Luminescent Immunoassays in 96 Well Plates

Judy Gibbs, Corning Incorporated, Science Products Division, Kennebunk, ME

Over the years, the enzyme immunoassay that Engvall and Perlman first described has taken many different forms. Today there are heterogeneous, homogeneous, cell-based, colorimetric, fluorescent and luminescent, to name just a few, versions of the original ELISA. They all have antibody-antigen complexes and enzyme reactions — colorimetric, fluorescent, and luminescent — in common. All ELISA’s, regardless of the detection system employed, require the immobilization of an antigen or antibody to a surface. They also require the use of an appropriate enzyme label and a matching substrate that is suitable for the detection system being used. Associated with the enzyme-substrate reaction are several requirements, such as timing and development conditions, that need to be optimized to result in a precise, accurate and reproducible assay.

Luminescence

Luminescent immunoassays, like fluorescent immunoassays, are variations of the standard ELISA. An enzyme converts a substrate to a reaction product that emits photons of light instead of developing a visible color. Luminescence is described as the emission of light from a substance as it returns from an electronically excited state to ground state. The various forms of luminescence (bioluminescence, chemiluminescence, and photoluminescence) differ in the way the excited state is reached. For example, photoluminescence is simply fluorescence; the excitation is initiated by light at a particular wavelength. Bioluminescence is characterized by the use of a bioluminescent compound, such as luciferin or firefly luciferase. Chemiluminescence is light produced by a chemical reaction. Both bioluminescence and chemiluminescence are widely used for immunoassays and will be discussed in this bulletin as “luminescence.” Luminescent assays, in particular enhanced luminescent assays, are very sensitive and have a wide dynamic range. It is believed that luminescence is the most sensitive detection method currently in use due to the ability of signal multiplication and amplification. Luminescent reactions are measured in relative light units (RLU) that are typically proportionate to the amount of analyte present in a sample.

Selecting the Appropriate Enzyme Label

As with colorimetric and fluorescent detection systems, the top three enzymes used for luminescence are peroxidase, alkaline phosphatase, and β-D-galactosidase. However, the most widely used enzyme for luminescent immunoassays is peroxidase. Peroxidase can be used with either bio- or chemiluminescent systems and is easily enhanced to allow prolonged detection of intense light (glow luminescence) which makes it compatible with 96 well plate assay formats.

Selecting a Suitable Substrate

A luminescent substrate should be chosen for its 1) low background luminescence in the ground state, 2) ability to produce intense light in its active state, 3) ability to produce stable light emission over a prolonged (minutes) period of time, and 4) commercial availability (quality and consistency). The substrate should be stable at room temperature throughout the duration of the assay.

The three most-used enzymes have one to several suitable substrates. Peroxidase has the most extensive list of suitable substrates, which include 1) luminol, 2) polyphenols and acridine esters, and 3) luciferin. For the luciferin substrate, peroxidase replaces the in vivo enzyme luciferase. Polyphenols are actually a class of substrates that include pyrogallol, purpurogallin, gallic acid, and umbelliferone. All polyphenols are known for their excellent signal-to-noise ratio and extremely rapid light decay. Polyphenol and acridine ester substrates can only be used in conjunction with luminescent detectors equipped to handle “flash” reactions. The most popular substrate used for immunoassays is luminol. Luminol is commercially available in a stabilized form. It is the most suited for clinical diagnostic tests due to its properties when used in an enhanced luminescence system. Commercially available luminol is provided with an enhancer (phenols, naphthols, aromatic amines, or benzothiazoles) that acts as an enzyme protector and allows the reaction to proceed for many minutes without substantial decay in light output. Typically, light emission stabilizes in less than 2 minutes, and sustained emission lasts for approximately 20 minutes or more. Enhanced luminescence is characterized by the following desirable features: intense light emission, prolonged light emission, low background, no preincubation step, and substrate can be added several minutes prior to detection. As long as commercial preparations of luminol are being used, control of the reaction pH is not a concern. However, if the substrate is a “home-brew” preparation, pH must be stabilized at 8.5 to allow optimal peroxidase activity (pH 5.5) and optimal light emission (pH 12.0) to occur. If the pH varies
above or below 8.5, either the enzymatic activity or the luminescent detection will be negatively affected. As mentioned, luminol-based chemiluminescence is well suited for 96 well immunoassays, plus this system is also recommended for the increasingly popular DNA probe assay.

There is an array of substrates for alkaline phosphatase and β-galactosidase available. The dioxetanes, CDP-Star® for alkaline phosphatase, and Galacton-Star® for β-galactosidase are among the most commonly used. Both substrates are generally used with enhancers to stabilize and increase signal intensity. CDP-Star® and Galacton-Star® are typically sold as kits that include enhancers and buffers matching to the substrate for optimal performance.

**Flash vs. Glow Detection Methods**

There are two distinct methods of detecting luminescence—flash and glow. Flash luminescence is transient in nature and reaches maximum light intensity within seconds or milliseconds. Due to the speed at which the reaction occurs, it is necessary to start the reaction while the reactants are in front of the photomultiplier or light detection device. Starting the reaction consists of adding substrate and complementary reagents or buffers and subsequent mixing of all the assay components. Of paramount importance is the requirement that there be a constant time interval between the addition of the starting reagents and the time that the measurement takes place. For a 96 well plate assay, this requirement is met by coordinating this step within the reading chamber. Reagent addition and light measurement takes place in a step-wise manner one well at a time.

An alternate detection method is glow luminescence, which is a steady-state kinetic approach to signal generation. Glow luminescence is actually a large number of transient signals that occur in sequence and result in a constant signal. Unlike colorimetric or fluorescent reactions, the light produced is not accumulated as color or fluorescence can be, so the light emitted must be intense and the enzyme reaction prolonged in order to obtain sufficient signal. The positive aspects of glow luminescence is that 1) the reaction can be started outside of the detection instrument, thus eliminating the need for internal injection and mixing within the reader, 2) the procedure is simple, 3) the results are sensitive, 4) excellent enhanced substrate systems are commercially available, and 5) the procedure is highly suited for 96 well plate assay formats. This type of luminescent reaction can be measured using a luminometer, captured on photographic film, or recorded via image analysis. Our experience with luminescence involves glow reactions. An area that we found to be crucial to obtaining reproducible results is the enzyme-substrate reaction time. Even though it is reported that glow reactions are expected to be stable for at least 20 minutes, we found our results to be more consistent from well-to-well and from plate-to-plate when our incubation time was short. We recommend allowing a 2-minute stabilization period after substrate addition and then immediately reading the plates. Both signal strength and precision can be improved by optimizing the enzyme-substrate reaction incubation time. Note: luminescent reactions do not require a stopping step.

**Opaque Plates to Reduce Crosstalk and Background**

Choosing the best plate for luminescence is crucial to developing a reliable assay. It is important to choose a plate that is specifically designed for luminescence. These plates are usually opaque white and can have solid white bottoms or clear bottoms. Both versions are designed to reduce crosstalk and background luminescence due to the specially formulated composition of the white resin.

White clear bottom plates are typically used for cell-based luminescent assays, such as luminescent cell proliferation assays. These plates allow visualization of the cells during attachment and growth and also prevent lateral light transfer from well-to-well during the detection step. Clear bottom plates are also useful for dual-assays that result in a colorimetric product for one analyte and a luminescent product for a second analyte. An example of the versatility of a white clear bottom plate is the ability to stain cells to observe and assess structural changes and to coordinate these observations with measurements of cell proliferation via luminescence—all in the same plate.

Why white plates? When these plates are made from a truly opaque, nonluminescent material, crosstalk and background can be almost eliminated. A properly formulated white material also increases assay sensitivity by reflecting emitted light into the detector. Note: luminescence is not cumulative, so it is important that each discreet and transient light photon reach the detector if sensitivity is to be realized. Sensitivity is compromised if light is allowed to escape from the plate undetected or is absorbed by the material chosen to make the plate. Opaque and highly reflective surfaces are the key ingredients for a quality plate designed for luminescence.

**Background RLU’s (Relative Light Unit) for Costar® White Assay Plates:**

<table>
<thead>
<tr>
<th>White Plates – Different Materials:</th>
<th>Costar® White Assay Plates:</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Solid Plate/Strips</td>
<td>17</td>
</tr>
<tr>
<td>White Clear Bottom Plates</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>This data indicates that neither the solid nor clear bottom plates or strips significantly contribute to background luminescent readings.</td>
<td></td>
</tr>
</tbody>
</table>

**Assay Background RLU’s for Solid White Plates – Different Materials:**

<table>
<thead>
<tr>
<th></th>
<th>Costar® Plate</th>
<th>Competitor #1</th>
<th>Competitor #2</th>
<th>Competitor #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Plates – Solid</td>
<td>17</td>
<td>72</td>
<td>327</td>
<td>2534</td>
</tr>
<tr>
<td>Different Materials:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As mentioned earlier, the choice for the white material is crucial to reducing background from the plastic itself and from light transfer from an adjacent well. Percent crosstalk from well-to-well can be significantly reduced using a solid white plate as opposed to a clear bot-
tom plate; however, one loses the advantage of visually assessing cell attachment, growth, and structure for cell-based luminescent assays on a solid bottom plate.

<table>
<thead>
<tr>
<th>Material</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Solid Strips</td>
<td>0.01%</td>
</tr>
<tr>
<td>White Solid Plates</td>
<td>0.02%</td>
</tr>
<tr>
<td>White Clear Bottom Plates</td>
<td>0.50%</td>
</tr>
</tbody>
</table>

RLU’s in wells adjacent to the blank wells reported above were approximately 100,000.

**Note:** A standard clear plate could not be evaluated for crosstalk due to the limitations of the luminometer, which cannot detect the presence of a clear plate and thus will not initiate reading. Our luminometer has injection ports such that the instrument can be used for flash luminescence and as a safeguard (to avoid reagent injection into an empty reading chamber), it will not function unless it senses the presence of a plate in the reading chamber.

It is important to choose a plate that meets the minimum criteria associated with background and crosstalk and is consistent from lot-to-lot.

**Equipment**

Due to innovations with photomultipliers that can be used as photon counters, 96 well plate luminometers are now readily accessible and can be relied on for accurate and consistent detection of luminescent output. When choosing an instrument we advise that the following features be considered:

1. Light sealed reading compartment.
2. Safeguard to prevent accidental injection of luminescent reagents into the reading chamber (flash luminometers only).
3. Control mechanisms for temperature drift.
4. Low instrument background at ambient temperatures.
5. Selectability of read time per well.
6. Optional for flash luminescence: equipped with precision auto-injectors. (Recommend a minimum of two injectors with back flush mechanisms to prevent dripping and liquid jet injection to ensure immediate and intensive mixing of reagents).
7. Optional for flash luminescence: equipped with an adequate mixing mechanism initiated at the time of injection.
8. Optional: adjusts to 384, 96, 48, 24, 12, and 6 well plate formats.

A quality luminometer can enhance the accuracy, sensitivity and consistency of assay results.