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TECHNICAL SERIES

CHEMILUMINESCENCE

Principle & Troubleshooting Aspects

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Foreword

Qualpro Diagnostics a division of **TULIP DIAGNOSTICS PVT. LTD.** based in Goa, India.

Tulip's commitment in building products of international standards, through indigenous R&D has accorded the company virtual leadership in most product segments in the Indian marketplace. Its state-of-art manufacturing facility conforms to the strictest FDA (India) and GMP regulations. In its efforts to build world-class Quality products, the group has recently received the ISO 9001(2000) certification from TUV. It is this commitment to Quality, which has given the group international acclaim

Publishing of **Technical Series** is one such initiative to make available to the Laboratory professionals and clinicians updated knowledge that is vital for them to set trends in their day-to-day practice.

Introduction

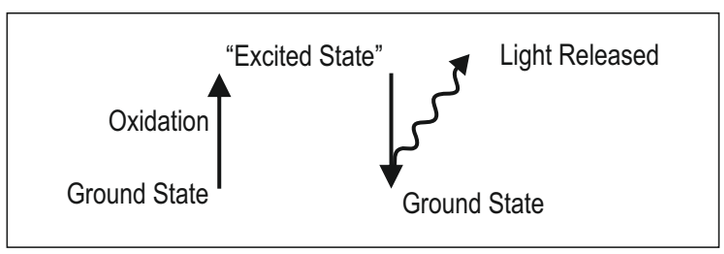
In recent years, chemiluminescence immunoassay (CLIA) has gained increasing attention in different fields, including life science, clinical diagnosis, environmental monitoring, food safety and pharmaceutical analysis because of its high sensitivity, good specificity, wide range of applications, simple equipment and wide linear range. Traditional immunoassay always needs a long incubation time, which in turn causes the whole analysis to take several hours for completion, so the throughput and application range are largely limited. Researchers have designed different methods to shorten the analysis time by improving mass transport and reaction kinetics. The immunoassay has expanded the applications of CLIA.

Chemiluminescence immunoassays are generally considered as 4th generation assays when compared to 3rd generation EIA's.This is because CLIA has significantly improved sensitivity & high signal to noise ratio.

What is Chemiluminescence ?

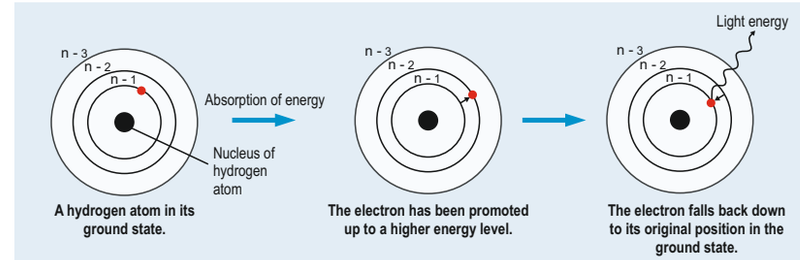
The term" Chemiluminesence" was first coined by Eilhardt Weidemann (1888). Chemiluminescence, which is the phenomenon observed when the vibronically excited product of an exoergic reaction relaxes to its ground state with emission of photons, can be defined in simplistic terms: chemical reactions that emit light. The chemical reaction produces energy in suficient amount (approximately 300 kJ mol 1 for blue light emission and 150 kJ mol 1 for red light emission) to induce the transition of an electron from its ground state to an excited electronic state. This electronic transition is often accompanied by vibrational and rotational changes in the molecule.

In organic molecules, transitions from a π bonding to a π* anti-bonding orbital (π→π*) or from a non-bonding to an anti-bonding orbital (n→π*) are most frequently encountered. Return of the electron to the ground state with emission of a photon is thus called chemiluminescence. The ex-cited molecule can also lose energy by undergoing chemical reactions, by collisional deactivation, internal conversion or inter-system crossing. These radiations less processes are undesirable from an analytical point of view when they compete with chemiluminescence. (see Figure below).



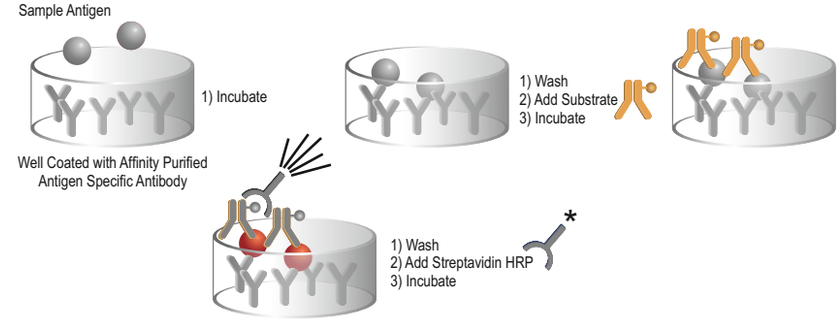
When two molecules react chemically so that there is a release of energy (an exothermic reaction), that energy sometimes manifests itself not as heat but as light. This occurs because the energy excites the product molecules into which it has been funneled. A molecule in this excited state either relaxes to the ground state, with the direct emission of light, or transfers its energy to a second molecule, which becomes the light emitter. This process is referred to as chemiluminescence. (see Figure below).

A + B -> AB* -> Products + Light



Principle of Chemiluminescence Immunoassay (CLIA)

In the presence of complimentary antigen and antibody, the paratope of the antibody binds to the epitope of the antigen to form an antigen-antibody or an immune complex. Estimating the levels of such immune complex by use of labeled antibodies form the basis of CLIA. It involves use of stationary solid phase coated either with the antigen or antibody of interest. This results in generation of light, the intensity of which is directly proportional to the amount of labeled complexes present and which indirectly aids in quantification of the analyte of interest. The intensity of light is measured in terms of Relative Light Units (RLU).(see Figure below).



The main advantage of this technology includes sensitivity and its ability to be unaffected by background signals. Also, the analyzers working under this principle are simple in design and operation

Basic Components & Prerequisites for Electra (CLIA)

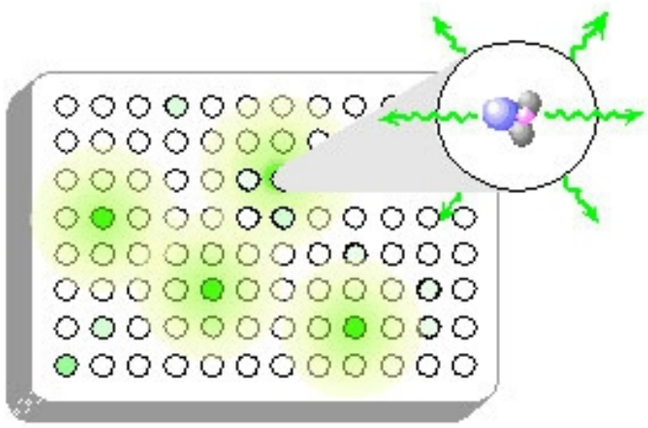
The basic components for a CLIA reaction and the corresponding prerequisites are:

1. Solid Phase: Where all reactions including the Ag-Ab and all subsequent reactions (till the generation of light) take place.

Prerequisite:

(a) Light emitted in CL reactions is isotropic- it is emitted equally in all directions. If CL reactions are conducted in wells of a transparent plate, light radiates out not only vertically in the direction of the detector, but also laterally to other wells. Light is easily transmitted through the plate material itself, a phenomenon termed light piping. **For this reason, CLIA should never be performed in clear plates. I**nstead, opaque plates must be used. Two kinds of opaque plates are available: Black & White. There is usually a 10-fold decrease in signal owing to light absorption, when using black plates.

The best option is to use white plates of uniform opacity. A recent breakthrough to minimize light piping and the resultant cross-talk between reaction cells is to coat the reaction cells with a layer of **Titanium. Titanium, which has a complex mesh work structure cuts down light piping and cross-talk drastically.** (see Figure below)



(b) The solid phase must allow efficient adsorption of Ag or Ab as capture.

© Optimum concentration of capture is critical: Prevents Hook Effect & ensures Sensitivity.

(d) High Specificity of capture prevents false positivity.

2. **Signal Reagent :** The Signal Reagent is the actual Chemiluminescent compound (e.g. Luminol, 1, 2-dioxetane, Acridinium ester). Produces light upon oxidation by the enzyme in the tracer. (May require the presence of an additional molecule like H202)

Prerequisite : The Signal reagent must have a combination of these compounds

(a) The Cl-Substrate Luminol I 1, 2-dioxetane (and also H202 if using luminol) to produce light.

(b) The Enhancer to enhance heighten the light produced.

3. **CLIA Substrate :** This is a critical factor in determining the CLIA efficacy. The CL-substrate, upon oxidation by the enzyme tagged to the tracer yields light. Quantum of light yield depends on the kind of substrate used. Several kinds of CL substrates are available. We take a look at two of the widely used CL substrates & the reactions involved therein:

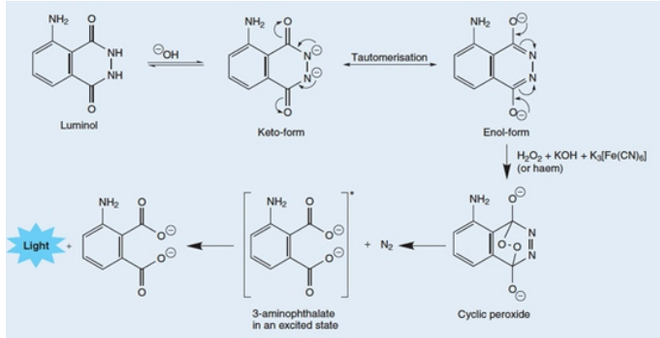
Luminol

In luminol-based assays oxidizer (OX:H202) & light-emitting material (LEM: Luminol) is mixed in liquid phase under a desired pH value. In presence of H202, Luminol is oxidized & decomposed into anIntermediate Compound-I, in the presence of the enzyme Peroxidase. Intermediate Compound-I reacts with an Enhancer (Iodophenol Compounds are strong enhancers that intensify luminol CL by 1000 times) to generate Intermediate Compound-II, which in turn is converted into 3- aminophthalate dianion with the concomitant emission of light.

H2O2ENHANCER

LUMINOI →INTERMEDIATE-1 →INTERMEDIATE-2→3-AMINOPHTHALATE+ CL

PEROXIDASE



Higher the signal generated in this step (light in CL) better the sensitivity. But, increase in signal should not be accompanied by increase in the noise (NSB). Discriminatory measurements add to the accuracy of results. Ideally the signal is stable for 10-15 mins.

4. The Enhancer : Enhancers are molecules, the addition of which increases the light output by several orders of magnitude. For example, HRPO, in the presence of H202, causes oxidation of luminol with the emission of light. This light output increases more than 1000- fold in the presence of 4-iodophenol. Most enhancers are substituted phenols & naphthols.

Enhancers improve the signal-to-noise ratio.

Enhanced CL is a glow rather than a flash of light which persists for hours, although reading is normally taken within 10-15 mins.

Examples of enhancers: Iodophenol, Phenothiazine.

Luminescence the ultimate result of the CL reaction

The ultimate result of a CL reaction is LIGHT. The intensity of all CL reactions is usually high (as enhancers are used).Depending on the duration of the light, CL can be of 2 types:

I- "FLASH"- type wherein addition of reagent(s) causes the immediate emission of light, which lasts for a few milliseconds.

II-"GLOW"- type where the emission of light builds up slowly and reaches a maximum after a substantial incubation time.

Measuring Luminescence

Light consists of billions of tiny packets of energy called photons. Light or Photons emitted from the reaction need to be measured or counted. Light from CL reactions are measured by an instrument:

Luminometer. The choice of a luminometer is dictated by the required dynamic range, sensitivity, reproducibility, temperature control, on-board software for data analysis.

ELECTRA : The Luminometer

All CL detection systems consist of the following components:

A. The Sample Chamber

B. The Detector

C. Single Processing Method & Signal Output Display

A. The Sample Chamber:-

a. Presents the luminescent sample to detector for measuring the quantity of light.

b. Must be sealed from ambient light in order to minimize potential interferences.

c. Must be positioned as close to the detector as possible to maximize optional efficiency.

(High Efficiency is desirable for an optional signal-to-noise ratio, which allows rapid, which allows rapid and precise measurements).

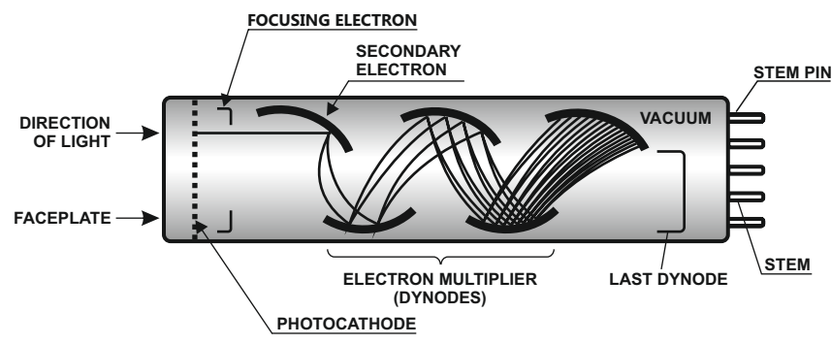
B. The Detector :-

The photo-multiplier tube (or PMT) is a versatile device that provides extremely high sensitivity and ultra-fast response. A typical photo-multiplier tube consists of a photo emissive cathode (photocathode) followed by focusing electrodes, an electron multiplier and an electron collector (anode) in a vacuum tube.

When light enters the photocathode, the photocathode emits photoelectrons into the vacuum. These photoelectrons are then directed by the focusing electrode voltages towards the electron multiplier where electrons are multiplied by the process of secondary emission. The multiplied electrons are collected by the anode as an output signal.

Because of secondary-emission multiplication, photomultiplier tubes provide extremely high sensitivity and exceptionally low noise among the photosensitive devices currently used to detect radiant energy in the ultraviolet, visible, and near infrared regions. The photomultiplier tube also features fast time response, low noise and a choice of large photosensitive areas.

This section describes the prime features of photomultiplier tube construction and basic operating characteristics.



A Photo Multiplier Tube

Process: Light which enters a PMT induces an output signal by the following process:

1. Light (Photons) enters through the input window and strikes the photocathode.
2. The Photocathode emits photoelectrons into the vacuum (due to photoelectric effect). The Photocathode is normally kept at a very high negative voltage, typically -500 to -1500 volts.
3. These photoelectrons are then directed by the focusing electrode voltages towards the electron multiplier (dynodes) where the electrons are multiplied (normally 106to 107) by a process of secondary emission. This secondary emission is repeated at each of the process of secondary emission. This secondary emission is repeated at each of the successive dynodes.
4. The multiplied electrons emitted from the last dynode are collected by the anode. Because of the secondary-emission multiplication, PMTs provide extremely high sensitivity & exceptionally low noise.

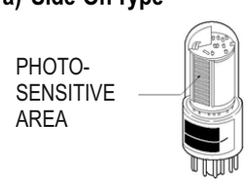
Construction:- The photomultiplier tube generally has a photocathode in either a side-on or a head-on configuration. The side-on type receives incident light through the side of the glass bulb, while in the head-on type, it is received through the end of the glass bulb. The head-on type (or the end-on type) has a semitransparent photocathode (transmission-mode photocathode) deposited upon the inner surface of the entrance window. The head-on type provides better spatial uniformity than the side-on type having a reflection-mode photocathode.

Other features of head-on types include a choice of photosensitive areas from tens of square millimeters to hundreds of square centimeters.

Variants of the head-on type having a large-diameter hemi-spherical window have been developed for high energy physics experiments where good angular light acceptability is important.

Figure : External Appearance

a) Side-On Type



b) Head-On Type

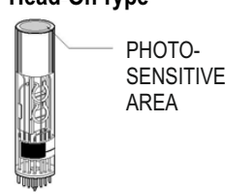
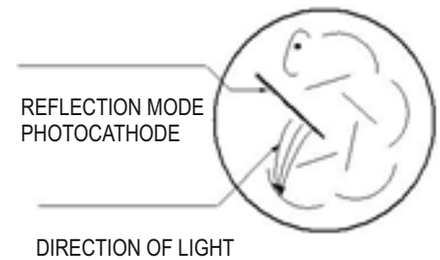
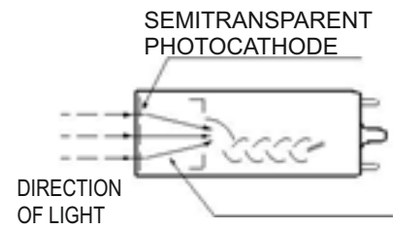


Figure: Types of Photocathode a) Reflection Mode



b) Transmission Mode



The superior sensitivity (high current amplification and high S/N ratio) of photomultiplier tubes is due to the use of a low-noise electron multiplier which amplifies electrons by a cascade secondary electron emission process.

C. Signal Processing Method & Signal Output Display.

In the signal processing method it is important to underline two very critical facts:

1. All electrons generated from the photocathode will not follow the same trajectory and hit the dynode. Some may deflect and become a source of NSB
2. Many times, output pulses are present even if no light falls on the PMT (dark current) owing to radiation & cosmic rays. This again contributes to high NSB.

Advantages of Chemiluminescence:-

Rated as the best detection technology, Chemiluminescence, has many advantages. We take a look.

1. **High Sensitivity:** The most important parameter that differentiates one detection technology from another is the analytical sensitivity. It is a well known fact, that higher the sensitivity, the better the technology.

Enhanced Chemiluminescence is upto 100,000 times more sensitive than absorption spectroscopy & at least 1000 times more sensitive than fluorometry.

2. High Linearity :

A higher linear range is limited in ELISA readers as they cannot read O.D.s higher than 3.0, thus compelling dilution.

In Chemiluminescence Immunoassays, samples of larger concentration ranges can be analyzed without dilution. Chemiluminescence offers linear measurement over a dynamic range of 10· or 10'. Furthermore, the higher dynamic range in CLIA enhances the resolution, making discrimination between positives and negatives easy. This is particularly useful in the diagnosis of infectious disease, where the discrimination between positives and negatives is absolutely essential for proper treatment.

3. High Signal To Noise Ratio :

In Chemiluminescence, there is better discrimination of signal from noise. This is possible by the use of enhancer, pulse height discriminator and photon counting.

4. Simplicity :

The attractiveness of ECL as an analytical tool is the simplicity of detection. Measurement of lightintensity is relatively simple, requiring only a photomultiplier or photodiode & the associated electronics to convert and record the signals.

5. Reproducibility :

Chemiluminescent assays are extremely robust and reproducible.

6. No Quenching:

Lower-than-expected quantum yield occurs in Fluorescence assays. This happens when amacromolecule such as an antibody heavily labeled with a fluorophor (FITC, fluorescein), is excited, and being spatially very close, radiation less energy transfer occurs. Also in aqueous environments quenching occurs due to collision. Inclusion of Enhancers excludes water from the site of CL production and increases emission efficiency.

7. No Inner Filter Effect :

Unlike in fluorescence, where the linear relationship between concentration and fluorescence emission holds as long as solutions are used that absorb less than 2% of the exciting light, in chemiluminescence, relationship between light emission and concentration is linear as no exiting light is required.

In fluorescence, as the absorbance of the solution increases above **this** amount the relationship becomes non-linear. The effect is called the inner filter effect and is caused by a loss of excitation intensity across the cuvet path length as the excitation light is absorbed by the fluorophor. Chemiluminescence is free from this error.

8. No Light Scattering Effects:

Rayleigh and Raman scattering common in Fluorescent measurements is not seen in Chemiluminescence as there is no excitation light source involved.

9. No Material & Solvent Effects:

In Fluorescence assays quartz glass and plastic materials containing ultraviolet absorbers may emit fluorescence and add to the background noise. This possibility is completely done away with as in Chemiluminescence, the light comes only from the reaction.

Also, some solvents like ethanol can cause appreciable fluorescence, but no chemiluminescence.

10. No Photodecomposition Effects :

Photodecomposition of analyte due to usage of intense light sources, which occurs in conventional and laser-induced fluorometry, does not occur in chemiluminescence.

11. No Dependence On Oxygen Concentration:

Electrochemiluminescence, a variant of chemiluminescence (wherein reactive species at the surface of an electrode emit light from stable precursors) is heavily dependent on oxygen availability.

The reaction usually involves oxidation-reduction type reaction with ruthenium (II) tris (bipyridyl) and triptopylamine. Chemiluminescence on the other hand, has no such dependence on oxygen availability as simple single-step reactions are involved in the generation of light.

12. No Pseudo-base Formation:

Pseudo-base formation is an inherent drawback in electro chemiluminscence, dictated by the complex chemistry involved.

Chemiluminescence, on the other hand has no such complex chemical intermediate formation, as the basic reaction involved is very simple. Also, flexibility to include enhancers in system does not affect overall Ag-Ab reaction.

13. Not Overly Prone To Interferences:

High background intensity observed in fluorescence and electro chemiluminecscence is not an issue when it comes to Chemiluminescence. Whatever little interferences arise in the PMT from stray electrons and radiations from the environment, can be taken care of, by the appropriate use pulse height discrimination mechanisms is a good luminometer.

14. Greater shelf-life:

Chemiluminescence substrates have a shelf-life of about a year, whereas those of fluorescence (containing fluorescin molecule) last only for a week. With so many advantages that it offers (as proven by numerous scientific studies), Chemiluminescence, definitely dominates over all other detection technologies. But the fact of the matter is, in India, Chemiluminescence is still a technoloqy accessible to a select few clinical laboratories. This marvel of a technology can do wonders if it becomes accessible to laboratories in all corners of the country. Near-patient testing and doorstep, high quality immunoassay results generated by Chemiluminescence will redefine immunosassays and benefit laboratories and patients alike.

Trouble shooting guidelines for CLIA

1. High RLU values of negative control

Possible Causes	Corrective Action
• Contamination of negative control cells preceding cell or positive control.	• When washing do not allow wells by to overflow.
• Contamination of negative control vial.	• Check pipette barrel for residual fluid or dried material. Remove if present. • Use new pipette tip for each sample. • Always format negative control wells before positive control. • Re-run with fresh reagents.
• Insufficient washing	• Make sure cells are completely filled. While washing ensure residual conjugate is removed from well. • Pipette all specimen and reagents in the center bottom of the microcell. Avoid contact with inner wall and rim. • Re-wash
• Wash buffer prepared incorrectly or not freshly prepared	• Rerun the assay with fresh wash buffer • Check the pH of the distilled water used for the preparation of wash buffer

2. Overall high RLU values

Possible Causes	Corrective Action
• Insufficient wash step.	• Repeat wash step. Ensure wells are filled completely but do not overflow.
• Contamination of reagents.	• Check pipette barrel for residual fluid or dried material. Remove if present. • Check that pipette tips are long enough to provide air space between top of tip and the barrel • Use new pipette tip for each sample • Always format negative control wells before positive control. • Re-run with fresh reagents.
• Wash buffer prepared incorrectly or buffer not freshly prepared	• Rerun the assay with fresh wash • Check the pH of the distilled water used for the preparation of wash buffer.

3. Low RLU values for positive control

Possible Causes	Corrective Action
• Reagent not at room temperature.	• Make certain all kit components are at R.T. (20 – 25°C).
• Test volume low.	• Ensure pipette tips are fitted correctly/ tightly. • Check pipette barrels for obstructions. • Check calibration of pipettes.
• Substrate A & B not freshly prepared or incorrectly prepared.	• Prepare substrate immediately before use. • Follow working reagent preparation chart
• Contamination of signal reagent	• Re-run the assay with fresh reagents.
• Incubation time too short.	• Check calibration of timers. • Record time of incubation.
• Moisture in pouches.	• Check whether desiccant in pouch is in working condition. • Seal unused wells in pouches. • Date pouches when first opened.

• Improper incubation temperature.	• Check incubator temperature/ Room Temperature (20 – 25°C).
• Room temperature too low for substrate incubation.	• Check temperature of the working area.
• Washing step too vigorous.	• Reduce pressure in wash system.
• Reagent not mixed before using.	• Mix the reagents before use.
• Cells allowed to dry after assay has started.	• Complete all assay steps without interruption.
• Insufficient tracer concentrate added in preparing working stock.	• Prepare conjugate accurately. • Follow working reagent preparation as described by the manufacturer.

4. Substrate "A" developing yellowish or brownish coloration

Possible Causes	Corrective Action
• The substrate is contaminated.	• Obtain fresh Substrate "A".

5. Poor assay reproducibility.

Possible Causes	Corrective Action
• Incorrect volume of reagent(s) added. Insufficient wash step.	• Be sure that hand wash technique is consistent. If using an automatic plate washer, perform regular Maintenance to ensure the machine is running properly and is clean.
• Incubation temperature.	• Try to maintain a consistent environment especially in regards to temperature. Refer to protocol for optimum reaction conditions.
• Protocol Variations	• Be sure to follow the written protocol as closely as possible to limit the variation that may be introduced by the environment, equipment, and technician.
• Dispensing/ pipetting error.	• Ensure pipette tips are fitted correctly / tightly. • Check pipette barrels for obstructions. • Check calibration of pipettes
• Improper wash step.	• Ensure all wash equipment is working properly and wells are filled completely but do not overflow.

6. Edge Effect

Possible Causes	Corrective Action
• Incubation chamber providing uneven temperature	• Maintain a consistent environment around the reaction vessel paying close attention to the temperature.

7. Assay drift

Possible Causes	Corrective Action
• Interruption(s) during assay set-up.	• Set-up of assays should be free of interruptions and be done in the shortest time possible while maintaining good technique. To prevent any delays/interruptions while setting up an assay prepare all reagents and samples prior to beginning.
• Reagents/samples are not at room temperature.	• Dispense samples/reagents only when all are at room temperature to prevent variation of temperature throughout the plate wells.

8. Automation results are different than manual method

Possible Causes	Corrective Action
• Automation application is not up to	• Set-up of assays should be free of interruptions and be done in the shortest time possible while maintaining good technique. To prevent any delays/interruptions while setting up an assay prepare all reagents and samples prior to beginning.
• Reagents/samples are not at room temperature.	• Dispense samples/reagents only when all are at room temperature to prevent variation of temperature throughout the plate wells.

TIPS FOR GOOD LABORATORY TECHNIQUES

Light reaction plates

The Light reaction plates are the platform on which the assay reaction proceeds so the correct type of plate is vital. Do not open and use any plate until it has reached room temperature. Once the pouch is opened, date the package, remove desired amount of strips, and seal remaining strips in the dated microplate pouch. Place the strips in the correct holder and place on a level surface for the remaining steps in assay setup. The strips should be inspected for any defects.

Micro-pipetting

The volume used in assays is very small so it is extremely important to calibrate the pipettes accurately and in accordance with the time frame suggested by the manufacturer. In addition to calibration of the pipetting instrument, be sure to use the correct tips required for the specific pipette; the tips should consistently draw/dispense the same volume and fit properly on the end of the pipette. To prevent contamination of any reagents used, change the pipette tip between dispensing of different samples/reagents. Further precaution can be taken by transferring only the amount of a sample/reagent needed to a separate container before dispensing.

Washing

The washing step is performed in order to remove the excess reagents from the well that now contains bound components. This step can either be done manually or with an automatic plate washer. The end result should be the same irrespective of the wash method used. The wash solution should flood each well 3-5 times and left to soak for 5-30 seconds between each wash, depending on the assay and the equipment being used. Many factors are taken into consideration when a wash solution's components are chosen so be sure to follow the suggested preparation. Here are some additional things to consider depending on the wash method of choice:

Manual – Fill each strip with the same volume of washing solution and keep the time it takes for each strip to be washed the same. No air bubbles should be trapped in the wells and the wells should not be overflowed with wash solution.

Plate Washers – Program the desired wash cycles and soak times for reproducible wash steps. Perform regular maintenance on the washers to ensure accurate dispensing volumes and that the Washing heads are clean.

Substrate

Always check the substrate for any coloration prior to use. Incubation time of the signal a vital point in the assay and time constraints should be kept according to those indicated in the protocol.

Reading environment

To ensure reproducible results run all assays in a stable environment. An ideal stable temperature is one with little to no air drafts. In tropical or cold climates, it is recommended that labs regulate the room temperature (20 - 25 °C) to ensure proper reactions. Vibrations/rotations will also affect the test so these should be avoided. The surrounding environment is not the only of concern; the immediate environment contained in the plate holder is also critical. All plates, samples/controls, conjugates, and other reagents should only be used when at room temperature. This will minimize any variations in temperature across the plate. The main goal is to keep the environment as stable and consistent as possible.