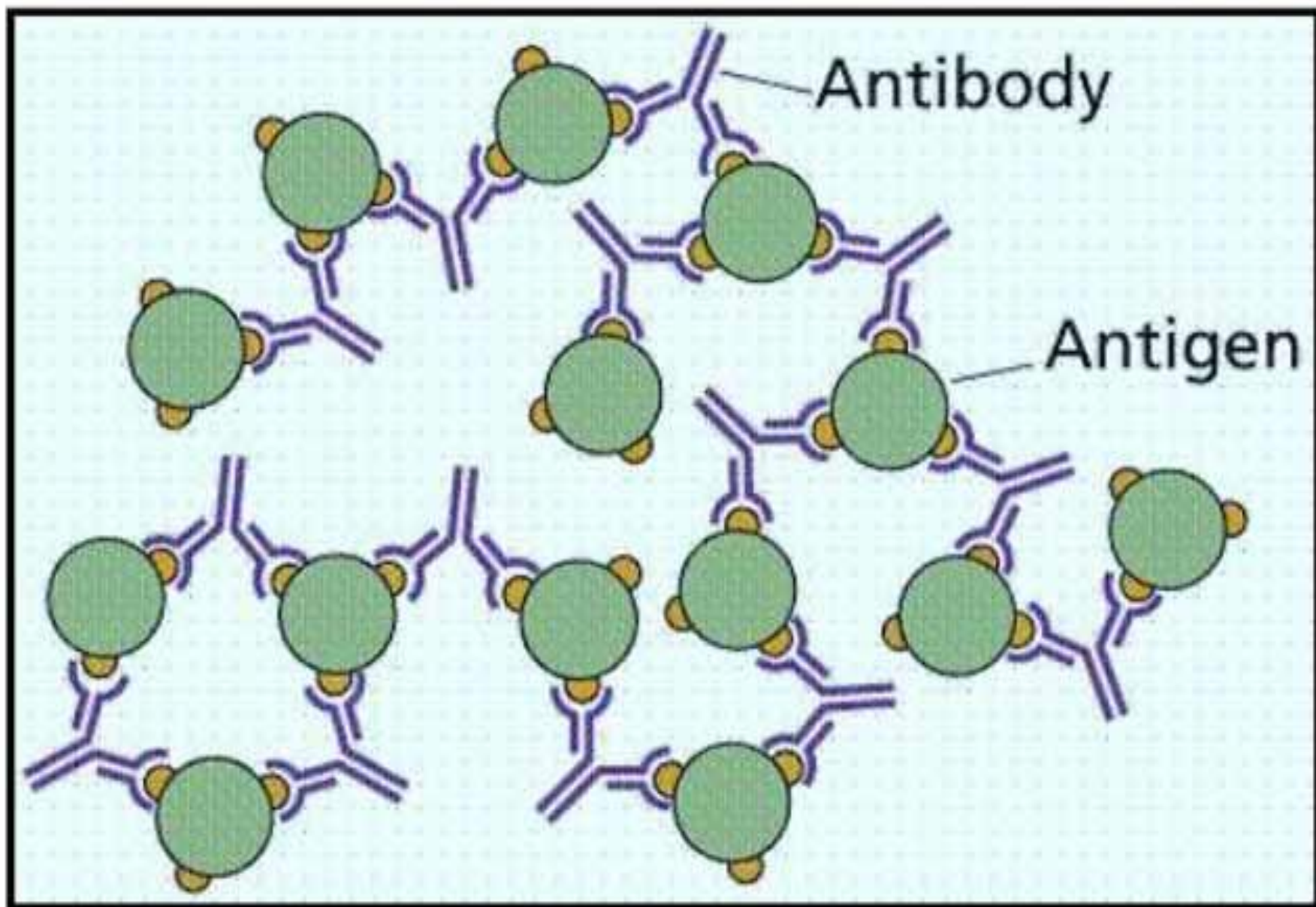
- Rapid and reversible attachment of Abs to particulate Ags



2. Lattice formation or aggregation or stabilization

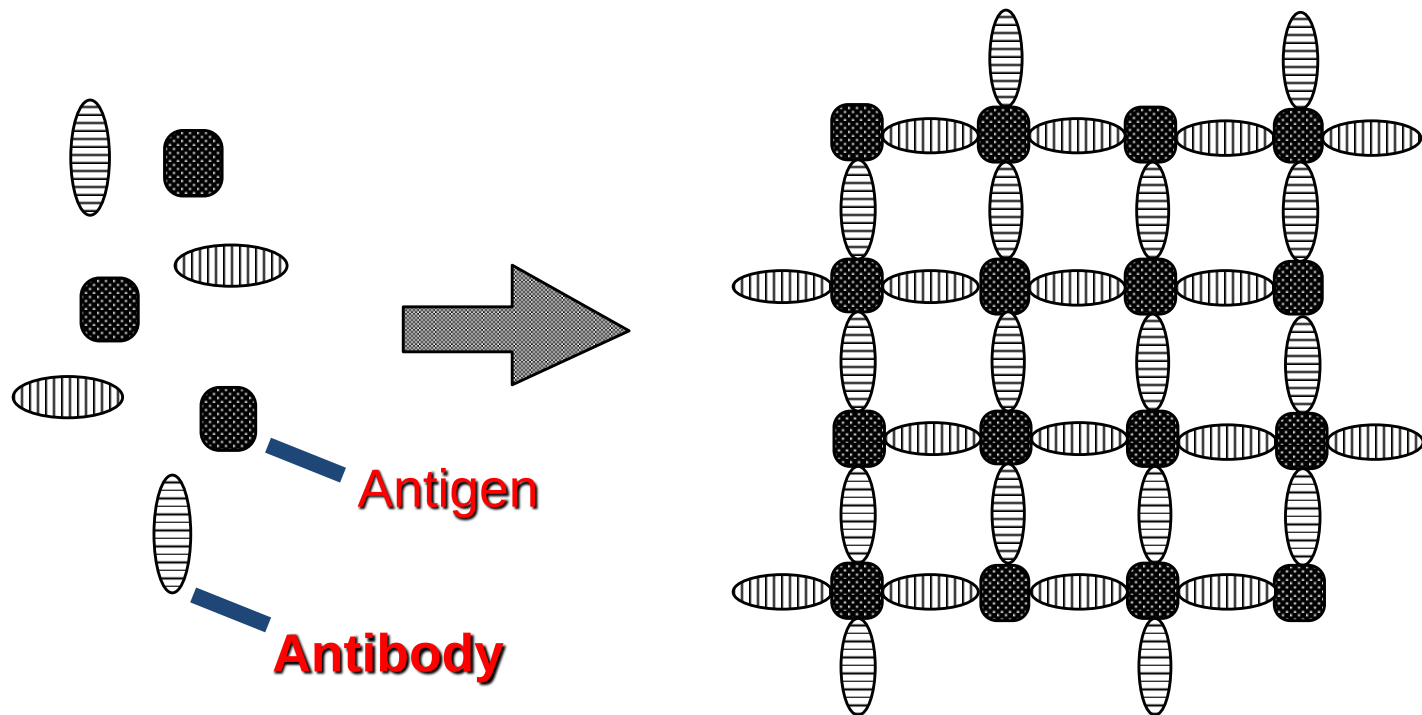
- Cross linking of particles and formation of visible enlarged aggregates

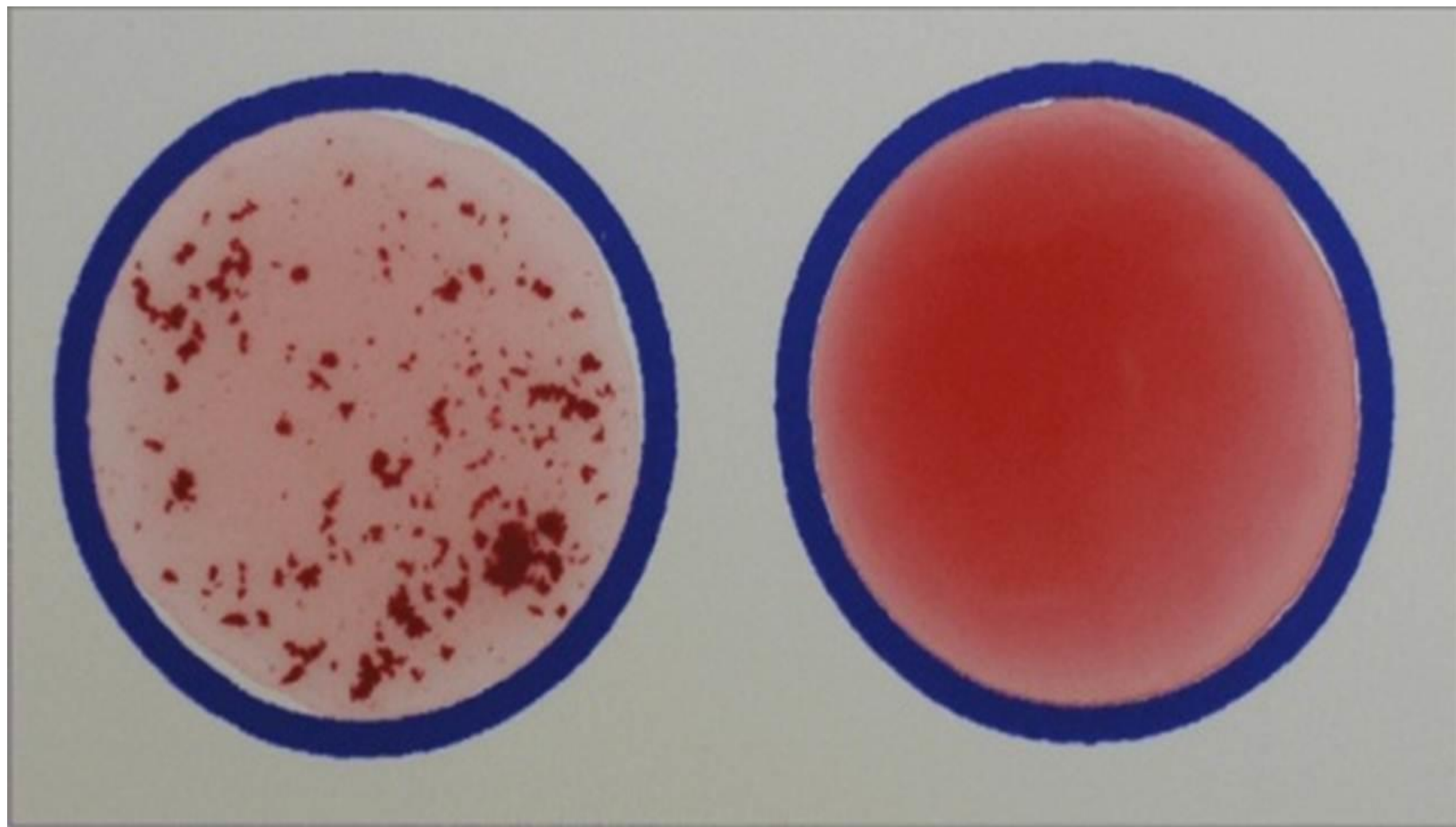




PRECIPITATION

- Is the reaction of soluble Ag with soluble Ab.
- The reaction results in the formation of Ag-Ab complexes (lattices)





+

-

Classification

(Based on Ag properties)

- **Active Agglutination Test**
- **Passive Agglutination Test**
- **Direct / Indirect Agglutination Test**

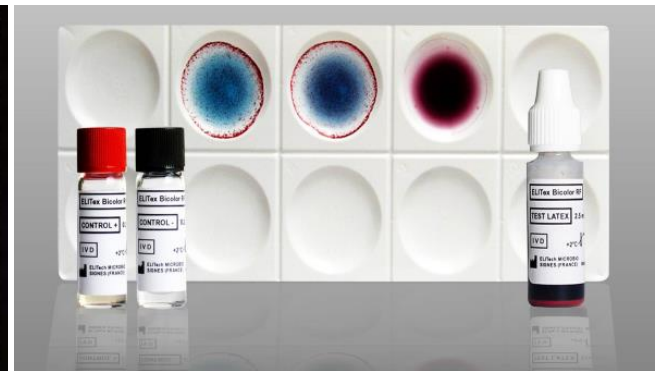
Classification

(Based on method of experiment)

- **Slide Agglutination Test (Rapid)**
- **Tube Agglutination Test (Standard)**
- **Microtiter plate Agglutination Test (Micro)**

Slid or Rapid Agglutination Test

- **Screening assay**
- **Qualitative** (Pos / Neg), (Pos: 1+, 2+, 3+, 4+): mostly used in passive agglutination tests
(Reactive/ Non-Reactive): blood grouping
- **Semi-quantitative** (Titer): Brucellosis (Wright), Sallomonellosis (Widal)

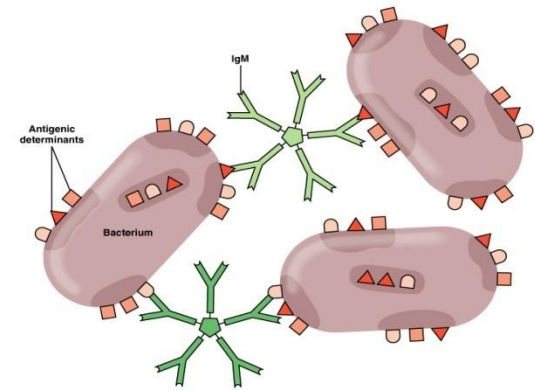


Tube Agglutination Test

- Also known as the **standard or Quantitative** agglutination test, e.g. Brucellosis (Wright)
- Serum is usually diluted in 2-fold manner
- Constant amount of Ag is added to each tube
- Incubation (overnight/ hours at RT or 37°C)
- Result is read as visible agglutination or precipitation of Ag clumps at the bottom of tubes

Active Agglutination

- Screening of Abs to surface Ags.
 - **Wright** and **Widal** tests
 - **Blood grouping (Back type)**
- Screening of surface Ags via specific Abs.
 - **Serotyping** of E. coli, Salmonella, Vibrio cholera, ... with specific antisera
 - **Blood groups (cell type)**



Serodiagnosis of Brucellosis as a Prototype

- **Brucellosis is the most common global zoonosis**
- **Serological diagnostic tests are well standardised and effective**
- **Serological diagnosis is based on whole cell Ag**
 - **B. abortus (Cross-reaction with B. melitensis & B. suis)**

Screening of Brucellosis

- Rapid screening test is performed on card or tile
- Antigen is rose bengal-stained suspension of smooth attenuated strain of *B. abortus*



Wright Tube Agglutination test

10	9	8	7	6	5	4	3	2	1	Tube
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.9	Saline
-									0.1	Serum
-	0.5 out	0.5 ←	0.5 ←	0.5 ←	0.5 ←	0.5 ←	0.5 ←	0.5 ←	0.5 ←	
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Ag
Control	1:5120	1:2560	1:1280	1:640	1:320	1:160	1:80	1:40	1:20	Titer

Wright Tube Agglutination test

In this case, the titre is 1/80

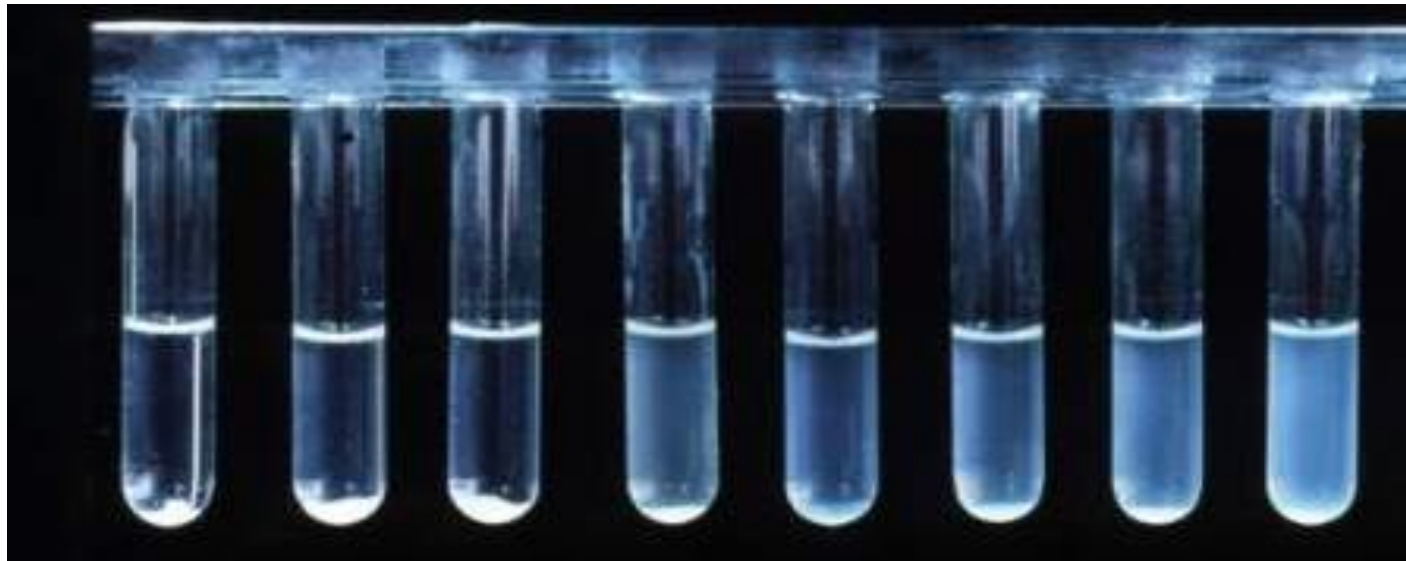
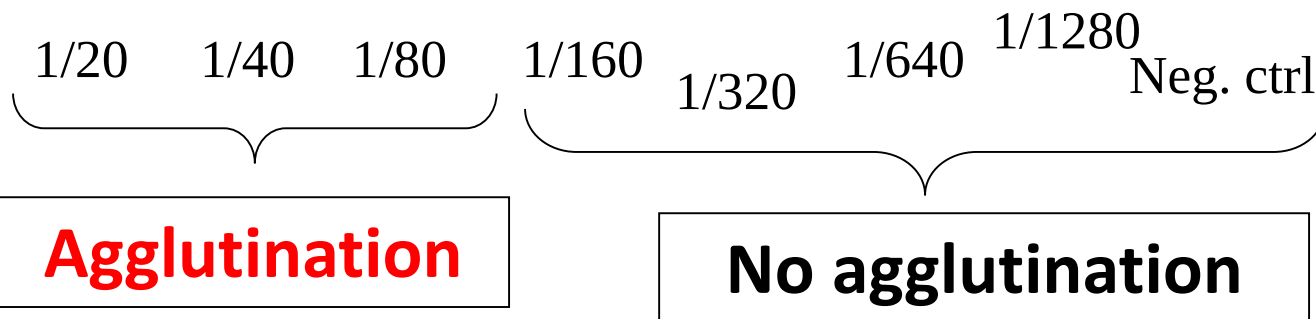


Table 9-1. Causes of False-Positive and False-Negative Reactions in Agglutination Testing

CAUSE	CORRECTION
False-Positive Reactions	
Overcentrifugation: Button is packed too tight and is difficult to resuspend.	Regulate centrifuge to proper speed and time.
Contaminated glassware, slides, or reagents: Contaminants (dust, dirt, or fingerprints) may cause cells to clump.	Keep supplies covered in storage and handle with care.
Autoagglutination: Test cells clump without specific antibody present; mainly a problem with red cells.	Use a control with saline and no antibody present. If the control is positive, test is invalidated.
Saline stored in glass bottles: Colloidal silica may leach out and cause agglutination.	Store saline in plastic containers.
Presence of cross-reactivity.	Use purified antigen preparations and specific monoclonal antibody whenever possible.
Presence of rheumatoid factor.	Test specifically for rheumatoid factor to rule out its presence. If rheumatoid factor is present, agglutination results must be interpreted carefully.
Presence of heterophile antibody: This occurs mainly when red blood cells are used as the carrier particle.	Preabsorb serum with red blood cells without specific antigen, or pretreat red blood cells to remove other antigens.
Delay in reading a slide test: Dried out antigen may look like agglutination.	Follow directions and read reactions immediately after incubation.

Table 9-1. Causes of False-Positive and False-Negative Reactions in Agglutination Testing

False-Negative Reactions

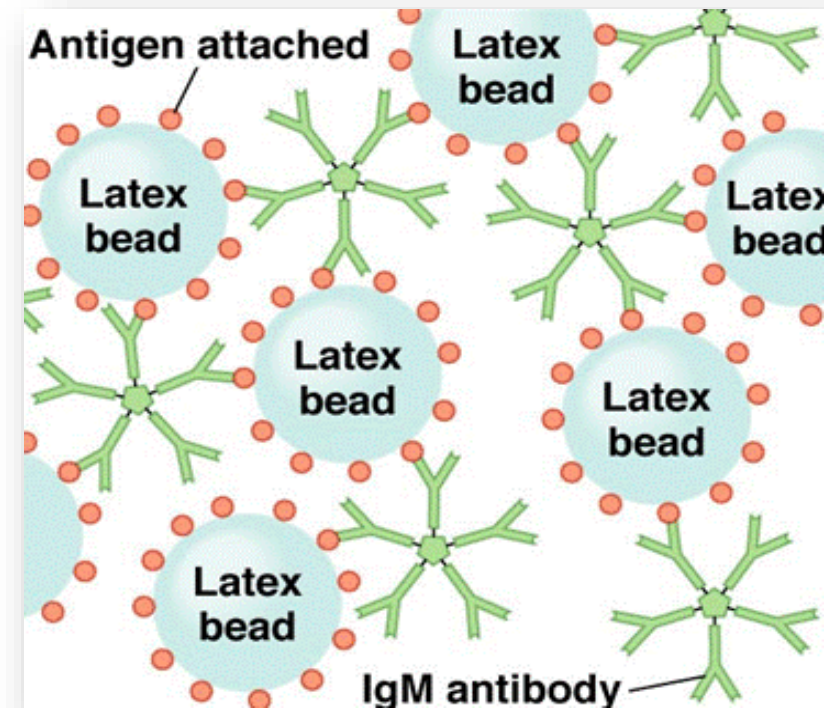
Under centrifugation: If sample is undercentrifuged, cells may not be close enough to interact.	Regulate centrifuge to proper speed and time.
Inadequate washing of cells, especially in antiglobulin testing: Unbound immunoglobulins may neutralize the antihuman globulin.	Wash cells thoroughly, according to the procedure being followed. Use control cells on negative reactions.
Reagents not active: This may be caused by improper storage.	Refrigerate antisera, but do not freeze because loss of activity may occur.
Delays in testing procedures: This especially pertains to antiglobulin testing. Antibody may be eluted from red blood cells.	Once a procedure is begun, follow through to the end without delay.
Incorrect incubation temperature: Too low a temperature may result in the lack of association of antigen and antibody.	Check temperature at which test is carried out.
Insufficient incubation time: Antigen and antibody may not have time for association.	Follow instructions carefully.
Prozone phenomenon: Too much patient antibody for amount of test.	If this is suspected, dilute antibody and repeat the test.
Failure to add antiglobulin reagent: This occurs mainly in direct and indirect antiglobulin testing.	Add check cells that are antibody-coated to see if agglutination occurs after a negative test. If there is still no agglutination, disregard the results and repeat.

TABLE 10.4 Causes of False-Positive and False-Negative Agglutination Test Reactions

Cause	Correction
False-Positive Reactions	
Contaminated equipment or reagents may cause particles to clump.	Store equipment and reagent in clean, dust-free environment, and handle with care. Use negative quality control (QC) steps.
Autoagglutination	Use a control with saline and no antibody as a negative control. If positive, patient's result is invalid.
Delay in reading slide reactions results in drying out of mixture.	Follow procedural directions and read reactions exactly as specified.
Overcentrifugation causes cells or particles to clump too tightly.	Calibrate centrifuge to proper speed and time.
False-Negative Reactions	
Inadequate washing of red blood cells in antihuman globulin (AHG) testing may result in unbound immunoglobulins neutralizing the reagent.	Wash cells according to directions. Use positive and negative QC steps.
Failure to add AHG reagent	Use positive QC steps.
Contaminated or expired reagents	Use positive and negative QC steps.
Improper incubation	Follow procedural protocol exactly. Use positive and negative QC steps.
Delay in reading slide reactions	Follow procedural protocol exactly. Use positive and negative control steps.
Undercentrifugation	Calibrate centrifuge to proper speed and time.
Prozone phenomenon	Dilute patient serum containing antibody, and repeat the procedure.

Passive Agglutination

- Agglutination reaction of inert particles coated with **soluble Ags** (Ags that are not normally found in the cell surfaces)
- **Inert Ag carriers:**
 - Red blood cells (O neg)
 - **Polystyrene latex**
 - Bentonite
 - Charcoal



Common Passive Agglutination tests

- **CRP** (C-reactive protein)
- **ASO** (Anti-streptolysin O)
- **Gravindex (HCG)**
- **RF** (Rheumatoid factor)
- If Ab is linked to Latex (e.g. **RF test**) the test is named **Reverse Passive Agglutination Test.**

Principle

The C-reactive protein rapid latex **agglutination** test is based on the reaction between patient serum containing CRP as the antigen and the corresponding antihuman (CRP) antibody coated to the treated surface of latex particles. The coated particles enhance the detection of an agglutination reaction when antigen is present in the serum being tested. The clinical applications of CRP evaluation include detecting inflammatory diseases, particularly infections. It is also a useful indicator in screening for organic disease, screening for inflammatory and malignant disease, and monitoring therapy in inflammatory diseases. Because CRP is more rapidly synthesized than other acute-phase proteins, assays of CRP are the measurement of choice in suspected inflammatory conditions.

See  instructor site for the procedural protocol.

Reporting Results

In patients who are free of inflammation and tissue necrosis, CRP is absent from the serum or present in concentrations below 0.5 mg/dL. Reference range mean values are 0.01 mg/dL in newborns and less than 0.05 mg/dL in adult men and nonpregnant women.

Positive Reaction

Agglutination of the latex suspension is a positive result. A positive reaction is reported when the undiluted specimen or the 1:5 diluted specimen demonstrates agglutination, or when both exhibit agglutination.

Negative Reaction

The absence of visible agglutination and the presence of opaque fluid constitute a negative reaction. A negative reaction is reported only when both the undiluted specimen and the 1:5 diluted specimen exhibit no visible agglutination.

Comments

Specimen collection and handling are important to the quality of the test. Strict adherence must be paid to technique, with a special emphasis on drop size, complete mixing, reaction time, and temperature of reagents.

The strength of a positive reaction may be graded as follows:

- 1+ Very small clumping with an opaque fluid background
- 2+ Small clumping with a slightly opaque fluid background
- 3+ Moderate clumping with a fairly clear fluid background
- 4+ Large clumping with a clear fluid background

Sources of Error

False-positive results may be observed if serum specimens are **lipemic**, hemolyzed, or heavily contaminated with bacteria. If the reaction time is longer than 2 minutes, a false-positive result may also be produced from a drying effect.

False-negative results may be observed in undiluted serum specimens because of high levels of CRP (antigen excess). A 1:5 dilution of serum is also tested for this reason.

Limitations

Because the latex slide agglutination test is a qualitative and semiquantitative procedure, other methods such as nephelometry should be used for the quantitative determination of the CRP level when indicated. The strength of the agglutination reaction is not always indicative of the CRP concentration. Weak reactions may be produced in samples with elevated or low CRP values. Results may vary, depending on the patient's condition.

This 2-minute slide latex agglutination test has a detection level of 1 mg CRP/dL; therefore patients with CRP values less than 1 mg/dL may go undetected. The sensitivity of the procedure has been assessed at 93%.

Clinical Applications

With the onset of a substantial inflammatory event (e.g., infection, MI, surgery), the CRP level usually increases significantly (>tenfold) above the reference range values for healthy individuals. The test is clinically useful for the early detection of inflammatory diseases (particularly infections), as an indicator in screening for organic diseases, and in monitoring patient progress.

Passive Reversed Agglutination

Ag + **Anti CRP Antibody** (Latex)



CRP Positive



CRP Negative



CRP Negative

TABLE 5.8 Examples of Clinically Useful Acute-Phase Proteins

Protein	Normal Concentration (g/L)	Concentration in Acute Inflammation (g/L)	Response Time (hr)
C-reactive protein	0.0008–0.004	0.4	6–10
α_1 -Antichymotrypsin	0.3–0.6	3.0	10
α_1 -Antitrypsin	2.0–4.0	7.0	24
Orosomucoid	0.5–1.4	3.0	24
Haptoglobin	1.0–3.0	6.0	24
Fibrinogen	2.0–4.5	10.0	24
C3	0.55–1.2	3.0	48–72
C4	0.2–0.5	1.0	48–72
Ceruloplasmin	0.15–0.6	2.0	48–72

Table 3-2 Characteristics of Acute-Phase Reactants

PROTEIN	RESPONSE TIME (HR)	NORMAL CONCENTRATION (MG/DL)	INCREASE	FUNCTION
C-reactive protein	4–6	0.5	1000X	Opsonization, complement activation
Serum amyloid A	24	5	1000X	Activates monocytes and macrophages
Alpha ₁ -antitrypsin	24	200–400	2–5X	Protease inhibitor
Fibrinogen	24	200–400	2–5X	Clot formation
Haptoglobin	24	40–290	2–10X	Binds hemoglobin
Ceruloplasmin	48–72	20–40	2X	Binds copper and oxidizes iron
Complement C3	48–72	60–140	2x	Opsonization, lysis

BOX 5.3 Major Applications of Acute-Phase Protein Measurements

- Monitoring the progress of diagnosed disease activity
- Assessing response to therapy in inflammatory diseases (e.g., rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, vasculitis, rheumatic fever)
- Detection of complications of a known disease (e.g., immune complex deposition, postsurgical infection)

Table 1

Acute-phase proteins

	Increased concentrations	Decreased concentrations
Protease inhibitors	alpha ₁ -antitrypsin antichymotrypsin	
Coagulation proteins	fibrinogen prothrombin factor VIII plasminogen	
Complement proteins	C1s, C2, C3, C4, C5 factor B C1 esterase inhibitor plasminogen	
Transport and storage proteins	haptoglobin haemopexin caeruloplasmin ferritin	transferrin
Miscellaneous	C-reactive protein procalcitonin serum amyloid protein fibronectin alpha ₁ -acid glycoprotein	albumin pre-albumin

Table 17.1 Levels of CRP and their common associations

Level of CRP	Common associations
Little or no change (<4 – 100mg/L)	Most viral infections Active SLE Systemic sclerosis and CREST Inactive Rha Myeloma Most tumours
Moderate elevation (100 – 200mg/L)	EBV/CMV infection Bacterial infection Active rheumatoid arthritis Polymyalgia rheumatic Temporal arteritis Lymphoma Hypernephroma
Large elevation ($>200\text{mg/L}$)	Severe bacterial sepsis Legionella Active vasculitis (Wegener's, rheumatoid)
Huge elevation ($>400\text{mg/L}$)	Overwhelming sepsis (deep tissue abscess) Fulminant legionella At this level, death is the usual outcome

Table 2. Discordant values in Hospitalized Patients

High ESR/Low CRP

- Infections (Bone and joint)
- Connective tissue disease (SLE)
- Ischemic stroke
- Malignancy
- Renal insufficiency
- Low serum albumin

High CRP/Low ESR

- Infections (urinary tract, gastrointestinal tract, lung and bloodstream)
 - Myocardial infarction
 - Venothromboembolic disease
 - Rheumatoid arthritis
 - Low serum albumin
-

Abbreviations: ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SLE, systemic lupus erythematosus.

hsCRP

- **The risk of developing cardiovascular disease is quantified as follows:**
- low: hs-CRP level under 1.0 mg/L
- average: between 1.0 and 3.0 mg/L
- high: above 3.0 mg/L

PROCEDURE: RAPID AGGLUTINATION

Principle

The principle of serologic testing for rheumatoid arthritis is based on the detection of macroglobulins collectively called *RF*. RF behaves like antibodies against human gamma globulin (IgG).

In rapid testing, sheep red blood cells (RBCs) or latex reagent consists of a stabilized RBC or latex suspension coated with albumin and chemically bonded with denatured human gamma globulin. This reagent serves as an antigen in the procedure. If RFs are present in the serum, macroscopic agglutination will be visible when the reagent is mixed with the serum.

The determination of RFs is important in the prognosis and therapeutic management of rheumatoid arthritis; however, biologically false-positive test results may be observed in a variety of disorders, such as SLE, Sjögren's syndrome, syphilis, and hepatitis.

Procedure Notes

The strength of a positive reaction may be graded as follows:

- 1+: Very small clumping with opaque fluid background
- 2+: Small clumping with slightly opaque fluid background
- 3+: Moderate clumping with fairly clear fluid background
- 4+: Large clumping with clear fluid background

Results

Qualitative Test Results*

- Negative: No agglutination or color change is seen in the reagent. The reagent should have a smooth appearance against a yellow/greenish background.
- Positive: The agglutinated reagent will become visibly blue against a yellow background.
- If a positive result is obtained with the undiluted specimen, the specimen should be diluted 1:10 to determine the relationship between the level of RF present and a particular disease state.
- If the 1:10 diluted specimen demonstrates readily visible agglutination, RF is present in the specimen at a level generally associated with RA. If the undiluted specimen demonstrates agglutination and the 1:10 diluted specimen demonstrates the absence of agglutination or a fine granular background, RF is present in the specimen at a low level, which may exist in disease states other than RA.

Semiquantitative Test Results

When positive samples are examined by serial dilution, the titer is the reciprocal of the last dilution that produced a positive result (agglutination).

Sources of Error

False-positive results may be observed if the following occurs:

- Serum specimens are lipemic, hemolyzed, or heavily contaminated with bacteria.
- The reaction time is longer than 3 minutes. A false-positive result may also be produced as a result of a drying effect.

Biological false-positive results can be manifested by disorders such as SLE, Sjögren's syndrome, syphilis, and hepatitis. A low rate of positive reactions has been observed in abnormalities such as periarteritis nodosa, rheumatic fever, osteoarthritis, tuberculosis, cancer, some diseases of viral origin, osteoarthritis, arthritis type undetermined, myositis, and polymyalgia rheumatica. Circulating RF appears to represent a phenomenon of aging, independent of disease.

Clinical Applications

RF is present in the serum of approximately 70% to 80% of patients with clinically diagnosed RA. Almost all patients with variants of RA (e.g., Felty's or Sjögren's syndrome) demonstrate positive results. The highest titers are often found in severe cases of RA. Although the latex agglutination procedure has a 95% correlation with a clinical diagnosis of probable or definite RA, RF is not exclusively limited to patients with RA.

In using latex tests for the detection of RF, a positive result can be expected in less than 5% of healthy individuals. In patients 60 years and older, as many as 30% may be seropositive.

Limitations

As with other diagnostic procedures, the results obtained by an assay yield valuable data that must be evaluated as a component of the total clinical information obtained by the physician. Approximately 25% of patients with definite RA may exhibit negative results for serum RF. Specimens from patients with JIA are usually negative for circulating RF. The strength of the agglutination reaction in the qualitative procedure is not indicative of the actual titer. Weak reactions may occur with slightly elevated or greatly elevated concentrations.

*Wampole Colorcard RF agglutination test (Wampole Laboratories, Cranbury, NJ).

TABLE 1 Summary of well-characterized autoantigens in rheumatoid arthritis

Class	Autoantigen
Citrullinated proteins	ACPA, Sa, vimentin, filaggrin, fibrin binding globulin
Articular cartilage	Collagen II
Serum protein	RF
Nuclear components	RA33
Enzymes	Calpastatin, glucose-6-phosphate isomerase
Stress protein	BiP
Glycoprotein	Fibronectin

TABLE 1 Current and future biomarkers for rheumatoid arthritis: diagnosis and prediction^a

Biomarker	Disease specificity	Mechanistic biomarker	Reference(s)
Current RA			
Rheumatoid factor	+++	++	5, 26, 32, 33
Anti-CCP			
ESR	++++	+++	
CRP	+	—	
	+	—	
Future RA			
ACPA subtypes	+++	+++	26, 30, 34–36
Novel autoantibodies	++	?	37–39
Multibiomarker disease activity test	++	++	40
Cytokines/chemokines	+	+++	28, 30, 41
MMPs	+	++	40
Genetic polymorphisms	+	+++	42–45
Functional cytometry	+	++	46

^aCCP, cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MMPs, matrix metalloproteinases.

Table 30-1 2010 ACR-EULAR Classification Criteria For Rheumatoid Arthritis*

Parameter	Score (Points)
A. Joint involvement^{†,‡}	
• One large joint [§]	0
• Two to ten large joints	1
• One to three small joints (with or without involvement of large joints) [‡]	2
• Four to 10 small joints (with or without involvement of large joints)	3
• >Ten joints (at least one small joint)	5
B. Serology (at least one test result is needed for classification)	
• Negative RF <i>and</i> negative ACPA	0
• Low-positive RF <i>or</i> low-positive ACPA	2
• High-positive RF <i>or</i> high-positive ACPA	3
C. Acute-phase reactants (at least one test result is needed for classification)[#]	
• Normal CRP <i>and</i> normal ESR	0
• Abnormal CRP <i>or</i> abnormal ESR	1
D. Duration of symptoms^{**}	
• <6 wk	0
• ≥6 wk	1



ANTISTREPTOLYSIN O LATEX TEST KIT

Principle

In the antistreptolysin O (ASO) latex test (Biotech Laboratories, Suffolk, England), the ASO reagent contains latex particles coated with streptolysin O (SLO) antigen. When the reagent is mixed with serum containing ASO, the particles will agglutinate, which is interpreted as a positive sample. Detection of ASO in serum may aid in the diagnosis of streptococcal infections.

Infections promoted by acute streptococcal infection result in the production of ASO antibodies because of the presence of the SLO antigen liberated by the bacteria.

Reference Intervals

Normal adult levels are less than 200 IU/mL. However, because values may vary with age, gender, diet, or geographic location, it is recommended that each laboratory establish its own reference range.

Limitations

Elevated levels of ASO have also been found in patients suffering from scarlet fever, acute rheumatoid arthritis, tonsillitis, and other streptococcal infections, as well as healthy carriers. Early infections and children aged 6 months to 2 years may cause false-negative results. A single positive ASO result does not provide much information about the state of the disease. Therefore it is advisable to perform titrations at biweekly intervals over 4 to 6 weeks to follow progression of the disease. The clinical diagnosis should be made in conjunction with clinical and laboratory data, not just on the findings of a single test result.

Clinical Applications

Detection of ASO in serum may aid in the diagnosis of streptococcal infections. Information on the extent and degree of infection can be obtained from the measurement of serum ASO levels. Increased ASO levels are also associated with rheumatic fever and glomerulonephritis.

TABLE 3 Host immune response to group A streptococcal infection

Streptococcal serological test	Antigen
Test for extracellular streptococcal antigen response	
Tests for nonspecific immunogenic antigens (extracellular antigens)	
Anti-streptolysin O (ASO)	Streptolysin O
Anti-DNase B	DNase B
Anti-streptokinase ^a	Streptokinase
Anti-streptococcal hyaluronidase ^a	Hyaluronidase
Anti-NADase ^a	NADase
Tests for specific immunogenic antigens (cellular components)	
Anti-streptococcal C5a peptidase ^a (anti-SCPA)	Streptococcal C5a peptidase (group A)
M protein antibody ^a	Type-specific M protein
Anti-A-carbohydrate ^a	Group A carbohydrate

^aNot commercially available but used in research laboratories.



PROCEDURE: PREGNANCY LATEX SLIDE AGGLUTINATION

Principle

The rapid, direct, monoclonal latex slide agglutination test for detection of hCG is based on the principle of agglutination between latex particles coated with anti-hCG antibodies and hCG, if present, in the test specimen.

See  instructor site for the procedural protocol.

Results

- Agglutination within 2 minutes represents a positive reaction.
- No agglutination represents a negative reaction.

Technical Sources of Error

Reagents should never be expired; latex reagent must be well shaken, and agglutination should be read within 3 minutes to avoid erroneous results caused by evaporation.

False-Positive Results

If a patient has been given an hCG injection (e.g., Pregnyl) to trigger ovulation or lengthen the luteal phase of the menstrual cycle, trace amounts can remain in the patient's system for as long as 10 days after the last injection. This will produce a false-positive result. Two consecutive quantitative hCG blood assays can circumvent this problem. If the hCG level increases by the second test, the patient is probably pregnant. Chorioepithelioma, hydatidiform mole, or excessive ingestion of aspirin may give false-positive results.

In men, a test identical to that used for pregnancy may be performed to detect the presence of a testicular tumor. If MAb against the β subunit is not used, other hormones with the same α unit may cross-react and cause a false-positive reaction.

False-Negative Results

Testing before reaching detectable levels of hCG will yield false-negative results.

Alternative Procedural Protocols

Latex agglutination slide tests have been replaced in many situations by one-step chromatographic color-labeled immunoassays for the qualitative detection of hCG in urine (e.g., Clearview hCG II and Clearview hCG Easy, Wampole Laboratories, Princeton, NJ). Another variation is a one-step chromatographic color-labeled immunoassay for use with urine or serum (e.g., Wampole PreVue hCG Stick or Cassette, Status hCG).



Principle

The BBLMonoSlide procedure (BD BBLMonoSlide, Becton Dickinson, Franklin Lakes, NJ) is based on the agglutination of horse erythrocytes by heterophile antibody present in infectious mononucleosis. Because horse red blood cells (RBCs) exhibit antigens directed against both Forssman and infectious mononucleosis antibodies, a differential absorption of the patient's serum is necessary to distinguish the specific heterophile antibody from those of the Forssman type.

The basic principle of the absorption steps in this procedure is comparable to that originally described by Davidsohn in the sheep agglutinin test. Serum or plasma is absorbed with both guinea pig kidney and beef erythrocyte stroma. Guinea pig kidney contains only the Forssman antigen, and beef erythrocytes contain only the antigen associated with infectious mononucleosis. Guinea pig kidney will absorb only heterophile antibodies of the Forssman type, and beef erythrocytes will absorb only the heterophile antibody of infectious mononucleosis. Agglutination of horse RBCs by the absorbed patient specimen indicates a positive reaction for heterophile antibody.

The BBLMonoSlide Test uses a disposable card, guinea pig kidney antigen for absorption, and specially treated horse erythrocytes (color-enhanced) to increase specificity and sensitivity and to enhance readability. No special equipment is required to read the BBLMonoSlide Test results.

Reporting Results: Qualitative Method

Positive

A positive infectious mononucleosis reaction will have dark clumps against a blue-green background, distributed uniformly throughout the test circle.

Negative

A negative reaction will have no agglutination but may have fine granularity against a brown-tan background. Peripheral color development associated with fine granularity should be interpreted as negative (e.g., giant blue-green halo on the periphery of the test circle should not be interpreted as a positive result).

Procedure Notes

If a positive qualitative result is demonstrated, a titration procedure may be performed to provide a quantitative indication of the level of heterophile antibody.

Sources of Error

For accurate results, only clear, particle-free serum or plasma specimens should be used. False-positive results can be caused by the following:

1. Observing agglutination after the observation time
2. Misinterpreting agglutination
3. Report of simultaneous occurrence of infectious mononucleosis and hepatitis

A result interpreted as a false-positive may be caused by residual heterophile antibody present after clinical symptoms have subsided.

Clinical Applications

Infectious diseases such as influenza, rubella, and hepatitis may cause clinical symptoms that mimic those of infectious mononucleosis and present problems in diagnosis. Although the final diagnosis of infectious mononucleosis depends on clinical, hematologic, and serologic findings, a positive test result indicates the presence of the heterophile antibody specific for infectious mononucleosis.

Limitations

The diagnosis of infectious mononucleosis should be based on the results of all clinical and laboratory findings. Some segments of the population do not produce detectable heterophile antibody (e.g., approximately 50% of children younger than 4 years old and 10% of adolescents). Detectable levels of heterophile antibody may persist for months and, more rarely, for years in some individuals.

NOTE: Other forms of rapid testing include Wampole Laboratories Colorcard Mono and Mono-plus.

PROCEDURE: PAUL-BUNNELL SCREENING TEST

Principle

The classic Paul-Bunnell test is a hemagglutination test designed to detect heterophile antibodies in patient serum when mixed with antigen-bearing sheep erythrocytes. Dilutions of inactivated patient serum are mixed with sheep erythrocytes, incubated, centrifuged, and macroscopically examined for agglutination. Positive reactions are preliminary.

See  instructor site for the complete procedural protocol and information related to the procedure.

Sources of Error

False-positive reactions have been observed in conditions such as hepatitis infection and Hodgkin disease. An improperly inactivated serum will produce hemolysis.

Clinical Applications

The Paul-Bunnell test is a useful test to screen for the presence of heterophile antibodies because it is simple and inexpensive. Although the specificity of the

heterophile assay is rated as good, negative results are demonstrated in individuals who do not produce infectious mononucleosis heterophile antibody. If negative results are displayed, however, EBV serology may be indicated.

Limitations

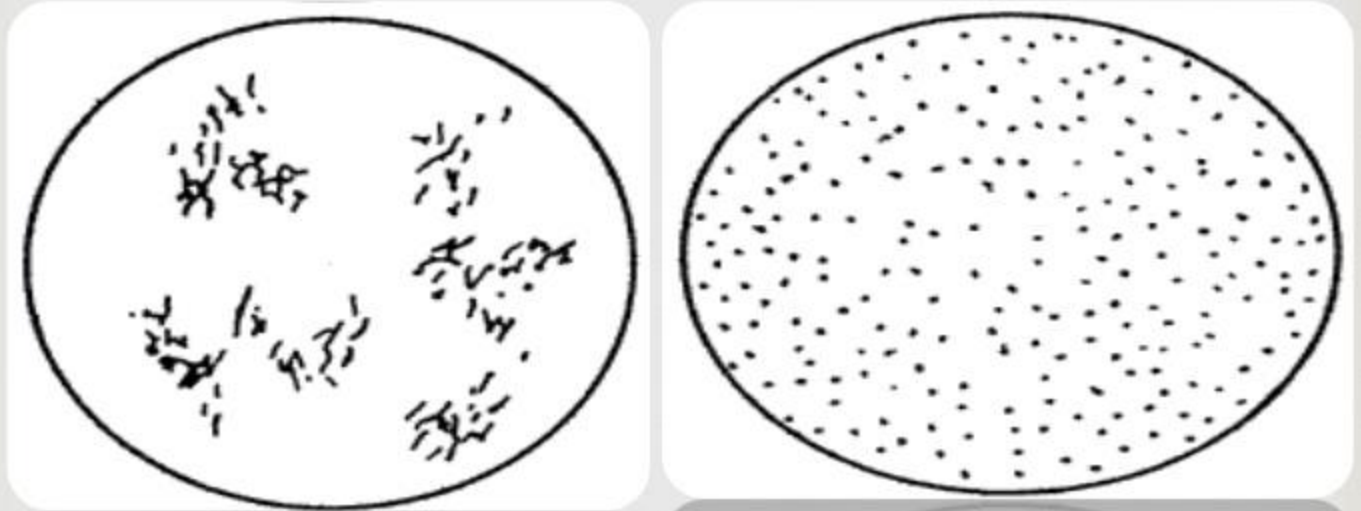
The test is only indicative of the presence or absence of heterophile antibodies. Demonstrating agglutination by using sheep erythrocytes does *not* distinguish between antibodies associated with infectious mononucleosis or serum sickness, or the Forssman antigen.

The heterophile antibody assay lacks sensitivity as a diagnostic criterion for infectious mononucleosis. Sheep erythrocytes are less sensitive than erythrocytes from other species. A patient may take as long as 3 months to develop a detectable heterophile titer.

Microscopic agglutination or Flocculation

- VDRL

VDRL



- ☞ Each preparation of antigen suspension should first be examined by testing with known positive or negative serum controls.
- ☞ The antigen particles appear as short rod forms at magnification of about 100x. Aggregation of these particles into large or small clumps is interpreted as degrees of positivity
- ☞ Reactive on left, non-reactive on right

Dr.T.V.Rao MD

TABLE 17.3 Tests for Syphilis Diagnosis

Test	Methodology	Comments
Direct Microscopy Observation		
Fluorescent	Fluorescent microscopy	
Darkfield	Darkfield microscopy	
Non-Treponemal Assay		
Quantitative		
RPR	Charcoal particle agglutination	CDC recommends for the screening and diagnosis of syphilis. Consider retesting in 3–12 months if patient remains in risk category. Reactive results reflex to titer and treponemal (e.g., TP-PA assay) for confirmation. Use to confirm reactive treponemal assay (e.g., CIAs, EIA) if using reverse algorithm testing Preferred test for monitoring treatment response in established syphilis RPR is being replaced by automated CIAs/EIAs.
VDRL	Flocculation	Acceptable screening or monitoring test for treatment of diagnosed cases of syphilis. May use to confirm reactive treponemal, if reverse testing is used.
TRUST (a modified VDRL)	Flocculation with red dye added	
Treponemal Test		
Qualitative/Semiquantitative		
CIAs/EIAs	Chemiluminescence	Popular for point-of-care testing but cannot distinguish between active disease and old disease (treated/untreated). CIAs/EIAs have high sensitivity but lower specificity than other methods. Studies to compare test performance with other serologic tests are lacking. Studies evaluating performance of EIA/CIA to detect IgM antibodies in early syphilis are lacking. Confusion regarding management of patients with discrepant serology (e.g., positive EIA/CIA and a negative RPR). All reactive EIA/CIA must be reflexly tested with a quantitative RPR.
ELISAs	Enzyme-linked immunosorbent assay	<i>T. pallidum</i> antibody IgG by ELISA is the recommended screening test in “reverse screening” protocol. Abnormal results require confirmation by nontreponemal assay (e.g., RPR or VDRL).
FTA-ABS	Fluorescent antibody absorption	Not an optimal reflex test; TP-PA is preferred.
IFA	Indirect fluorescent antibody	May assist in workup of tertiary syphilis. May be considered if suspicion of neurosyphilis remains after VDRL testing.
TP-PA	Particle agglutination	CDC recommends as confirmatory test for syphilis, if initial screening (e.g., RPR or VDRL) is reactive.
MBIAs	Microbead immunoassay	
MHA-TP	Microhemagglutination	Used less commonly.

CDC, Centers for Disease Control and Prevention; CIAs, chemiluminescence immunoassays; EIA, enzyme immunoassay; FTA-ABS, fluorescent treponemal antibody absorption; IgG, immunoglobulin G; IgM, immunoglobulin M; MBIAs, microbead immunoassays; MHA-TP, microhemagglutination assay-Treponemal pallidum; RPR, rapid plasma reagin; TP-PA, Treponemal pallidum particle agglutination assay; TRUST, Tolidine Red Unheated Serum Test; VDRL, Venereal Disease Research Laboratory.

Note: VDRL is the preferred test for cerebrospinal fluid (CSF). Treponemal tests (TP-PA or FTA) are *not* recommended for CSF. FTAs on CSF may be tested, but TP-PA *cannot* be tested on CSF.



CLASSIC VENEREAL DISEASE RESEARCH LABORATORY PROCEDURE: VENEREAL DISEASE RESEARCH LABORATORY QUALITATIVE SLIDE TEST

Principle

During the period of infection with syphilis, reagin, a substance with the properties of an antibody, appears in the serum of infected patients. Reagin has the ability to combine with a colloidal suspension extracted from animal tissue and clump together to form visible masses, a process known as *flocculation*.

In the VDRL procedure, the patient's heat-inactivated serum is mixed with a buffered saline suspension of cardiolipin-lecithin-cholesterol antigen. This serum-antigen mixture is microscopically examined for flocculation. Positive or reactive sera can be serially diluted and titrated. Syphilis and disorders such as pinta, yaws, bejel, and other treponemal diseases can produce positive reactions.

Procedure

The procedural protocol is posted on the website.

Sources of Error

False-negative reactions can occur in a variety of situations. These include the following:

1. Technical error (e.g., unsatisfactory antigen or technique)
2. Low antibody titers

Patients may have syphilis, but the reagin concentration is too low to produce a reactive test result. A low concentration of reagin may be caused by several factors, such as an infection that is too recent to have produced antibodies, the effects of treatment, latent or inactive disease, and patients who have not produced protective antibodies because of immunologic tolerance. These seronegative patients may demonstrate a positive reaction with more sensitive treponemal tests such as the FTA-ABS.

1. Presence of inhibitors in the patient's serum
2. Reduced ambient temperature (below 23°C to 29°C [below 73°F to 84°F])
3. Prozone reaction

A prozone reaction is encountered occasionally. This type of reaction is demonstrated when complete or partial inhibition of reactivity occurs with undiluted serum and minimal reactivity is obtained only with diluted serum. The prozone phenomenon may be so pronounced that only a weakly reactive or rough non-reactive result occurs in the qualitative test by a serum that will be strongly reactive when diluted. It is recommended that all sera producing a weak reaction

or rough nonreactive results in qualitative testing be retested with a quantitative procedure before a final report of the VDRL slide test is issued.

Weakly reactive results can be caused by the following:

1. Very early infection
2. Lessening of the activity of the disease after treatment
3. Improper technique or questionable reagents

False-positive reactions can also occur. Of all positive serologic tests for syphilis, 10% to 30% may be false-positive biological reactions. Nonsyphilitic positive VDRL reactions have been reported with the cardiolipin type of antigen in the following:

1. Lupus erythematosus
2. Rheumatic fever
3. Vaccinia and viral pneumonia
4. Pneumococcal pneumonia
5. Infectious mononucleosis
6. Infectious hepatitis
7. Leprosy
8. Malaria
9. Rheumatoid arthritis
10. Pregnancy
11. Older individuals

Contaminated or hemolyzed specimens can also produce false-positive results.

Clinical Applications

The purpose of the VDRL procedure is to demonstrate reagin in cases of syphilis. The procedure may also be positive in treponemal diseases such as yaws and pinta. Reagin, however, is found in some patients who are not infected with treponemes, which can be partially explained by the necrotizing effect of spirochetes on tissues and in other conditions and disorders. It is important that results of the VDRL procedure be correlated with the patient's history and with signs and symptoms.

Limitations

The VDRL procedure is not specific for syphilis but may demonstrate positive reactions in other reagin-producing disorders, autoimmune disorders, infectious diseases, and in pregnancy or aging in normal physiology.



RAPID PLASMA REAGIN CARD TEST

Principle

The RPR test is designed to detect reagin, an antibody-like substance present in serum. In this procedure, serum is mixed with an antigen suspension of a carbon particle cardiolipin antigen. If the specimen contains antibody, flocculation occurs with a coagglutination of the carbon particles of antigen. This flocculation appears as black clumps against the white background of a plastic-coated card. The cards are viewed macroscopically.

This is a nontreponemal testing procedure for the serologic detection of syphilis; however, pinta, yaws, bejel, and other treponemal diseases may produce positive results. Positive reactions are occasionally observed with other acute or chronic conditions.

Sources of Error

Error can be introduced into test results because of factors such as contamination of rubber bulbs or an improperly prepared antigen suspension.

False-positive biological reactions have been reported with cardiolipin type of antigens in the following conditions:

- Lupus erythematosus
- Rheumatic fever
- Vaccinia and viral pneumonia
- Pneumococcal pneumonia
- Infectious mononucleosis
- Infectious hepatitis
- Leprosy
- Malaria
- Rheumatoid arthritis
- Pregnancy
- Aging individuals

False-negative reactions can result from the following:

- Poor technique

- Ineffective reagents
- Improper rotation

Again, if mechanical rotation is below or above the 95- to 110-rpm acceptable range, the clumping of the antigen tends to be less intense in procedures with undiluted specimen; thus some minimal reactions may be missed. In quantitative tests, rotation above 110 rpm tends to produce a decrease in titer, approximately one dilution lower.

Limitations

A diagnosis of syphilis cannot be made based on a single reactive result without clinical signs and symptoms or history. Plasma specimens should not be used to establish a quantitative baseline from which changes in titer can be determined, particularly for evaluating treatment.

The RPR cards should not be used for testing CSF. Little reliance should be placed on cord blood serologic testing for syphilis. The RPR procedure has adequate sensitivity and specificity in relation to the clinical diagnosis.

Clinical Applications

The purpose of the RPR procedure is to demonstrate reagin in cases of syphilis. The test results may also be positive in treponemal diseases such as yaws and pinta. Reagin, however, is found in some patients who are not infected with treponemes, which can be partially explained by the necrotizing effect of spirochetes on tissues and in other conditions and disorders. It is important that results of the procedure be correlated with patient history and with signs and symptoms.



FLUORESCENT TREPONEMAL ANTIBODY ABSORPTION TEST

Principle

The FTA-ABS test is a direct method of observation. Although not recommended for screening, it is the most sensitive serologic procedure in the detection of primary syphilis.

Limitations

The FTA-ABS test is recommended as a confirmatory procedure for syphilis. Its use is discouraged as a screening test. A reagin test such as the RPR is recommended for screening.

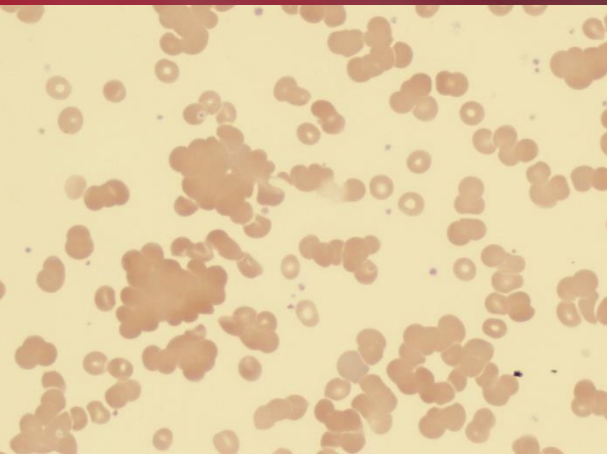
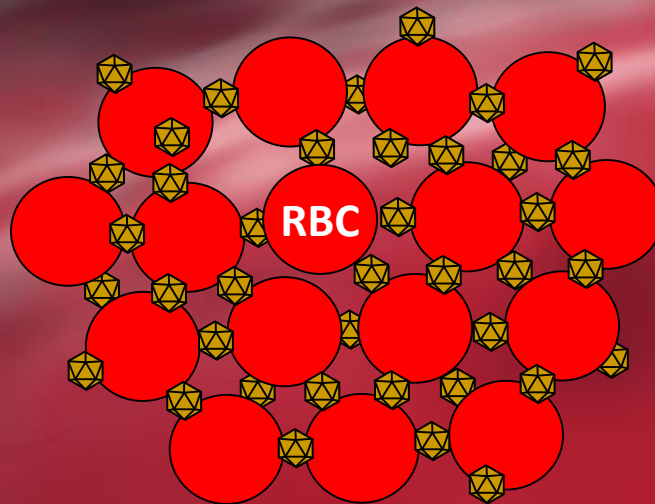
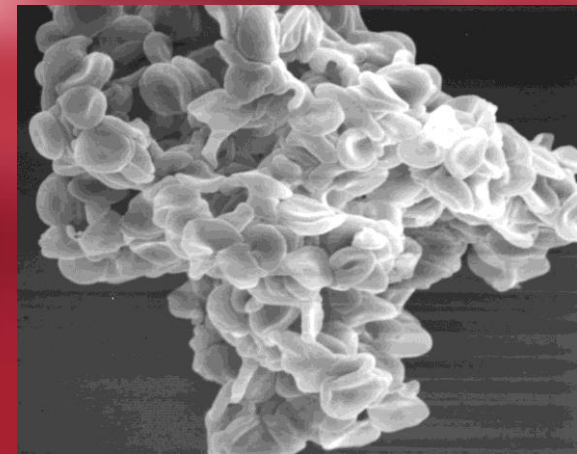
Clinical Applications

If a patient has two borderline test results, it is impossible to conclude definitively that the patient has or does not have serologic evidence of syphilitic

infection. The attending physician should review the patient's history and physical findings. Diagnosis will rely on the clinical evidence in conjunction with the borderline serologic findings.

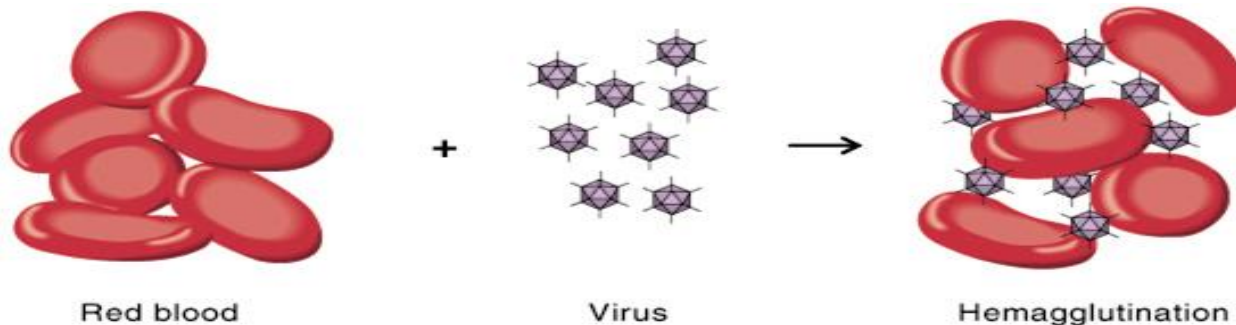
The false-positive rate of this test is very low, but it can be associated with autoimmune disorders such as systemic lupus erythematosus. False-positive FTA-ABS results occur in patients with other treponematoses (pinta, yaws, bejel) and in those who have a high titer of antinuclear antibodies or rheumatoid factor. Evidence indicates that pregnant women occasionally have false-positive FTA-ABS test results.

Heamagglutination



Heamagglutination

- Agglutination of RBCs may occur by:
 - Antibodies to RBC surface Ags
 - Proteins that interact with RBC surface antibodies (Heamagglutinins)



Application of Heamagglutination

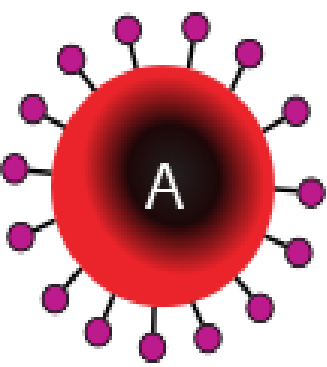
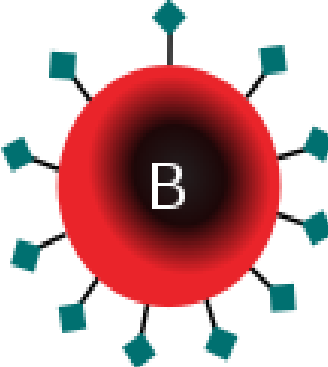
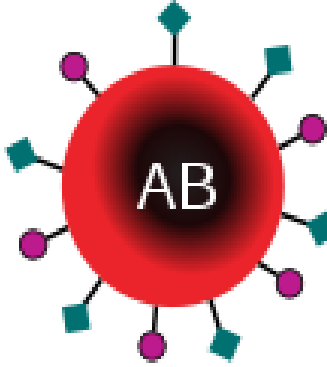
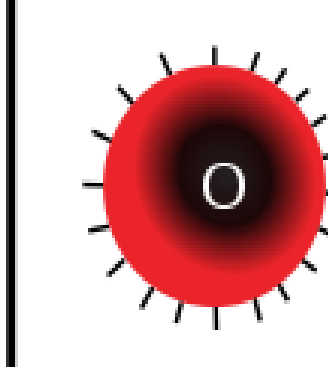
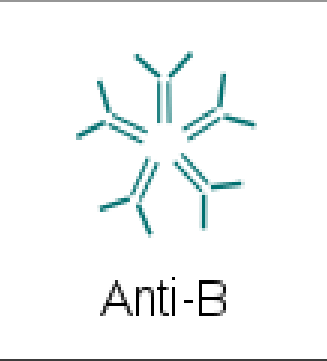
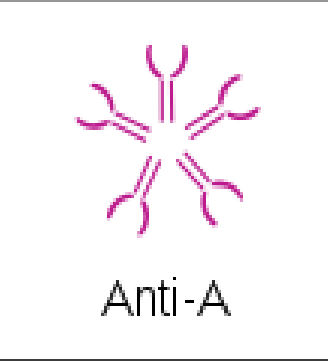
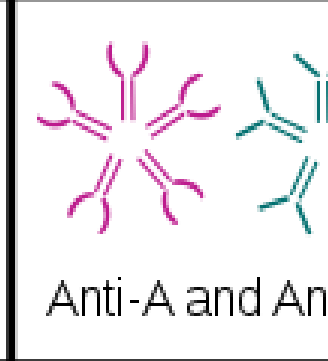
- **Blood grouping**
 - Cell type / Cell grouping
 - Back type / Serum grouping
- **Cross match** (for transfusion purposes)
 - Major and Minor
- **Coombs tests** (for immune anti-RBC antibody)
 - Direct and Indirect
- **Other diagnostic tests**
 - **Mono test** (Heterophil Abs)
 - **Virology tests** (anti-Heamagglutinin)

Classification of Blood Group Antibodies

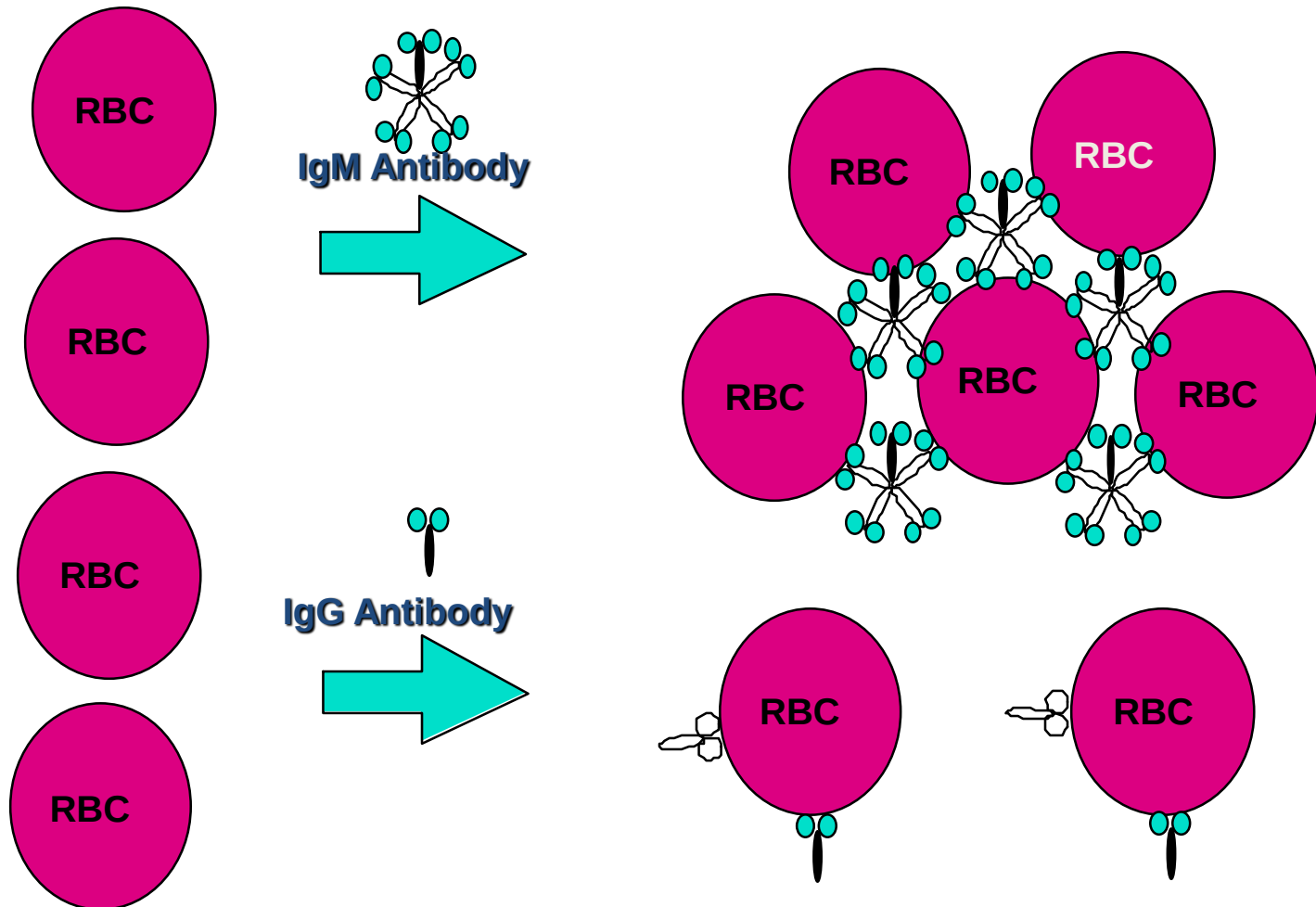
- **Natural and immune antibodies**
 - Natural Abs: production does not need pre-sensitization
 - Immune Abs: produced following sensitization
- **Cold and warm antibodies**
 - Thermal range of optimal reactivity
 - Most of natural Abs are cold and have less clinical importance
 - Most of immune Abs are warm and can destroy RBCs *in-vivo*
- **Complete and incomplete antibodies**
 - Depends on agglutination of washed RBCs (away from proteins)
 - IgM is complete; IgG and IgA are incomplete Abs
 - Natural antibodies are usually IgM
 - Immune Abs are usually IgG and IgA

Cell type / Back type

- **Cell type (detection of RBC Ags)**
 - ABO: (washed) RBC + Anti-A & Anti-B (& Anti AB)
 - Rh: (washed) RBC + Anti-D (& Anti C ,)
- **Back type (evaluation of serum Abs)**
 - Performed for ABO system (Natural Abs)
 - Defined washed A and B cells + patient's serum

	Group A	Group B	Group AB	Group O
Red blood cell type	 <p>A red circular cell with a black center containing the letter 'A'. It is surrounded by 12 purple circular antigens, each on a short black stalk.</p>	 <p>A red circular cell with a black center containing the letter 'B'. It is surrounded by 12 teal diamond-shaped antigens, each on a short black stalk.</p>	 <p>A red circular cell with a black center containing the letters 'AB'. It is surrounded by 12 antigens: 6 purple circles and 6 teal diamonds, each on a short black stalk.</p>	 <p>A red circular cell with a black center containing the letter 'O'. It is surrounded by 12 short black stalks without any antigens.</p>
Antibodies in plasma	 <p>Five Y-shaped antibodies in teal, arranged in a circle. Each has two arms pointing outwards.</p> <p>Anti-B</p>	 <p>Five Y-shaped antibodies in purple, arranged in a circle. Each has two arms pointing outwards.</p> <p>Anti-A</p>	<p>None</p>	 <p>Five Y-shaped antibodies in purple and five in teal, arranged in a circle. Each has two arms pointing outwards.</p> <p>Anti-A and Anti-B</p>
Antigens in red blood cell	 <p>A single purple circular antigen on a short black stalk.</p> <p>A antigen</p>	 <p>A single teal diamond-shaped antigen on a short black stalk.</p> <p>B antigen</p>	 <p>Two antigens on short black stalks: one purple circle and one teal diamond.</p> <p>A and B antigens</p>	<p>None</p>

Agglutination



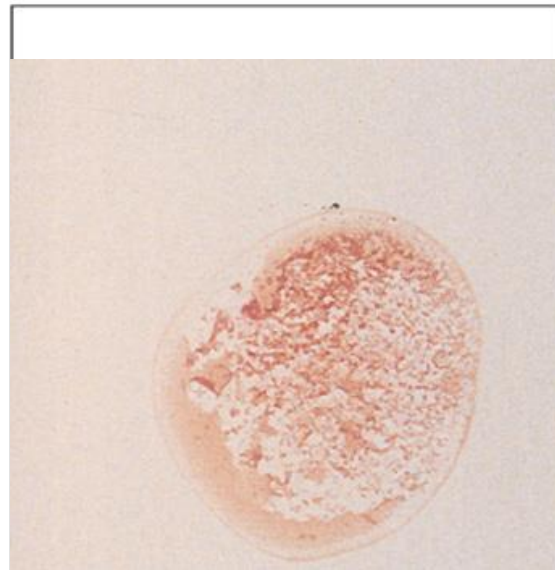
Coomb's tests

- **Coomb's** test is useful for detection of some incomplete Abs specially Anti-Rh antibodies
- **Direct Coomb's** test is usually used for evaluation of presence of bond antibodies on RBCs of Rh+ children born to Rh- mothers
- **InDirect Coomb's** test is usually used for evaluation of anti-Rh antibodies in serum of mothers who give birth to newborns with jaundice or other immune mediated hemolytic diseases

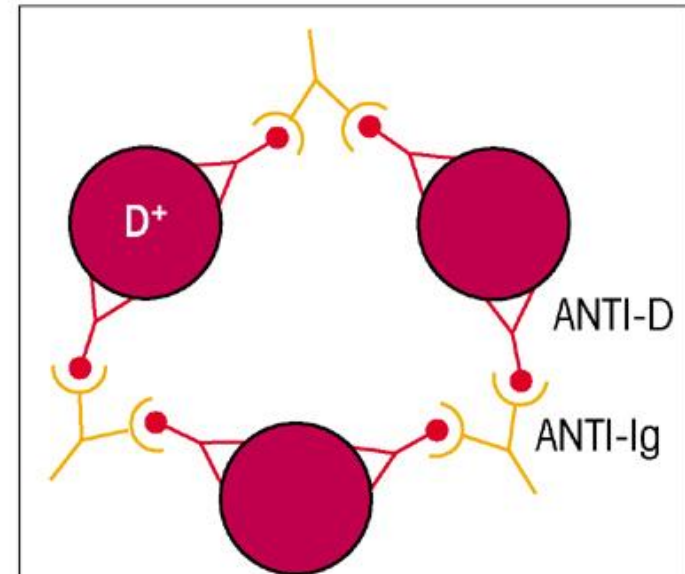
Antiglobulin (Coombs) Test



a Negative Coombs' reaction



b Positive Coombs' test

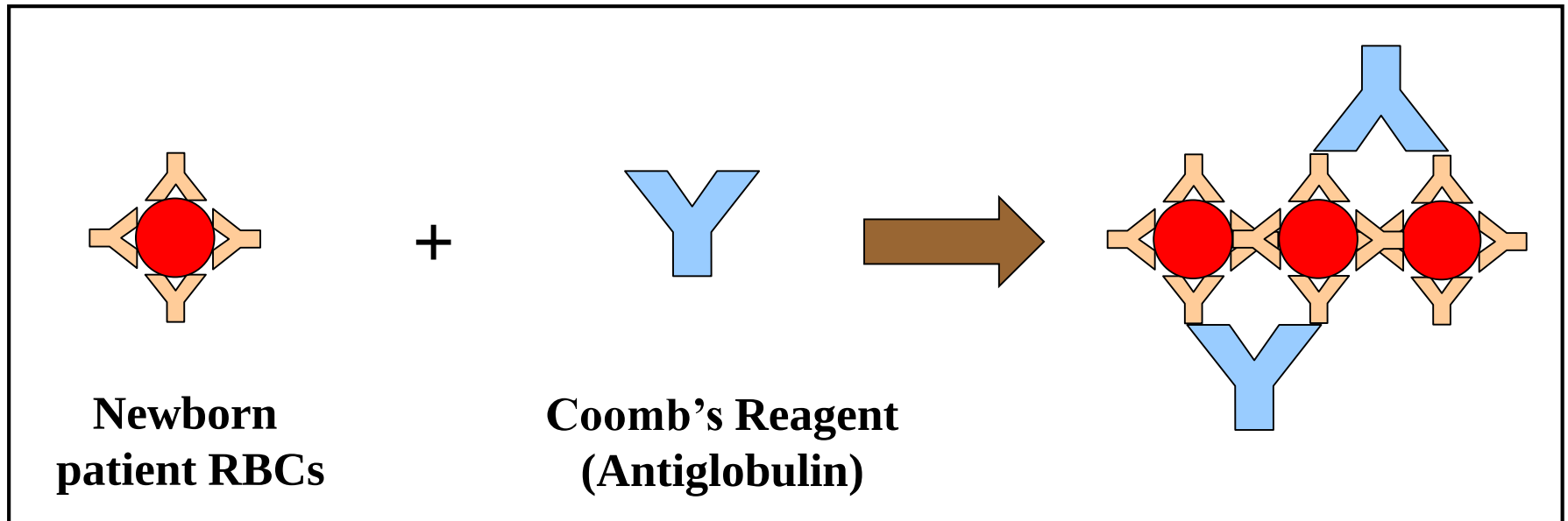
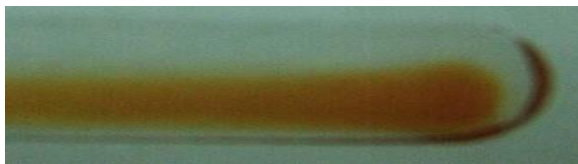
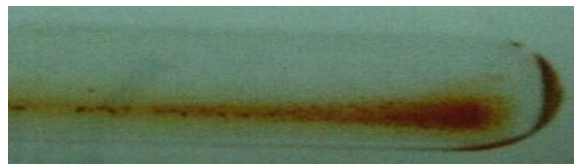
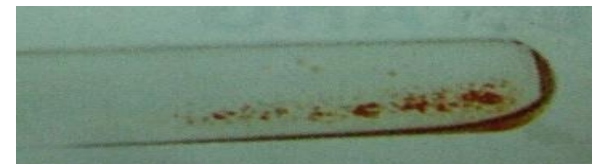


c Mechanism

Coomb's (Anti-globulin) Tests

Direct Coomb's Test

Detects antibodies on erythrocytes

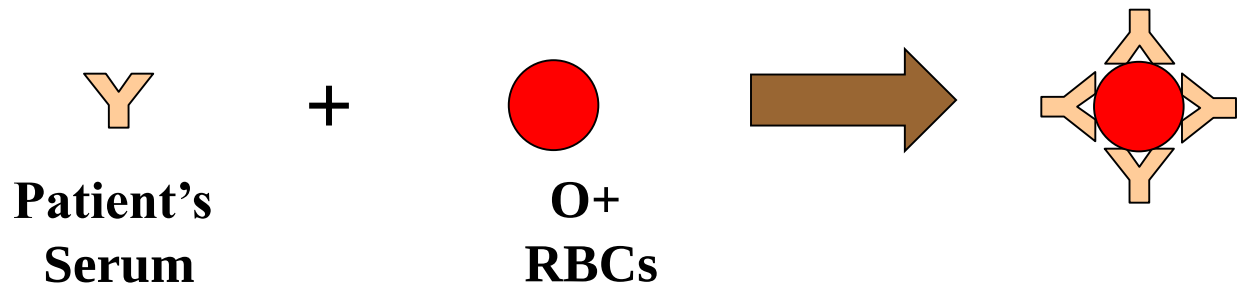


Coomb's (Anti-globulin) Tests

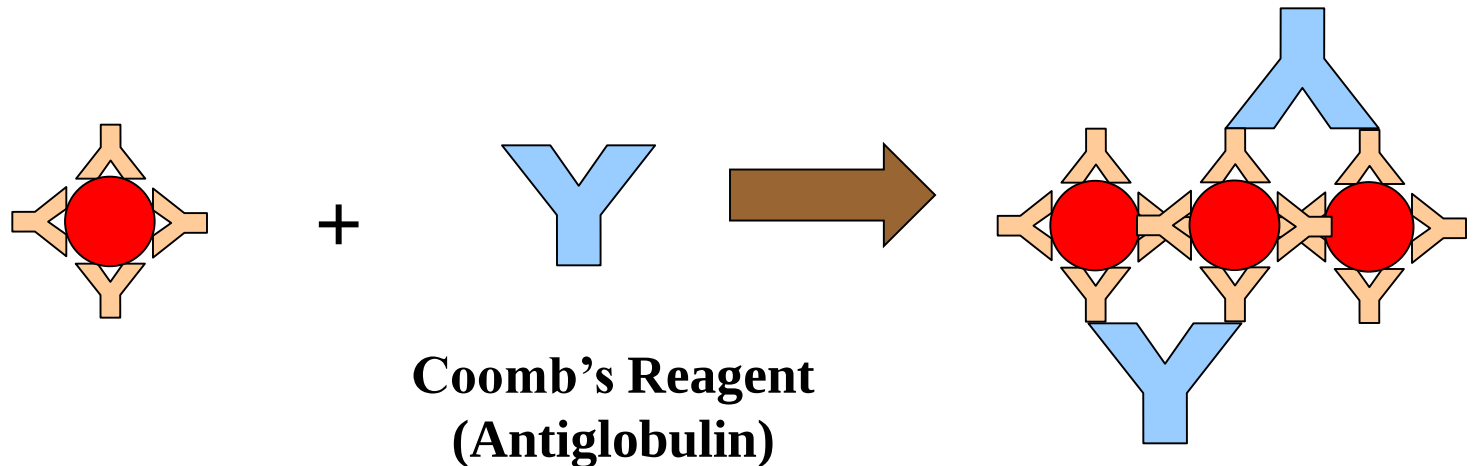
Indirect Coomb's Test

Detects anti-erythrocyte antibodies in serum

Step 1



Step 2

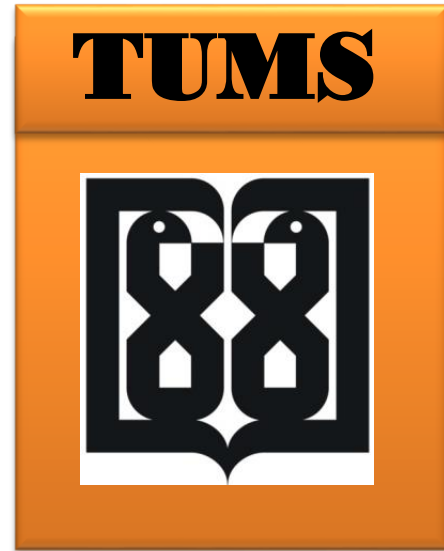


Cross Match Test

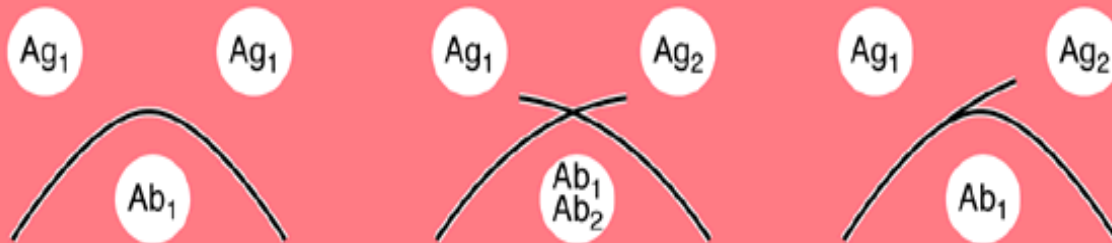
- **Types: Major and Minor**
- **Major: Donor RBC + Recipient Serum**
 - 1- RT
 - 2- Albumin (37 °C)
 - 3- Anti-Human (37 °C)
 - After 1 h the mixture is washed 3 times with saline and one drop of anti-human is added and centrifuged
- **Minor: Recipient RBC + Donor Serum**

Immunoprecipitation Reactions

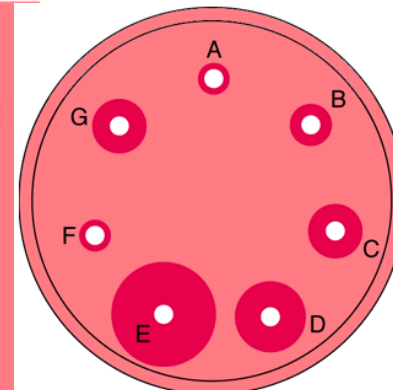
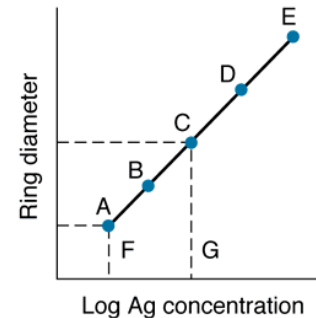
Immunology Department



DID



SRID



Immunoprecipitation Reactions

- **Immunoprecipitation in semisolid media**
 - **SRID**
 - **DID**
 - **Immuno-electrophoresis**
- **Immunoprecipitation in fluids**
 - **Ring test**
 - **Turbidimetry**
 - **Nephelometry**

Immunology/Serology

Precipitation Reactions

I- Precipitation in Solution

- a) Bottom Precipitate
- b) Ring Precipitate (Ring Test)

II- Simple Immunodiffusion (ID)

- a) Double ID (Ouchterlony)
- b) Single Radial ID (RID) (Mancini)

III- Electro-Immunodiffusion

- a) Immunoelectrophoresis (IEP)
- a) Immunofixation
- b) Rocket Electroimmunodiffusion (EID)
- c) Counterimmunoelectrophoresis (CIEP)

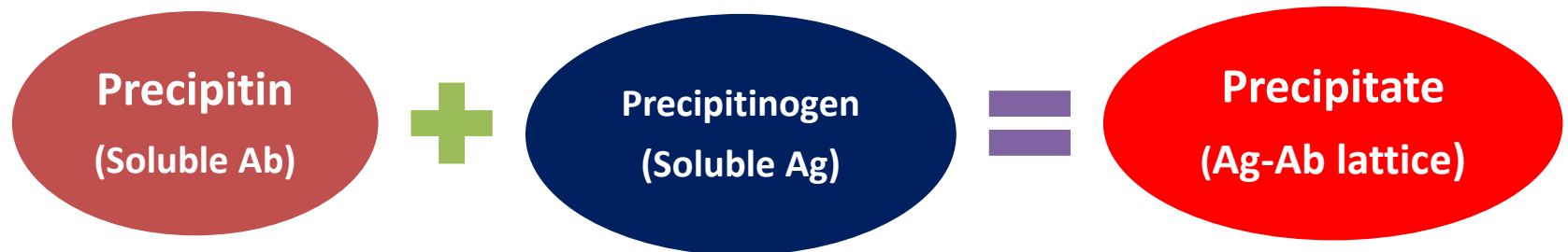
IV-Measurement of Precipitation by Light

- a) Turbidimetry
- b) Nephelometry

Principle of Immunoprecipitation

Immunoprecipitation is the result of the reaction between **soluble antigens (Ag)** and **soluble antibodies (Ab)** to form a **lattice** until the complex is large enough to become insoluble.

Immunoprecipitation could be studied in semisolid media or fluids.

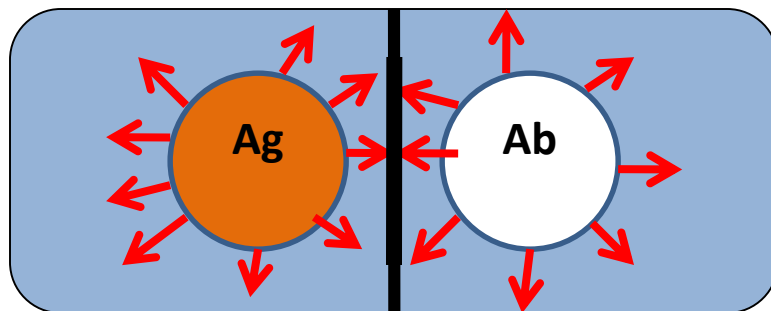


Semisolid Media

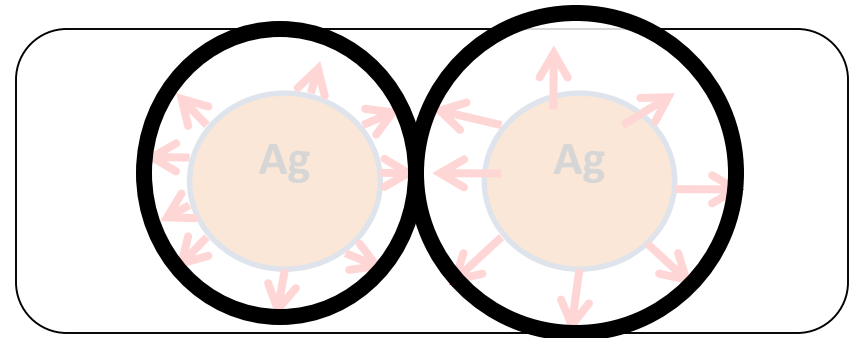
1. **Agar** - High molecular weight complex polysaccharide from seaweed
 2. **Agarose**- Purified agar
 - Agar , Agarose : 0.3 – 1.5 %
- Semisolid media help to stabilize the diffusion process and allow visualization of precipitin bands
 - Soluble molecules could be washed latter on by physiologic buffers (low salt buffers)

Immunoprecipitation appearance

- Immunoprecipitation in **semisolid media** results in visible **precipitation line or ring**.
- Immunoprecipitation **in fluids** result in significant **opacity or cloudiness** of the fluid.



DID



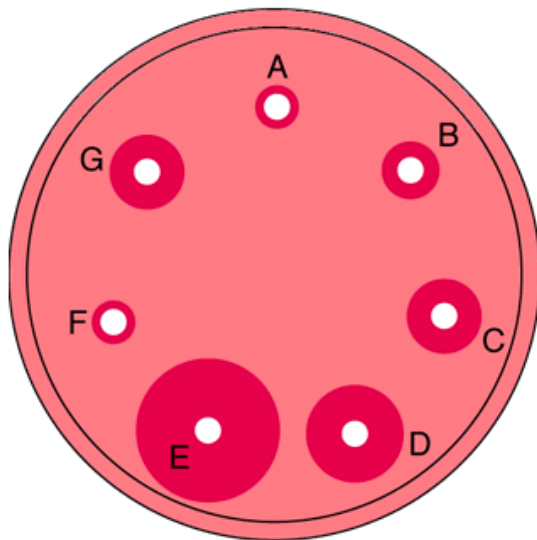
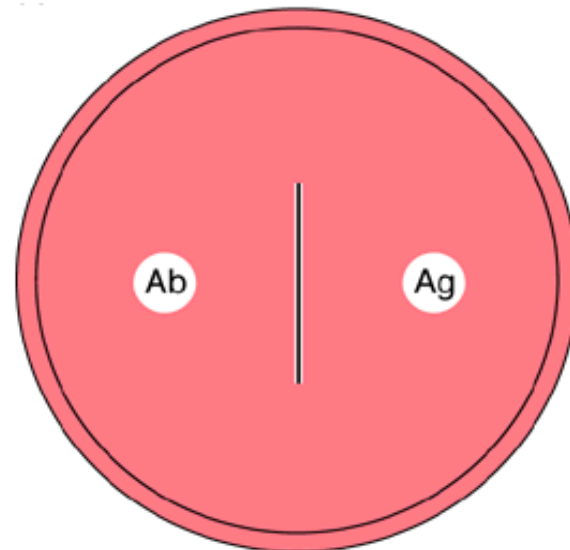
SRID

Immunoprecipitation in gel

- **Passive immunodiffusion**
 - Molecules move by **diffusion**
 - SRID and DID are the most common tests
- **Active immunodiffusion**
 - **Electrical current** is used to speed up the movement and interaction of molecules
 - Rocket technique, Immuno-electrophoresis and Western blotting are more common

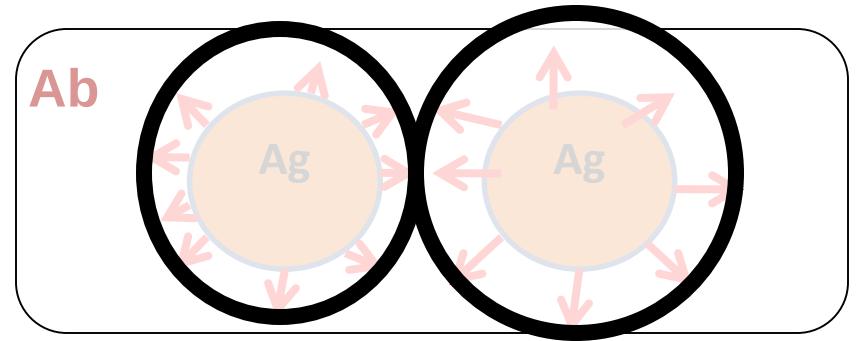
Classification of Passive Immunodiffusions

- Classification according to **number of reactants** and **direction of diffusion**
 - **Single diffusion:** Only one of components diffuse
 - **Double diffusion:** Both of components diffuse

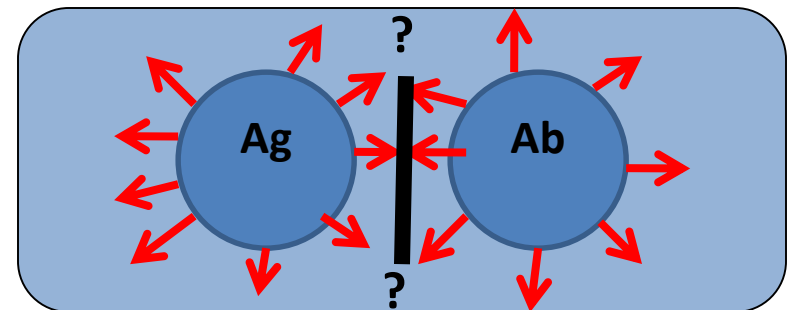


Passive Immunodiffusion in gel

1- Single Radial ImmunoDiffusion (SRID)

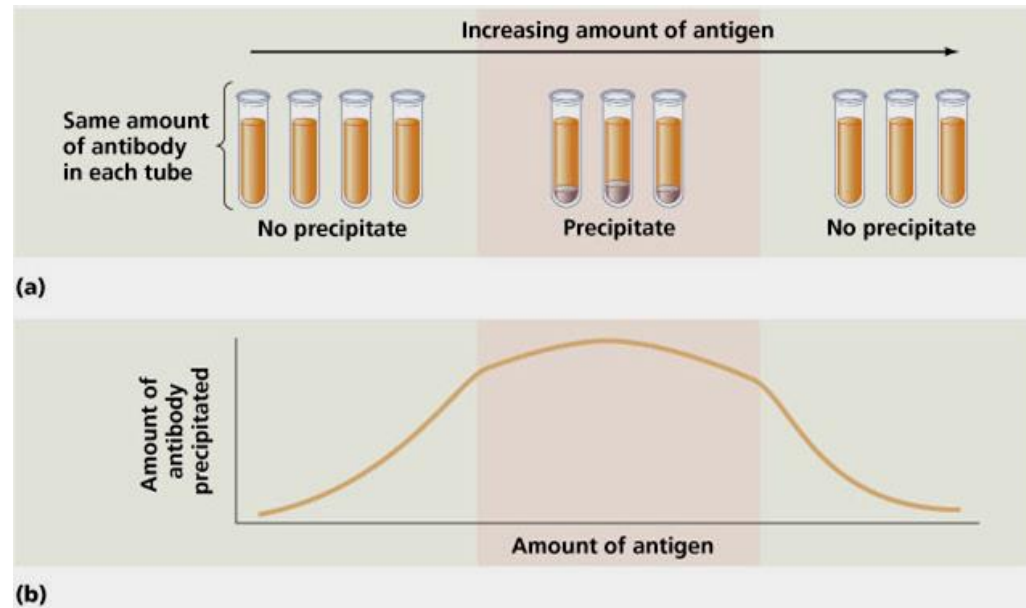


2- Double ImmunoDiffusion (DID)



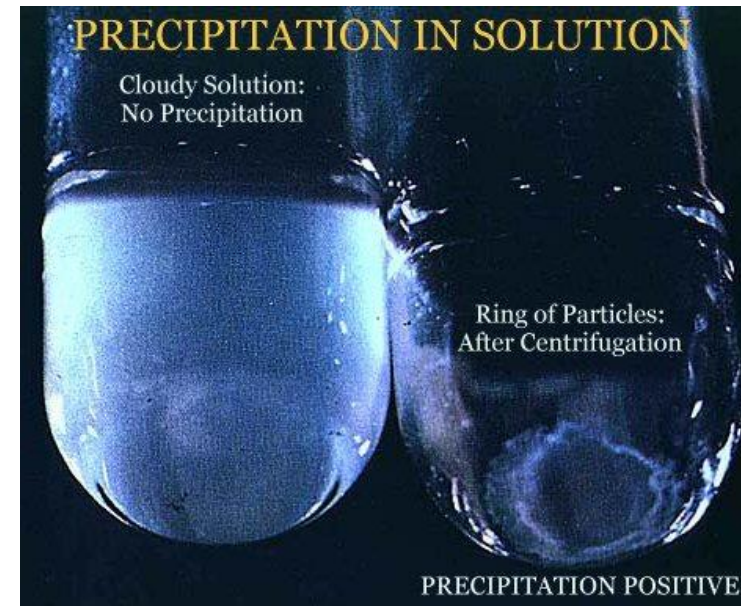
I- Precipitation in Solution

Precipitation reactions in fluids follow the same roles of precipitation curve zones.



a)Bottom Precipitate

Occurs when **Soluble Ag** interact with **soluble Ab** and **form a visible precipitate** that give **bottom ppt** after centrifugation.

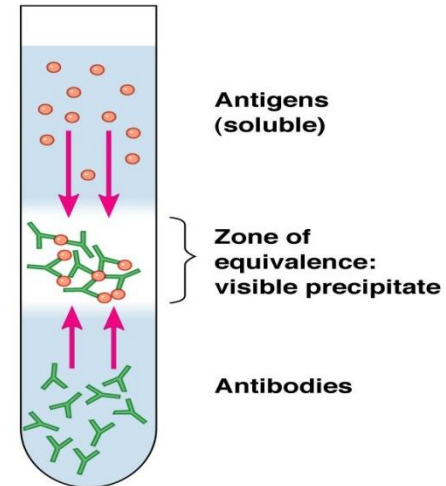


I- Precipitation in Solution (cont.)

b) Ring Precipitate (Ring Test) (Tube Precipitation test)

- Involve soluble antigens with antibodies in tubes (test or Capillary tubes).
- Layer Ag over Ab
- Precipitate occurs at the interface of the two reagents, forming a ring.
- Simplest test
- Qualitative
- Can be done in Agarose.

Test Tube reaction



Capillary Tube reaction

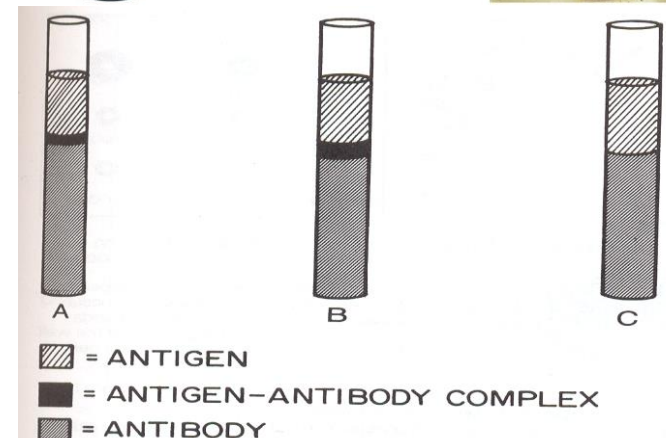


FIGURE 11-3

The antigen and antibody solutions are placed on top of each other in a capillary tube. After diffusion the antigen and antibody precipitate in the zone of equivalence. In samples **A** and **B**, some precipitate is formed at the interface, with **B** having more than **A**. In sample **C** there is no precipitate.

II- Simple Immunodiffusion (ID)

- Immunodiffusion procedures are carried out in an agar gel medium.
- The precipitate is easily seen in gels  visible precipitin lines
- But no visible precipitate forms in regions of Ab or Ag excess.

a) Single Radial ID (Mancini Reaction)

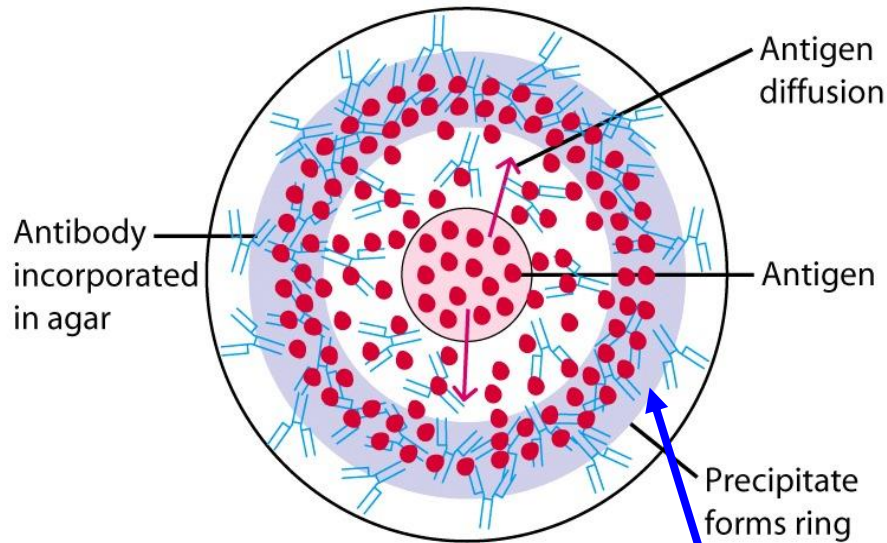
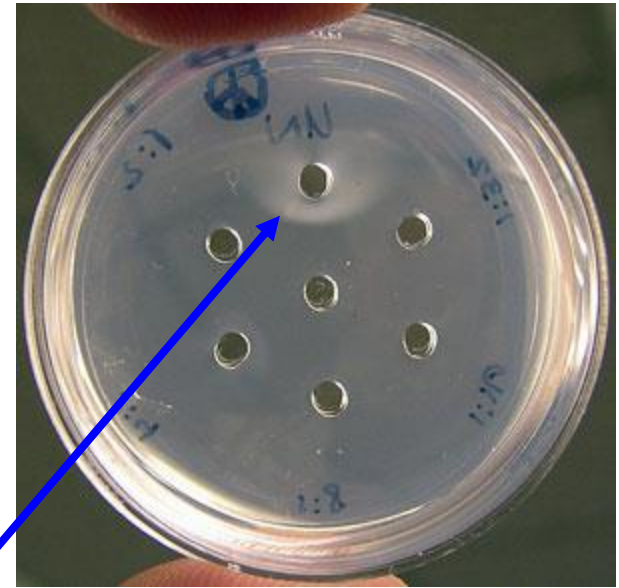
Ab is put into a gel and Ag is put in a well cut into the gel and a precipitin ring formed when Ag diffuses out in all directions.

b) Double ID (Ouchterlony Reaction)

Both Ab and Ag diffuse from wells into a gel medium

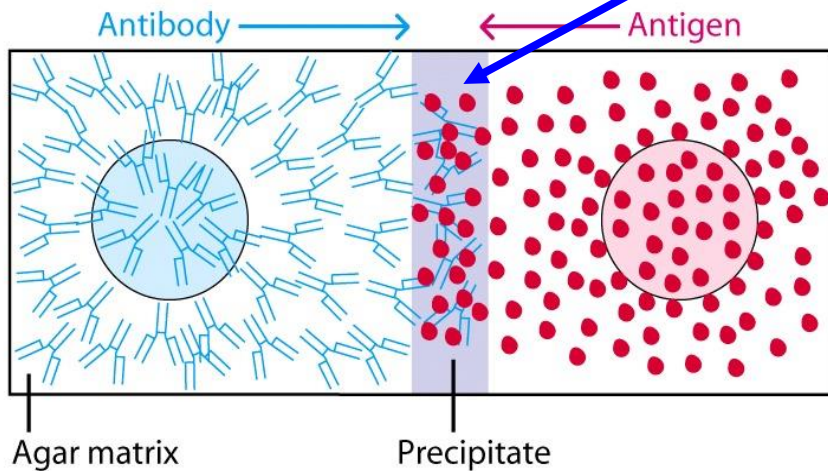
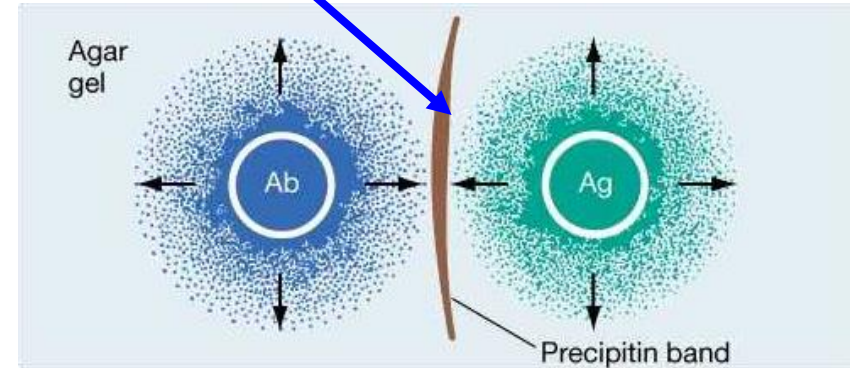
Simple Immunodiffusion Reactions

RADIAL IMMUNODIFFUSION



The region of equivalence

DOUBLE IMMUNODIFFUSION



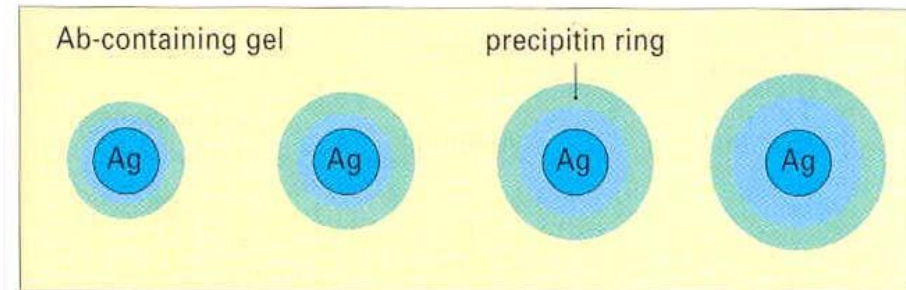
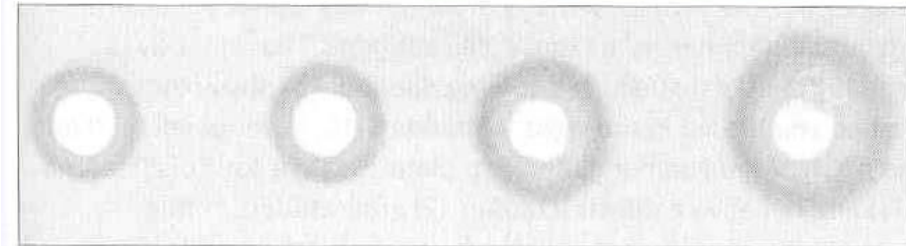
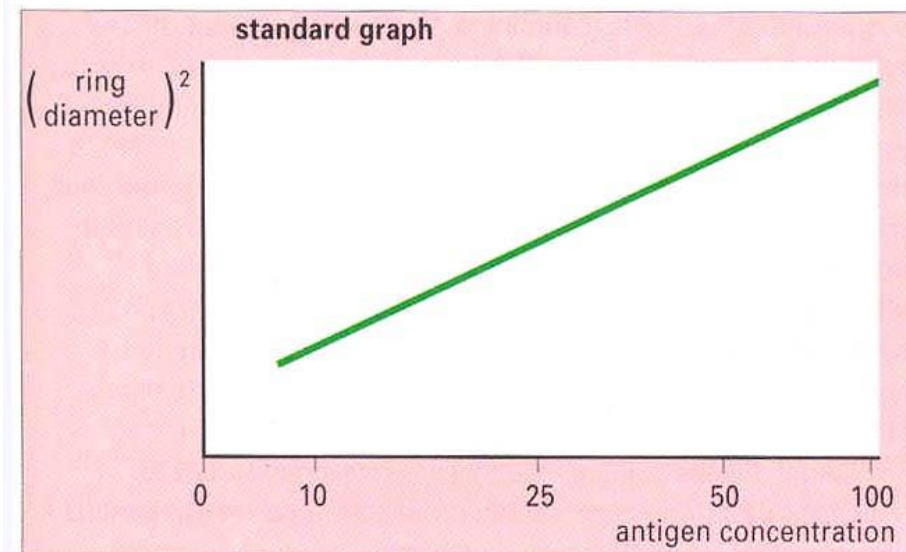
Diagrammatic representation of radial & double immunodiffusion.
(Precipitation reactions in gels yield visible precipitin lines; no visible precipitate forms in regions of Ab or Ag excess.)

II- Simple Immunodiffusion (ID)

a) Single Radial ID

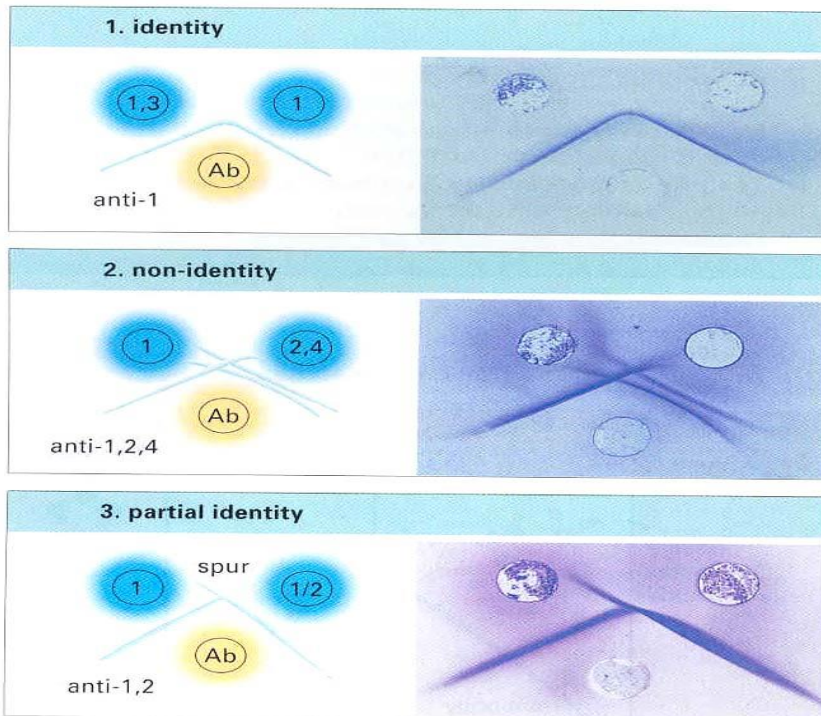
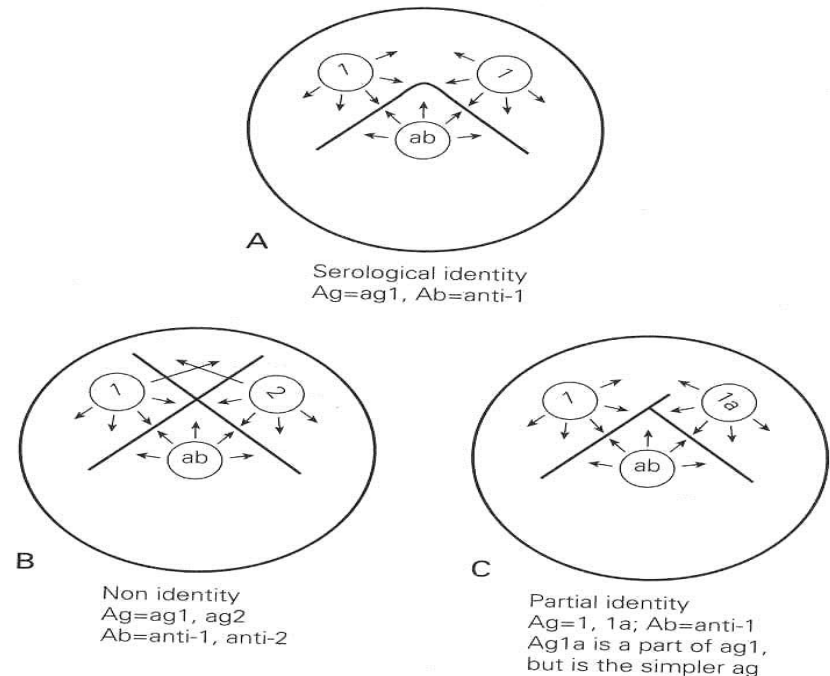
- The technique is quantitative
- is based upon the reaction between an Ag, and a specific Ab during a diffusion period.
- Ag placed in a well diffuses into an agar containing the Ab (anti-IgG looking for serum IgG).
- The Ag-Ab interaction is manifested by a well-defined ring of precipitation around the Ag well.

Interpretation : Diameter of ring is proportional to the Antigen concentration.



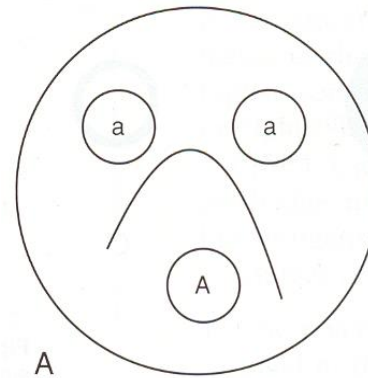
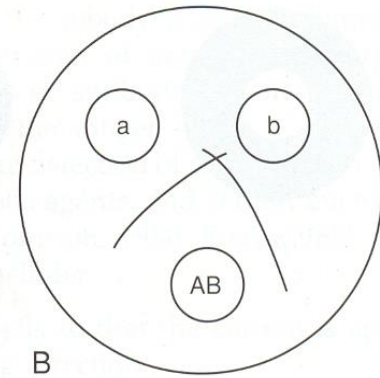
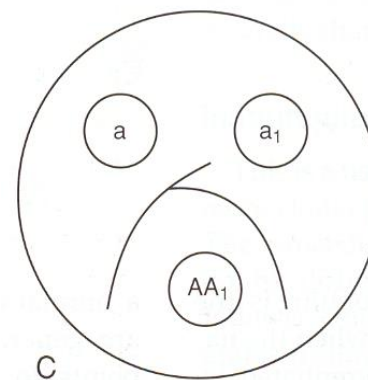
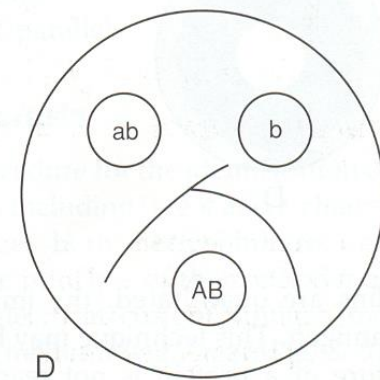
II- Simple Immunodiffusion (ID) (cont.)

- **b) Double ID (Ouchterlony)**
- Both antigen and antibody can diffuse independently
- It is based upon the simultaneous application of Ag and Ab in separate but adjacent wells of an agar plate.
- As the materials diffuse toward one another, ppt. lines form resulting from the Ag-Ab interactions (i.e. it is Qualitative).
- If multiple wells of Ag are positioned around an Ab well on the same plate, several patterns of reactivity may be observed.



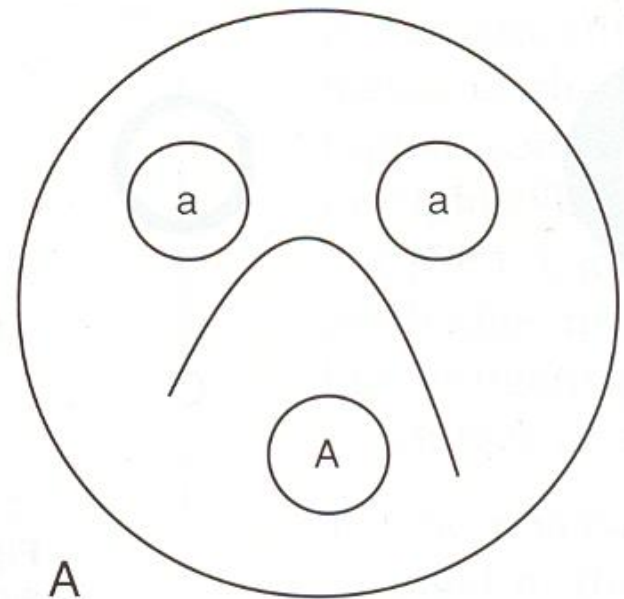
Ouchterlony double diffusion (Immunodiffusion) Qualitative test

- It is based upon the simultaneous application of Ag and Ab in separate but adjacent wells of an agar plate.
- As the materials diffuse toward one another, ppt. lines form resulting from the Ag-Ab interactions.
- If multiple wells of Ag are positioned around an Ab well on the same plate, several patterns of reactivity may be observed.



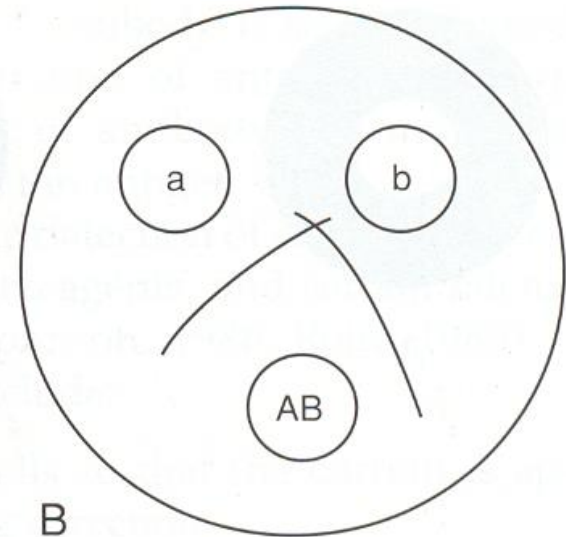
Ouchterlony double diffusion (Immunodiffusion)

- If the Ag A (patient) is the same as the Ag A (control), the reaction with the Ab will be the same and the result is a solid, continuous, smooth line of identity between the Ag wells and the Ab well.



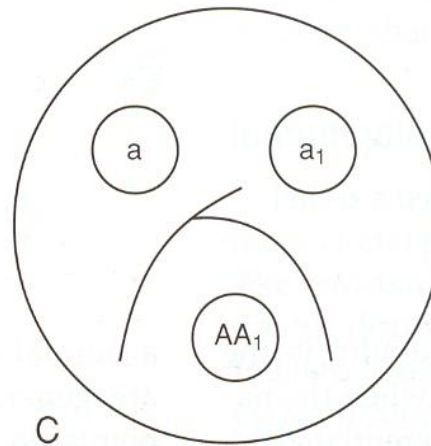
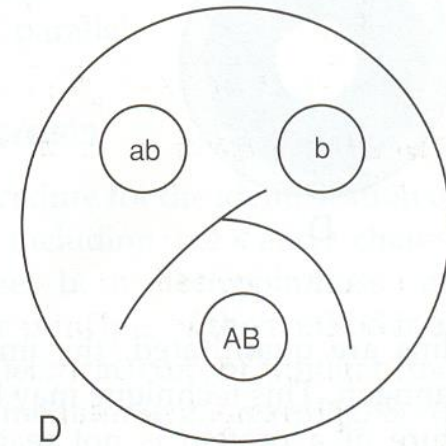
Ouchterlony double diffusion (Immunodiffusion)

- If Ag A (patient) is different from Ag B (control), and both react with the Abs to A & B, the precipitin lines cross and a double spur is formed; this is a line of nonidentity.



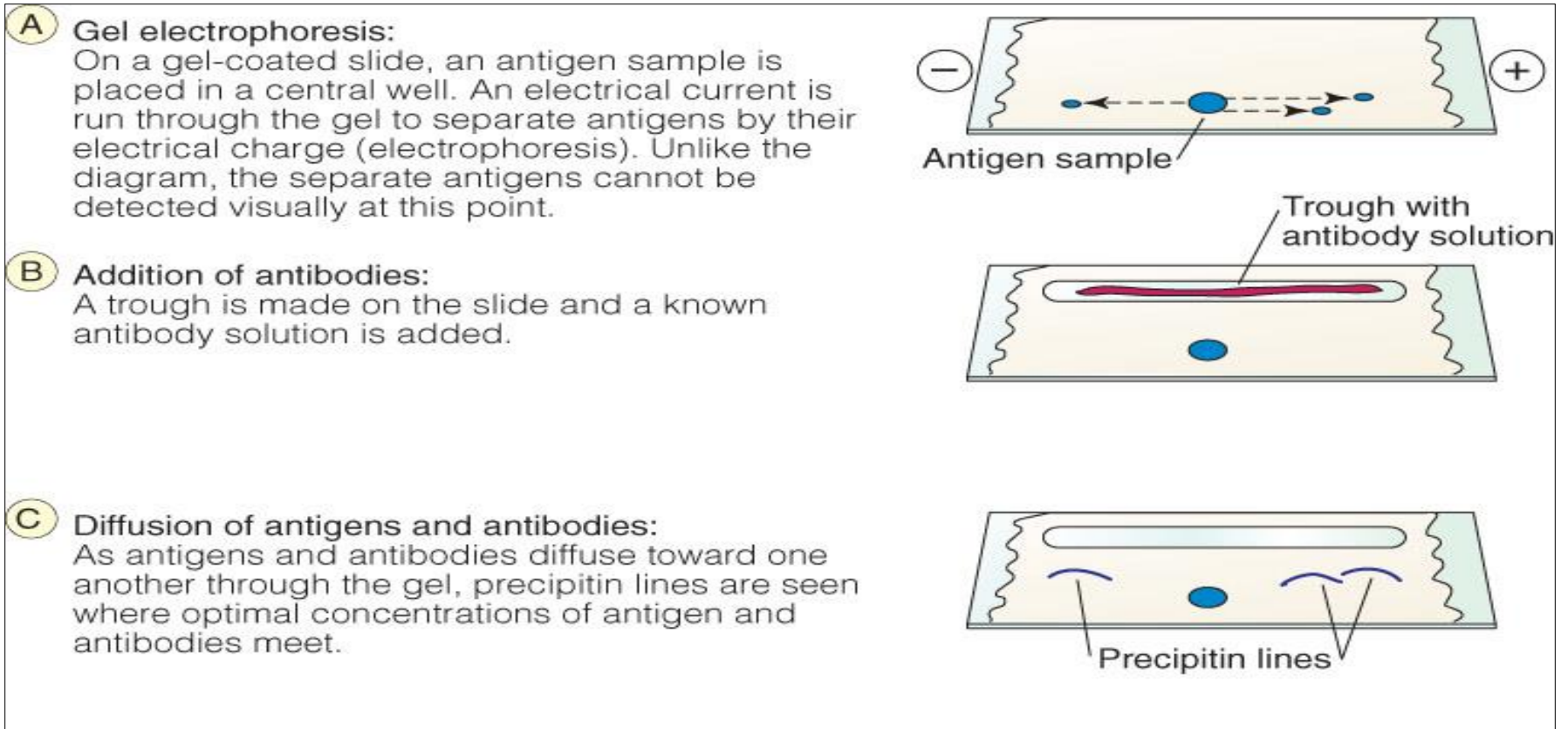
Ouchterlony double diffusion (Immunodiffusion)

- If Ag A (patient) and Ag A₁ (control) share a common element but are not exactly the same (Abs to A), a single spur is formed.
- This is the line of partial identity.



III- Electro-Immunodiffusion

- In **electro-immunodiffusion**, diffusion is combined with electrophoresis
- **Electrophoresis** separates antigen molecules according to differences in their electrical charges and molecular weight then specific **antibodies diffuse** and react with separated antigen forming precipitin bands.

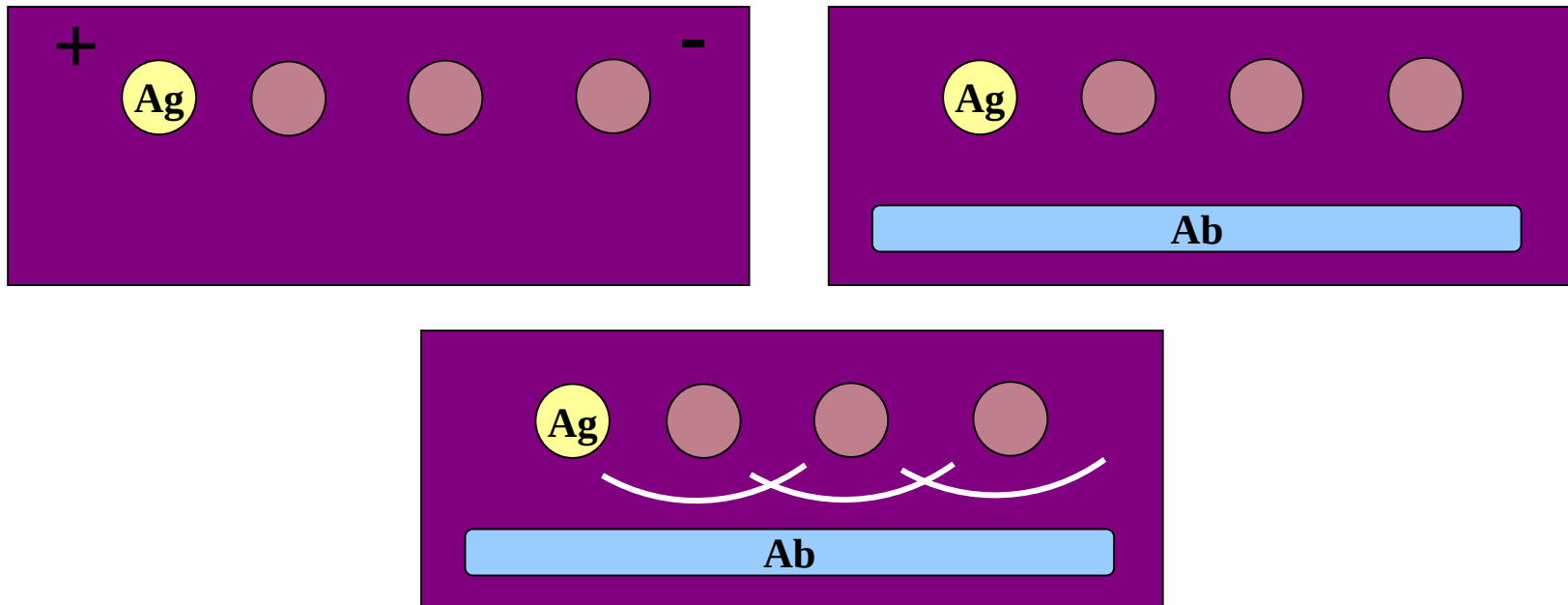


III- Electro-Immnodiffusion

- a) Immuno electrophoresis (IEP)**
- a) Immunofixation**
- b) Rocket Electroimmunodiffusion (EID)**
- c) Counterimmuno electrophoresis (CIEP)**

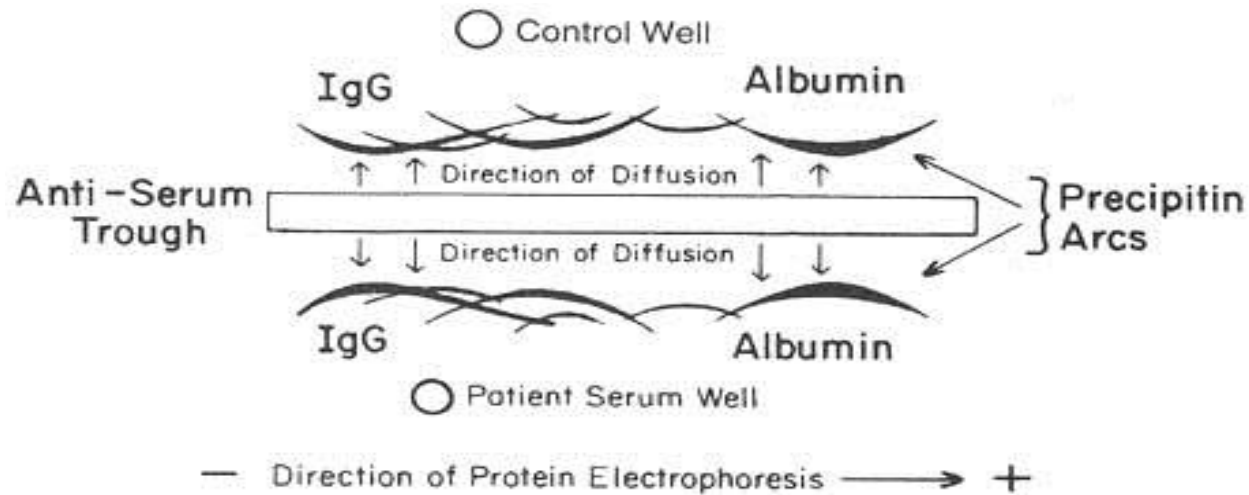
a) Immunelectrophoresis

- Method
 - Ags are separated by electrophoresis
 - Ab is placed in trough cut in the agar

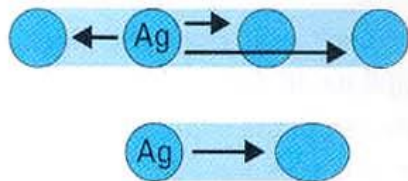


- Interpretation- **Precipitin arc** represent individual antigens
- **Qualitative** - Relative concentration

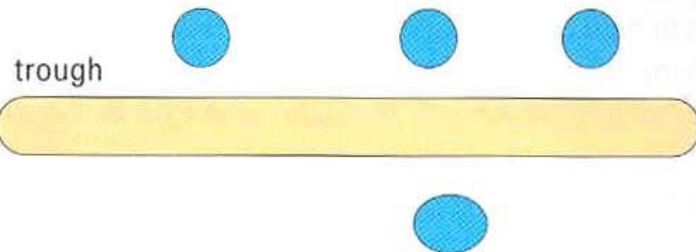
a) Immunoelectrophoresis (cont.)



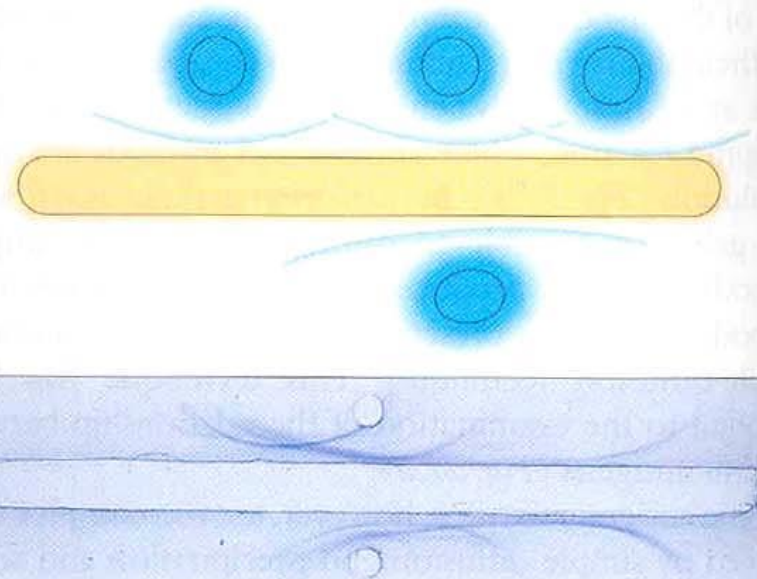
1. separation of antigens



2. antiserum in trough



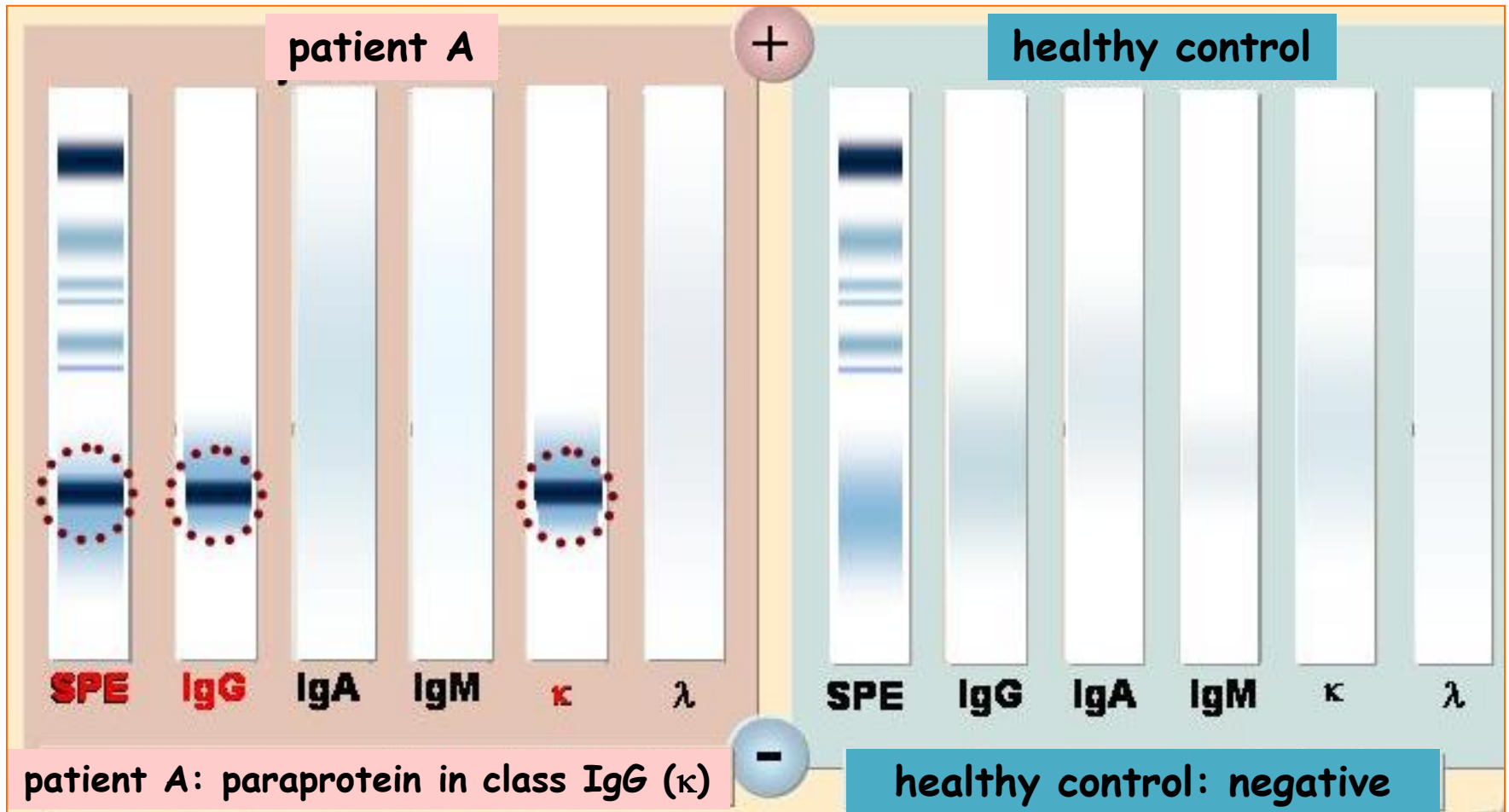
3. diffusion and precipitation



b) Immunofixation

- serum electrophoresis  several bands
- Anti-immunoglobulin antibodies application
(anti-IgG, -IgA, -IgM, - κ , - λ)
- Ag/Ab precipitates are formed
- staining to visualize the precipitation bands

b) Immunofixation (cont.)

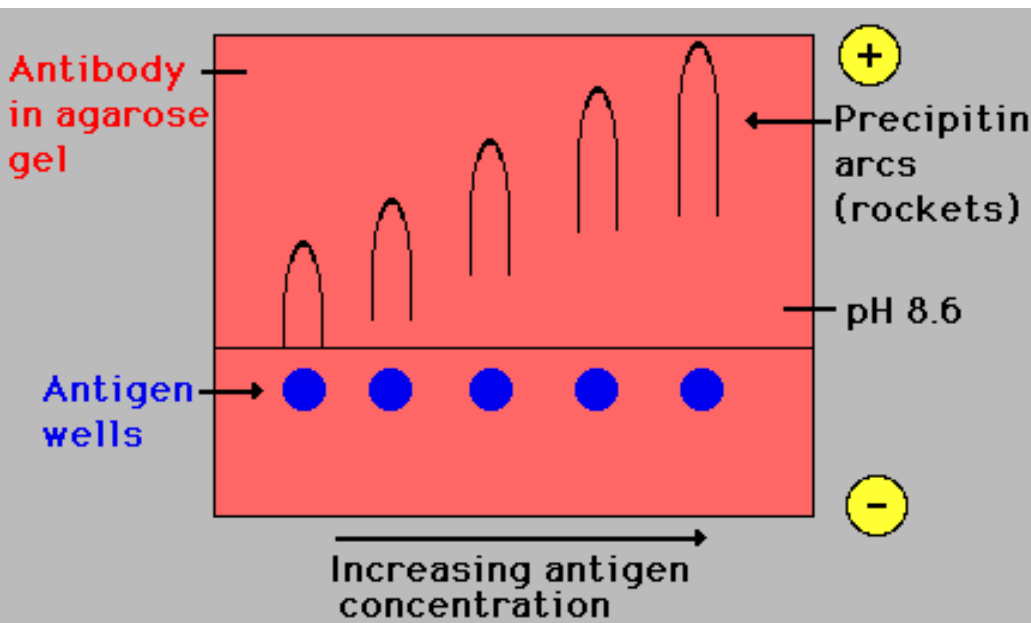
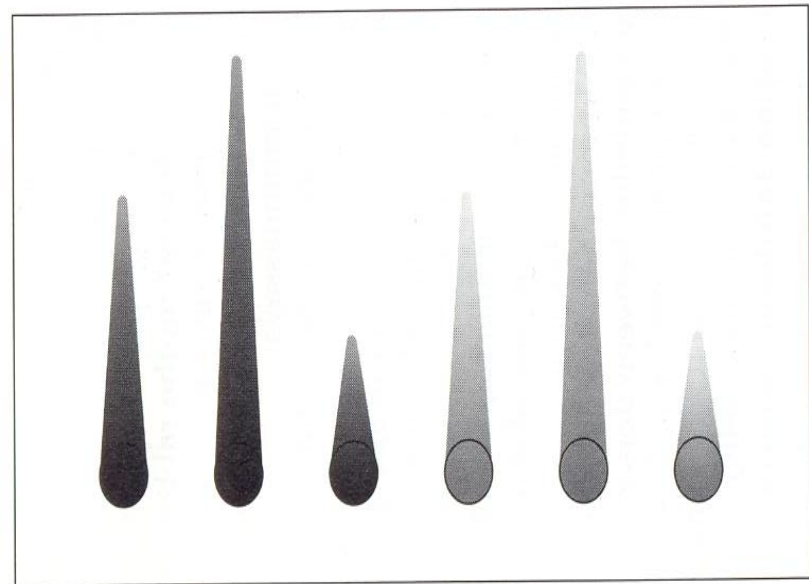


Usage of immunofixation

- Diagnostic for monoclonal gammopathies (multiple myeloma, macroglobulinemia, etc..)
- **Monoclonal immunoglobulin is synthesized by single clones of myeloma cells (neoplastic proliferation of B lymphocytes)**

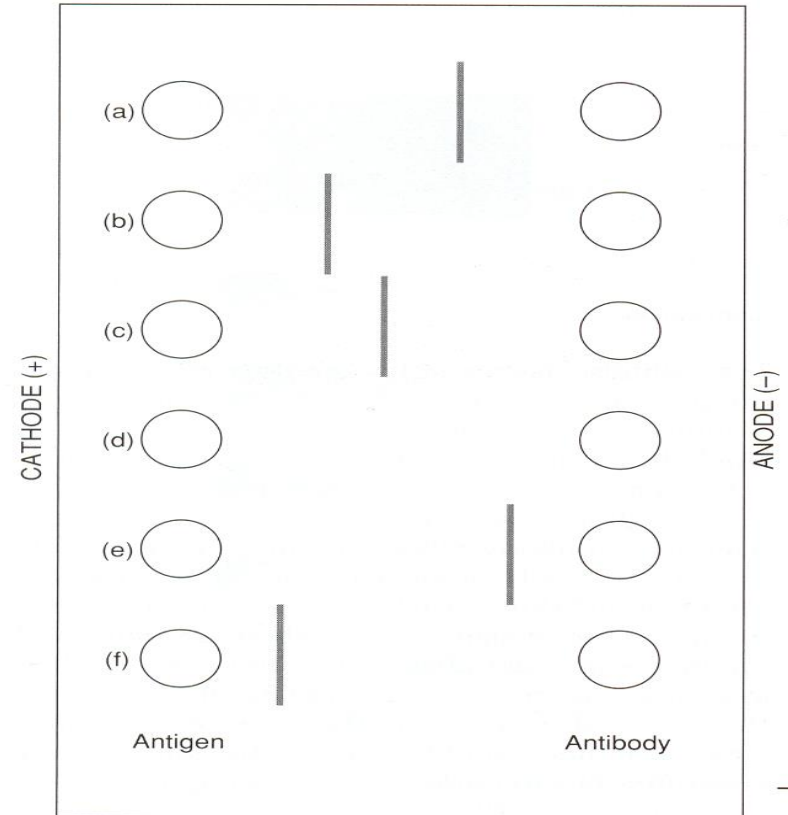
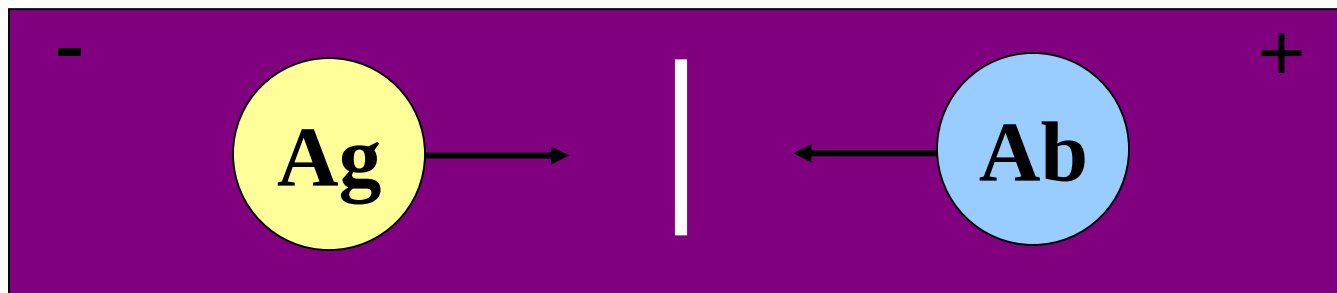
c) Rocket Electroimmunodiffusion

- **Antigen is electrophoresed into gel containing antibody.**
- The precipitin reaction results in a rocket-shaped precipitin formation.
- **The distance from the starting well to the front of the rocket shaped arc is related to antigen concentration.**



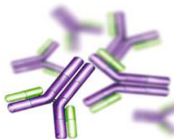
d)Counter immunoelectrophoresis

- Reactions occur between migrating Ag's and Ab's during electrophoresis (Ag and Ab migrate toward each other by electrophoresis)
- Used only when Ag and Ab have opposite charges
- Pairs of wells are punched in agarose plates in which Ag is placed in one well of each pair and Ab in the other.
- Following electrophoresis, precipitin lines will be visible between the wells of a pair of wells of matching specificity.
- **Qualitative**
 - Its major advantage is its speed.



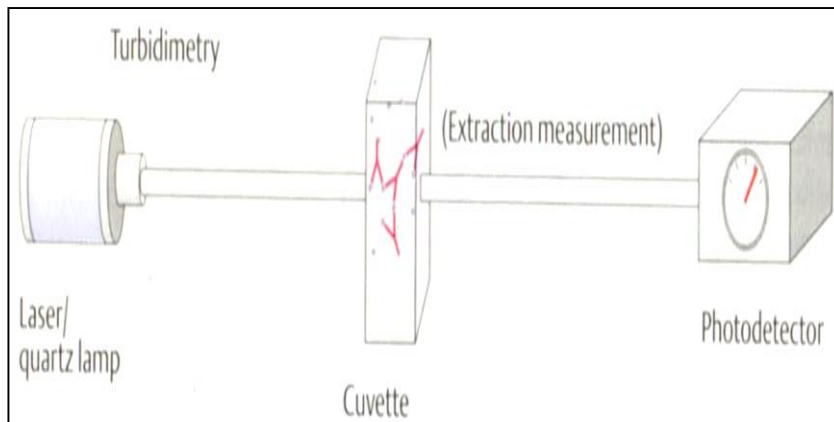
IV-Measurement of Precipitation by Light

- **Principle:** Antigen-antibody complexes, when formed, will precipitate in a solution resulting in a turbid or cloudy appearance that can be measured by:
 - **Turbidimetry**
 - Passing light through a cloudy solution. (Net decrease in light intensity)
 - **Nephelometry**
 - Measuring light scattered at a particular angle after being passed through a solution i.e. indirect measure.
 - Amount of light scattered correlates to the concentration of the solution
- **Evaluation:** Ag concentration is proportional to the turbidity of complexes („end point“)



Turbidimetry

- precipitation in solution
- measurement of light extraction
(precipitate absorption)
- standard curve



Nephelometry

- precipitation in solution
- measurement of scattered light (proportional to number of insoluble complexes)
- standard curve

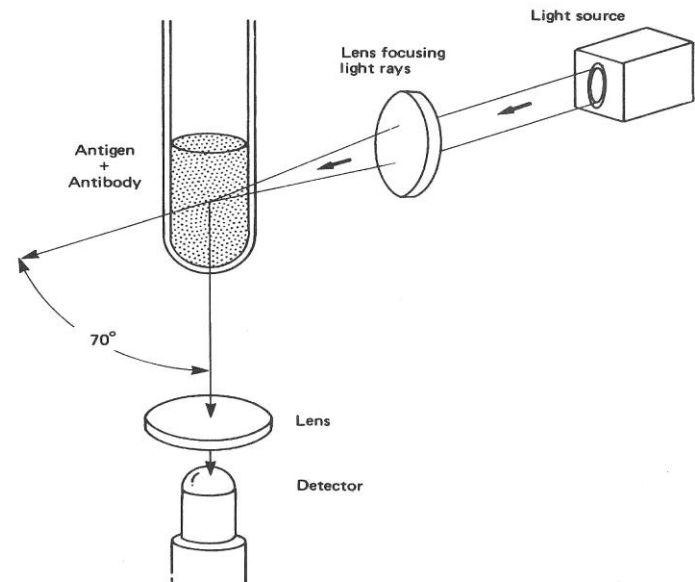


Figure 8-3 Principles of nephelometry. The light detection device is at an angle to the incident light, in contrast to turbidity, which measures light rays passing directly through the solution. (From Rodgers, RPC,¹⁴ p 171, with permission.)

Usage of turbidimetry and nephelometry

- **measurement of serum proteins' concentration**
(immunoglobulins, acute-phase proteins, complement components C3, C4, transferrin, albumin,...)
- **rapid,**
- **fully-automated techniques**
- **for large quantity of samples**