Selecting the Detection System - Colorimetric, Fluorescent, Luminescent Methods

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Introduction

Over the years, the enzyme immunoassay that Engvall and Perlmann 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 in common. In this technical bulletin,, the fifth in the series, we will focus on the enzyme linked immunosorbent assay and discuss three types of detection systems — colorimetric, fluorescent, and luminescent.

All ELISA, regardless of the detection system employed, require the immobilization of an antigen or antibody to a surface (Corning Life Sciences *ELISA Technical Bulletin No. 1*). 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.

Colorimetric Assays

Colorimetric assays result in a colored reaction product that absorbs light in the visible range. The optical density of the reaction product is typically proportional to the amount of analyte being measured.

Selecting the Appropriate Enzyme Label

The most common enzymes used as labels for ELISA are 1) horseradish peroxidase, 2) calf intestine alkaline phosphatase, and 3) *E. coli* ß-D-galactosidase. These enzymes are typically used because they each meet most, if not all, of the criteria necessary to produce a sensitive, inexpensive, and easily performed assay.

These criteria include:

- stability at typical assay temperatures: 4°C, 25°C, and 37°C,
- greater than six months shelf life when stored at 4°C,
- commercially available,
- capable of being conjugated to an antigen or antibody,
- inexpensive,
- easily measurable activity,
- high substrate turnover number,
- unaffected by biological components of the assay.

By far, the two most popular enzymes are peroxidase and alkaline phosphatase. Each has their advantages and disadvantages. Both are quite stable when handled and stored properly, and both can be stored at 4°C for greater than 6 months. Both are also commercially available as free enzymes and as enzyme conjugates (enzyme labeled antibodies, etc.) and are relatively inexpensive. However, there are some differences between these two enzymes that should be considered when choosing one for an assay.

Peroxidase is a small molecule (MW ~40,000) that can usually be conjugated to an antibody in a 4:1 ratio. Due to its small size, it rarely causes steric hindrance problems with antibody/antigen complexes bound on a surface. Peroxidase is very inexpensive compared to alkaline phosphatase. Several substrates, yielding either soluble or insoluble reaction products, are commercially available for peroxidase. Since all peroxidase reactions require hydrogen peroxide, purchasing commercially available substrates is recommended because these preparations contain stabilized hydrogen peroxide which adds to their value and usefulness.

The major disadvantage associated with peroxidase is that it is incompatible with many preservatives, such as sodium azide, that are used to reduce microbial contamination in many biological buffer solutions. Sodium azide, even in low concentrations, inactivates peroxidase activity. Other compounds or elements that interfere with peroxidase activity are metals found in water and endogenous peroxidases found in biological specimens. These disadvantages can be overcome by using sterile buffers without preservatives, using reagent grade type II water, and pretreating specimens suspected of having high peroxidase levels with hydrogen peroxide prior to use in an assay. Typically, non-bound biological components are washed away prior to the addition of the enzyme, so endogenous peroxidase activity is usually not an issue.

Alkaline phosphatase is approximately double the size of peroxidase (MW ~86,000). This means that one will typically see a lower enzyme to antibody conjugation ratio. It also means that the larger molecular size of alkaline phosphatase can cause steric hindrance issues due to close packed antigen-antibody complexes. This can result in lower activity than expected for the estimated number of bound enzyme molecules (which is sometimes considered responsible for the "high dose hook" phenomenon). Alkaline phosphatase is slightly more expensive than peroxidase, but is considered to be more stable. Substrates for alkaline phosphatase range from soluble to insoluble; many can be signal enhanced to increase sensitivity.

The major disadvantage associated with using alkaline phosphatase is that it is inactivated by chelating agents, acidic pH (< 4.5), or inorganic phosphates. This means that buffers must be specific for alkaline phosphatase, and one cannot use standard assay phosphate buffered saline

solutions as diluents or wash solutions that come in contact with the enzyme during an assay. However, chelators (EDTA) and acidic pH are typically used as convenient and inexpensive stopping reagents for alkaline phosphatase reactions.

ß-galactosidase is the least used of the three top enzymes for ELISA. This enzyme is quite large; its four subunits combined have a molecular weight of greater than 300,000. Its size is most likely the reason why it is the least popular. For unexplained reasons, ß-galactosidase also suffers from antibody-induced inhibition. An advantage of ß-galactosidase is its enhanced reaction rate in the presence of alcohols, which lends itself as a suitable enzyme for assays performed on hydrophobic membrane surfaces (i.e., dot blot applications) that require alcohol to wet out.

For colorimetric assays, either alkaline phosphatase or peroxidase is a suitable enzyme. Both enzymes have a wide range of substrates that yield qualitative and quantitative results.

Selecting a Suitable Substrate

For all enzyme-linked immunoassays, the final stage is the addition of the enzyme substrate. The substrate is chosen for its quantitative yield of a colored, fluorescent or luminescent reaction product. For colorimetric assays the rate of color development is proportional, over a certain range, to the amount of enzyme conjugate present.

A suitable substrate must be chosen to meet the assay requirements of the assay being performed. Substrates can produce either insoluble or soluble colored reaction products. Typically, insoluble reaction products are desired for membrane-based assays, such as dot blots. An insoluble colored dot is produced at the site of the reaction. Along with being a visual and sometimes permanent record, the intensity of the colored product can be measured using densitometry. However, insoluble reaction products are not practical for solution immunoassays performed in multiple well assay plates. Substrates that form soluble reaction products are better suited for ELISA.

Both peroxidase and alkaline phosphatase have substrates that yield soluble colored reaction products. The decision as to which substrate is the best for any type of assay depends on the sensitivity desired, the timing requirements, and the detection device to be used. For assays that need to be very sensitive (able to detect low amounts of analyte), the most desirable substrates produce intensely colored reaction products at very fast reaction rates. For assays that require a large dynamic range (typical analyte amounts span a wide range of concentrations), substrates that produce reaction product over a long time (15 to 30 minutes) and result in a broad range of analyte-dependent color intensities are the most desirable. For assays with a timed endpoint, a chemical inhibitor is added to the reaction after a defined time that stops further color development. This allows detection to be performed within a reasonable time; for this, a substrate that has a "slow" reaction rate (15 to 30 minutes to completion) is optimal. This "slow" reaction rate allows the technician (or automated equipment) to start the reaction and stop the reaction at a reasonable pace. However, when kinetic analysis of the enzyme-substrate reaction is used, a substrate that has a "fast" reaction rate (5 minutes or less) should be used. In this case, the substrate is added, and the rate of conversion of substrate to colored reaction product is immediately measured. The reaction is usually measured over discreet and short time intervals (i.e., 10 seconds) for 2 to 5 minutes.

The following are the most commonly used substrates for peroxidase and alkaline phosphatase:

Peroxidase: The three most common substrates that produce an insoluble product are TMB (3,3',5,5' tetramethylbenzidine), DAB (3,3',4,4' diaminobenzidine), and 4CN (4-chloro-1-naphthol). The most common substrates that produce soluble reaction products are TMB (dual function substrate), ABTS (2,2'-azino-di [3-ethylbenzthiazoline] sulfonate), and OPD (o-phenylenediamine). TMB is a highly sensitive substrate. Due to its rapid reac-

tion rate, it is ideally suited for on-line kinetic analysis. It produces a blue color measurable at a wavelength of 650 nm. TMB can also be used in endpoint assays by stopping the reaction with 1M phosphoric acid. A yellow reaction product is formed upon acidification that is measurable at 450 nm. ABTS is considered an all-purpose substrate. Although it is less sensitive than either TMB or OPD, it has the widest working range of any substrate currently available for peroxidase or alkaline phosphatase. The reaction product for ABTS is a blue-green compound measurable at 405 to 410 nm. Its reaction rate is suitable for endpoint assays and is easily stopped with 1% SDS (sodium dodecyl sulfate), which does not change the color or the absorbance of the reaction product. OPD was once the most popular substrate for peroxidase. It is slightly less sensitive than TMB. Its reaction product is yellow and can be read at 490 nm.

Alkaline phosphatase: The most common substrate that produces an insoluble reaction product is BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium). It is recognized as the most effective substrate for immunoblots due to its stability and resistance to fading when exposed to light. The most widely used substrate that produces a soluble reaction product is p-NPP (p-nitrophenylphosphate). It produces an intense yellow color measurable at 405 to 410 nm. An advantage of this substrate is that it can be allowed to develop for extended periods to obtain a corresponding increase in sensitivity. Normally p-NPP has a slow reaction rate which should be allowed 30 to 60 minutes to reach optimal color development before being stopped with 1N NaOH. It is not recommended for kinetic analysis.

Reaction Requirements

Many factors affect the measurement of enzymatic activity. Some of the most obvious are:

- temperature,
- pH,
- ionic strength,
- buffer composition,
- substrate depletion,
- build-up of product inhibitors,
- increasing back-reaction as product concentration increases,
- denaturation of the enzyme, and in some cases,
- exposure to light.

The ones that are of most concern for ELISA today are reaction time, temperature and exposure to light. The factors, such as pH and substrate depletion, have been addressed, and commercially available reagents have been optimized for composition and concentration in order to control these parameters.

Timing the Reaction

In order to have an endpoint assay that provides reliable and consistent results, it is important that the timing of the reaction in each and every well, in each and every plate, and in each and every set of plates be controlled as precisely as possible. Since enzyme-substrate reactions are kinetic, timing from the start to the end of the reaction can and will affect the final concentration of product developed. To ensure precise timing, we follow this scheme for every assay that we perform — regardless if only a few wells, an entire plate, or ten plates are involved in the assay:

1. Set timer to the desired and predetermined substrate incubation time.
2. Start timer with the addition of substrate to the first well or set of wells.
3. Use a rhythmic pipetting pattern to add substrate to all the wells.
4. When the timer signals the end of the incubation period, stop the reaction using the same pipetting pattern and rate that was used to add the substrate.

This scheme assures that all the wells see active substrate for the same amount of time and adds consistency to the assay results. We use a rhythmic pipetting pattern with a 12 channel pipettor for dispensing substrate and/or stop solution from row A through row H in a 96 well plate that takes approximately 30 seconds per plate to complete.

The incubation time associated with the substrate step in an assay must be predetermined so that the color formed for the lowest analyte concentration is significantly higher than the background and the color formed for the highest analyte concentration is less than the reader cut-off value. (This value usually ranges from 2.0 to 4.0 optical density (OD) units depending on the reader used.) A good rule of thumb is to choose a high level OD of approximately 1.0. The following is a method that can be used to determine optimal incubation times for the substrate step:

1. Coat the plate with the optimal antigen or antibody dilution.
2. After rinsing away non-bound reagent, block the surface.
3. Add the standard or sample containing the highest concentration of analyte to be detected.
4. Incubate as appropriate; wash away non-bound analyte.
5. Add the enzyme conjugate. Incubate as appropriate. Wash.
6. Add the substrate solution.
7. Monitor color development.
8. Stop the reaction when the OD is approximately 1.0.
9. Record the time required to reach an OD of 1.0. This is the optimal substrate incubation time.

Development Conditions

As mentioned earlier in this bulletin, temperature and light can affect the enzyme-substrate reaction. These two assay parameters can be the cause of "edge effect"; where OD's in edge wells are higher or lower than center wells. All enzyme reactions are temperature dependent. This means that temperature during the enzyme-substrate reaction step must be kept constant in all the wells. Typically, this reaction step can be performed at room temperature. Some assays require this step to be performed at elevated temperatures to increase enzyme activity. Regardless of the assay temperature, the temperature of the substrate prior to addition to the plate should be equivalent to the intended incubation temperature. Room temperature incubations are the most common and are the easiest to control. All plates being processed at room temperature should be placed in a location such that none of the plates is situated near a source of heat or cold. For assays at elevated temperatures, controlling edge effect is more difficult. Since polystyrene is a poor conductor of heat, incubations performed in an incubator usually result in edge effect problems – the outer wells reach the desired temperature up to 30 minutes before the center wells do. This results in higher OD's in the edge wells due to increased enzyme activity in these wells. In order to maintain constant temperatures in all the wells, elevated temperature incubations should be performed on a heat block that allows all wells to reach the critical temperature simultaneously. The easiest way to avoid temperature related edge effect problems is to incubate at room temperature – which may mean simply increasing the incubation time to reach the OD acquired at the higher temperature incubation.

Although many substrate systems are believed to be stable in the presence of light, we firmly recommend that the substrate incubation step be performed in the dark to avoid edge effects caused by variations in incident light from well to well. Typically, outer wells receive a higher degree of incident light than center wells, which results in lower or higher than average OD's in these wells. Plates that are incubated in a drawer or cabinet that has a light leak can also be adversely affected by incident light (wells closest to the light leak exhibit higher or lower OD's). Performing incubations in the dark is the easiest method of assuring that incident light is not affecting the assay's outcome. Kinetic assays are usually not a problem since the incubation is performed within the reading chamber of the detection instrument which has to be light-proof in order to function properly.

If temperature and light exposure is controlled, the probability that the assay will suffer from edge effect problems is greatly reduced.

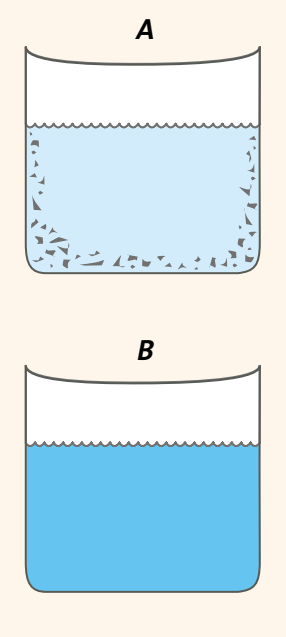


Figure 1. Effect of Shaking vs. Non-shaking

Mixing the Chromophore

After the addition of stop solution and/or prior to reading the OD's, it is important to adequately mix the well contents. This will assure both complete cessation of the reaction (endpoint assay only) and even dispersion of the colored reaction product. Well-to-well precision can be dramatically improved by adding this step to the protocol.

The physical effect that shaking the plate prior to reading has on the resulting optical density is depicted in Figure 1. Microplate colorimeters only read the immediate center of the well. This means that an unshaken plate will have wells that resemble Well A in the diagram. Colored reaction product will be concentrated at the surface of the well where the enzyme is located. Dissolution of the reaction product is slow if not mechanically stirred or mixed. The reader will not be measuring the true optical density within Well A. With good mixing, the colored reaction product is evenly dispersed through the solution in the well as seen in Well B. The reader can then detect the true optical density of the colored reaction product resulting from the enzyme-substrate reaction occurring at the surface.

The data in Table 1 clearly shows the increased precision that is possible by simply shaking the plate prior to reading. Out of 26 plates that were shaken before reading the OD's, all of the plates pass our certification criteria for CV's less than 3.0% and high and low wells less than 8% from the mean OD. Over half of the plates that were not shaken do not pass our certification criteria. The major problem with these plates is low wells suspected of being caused by a lower concentration of colored reaction product in the center of the wells (see Figure 1) as opposed to the edge of the wells. (*Note:* our readers only read the center of the well).

There are several ways to accomplish the mixing step. Microplate shakers are available that hold 2 to 4 plates at a time so that plates can be shaken as a group prior to being loaded into a reader. Many microplate readers have built-in shaking mechanisms that allow plates to be shaken just prior to being read. Both of these methods (built-in versus remote shakers), when used properly, can have very positive effects on assay precision.

Detection Methods

For the purpose of this bulletin, we will focus on the detection methods and equipment used for soluble reaction products that absorb light in the visible wavelength range (> 405nm). There is a wide choice of microplate readers available today that range from simple, single wavelength colorimeters to sophisticated multi-wavelength spectrophotometers. The main function of a microplate reader is to measure light absorbed at particular visible wavelengths of light by a substance, which in most cases is a colored solution. Some of the options available make the measurement easier to attain and allow analysis of samples that cannot be measured in the simplest models.

Some readers are capable of only single wavelength measurements. These are adequate for standard ELISA that have little or no interference from subtractable background noise. Most readers have the ability to read at dual wavelengths. This option is necessary when background noise from attached cells or minute scratches on the optical surface interfere with accurate detection of the colored reaction product. Dual wavelength allows one to read the plate at a wavelength specific for the reaction product and at a second wavelength that is out of the range of absorbance for the reaction product. The instrument subtracts the second wavelength from the first, effectively subtracting out the background noise that is usually not wavelength specific. The

Table 1. Effect of Shaking vs. Non-shaking

Shake vs. Non-shake	# of Plates Run	# of Plate Out-of-Spec	CV	Low Well	High Well
Shake	26	0	2.0	5.7	6.0
Non-shake	23	14	3.1	11.0	6.9

resulting OD is specific for the reaction product. The added cost of dual wavelength capability is minimal and worth purchasing with any microplate reader.

All readers are capable of endpoint analysis. This is adequate for most assays. However, for increased sensitivity and dynamic range, a reader capable of kinetic analysis is required. Endpoint assays require that the reaction be stopped when a certain predetermined high dose OD is reached. These assays many times lack the sensitivity to distinguish minor changes in analyte concentration above a certain limit — typically due to substrate depletion. When kinetic analysis of the enzyme-substrate reaction is employed, this limitation is minimized. Since reaction rates are measured in mOD/minute as soon as the reaction is initiated, minor differences in analyte concentrations are easily distinguishable even at the high dose end. Readings are performed before substrate depletion is an issue.

The newest readers available are microplate spectrophotometers. These readers are capable of measuring absorbance at several wavelengths and can even perform continuous scans on samples over the whole range of available wavelengths (including UV in some cases). These readers are probably more sophisticated than what is necessary for a standard ELISA; however, there are a number of applications (total protein and DNA determinations) that can benefit from this feature.

Fluorescent Assays

Fluorescent immunoassays (ELFIA) are simply a variation of colorimetric ELISA. An enzyme converts a substrate to a reaction product that fluoresces when excited by light of a particular wavelength. The relative fluorescence units (emitted photons of light) that are detected are typically proportional to the amount of analyte being measured. In comparison to the colorimetric ELISA, fluorescent immunoassays are only slightly more sensitive. However, they widen the dynamic range of the assay by allowing very high readings to be accurately measured as opposed to the 2.0 to 4.0 OD limit imposed on colorimetric assays.

Selecting the Appropriate Enzyme Label

The three main enzymes that were described in the section for colorimetric assays are the same three that are used for ELFIA. However, the order of popularity is different. Alkaline phosphatase is the most widely used enzyme for fluorescent applications. ß-galactosidase is also used more frequently due to its greater theoretical sensitivity when used with a fluorogenic substrate. Peroxidase is rapidly gaining popularity as an enzyme label for fluorescent-based immunoassays. Stabilized substrates for all three of the top enzymes are commercially available.

Selecting a Suitable Substrate

A fluorogenic substrate is chosen for its quantitative emission of light following excitation. The rate of light emission should be proportional to the amount of enzyme conjugate present. The substrate should be stable at room temperature and in the presence of normal room lighting. The resulting enzyme-substrate reaction product should also have distinctly separate excitation and emission wavelengths, plus the substrate itself should be non-fluorescent.

The three main enzymes each have one or two major fluorogenic substrates that are suited for ELFIA. Alkaline phosphatase is usually paired up with 4-MUP (4-methylumbelliferyl phosphate), which is converted to 4-methylumbelliferone with an excitation wavelength of 360 nm and an emission wavelength of 440 nm. This substrate is dissolved at 0.1 to 0.2 mg/ml in 100mM diethanolamine, 1 mM $MgCl_2$, pH 9.6. Commercial liquid preparations of 4-MUP are available; however, our results have indicated that the liquid version results in a much higher background as compared to freshly prepared substrate solutions.

A suitable fluorogenic substrate for ß-galactosidase is MUG (4-methylumbelliferyl galactoside), which is converted to 4-methylumbelliferone. (This is the same product that results from the conversion of 4-MUP, the substrate used with alkaline phosphatase.) There is an increased use of 4-MUG to detect reporter gene expression; a ready-to-use form of this

substrate is available for other non-ELISA applications.

Two fluorogenic substrates are currently used for ELFIA utilizing peroxidase as the enzyme label. These are HPA (hydroxyphenylacetic acid) and HPPA (3-p-hydroxyphenylproprionic acid). Both require, as expected, the addition of hydrogen peroxide in order to produce a fluorescent product. HPPA is the most widely used fluorogenic peroxidase substrate. Its fluorescent product has an excitation wavelength of 320 nm and an emission wavelength of 404 nm. Stabilized fluorescent substrates for peroxidase are now commercially avaialable.

Methods to Improve Signal

Fluorometric assays are subject to several problems that either non-specifically reduce or enhance the signal output. Detection of fluorescence is susceptible to changes in pH, temperature, ion concentration, detergent concentration, drying, and the solid matrix, which lead to light scattering, high background, quenching and bleaching issues.

Light scattering is a phenomenon caused by the emitted fluorescent light being bounced around as it encounters molecules and/or particles in solution, or the surface of the microplate. It is important to use high quality chemicals; however, even the purest of reagents have particles that lead to light scattering. Opaque black plates and strip plates of the highest quality are designed to reduce the scatter caused by light bouncing off the surface by absorbing this light. Only light directed toward the detector should be measured. Aside from controlling the purity of reagents and diluents, and using a quality microplate to minimize the matrix effect, the only other assay technique that can reduce light scatter is preventing bubbles in the well contents.

Background fluorescence and/or autofluorescence has several sources and is a major obstacle in the development of 96 well fluorescent assays. Sources of background include:

- sample components (hemoglobin, bilirubin, cellular debris, drugs),
- diluent components (metal ions),
- plate material (type of plastic used),
- miscellaneous contamination (dust particles, fingerprints).

Background can be combated via three approaches: assay design, instrumentation, and cleanliness. Several assay design features can help reduce background. For homogeneous assays, sample dilution is the key. It is important that any interfering substances present in the sample be diluted to a degree that background from these substances is minimized. When a low enough dilution cannot be feasibly achieved, employing kinetic analysis of the production of a fluorescent reaction product is a good alternative. The interfering substances will produce only static fluorescence, while the specific assay reaction will be kinetic and easily discernible from the background. Time-resolved fluorometry is also a successful detection method that reduces non-specific background from being measured.

For heterogeneous assays, background interference from the sample components is rarely an issue since this type of assay employs a separation step. However, this separation step must be adequate to remove all the interfering substances prior to the addition of substrate. It is imperative that all reagents used in the assay are of high quality and filtered through a 0.45 µm (non-particle shedding) membrane prior to use. It is also imperative that the microplate used be molded from non-autofluorescing material. Opaque black plates and strips are typically used for fluorescent assays due to their low background fluorescence. The type of plastic used is critical. Some plasticizers that may leach into solution can auto-fluoresce. Most quality fluorometers are equipped with features that aid in the reduction or elimination of machine-related background. These features include (i) filters, (ii) adjustable slit-width, and (iii) adjustable gain. Of these, the one that allows the end-user to most easily adjust for (subtract) background is gain. If the gain is adjustable, it can be used to achieve the best signal-to-noise ratio.

In general, lack of cleanliness is probably the most common cause of occasional background. Physical items, such as fingerprints and dust, are notorious for increasing background fluorescence. The fogging of plate bottoms or instrument optics by condensation from temperature differences can also increase non-specific background. Typically, high background from these physical causes results in poor well-to-well precision.

Quenching is a problem characterized by a non-specific reduction in signal. This phenomenon is caused by the absorption of the emission by dissolved oxygen. Reagents can be degassed prior to use to alleviate this effect.

Bleaching or fading is also characterized by a reduction in signal. It is caused by an excessively long excitation step. Typically this is not a problem due to the low power and short excitation exposure times associated with today's fluorometers. However, if substrate incubation steps are performed under bright light, bleaching can occur. It is recommended that this step be performed in the dark. Other sources of interference that can distort fluorescent readings include: (i) temperature variations, (ii) light source stability, and (iii) slit-width. Fluorescence behaves opposite to most other detection systems. Fluorescence increases with decreasing temperature. It is important to maintain both a constant intra-assay and inter-assay temperature. Variations in temperature from one assay to another can cause changes in assay sensitivity. We recommend that assays be performed at room temperature and include a positive and negative control (as a minimum) on each multiplate per assay.

Opaque Plates to Reduce Crosstalk and Background

Since some of the plastics used to manufacture microplates and 8 well strips are autofluorescent, it is important to choose a plate that is made specifically for fluorescent assays. These plates are usually opaque black (although white plates can be used for some fluorescent applications such as TRF). These black plates can have solid black bottoms or clear bottoms. Both are designed to reduce well-to-well crosstalk and background fluorescence.

Clear bottom black plates are typically used for cell-based fluorescent assays to allow visualization of the cells during attachment and growth while reducing lateral light transmission or crosstalk during the detection step. With the advent of microplate fluorometers that are capable of reading from the top or the bottom of the well, these plates have allowed increased versatility associated with assay design. Using a clear bottom plate, a dual assay resulting in a colorimetric product for one analyte and a fluorescent product for a second analyte can increase productivity and reduce sample requirements.

Why black plates? When an appropriate material is selected for the manufacture of these plates, background noise can be significantly reduced and crosstalk virtually eliminated. The black material reduces background by being non-autofluorescent and absorbing stray light (reducing the background caused by light scatter).

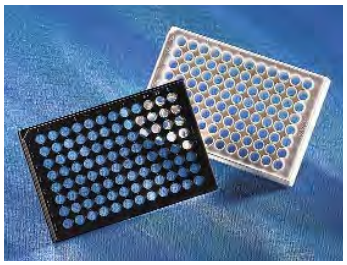
Background relative fluorescence units (RFU) at excitation 485 nm and emission 530 nm for Corning's assay plates and strips

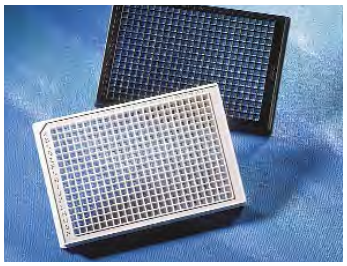
Plate Type	RFs
Standard Clear Plate	4
Black Clear Bottom Plate	2
Solid Black Plate/Strips	1

Although background is relatively low even for the standard clear plate, it is evident that the black material used for the clear bottom plate and solid plate and strips reduce background by 50% to 75%, respectively.

Percent crosstalk from well to well can be significantly reduced by using high quality black plates

Plate Type	Percent cross Talk
Standard Clear Plate	0.30%
Black Clear Bottom Plate	0.05%
Solid Black Plate/Strips	0.03%





Corning assay plates are available in a variety of sizes and materials.

As with background fluorescence, crosstalk reduction is dependent on the material used to manufacture the plates or strips. It is important to choose a plate that meets the minimum requirements associated with both background and crosstalk and is consistent from lot-to-lot.

Equipment

There are currently many different microplate fluorometers on the market. These devices vary greatly in performance. When choosing an instrument we advise that you ask for the following features:

- Light sealed reading compartment.
- Adjustable light detector. (The detector must be positioned as close to the plate as possible to obtain accurate readings. Since plates from different manufacturers vary in dimensions, it is important to have the ability to adjust for these variations).
- Numerically adjustable gain. (Choose a reader that has a gain adjustment that is numerical and not just low, medium, or high light intensity options).
- Thermal consistency in the reading chamber. (Since fluorescent output is affected by temperature variations, it is imperative that the reading chamber be temperature controlled such that each plate has even heating or cooling. A plate that is warmer on one side versus the other will exhibit inconsistent fluorescence from well to well).
- Internal background control mechanisms.
- Mix and match excitation and emission wavelengths.
- Selectability of number and duration of read times per well.
- Optional: choice of top plus bottom detection.
- Optional: adjusts to 1536, 384, 96, 48, 24, 12, and 6 well plate formats.
- Features 1 to 5 are necessary if accuracy and consistency are to be realized.

Luminescent Assays

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, 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 and 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 the previous two detection systems described in this bulletin, the top three enzymes used for luminescence are peroxidase, alkaline phosphatase, and ß-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 all size microplate assay formats.

Selecting a Suitable Substrate

A luminescent substrate should be chosen for its:

- low background luminescence in the ground state,
- ability to produce intense light in its active state,

- ability to produce stable light emission over a prolonged (minutes) period of time, and
- commercial availability (quality and consistency).

The substrate should be stable at room temperature during 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 (i) luminol, (ii) polyphenols and acridine esters, and (iii) luciferin. The reaction of peroxidase with luciferin is considered bioluminescence. In this reaction, peroxidase replaces the *in vivo* enzyme, luciferase. The other substrates are chemiluminescent compounds. 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 that can be added several minutes prior to detection. As long as commercial preparations of luminol are used, control of the reaction pH is not a concern. However, if the substrate is a "home-brew" preparation, pH must be stabilized at about 8.5 to allow both peroxidase activity (optimal at pH 5.5) and light emission (optimal at pH 12.0) to occur. If the pH varies much 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 microplate-based immunoassays; in addition, this system is also recommended for DNA probe assays.

Alkaline phosphatase and galactosidase each have one preferred substrate. AMPPD (3-(2'-spiroadamantane)-4-methyl-4-(3'-phosphoryloxyphenyl-1, 2-dioxetane, disodium salt) is the substrate most commonly used with alkaline phosphatase. A similar substrate, AMPGD (3-(2'-spiroadamantane)-4-methoxy-4-(3'-ß-D-galactopyranosyloxyphenyl-1,2-dioxetane), is routinely used with ß-galactosidase. Both substrates are compatible with commercially available enhancers. AMPPD is typically sold as a kit that includes additional buffers and reagents matched to the substrate for optimal performance.

Glow versus Flash Detection Methods

There are two distinct methods of detecting luminescence — flash and glow.

Flash luminescence is transient in nature and reaches maximal 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 tube or other 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 that there be a constant time interval between the addition of the starting reagents and the time that the measurement takes place. For a microplate-based assay, this requirement is met by coordinating this step within the reading chamber. Reagent addition and light measurement takes place in a stepwise 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

- the reaction can be started outside of the detection instrument, thus eliminating the need for internal injection and mixing within the reader,
- the procedure is simple,
- the results are sensitive,
- excellent enhanced substrate systems are commercially available,
- the procedure is highly suited for microplate-based 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. Although it is reported that glow reactions are expected to be stable for at least 20 minutes, we found our results 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. As with the opaque black plates discussed earlier, these white plates 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 a luminescent cell proliferation assay. These plates allow visualization of the cells during attachment and growth and 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, non-luminescent 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. An opaque and highly reflective surface are the key ingredients for a quality plate designed for luminescence.

Background in relative light units (RLU) for Corning's white assay plates

Plate Type	RLU
White Solid Plate/Strips	17
White Clear Bottom Plates	17

These data indicate that neither the solid or clear bottom plates or strips significantly contribute to background luminescent readings.

Assay background in relative light units (RLU) for solid white plates — different materials

Plate Type	RLU
Corning Plate	68
Competitor #1	72
Competitor #2	327
Competitor #3	2534

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. The RLU values in wells adjacent to the blank wells reported above were approximately 100,000.

Percent crosstalk from well to well can be significantly reduced using a solid white plate as opposed to a clear bottom plate. However, one loses the advantage of visually assessing cell attachment, growth, and structure for cell-based luminescent assays on a solid bottom plate.

Plate type	Percent crosstalk
White Solid Strips	0.01%
White Solid Plates	0.02%
White Clear Bottom Plates	0.50%

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. 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.

Equipment

Due to innovations with photomultiplier tubes 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:

- light sealed reading compartment,
- safeguard to prevent accidental injection of luminescent reagents into the reading chamber (flash luminometers only),
- control mechanisms for temperature drift,
- low instrument background at ambient temperatures,
- selectability of read time per well,
- optional for flash luminescence: 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.)
- optional for flash luminescence: an adequate auto-mixing mechanism initiated at the time of injection.
- optional: easy conversion to 1536, 384, 96, 48, 24, 12, 6 well plate formats.

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

Conclusion

Regardless of the detection technique one chooses, several parameters always need to be controlled:

- One must always choose an enzyme label that is suitable for the application and detection method being employed.
- Following the enzyme selection, a substrate that matches both the enzyme and the detection method must be chosen. It must also meet the assay's requirements for sensitivity, dynamic range, and reaction speed, plus if possible, be commercially available as a stabilized tablet or solution.
- Enzyme-substrate reaction requirements, such as incubation time and development conditions (temperature, light, etc.) need to be optimized.
- The reaction product, be it colorimetric, fluorescent or luminescent, must be adequately mixed prior to detection to ensure accurate and precise readings.
- The proper microplate must be chosen — clear for colorimetric, black for fluorescence and white for luminescence — and verified as a quality product.

- Finally, the detection device must be selected, keeping in mind that special options may actually be critical requirements; such as a temperature controlled reading chamber.

Each detection method has its own set of special requirements, but if the general assay precautions outlined in this bulletin are followed, a precise, sensitive and reproducible assay can be developed and consistently performed.

Technical Assistance

For additional ELISA technical support and bulletins or product information, please visit the Corning Life Sciences web site at www.Corning.com/lifesciences or call 1-800-492-1110.

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Understanding the Relationship between Automation/Instrumentation and Microplates

SnAPPShots

A brief technical report from the Corning Applications Group

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Introduction

The past 30 years have seen an astonishing rate of growth in automation to aid in research and drug development. From instruments such as basic microplate readers, liquid handlers, grippers, and incubators, to highly advanced instrumentation such as high content imagers, the microplate-automation relationship has always played a critically important role.

The relationship between microplates and the equipment used to run the experiment is fundamental to any assay ranging from low to high throughput. By not appreciating this dynamic, resulting data will be less robust, CVs will be increased and there will be more opportunities for false results. For instance, if a researcher wants to perform a fluorescence-based assay, selecting a clear microplate would result in not only high background but also crosstalk between wells resulting in inferior data. Additionally, if the microplate reader is optimized for a 384 well normal volume microplate and a researcher attempts to analyze a 384 well low volume microplate, the microplate dimensions and focal plane will be inaccurate leading to suboptimal results. Factors such as those listed above and others (e.g., liquid handling) are critical to understand when optimizing any assay.

The following describes the importance of choosing the correct microplate for an assay, as well as optimizing the reader for the microplate. Additionally, the importance of proper liquid handling and how inaccurate dispensing of a sample may negatively impact the results is presented. The goal is to arm researchers with the tools needed to develop successful assays by providing a better understanding of the relationship between microplates, assays, and instrumentation.

Materials and Methods

- Multitox-Glo was purchased from Promega (Cat. No. G9272), and the protocol was followed as outlined in the manual.
- Fluorescein was purchased from Sigma (Cat. No. F6377)
- The following Corning® microplates were used:
  - 384 well Black Solid Bottom (BSB) Tissue Culture Treated (TCT) microplate (Cat. No. 3571)
  - 384 well White Solid Bottom (WSB) TCT microplate (Cat. No. 3570)
  - 384 well BSB Not Treated (NT) Standard microplate (Cat. No. 3573)
  - 384 well BSB NT Low Volume (LV) microplate (Cat. No. 3821)
- Plates were screened using an EnVision® multimode reader (Perkin Elmer).

Results and Discussion

The Value of Choosing the Correct Microplate

In any assay, selecting the correct microplate is critical for assay success; it is essential to understand that each well contains its own microenvironment for any biochemical or cell-based assay. Microplate dimensions (e.g., well geometry, depth, and surface area), surfaces and color all play important roles in achieving optimal results. Therefore, when designing an assay the following questions should be addressed (Fig. 1):

1. What type of assay will be performed? (i.e., What type of surface will be needed?)
   a. Biochemical:
      i. Not treated
      ii. Nonbinding
      iii. Medium binding
      iv. High binding

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b. Cell-Based:
   i. Tissue culture treated (TCT)
   ii. Corning® CellBIND® Surface treated
   iii. Poly D-lysine (PDL) coated
   iv. Ultra-Low Attachment Surface
   v. Biological coated microplates (collagen, laminin, etc.)

2. What type of detection is required?
   a. Fluorescence
      i. Fluorescence intensity
      ii. Time resolved fluorescence-like assays
   b. Luminescence
   c. Absorbance
   d. Label-free (e.g. Epic® technology)
3. What type of reader is required?
   a. Top reader
   b. Bottom reader
   c. Top/bottom reader
   d. High content imager
4. Microplate density and type?
   a. 96 well normal volume or 96 half area microplates
   b. 384 well normal volume or low volume microplates
   c. 1536 well microplates
   d. Polystyrene
   e. Polypropylene cyclic olefin copolymer (COC) – storage and chemical resistance

As mentioned above, selecting the correct microplate for the assay is critical for a successful experiment; it is important to understand when to use a black, white, or clear microplate (Table 1). Typically for luminescence-based assays, due to the low energy produced by the biochemical reaction releasing light, the recommendation is to use a white colored microplate. The white colorant allows for increased reflective capacity for data capture.

It is widely accepted that for fluorescence intensity assays, a black colored microplate is the optimal choice. Fluorophores, when excited by an energy source, release a large amount of energy in the form of light. The black colorant used in

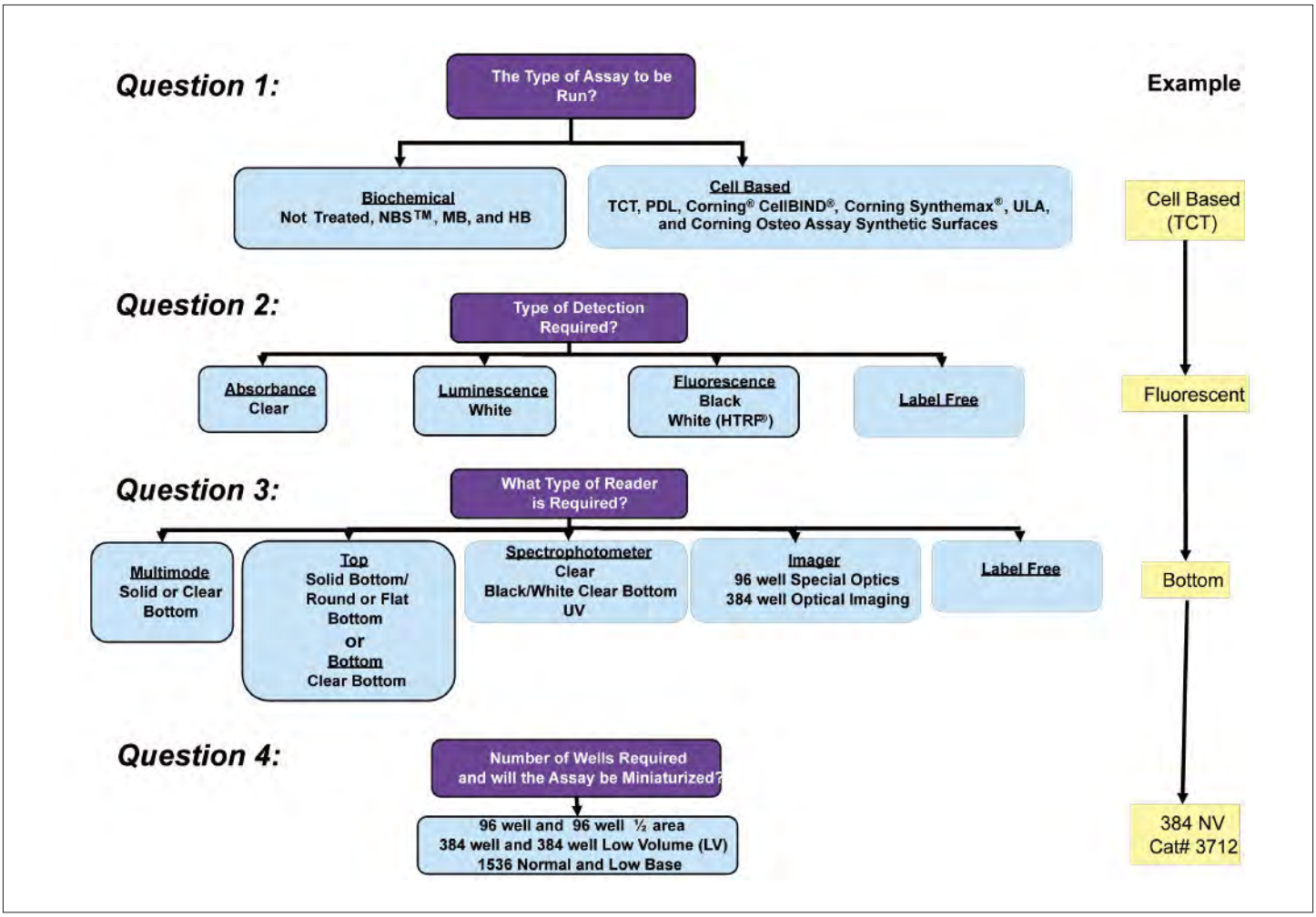


Figure 1. Flow chart designed to select the best microplate for the assay and instrument.

the microplates helps to reduce background by absorbing some of the emission energy as well as preventing cross-talk between wells. Background or noise is of critical concern in any fluorescent assay. Utilization of a clear microplate for a fluorescence-based assay may lead to photobleaching, photochemical destruction of a fluorophore, and high crosstalk levels between wells resulting in a low signal to noise ratio. For any assay, a high signal to noise ratio is preferred to reduce the possibility of false-positives and negatives. A low signal to noise ratio may mask subtle differences in the fluorescent signal leading to the potential of false negatives.

In most cases, a white microplate for a fluorescence-based assay may also lead to a poor signal to noise ratio because the overall signal (including background) will be amplified. However, this result is dependent on whether the fluorescent dye is a high or low energy emitter. A low energy emitter yields better results in a white microplate compared to a black microplate. For example, in Time-Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) assays a stable, low energy emitter fluorescent molecule is used to examine protein-protein interactions. As a result, a white microplate would be preferred due to its ability to amplify the low signal produced by the protein interactions. Fig. 2 depicts another instance when selecting a white microplate would be optimal to achieve the highest fluorescent signal.

Understanding the impact of surface area, well depth, and well number is important when choosing a microplate. When performing cell-based assays it is vital to know the surface area of that given microplate. Surface area is a distiguishing feature between vendors who sell microplates. It is highly recommended that when seeding microplates for any cell-based assay that seeding is performed by surface area (cells/cm^2) rather then cells/well. Not all microplates have the same surface area despite having the same number of wells (e.g., normal volume (NV) vs. LV microplates). For detailed information on microplate dimensions and compatibility with various readers please visit http://www.corning.com/lifesciences/us_canada/en/technical_resources/product_guid/eq_cp.aspx. It may be beneficial to consider miniaturizing an assay into a LV microplate so that the same microplate format can be used while reducing reagent consumption, cost, and still minimizing impact on automation.

Lastly, when selecting a microplate for an assay, it is essential to understand how the microplate surface can impact the results. As mentioned above, there are different surface types based on the assay that is being performed (biochemical or cell-based). When performing biochemical assays such as an enzymatic assay, it may be best to use Corning's NBS™ microplate. This microplate is designed to significantly reduce (<2 ng/cm^2) protein and nucleic acid binding thus providing researchers with maximum accessibility of assay components in solution compared to other microplates. However, due to the nature of the coating, the NBS microplate is not recommended for cell-based assays. For biochemical reactions where proteins (antibodies, antigens etc.) are required to bind to the surface, the better option is to use of the High Binding (HB) surface microplate. The HB microplate enhances binding of medium to large biomolecules (>10 kD) that are positively charged, with or without hydrophobic regions. Furthermore, when performing cell-based assays it is necessary to use a microplate that is designed for cell attachment (e.g. TCT, Corning® CellBIND® Surface or PDL). While some cells adhere well under normal conditions, the cells may detach during an assay with multiple wash steps. If cells are detaching during an assay one option would be to switch to a microplate precoated with PDL or Corning® CellBIND® Surface treated. Alternatively, other biological coatings could be used (e.g., collagen and laminin); however, the coatings and concentrations would need to be optimized for each cell line. Figure 3 provides an example

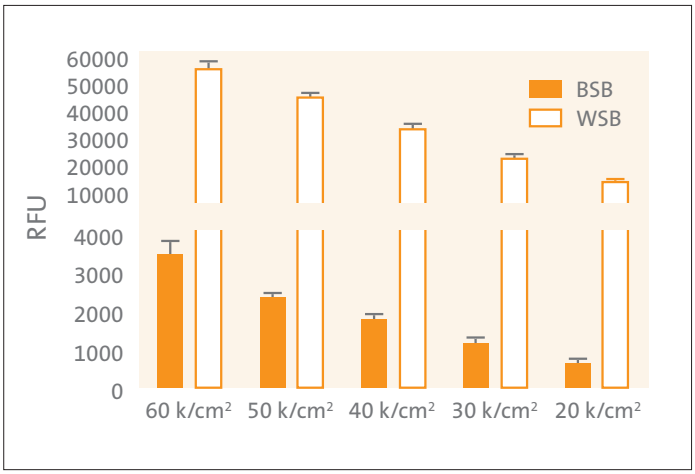


Figure 2. CHO-K1 cells were plated at various seeding densities for 24 h in phenol red- free 2% FBS containing media. Cells were incubated with MultiTox-GLO GF-AFC reagent for 1 h at 37°C. Fluorescent signal was measured with the Perkin Elmer EnVision® instrument λ_{ex} 400 nm, λ_{em} 515 nm). The results demonstrate that a white solid bottom (WSB) microplate yields higher relative fluorescence units (RFU) than a black solid bottom (BSB) microplate.

Table 1. Selecting the Best Microplate for the Assay

Detection Type	Sensitivity	Background	Handling	Plate
Colorimetric	Low	Sometimes	Very easy	Clear
Radiometric	Very high	No	Difficult	White
Luminometric	Very high	No	Easy	White
Fluorometric	Medium to High	Varies	Easy	Black*
Label Free	Very high	No	Easy	Black with optical biosensors

*Note that some assays require a white microplate for optimal results.

on how selecting the correct microplate surface is important for assay optimizations. For more detailed information on recommended microplate surfaces please visit http://www.corning.com/lifesciences/us_canada/en/technical_resources/surfaces.aspx.

Importance of Instrumentation Settings

An essential part when setting up an experiment and validating all the necessary components (reagents, microplate compatibility, instrument settings, etc.), is to confirm the instrument settings as they relate to the microplate. Settings to consider include, but are not limited to, understanding the various attributes of the instrument (bottom reader vs. top reader, etc.), Z-height, and microplate dimensions (e.g., depth of well, well position, length and width of microplate).

Z-height is defined as the height between the well bottom and focal plane. Well bottom elevation and the volume of liquid will impact the optimal Z-height for an experiment (Fig. 4A). Therefore, for each experiment a researcher would want to re-optimize the Z-height if there is a change in either the microplate type (well height and/or well geometry) or liquid volume. Often minor changes in the Z-height can result in a decrease in signal and a potential increase in the overall microplate CV (coefficient of variance, a statistical measurement that analyzes the distribution of data points around the mean) (Fig. 4B). In the example shown in Fig. 4B, the optimal Z-height is 4 mm. By either increasing or decreasing the height, the signal in this example expressed as relative fluorescence units (RFU), and the CV are greatly impacted.

Confirming and validating the correct microplate dimensions in an instrument is important to achieving optimal results. For example, analyzing a 384 well LV microplate under the settings for a 384 well NV microplate may result in less then optimal results due to higher then expected microplate CVs (Fig. 5A). Figure 5B shows the differences in well geometry dimensions between the Normal and low volume 384 well microplates. Proper alignment and optimization is critical when changing microplate formats. As depicted in the figure, there is a significant difference between the well depth of the NV microplate (11.43 mm) and the well depth of the LV microplate (9.39 mm). This difference in well depth will greatly impact the optimal Z-height.

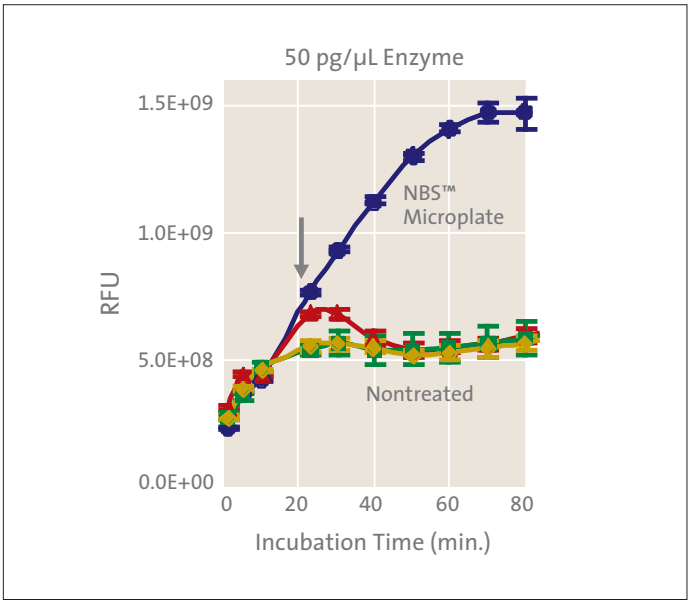


Figure 3. Selecting the correct microplate surface is important for all assays. In the example depicted above two different microplate surfaces were used for an enzymatic assay, NBS™ and Not treated. The data demonstrate that the NBS microplate greatly enhances enzymatic activity compared to the not treated surface. The increase in activity with the NBS microplates is an effect of the lack of protein and nucleic acid binding to the microplate thereby providing researchers with maximum accessibility of the components in solution.

Importance of Proper Liquid Handling Techniques

In addition to optimizing Z-height, accurate dispensing of liquids into a microplate is important for obtaining high quality data (e.g., excellent CVs). If each well in a microplate had a different volume as a result of poor liquid handling, the optimal Z-height for each well would be different. As a result, the data would be inconsistent from well to well leading to high CVs and the possibility of false-positives and negatives.

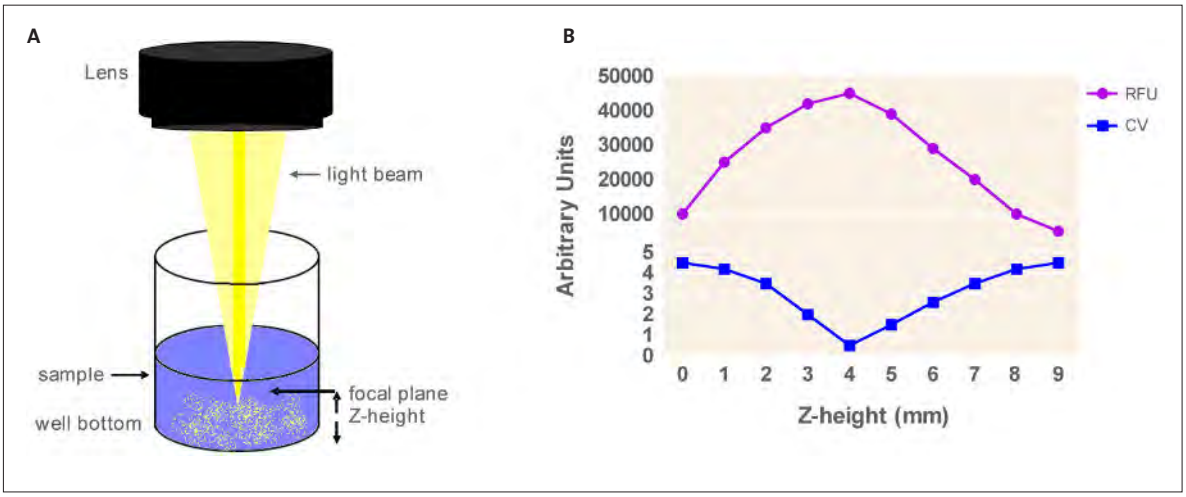


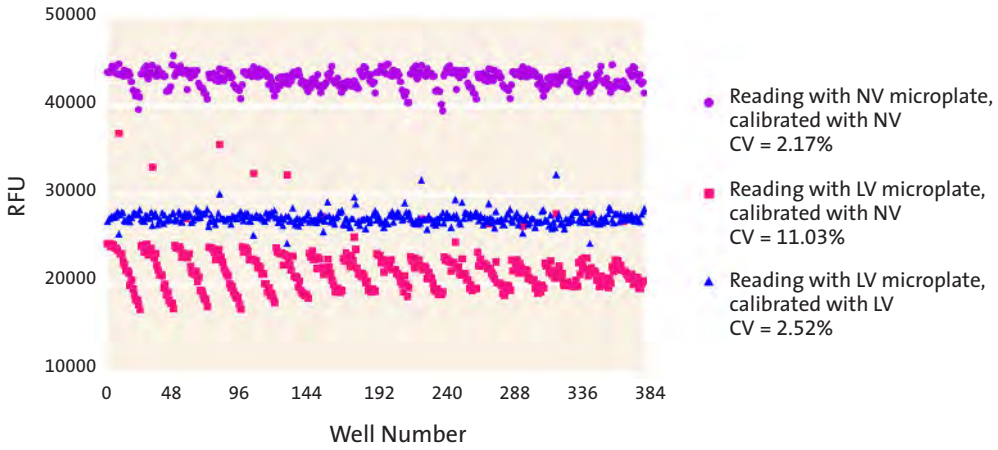
Figure 4. (A) Z-height is the distance between the lens and the sensing volume and determines the focal plane. (B) Performing an assay with suboptimal Z-height can result in a decrease in signal and increase in CV.

Fig. 5C demonstrates that the same XY coordinates used for a larger well microplate (e.g., 384 NV) may not work for a smaller well microplate (e.g., 384 LV) due to the smaller well diameter. Additionally, for label-free assays accurate liquid handling parameters are essential for optimal results. Taken together, microplate readers, handlers and liquid dispensers should be optimized for each new microplate type, and it is highly recommended that the instrument be re-optimized prior to setting up any new assay.

Summary

- Each microplate needs to be carefully selected for the assay.
- Instrumentation settings for each assay need to be optimized to the microplate being used.
- Proper liquid handling is important for achieving optimal CVs.
- Collectively, each of these areas needs to be evaluated before a successful assay can be attempted.

A



B

	Representative microplate (Corning Cat. #)	Well Volume (uL)	Well Depth (mm)	Well Diameter (top/bottom) (mm)	Plate Length (mm)	Plate Width (mm)	Plate Height (mm)	A1 Row Offset (mm)	A1 Column Offset (mm)	Well center to well center spacing (mm)	Flange or skirt height (mm)	Stack Height	Well Bottom Elevation (mm)	Well Bottom Thickness (mm)	Well Bottom Area (cm2)	Distance to bottom of plate
384 Well Microplates																
Solid black, white, and clear polystyrene plates																
384 low flange flat bottom	3570, 3571, 3572, 3573, 3574, 3575	112	11.43	3.63 / 2.82 (width)	127.8	85.5	14.2	8.99	12.12	4.5	2.41	12.95	2.794	1.4	0.062	1.37
384 low volume flat bottom	3820, 3821, 3822, 3824, 3825, 3826	50	9.39	3.30/2.00	127.8	85.5	12.19	8.99	12.12	4.5	2.41	10.49	2.79	0.635	0.0314	2.165

A1 Row Offset (mm) = Distance from top of plate to center of A1 well, A1 Column Offset (mm) = Distance from left edge of plate to center of A1 well

C

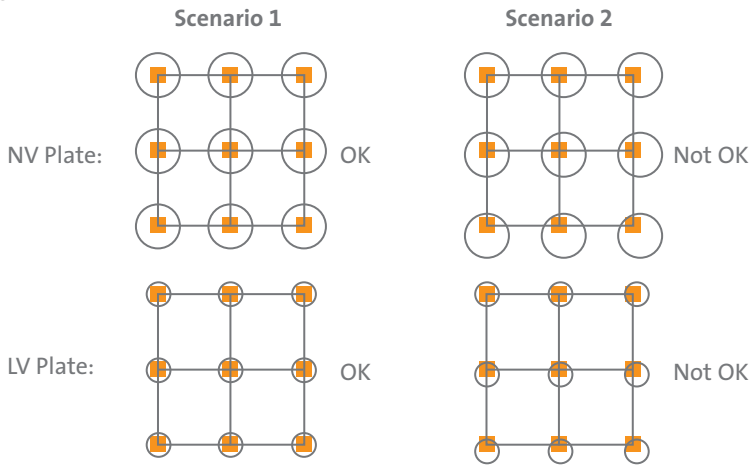


Figure 5. (A) Reading a LV microplate calibrated to a NV microplate results in a more dispersed signal across the microplate and a higher CV value. As demonstrated above a LV microplate calibrated to LV settings (red triangles) results in a uniform signal across the microplate. However, the same microplate calibrated to NV settings (green squares) leads to a high degree of dispersion across the microplate. This effect is due to the differences in the well diameter and depth between the two microplates. (B) Microplate dimensions for a 384 NV and LV microplate. (C) Dispensing liquid with the proper XY coordinates is more important in a LV microplate compared to a NV microplate due to the smaller area of the well. Poor liquid handling may lead to a scattering of data points across a microplate as seen in (A).

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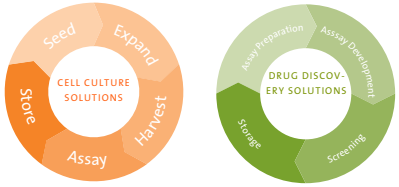

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