Swayam Course - **Analytical Techniques**

Week: 15, Module 38 - **CLIA and its diagnostic applications**

Content Writer - **Dr. Archana Singh, Assistant Professor, Department of Biochemistry, All India Institute of Medical sciences, New Delhi.**

1. Objectives:

-To elucidate Principle and theory of chemiluminiscence based Immunoassay (CLIA)

-To mention advantages of CLIA over other Immunoassay

-To describe working of CLIA autoanalyser

-To state diagnostic applications of CLIA

2. Introduction: Bioluminescence is a phenomenon which occurs widely in nature and has been studied for many years. However use of luminescence for analytical application is quite recent and this application came to light after the discovery that ATP was a cofactor for luciferase catalysed luminescence of firefly luciferin. At present various synthetic molecules are available like luminol and bis acridinium salt which gets oxidised to give luminescent reaction. The basic principle of chemiluminescence is when an excited product of an exogenic reaction comes back to its ground state it emits photons which is detected by a photodetector and expressed as readouts in terms of concentration. These synthetic molecules can be labelled to study antigen antibody reactions. These immune reactions can be studied in complex mixtures such as serum/plasma. Conventional enzyme labels or fluorescent derivatives have detection limits up to 10^{-14} which is even enhanced in chemiluminescent labels for as low as 10^{-18} thus matching or even surpassing detection limits by radioimmunoassay.

Immunological reactions between antigen and antibodies forms basis of many assays which are more sensitive and specific over a simple biochemical reaction. The specificity and high affinity of antibodies to combine with their antigens allow the identification and quantification of an analyte.

3. Basic concept in antigen-antibody reaction

Antibody binding to its complimentary epitope on an antigen forms basis of all immunochemical techniques including Immunoassays.

3.1 Antibodies: Immunoglobulins binding to a wide variety of antigens like protein, carbohydrates, nucleic acid, lipids and others. It comprises of 5 classes namely IgG, IgA, IgM, IgD and IgE. IgG is most commonly used in reagents for immunochemical reactions.

3.2 Antigens/Immunogens: An Immunogen when introduced in a host mounts an immune response and leads to generation of antibodies of different types.

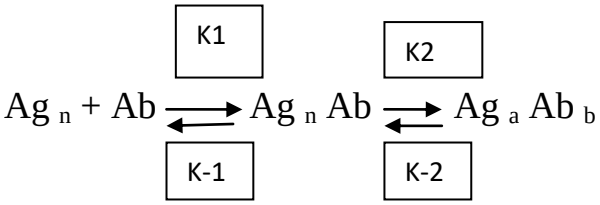
The strength of interaction between an antigen and antibody is described by their affinity and avidity. Affinity is combined strength of the noncovalent interactions between a single antigen-binding site on an antibody and a single epitope. Ability of an antibody to bind to multiple identical epitopes of the antigen simultaneously is defined as its avidity.

3.3 Avidity and Affinity

Avidity refers to sum of all binding site on an antibody with the antigen. IgM has more avidity compare to IgG as it has 10 antigen binding site compared to 2 antigen binding site of IgG. Affinity is the property of bound antigen and avidity is property of binder antibody. The High avidity can compensate for low affinity.

3.4 Antigen-antibody binding:

Antigen-antibody binding strength depends upon vander waals interactions, hydrophobic interactions and coulombic bonds. The binding of antigen to antibody occurs in an equilibrium reaction as shown:



Where K1 >>> K2, n is the number of epitopes per molecules, and a and b are the numbers of antigen and antibody molecules per complex. The initial reaction occurs very rapidly to form antigen antibody complexes leading to precipitation of complex after a critical size is reached. The speed of reaction

depends upon electrolyte concentration, pH, temperature, antigen structure, antibody class and binding affinity of the antibody.

Factors affecting antigen-antibody binding: antigen and antibody binding is influenced by ion species, ionic strength and polymers used in the solution. Cationic salts inhibit antigen-antibody binding. Narrow ionic radius or an increased radius of hydration is favourable condition for antigen-antibody interaction. A high molecular weight, linearity and aqueous solubility of a polymer are desirable to enhance antigen-antibody reactions.

4. Types of antigen –antibody reactions

Precipitin reaction: In a situation where antibody combining sites are greater compare to antigen epitope sites, antigen binding sites are saturated with formation of small insoluble antigen-antibody complexes which precipitates thus giving the reaction. Antigen or antibody excess reduces precipitation as now soluble antigen and antibody remain in solution. Examples of precipitin reaction are precipitation in solution, precipitation in agar and precipitation in agar with electric field. Precipitation in solution comprise of ring test, slide flocculation test for VDRL, tube flocculation for syphilis. Precipitation in agar is termed as immunodiffusion. Precipitation in agar over an electric field is Immunodiffusion electrophoresis which comprise of rocket immunoelectrophoresis, counterimmunoelectrophoresis.

4.1 Reactions at solid liquid interface: Here antibody/antigen is bound to a solid phase such as polystyrene or cellulose. This situation favours formation of both low and high avidity antigen and antibody complexes improving detection at lower concentration. Many immunoassays have been developed using antigen antibody reactions at solid liquid interface.

4.2 Immunoassays: A measurement technique which uses an antibody (or antigen) in combination with a sensitive detection signal for the quantitive (or qualitative) determination of an antigen (or antibody) in a patients. Measurement assays on these techniques are very sensitive and specific due to exquisite sensitivity and high specificity of antibodies against their antigens.

5. Immunoassays could be of two types:

Qualitative: passive gel diffusion, immunoelectrophoresis, western blotting, dot blotting

Quantitative: Radial immunodiffusion, turbidimetric and nephelometric assays and labelled immunochemical antibody assays.

5.1 Labelled immunochemical assays:

Types of labels

Examples of labelled immunoassay

- Radioactive (1959): radioisotopes I^{125}, I^{131}
- Enzymes (1971): alkaline phosphatase, horseradish peroxidase, firefly luciferase
- Fluroscence (1973): fluorescein
- Chemiluminescence (1978): acridium ester, sulfonyl acridinium ester, isoluminol

Others

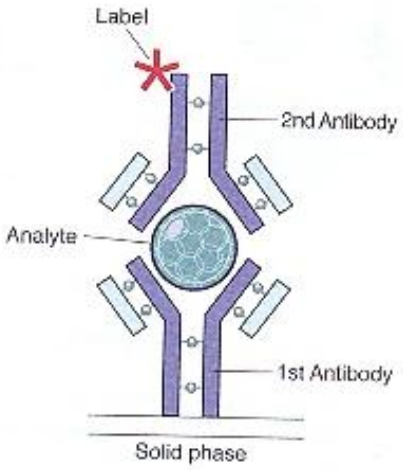
- Cofactor: adenosine triphosphate, flavin adenine dinucleotide
- Metal particle: gold sol, selenium sol, silver sol

6. Chemiluminescence based Immunoassay:

Chemiluminiscent based immunoassay is a variation of enzyme immunoassays based on noncompetitive sandwich ELISA. In ELISA enzyme labelled antibodies and antigens are used which gives a color at the end of reaction process whose intensity is measured and expressed into concentration. Similarly in chemiluminescence immunoassays luminescence generated during a chemical reaction which is emitted light when a substance returns from an excited state to a ground state is measured. The chemiluminiscent substance can be excited by an oxidation reaction forming an intermediate . this intermediate when returns to a ground state emits photon which is detected by luminescent signal instrument.

Principle of Sandwich ELISA (double antibody sandwich method): Antibody attached onto solid phase captures antigen (test analyte). After analyte

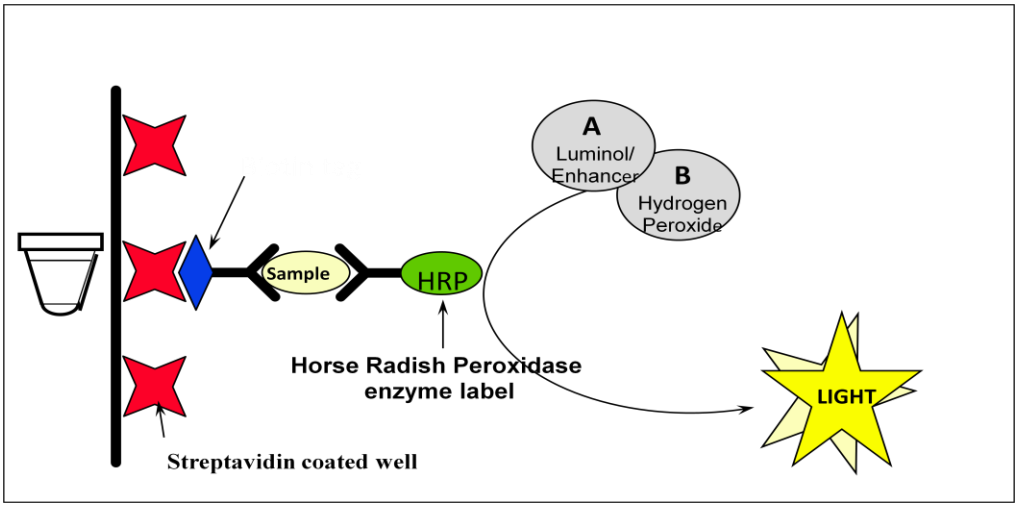
binding, second antibody added. This second antibody is labelled with an enzyme. A suitable conjugate is then added, this conjugate is converted to a colored product by the enzyme. Strength of signal directly proportional to the color development and hence analyte concentration.



Basics of the sandwich (noncompetitive) immunoassay

6.1 **Principle of Chemiluminescence based immunoassay:**

- Analyte to be measured is reacted with biotinylated antibody (sheep anti analyte). The antigen-antibody complex is captured by streptavidin on the wells. Any unbound material is removed by washing
- In a second incubation a horseradish peroxidase labeled antibody conjugate (mouse monoclonal anti analyte) binds to immobilized analyte. Unbound material is again removed by washing.
- Next a luminogenic substrate (luminol) and an electron transfer agent (acetanilide) is added to the wells. The bound HRP conjugate catalyzes the oxidation of luminol derivative producing light. The electron transfer agent increases the level of light produced and prolongs its emission. The light signals are read by the system.
- The amount of HRP conjugate bound is directly proportional to the concentration of analyte present.



6.2 Chemiluminiscence vs other immunoassays

- **Radiolabels: RIA**
    - Slow processes
    - Difficult to automate
    - Short shelf life
    - Regulatory scrutiny and disposal hazard
- **Colorimetric: EIA**
    - Limited sensitivity
- **Fluorescence: FPIA**
    - Can suffer from background interference
    - Limited sensitivity
    - Limited to small molecules

6.3 Advantages of CLIA

- Ultrasensitive: can detect small amounts
- Wider dynamic range: maintains linearity between luminous intensity and concentration of the measured substance
- Latest Technology

- Lasting luminescence
- Faster TAT than ELISA
- Increased sensitivity
- High precision when automated
- Wider detection limits
- Reaction can be enhanced with an enhancer: this allows the reaction to take place for a longer period of time and gives an intense light emission.

6.4 Limitations

- Limited suppliers
- High capital investment

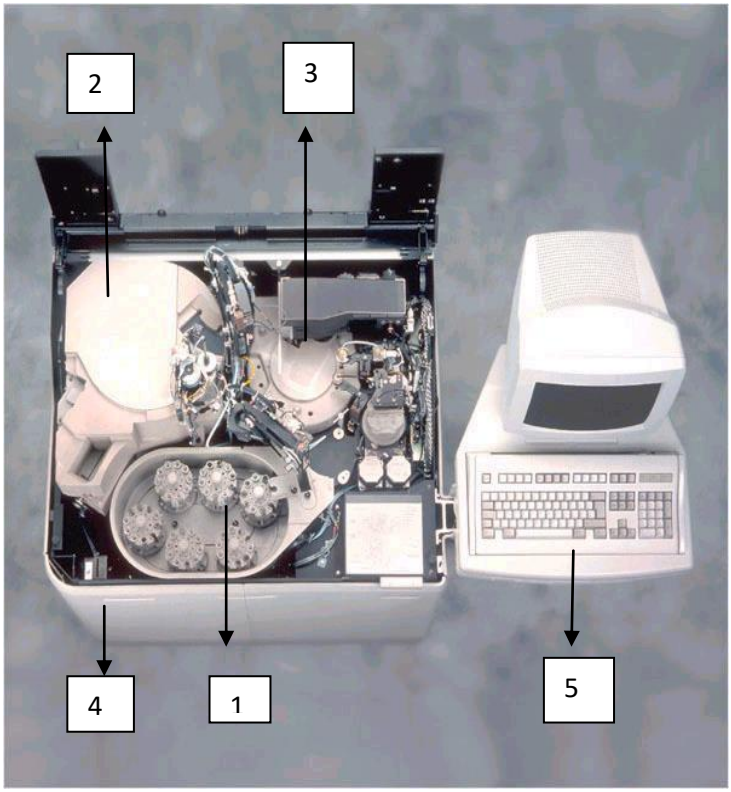
6.5Types of CLIA

- **Direct "Flash"**
    - Label is the substrate; linked to antibody/antigen
    - One oxidation event liberates one molecule of label with release of set number of photons.
    - Examples include: Acridinium esters, Ruthenium-E
- **Indirect "Glow"**
    - The label is an enzyme
    - Amplify by turnover of the chosen substrate
    - A single enzyme label can convert $>10^7$ molecules per minute
    - Represents a million-fold increase over Direct reactions
    - Examples include Luminol, HRPO

6.6 Enhanced Chemiluminiscence

- Horseradish Peroxidase (HRP) is the label
- Luminol is the substrate: Together with H_2O_2
- Enhancer (acetanilide) act as catalysts
- The Enzyme HRPO oxidises Luminol in the presence of Hydrogen Peroxide and enhancer 4 Chloro 3 Hydroxy Acetanilide.
- The oxidized Luminol produces a glow of light.
- The light is measured using Luminometer.
- Enhancers speed the oxidation of the luminol by HRP by as much as 1,000 times.
- Enhancer enhances the light intensity of each luminol molecule and sustains light production so that resulting light output is transformed from flash to glow

7. Instrumentation: Parts of chemiluminescent Immunoassay analyser

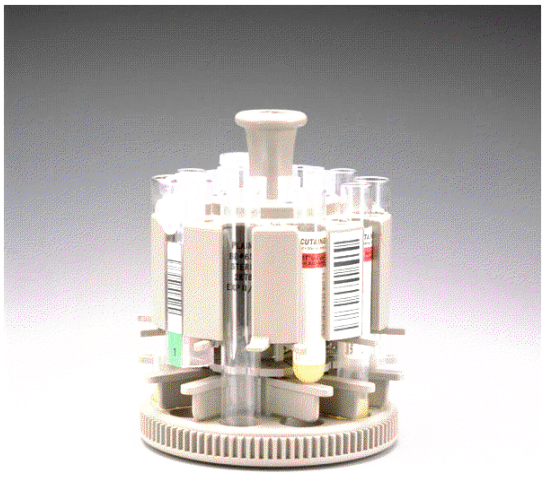


1. Sampling Center

2. Reagent Management Center
3. Processing Center
4. Supply Center
5. System Command Center

7.1 Sampling centre

Universal sample tray



- Continuous loading facility for samples without stopping machine
- Universal sample tray can accommodate multiple sample tube size or sample cups
- Sample metering to detect amount of sample, clot, lipemic sample, bubble or hemolysed sample

• Sample volume as low as 10 microlitre could be used.

•onboard auto dilution can be done in cases of high concentration

•Auto sample retest (same assay)

7.2 Reagent management centre

• On-board reagent stability : on-Board Refrigeration

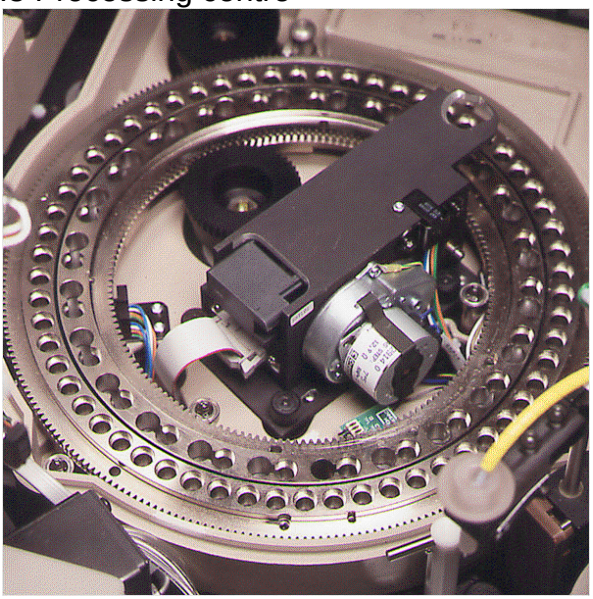
• Variable On-board test capacity

•Automatic inventory management

•Ability to load/unload reagents during system operation

Ready-to-use reagents
•Calibration stability of 28 days for all assays
•Magnetic Card data upload
•90-100 Tests per pack

7.3 Processing centre



•Micro-well wash subsystem
•Signal reagent subsystem

7.4 Supply centre

Universal wash reagent

•Less solid waste to manage

•No special maintenance

•Elimination of external plumbing and drains

•Automatic inventory management

Command centre

Color-coded touch screen

•Cross-functional navigation

•Full inventory management

•On-board QC

•Active HELP – Operator's Manual

- Storage of patient's results

Sample preparation

Both serum and plasma samples along with other body fluids after protein removal can be run in CLIA.

8. Test profile on CLIA

- Hormones: reproductive, endocrine, thyroid : Anti Tg, Anti TPO, Anti TSH receptor, free and total T3 and T4, TSH, thyroglobulin, ACTH, cortisol, estradiol, LH, progesterone, FSH, insulin, testosterone, DHEA-S
- Anemia markers: Ferritin, folate, RBC folate, vitamin B12
- Tumour markers:AFP, CA 125, CA 19.9, CA 15.3, CEA, PSA, freePSA, NSE, calcitonin
- Infections: hepatitis virus (HBe Ab, HBs Ab HBs Ag, HCV, HAV), HIV, H Pylori, syphilis
- Bone metabolism: PTH, vitamin D
- Cardiac markers: CK-MB,BNP, myoglobin, NT pro-BNP, troponin, galectin-3
- Drugs: cyclosporine, tacrolimus, digoxin, valproic acid, carbamazepine, Phenobarbital, phenytoin

Summary

The basic principle of chemiluminescence is when an excited product of an exogenic reaction comes back to its ground state it emits photons which is detected by a photodetector and expressed as readouts in terms of concentration. This enhances the detection limits of an analyte. It is a type of labelled immunoassay which is chemiluminescent like acridium ester, sulfonyl acridinium ester, isoluminol It provides higher sensitivity and specificity over

ELISA. Tests which were previously done with ELISA are now shifted to CLIA. CLIA is used where sample load is more in bigger laboratories. As it is an automated system the turnaround time is high. Application of CLIA includes estimation of various hormones, tumour markers, anaemia markers, infectious disease, bone metabolism, cardiac markers and drugs.