Chemiluminescence

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Fluorescence

- Fluorescence occurs when a substance **absorbs photons** (light particles) at one wavelength and then quickly emits photons at a longer wavelength, typically within nanoseconds to microseconds.
- Fluorescence emission is transient and typically lasts only as long as the substance is **excited by the external light source**.
- **Fluorescent dyes** used in microscopy and fluorescence-based assays exhibit fluorescence when excited by specific wavelengths of light.

Phosphorescence

- Phosphorescence occurs when a substance **absorbs photons** and undergoes a transition to a higher energy state, but the **return to the ground state is delayed**, resulting in the emission of photons over a longer timescale.
- Phosphorescence emission persists after the removal of the external light source and can last **from microseconds to hours or even days,** depending on the substance.
- **Glow-in-the-dark materials**, such as certain phosphorescent paints or stickers, exhibit phosphorescence when exposed to light and continue to emit light in the dark.

Luminescence

- Luminescence is a broader term that **encompasses both** fluorescence and phosphorescence, as well as other forms of light emission resulting from non-thermal processes.
- Luminescence can refer to both **transient emissions (fluorescence) and persistent emissions (phosphorescence) depending on the specific mechanism** involved.
- **Bioluminescence** (light emitted by living organisms), **chemiluminescence** (light emitted by a chemical reaction), and **electroluminescence** (light emitted by the passage of an electric current through a material) are all examples of luminescence

Enzyme Linked ImmunoSorbent Assay

- Enzyme-linked immunosorbent assay is commonly known as ELISA where **Ag-Ab interaction** is monitored by enzyme measurement.

- It is similar in principle to Radio Immuno Assay (RIA) but depends on an **enzyme** rather than a radioactive label.

- An enzyme conjugated with an antibody reacts with a **colorless substrate** to generate a **colored reaction product**. Such a substrate is called a chromogenic substrate.

PRINCIPLE

- ELISA use an enzyme to detect the **binding** of antigen (Ag) antibody (Ab).
- The enzyme **converts** a colorless substrate (choromogen) to a colored product, indicating the presence of Ag: Ab binding.
- An ELISA can be used to detect either the presence of antigens or antibodies in a sample depending how the test is designed.

- A number of enzymes have been employed for ELISA, including
  1. **Alkaline phosphatase**
  2. **Horseradish peroxidase**
  3. **Galactosidase**
- These assays approach the sensitivity of RIAs and have the advantage of being **safer** and **less costly.**

TYPES OF ELISA

- A number of **variations** of ELISA have been developed, allowing **qualitative detection** or **quantitative measurement** of either antigen or antibody.

- Each type of ELISA can be used **qualitatively** to detect the presence of antibody or antigen.

- Alternatively, a **standard curve** based on known concentrations of antibody or antigen isprepared, from which the **unknown concentration of a sample can be determined**.

INDIRECT ELISA

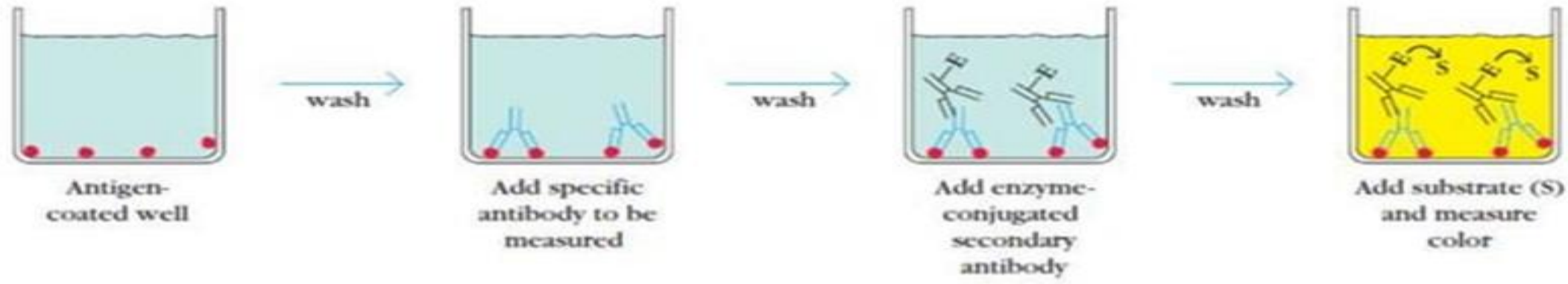
- Antibody can be **detected** or' **quantitatively determined** with an indirect ELISA.

- Serum or some other sample containing primary antibody is added to an **antigen-coated microtiter well** and allowed to react with the antigen **attached** to the well.

- After any free antibody is **washed** away, the presence of antibody bound to the antigen is **detected** by adding an **enzyme-conjugated secondary anti-isotype antibody** (Ab2), which binds to the primary antibody.

- Any **free Ab2** then is washed away and a **substrate** for the enzyme is added.

- The amount of colored reaction product that forms is measured by **specialized spectrophotometric plate readers,** which can measure the absorbance of all of the wells of **96 – well plate** in seconds.

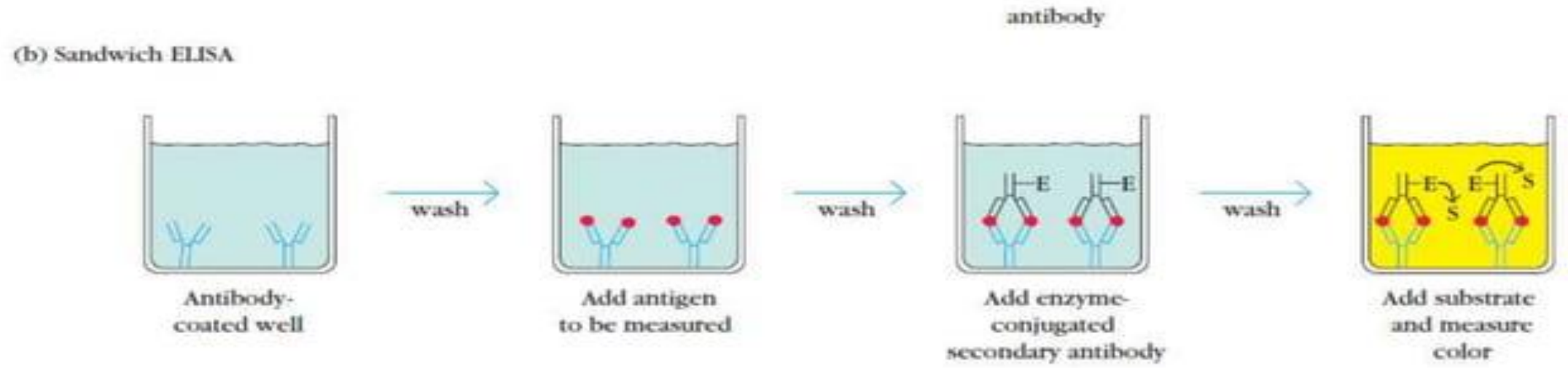


- Indirect ELISA detects the presence of serum **antibodies** against human immunodeficiencyvirus (**HIV**), the causative agent of AIDS.
- In this assay, recombinant envelope and core proteins of HIV are adsorbed as solid-phase antigens to microtiter wells.
- Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins.
- Generally, serum antibodies to HIV can be detected by indirect ELISA **within 6 weeks of infection.**

SANDWICH ELISA

- **Antigen** can be detected or measured by a sandwich ELISA.
- **In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well.**
- A sample containing antigen is added and allowed to react with the immobilized antibody.
- After the well is washed, a **second enzyme- linked antibody** specific for a different epitope on the antigen is added and allowed to react with the bound antigen

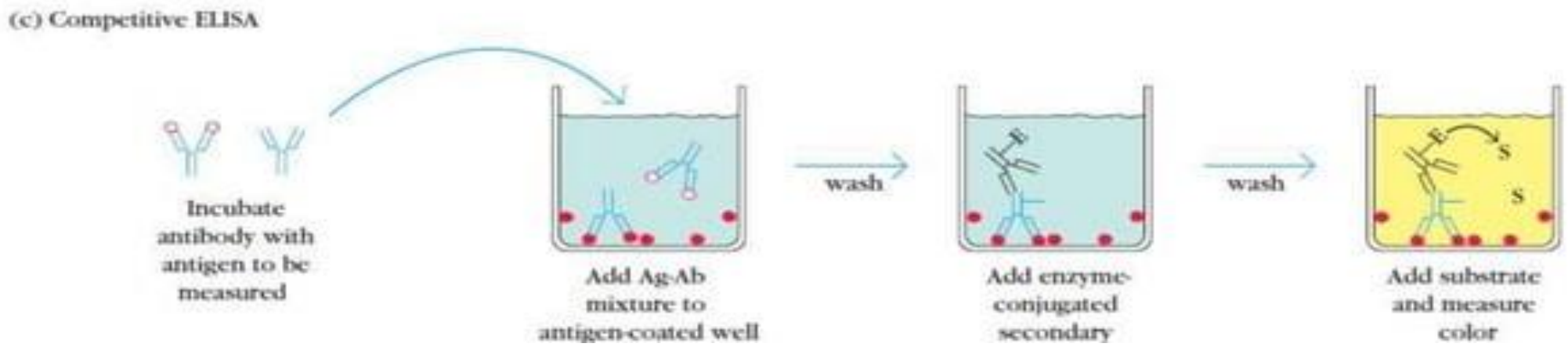
- After any free second antibody is removed by washing, **substrate** is added, and the colored reaction product is measured.



COMPETITIVE ELISA

- **Another variation for measuring amounts of antigen is competitive ELISA.**
- In this technique, antibody is **first incubated** in solution with a sample containing antigen.
- The **antigen-antibody mixture** is then added to an **antigen-coated microtiter well**.
- The **more antigen present in the sample**, the **less free antibody will be available** to bind to the antigen-coated well.

- Addition of an **enzyme-conjugated secondary antibody (Ab2)** specific for the isotype of the primary antibody can be used to determine the amount of **primary antibody** bound to the well as in an indirect ELISA.
- In the competitive assay however, **higher the concentration of antigen** in the original sample, the lower **will be the absorbance.**



Commonly used enzymatic markers

- **OPD** (*o*-phenylenediamine dihydrochloride) turns **amber** to detect HRP (horseradish peroxidase), which is often used to as a conjugated protein.

- **TMB** (3,3',5,5'-tetramethylbenzidine) turns **blue** when detecting HRP and turns yellow after the addition of sulfuric or phosphoric acid.

- **ABTS** (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) turns **green** when detecting HRP.

- **PNPP** (*p*-Nitrophenyl Phosphate, Disodium Salt) turns **yellow** when detecting alkaline phosphatase.

COMMONLY USED LABELED ANTIBODIES IN IMMUNOASSAYS:

- **Enzyme-labeled antibodies (ELISA):** horseradish peroxidase (HRP) or alkaline phosphatase (AP)
- **Fluorescent-labeled antibodies:** as fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC). flow cytometry and immunofluorescence microscopy
- **Radio-labeled antibodies:** radioactive isotopes such as iodine-125 (^125I) or tritium (^3H). Radioimmunoassay (RIA)

- **Gold nanoparticle-labeled antibodies:** lateral flow assays and other point-of-care diagnostics.
  gold nanoparticles. visible signal when bound to the target antigen, producing **a colored line** or **spot on the test strip**.

- **Biotin-labeled antibodies:** biotin, a small molecule that binds strongly to avidin or streptavidin.

GENERATIONS OF ASSAYS

- The concept of "generations" in immunoassays is based on **enhancements in sensitivity and specificity**.
- Terms like first, second, and third generation are commonly used, particularly in **assays for thyroid-stimulating hormone (TSH).**

Background and Origin:

- **Nicoloff** and **Spencer** introduced the classification based on functional sensitivity in 1990.
- Functional sensitivity is defined as **the lowest concentration measurable with a CV of 20% or less.**

First Generation:

- Introduced in the 1960s.
- Based on **competitive binding using one polyclonal antibody and a radioactive label.**
- Functional sensitivity: **1-2 mIU/L**.

Second Generation:

- Emerged in the 1970s.
- Utilized **noncompetitive** or two-site immunometric methods.
- Employed **polyclonal antibodies initially**, **later** incorporating **monoclonal antibodies**.
- Functional sensitivity: **0.1-0.2 mIU/L.**

Third Generation:

- Introduced with **monoclonal antibody** designs.
- Utilized highly sensitive labels like **chemiluminescent tags and fluorophores.**
- Functional sensitivity: **0.01-0.02 mIU/L.**

Advancements and Clinical Relevance

- Monoclonal antibodies coupled with sensitive labels lead to a **100-fold improvement** over first-generation assays.
- Clinically relevant for **diagnosing various thyroid conditions and other illnesses.**

Chemiluminescence

- Chemiluminescence is the emission of light when an electron returns from an excited or higher energy level to a lower energy level.

- The excitation event is caused by a chemical reaction and involves the oxidation of an organic compound, such as luminol, isoluminol, acridinium esters, or luciferin, by an oxidant (e.g., hydrogen peroxide, hypochlorite, oxygen; light is emitted from the excited product formed in the oxidation reaction.

- These reactions occur in the presence of catalysts, such as enzymes (e.g., alkaline phosphatase, horseradish peroxidase), metal ions, or metal complexes (e.g., hemin).

principles of chemiluminescence

- 1. Chemical Reaction:
  The conversion of chemical energy into light energy. This typically occurs when reactants undergo a chemical reaction, resulting in the formation of an excited-state molecule.

- 2. Excited-State Molecule Formation: During the chemical reaction, some molecules become temporarily elevated to an excited electronic state. This state is characterized by higher energy levels than the ground state.

3. Light Emission: As the excited-state molecules return to their ground state, they release energy in the form of photons (light particles). This energy release results in the emission of light with specific wavelengths characteristic of the reaction.

4. Detection: The emitted light can be detected and measured using photodetectors, such as photomultiplier tubes or photodiodes, which are sensitive to low levels of light.
The intensity of the emitted light is proportional to the concentration of the chemiluminescent species formed during the reaction.

essential components required for performing chemiluminescence assays

- Target Analyte:
  - molecule of interest that the assay aims to detect or quantify.
  - Eg: Antigen, Antibody, Enzyme, Nucleic Acid,
    Or Other Biomolecule Relevant To The Assay's Purpose.
- Capture and Detection Reagents:
  - antibodies or nucleic acid probes, are used to immobilize the target
    analyte onto a solid-phase support, microplate well or membrane.
  - Detection reagents, often labeled with a chemiluminescent tag or enzyme, bind specifically to the captured analyte and facilitate signal generation.

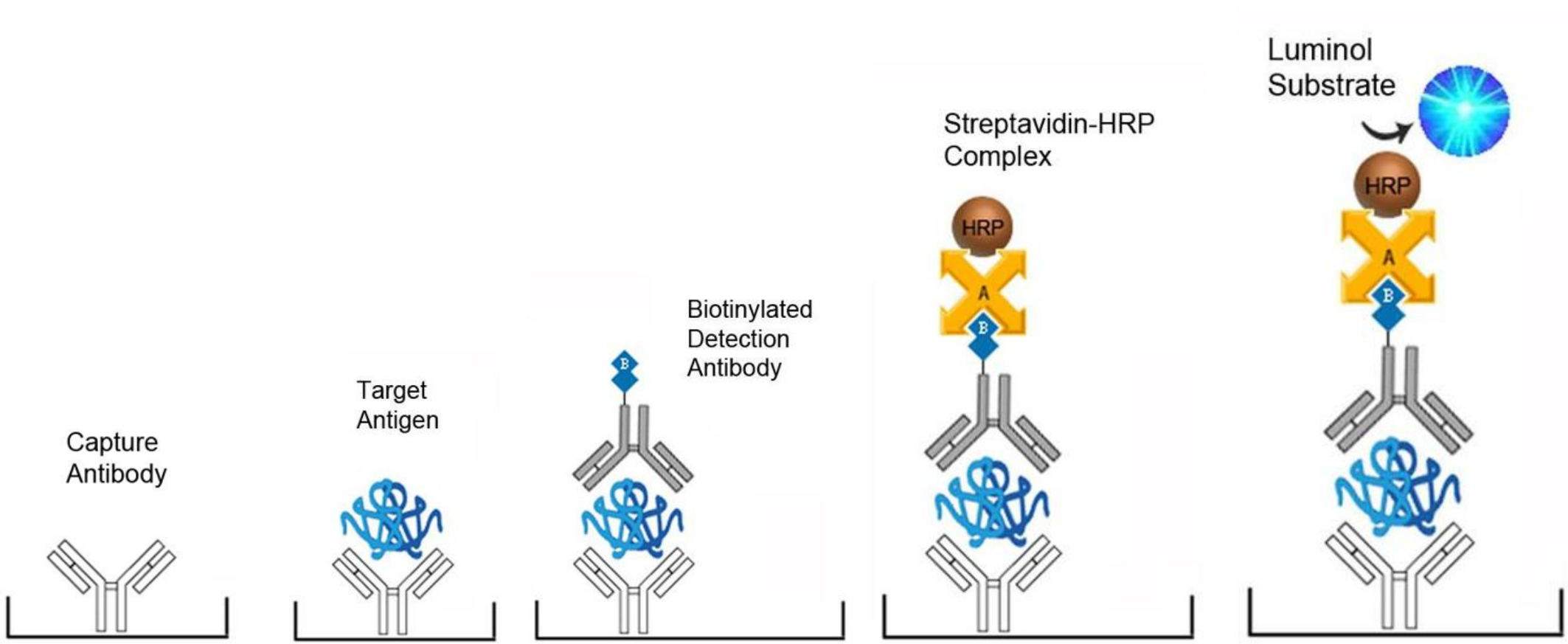
3. Chemiluminescent Substrate:

- These substrates emit light upon reacting with the enzyme-labeled detection reagent in the presence of cofactors or co-substrates.
- luminol, acridinium esters, and dioxetanes.

4. Enzyme or Catalyst:

- an enzyme or catalyst is conjugated to the detection reagent to catalyze the chemiluminescent reaction with the substrate.
- Horseradish Peroxidase (HRP), Alkaline Phosphatase (AP), And Luciferases.
- These enzymes catalyze the conversion of the chemiluminescent substrate into a product that emits light.

- 5. Reaction Buffer and Cofactors:
  - A reaction buffer is used to create optimal conditions for the chemiluminescent reaction to occur including maintaining appropriate pH, ionic strength, and cofactor concentrations.
  - hydrogen peroxide (H2O2), ATP, and magnesium ions
- 6. Detection Instrumentation:
  - detecting and quantifying the emitted light.
  - luminometer or a photodetector system equipped with sensitive detectors capable of measuring low levels of light emission.
  - Detection instrumentation may also include automated sample handling and data analysis capabilities.


Capture
Antibody
Target
Antigen
Biotinylated
Detection
Antibody
B
Streptavidin-HRP
Complex
HRP
A
B
Luminol
Substrate
HRP
A
B

Matrix effect :

- Matrix effects (MEs) are changes in the detection or quantification of an analyte when other substances are present in a sample. These effects can cause poor accuracy in results, such as immunoassays, and reduced precision in results. MEs are often observed in modern analytical routines, such as GC, HPLC, and ICP.

- In chemiluminescence, matrix effects can occur in cryogenic matrices of nitrogen and inert gases.

- MEs are caused by co-eluting matrix components that alter the ionization and chromatographic response of target analytes. This can lead to reduced or increased sensitivity of the analysis.

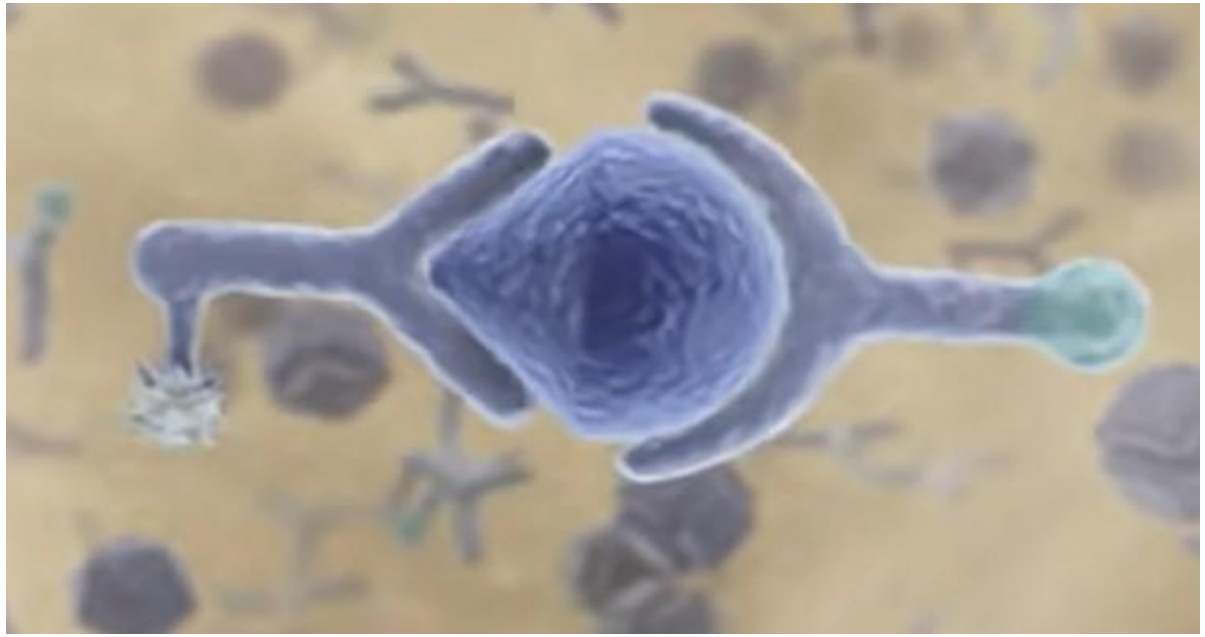
- The most common approach for accounting for MEs is to build a calibration curve using standard samples with known analyte concentration.

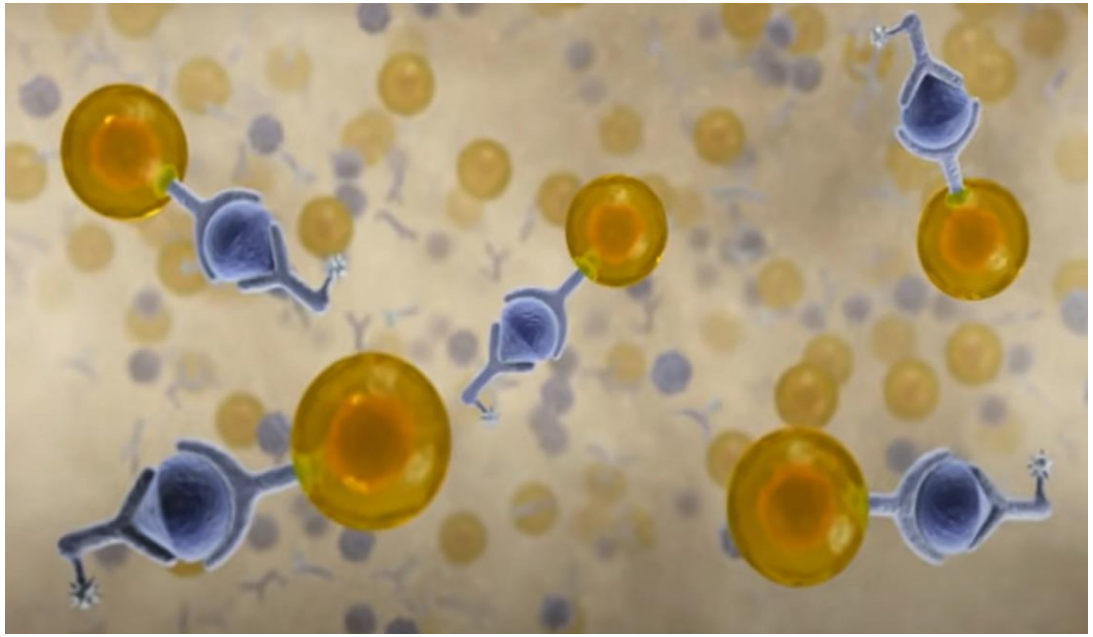
ELECTROCHEMILUMINESCENCE (ECL)

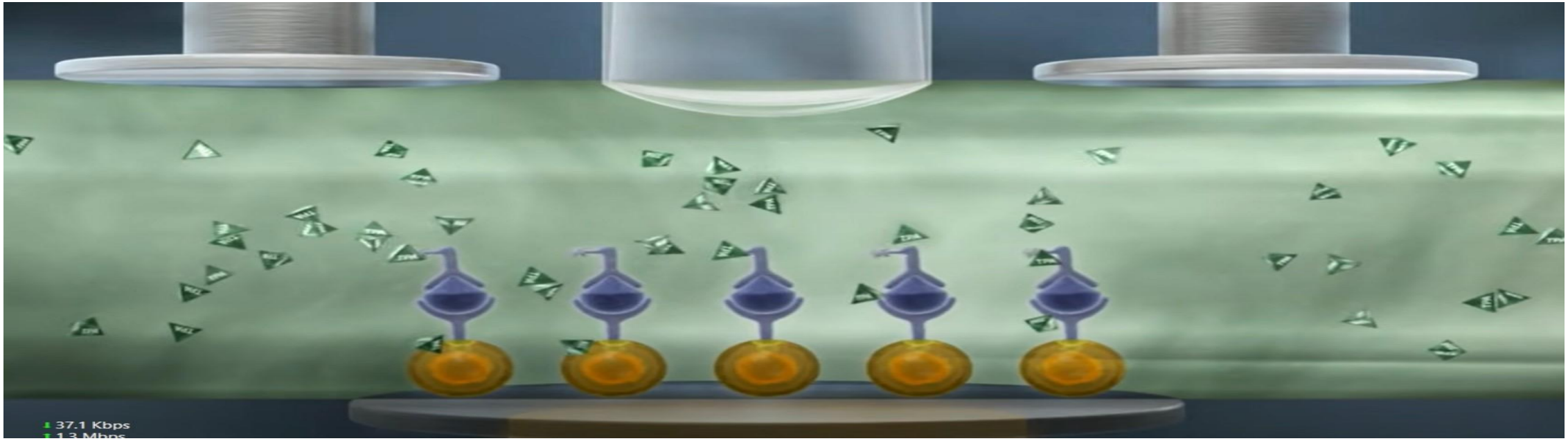
- ECL: primary and secondary antibody is labelled with Biotin and Rutherium resp.
- Highly sensitive.
- Microbeeds coated with streptavidin. (Streptavidin-Biotin bounded)

- TPA (tripropylamine)

- TPA is oxidized to a radical cation that spontaneously loses a proton. The resulting TPA radical reacts with oxidized ruthenium, resulting in the excited state of the ruthenium label, which decays with the emission of a photon (620 nm)





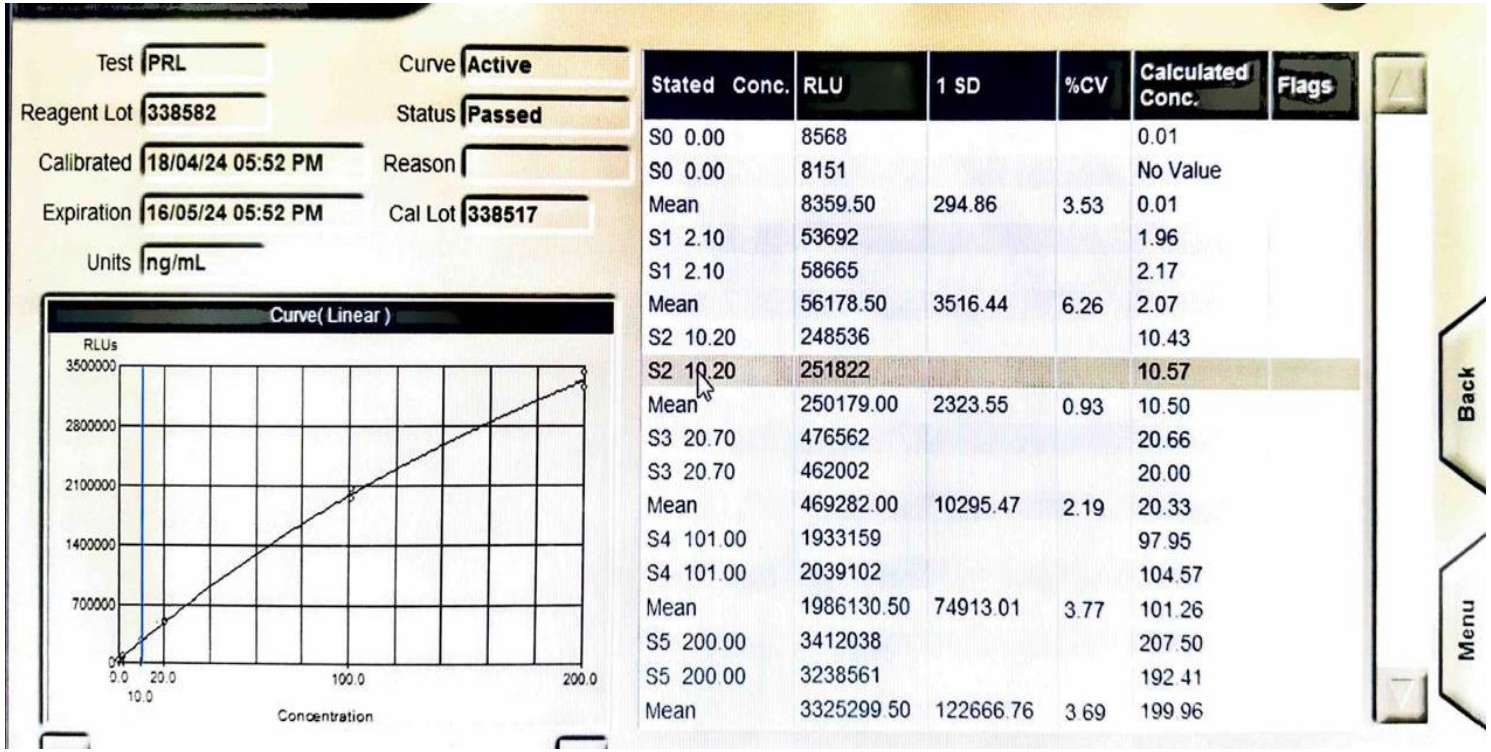

37.1 Kbps

What is RLU (Relative Light Units)

- we are essentially measuring the number of photons emitted. It turns out that accurately quantifying photons is hard.
- instrument contains a photomultiplier tube (PMT) that amplifies the signal so it can be measured. Electrons are produced when photons strike the detection window, generating an amplified electrical signal.
- The more photons, the more electrical signal. The software in the instrument then attempts to quantify the electrical signal and reports a numerical value.

How Simple is the Measurement?

- The numbers are calculated in proportion to the electrical signal, they are relative to the amount of electrical signal produced: the relative in the RLU.



Stated Conc.	RLU	1 SD	%CV	Calculated Conc.	Flags
S0 0.00	8568			0.01	
S0 0.00	8151			No Value	
Mean	8359.50	294.86	3.53	0.01	
S1 2.10	53692			1.96	
S1 2.10	58665			2.17	
Mean	56178.50	3516.44	6.26	2.07	
S2 10.20	248536			10.43	
S2 10.20	251822			10.57	
Mean	250179.00	2323.55	0.93	10.50	
S3 20.70	476562			20.66	
S3 20.70	462002			20.00	
Mean	469282.00	10295.47	2.19	20.33	
S4 101.00	1933159			97.95	
S4 101.00	2039102			104.57	
Mean	1986130.50	74913.01	3.77	101.26	
S5 200.00	3412038			207.50	
S5 200.00	3238561			192.41	
Mean	3325299.50	122666.76	3.69	199.96	

The Best Application- CLIA

- Chemiluminescent immunoassays have been developed for several applications by measuring RLU.
- The level of light produced in enhanced chemiluminescence reactions is about 100-fold higher.
- The highest OD reading achieved in ELISA was 0.3-0.4 OD.
- In contrast, the CLIA ranges between 0 and 800 RLU.

CLIA VS ECL

Feature	Chemiluminescence	Electrochemiluminescence
Duration of Signal	Typically shorter duration of light emission	Longer duration of light emission
Activation	Typically triggered by mixing reactive substances	Requires electrical potential for activation
Reaction Speed	Reaction rates may vary, usually slower	Faster reaction rates
Environmental Sensitivity	Susceptible to environmental factors	More robust against environmental factors
Multiplexing Potential	Can be challenging to multiplex	Allows for easier multiplexing
Cost	Often lower cost due to simpler instrumentation	Higher cost due to specialized equipment
Sample Requirements	Can require specific sample preparation	Less stringent sample requirements
Stability	Generally less stable signals	More stable signals
Electrode Dependency	Not dependent on electrodes	Requires electrodes for electrochemical
		reactions

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THANK YOU