Introduction

What is Chemiluminescence?
Chemiluminescence is the conversion of chemical energy to light energy without energy addition. The reaction is catalyzed by an enzyme that converts a substrate to a product that is in an excited, light emitting, state. The emitting product is chemically different from the initial substrate. Each enzyme can initiate multiple chemiluminescent reactions but each product molecule can luminesce only once. Luminescence assays can reach sensitivities equivalent to those achieved with radioimmunoassays because of very low background luminescence in samples and reagents. It is also common for a luminescence assay to have a linear dynamic range of 6 or 7 decades.

Common Applications Using Chemiluminescence
The most widely used application for luminescence is the measurement of gene expression using reporter gene assays. There are several available reporter gene systems including luciferase, β-galactosidase, and alkaline phosphatase. The reporter gene is placed under control of a promoter, transfected into a cell, and upon activation produces a measurable enzyme protein. After a set time, a substrate specific for the reporter enzyme is added to the well and a luminescence measurement is taken.

Glow-Type vs. Flash Chemiluminescence
There are substrates available that enhance the luminescent signal. Some enzymes and their substrates react within seconds and emit a single, brief flash of luminescence. These reactions must be measured using instruments equipped for nearly simultaneous fluid injection and reading. Other enzymes and substrates react to emit a stable glow over several minutes or hours. All Criterion™ products are suitable for use with glow luminescence. This note applies to Analyst, Analyst AD, and Analyst HT.

Guidelines for Assay Development
This application note presents data evaluating two glow-type substrates in the luminescence mode of Analyst. The data support the following guidelines for development of the luminescence assay:

- Determine the luminescence assay parameters with purified enzyme
- Use white plates and dark adapt
- Optimize assay incubation time
- Minimize the distance between the read head and plate
- Determine necessary sample volume
- Optimize integration time
- Determine the assay reproducibility
Materials and Methods

Materials Required

The following materials are required for the luminescence assay:

- Criterion™ HTS Assay Detection System (Molecular Devices P/N 042-000-0024)
- Galacto-Star™ luminescence kit (Tropix P/N ABS180RS)
- β-Galactosidase (β-Gal) enzyme (Sigma P/N G4155)
- SuperSignal™ LBA (Pierce P/N 37070)
- Horseradish Peroxidase (HRP) enzyme (Pierce P/N 31490)
- Microplates
  - White 96-well (Packard OptiPlate P/N 6005190)
  - Black 96-well (Packard HTRF plate P/N 6005207)
  - White 384-well (Packard OptiPlate P/N 6005214)
  - White 96-well High Efficiency (HE) plate (Molecular Devices P/N 42-000-0071)

General Method

The two reagent kits, SuperSignal LBA from Pierce and Galacto-Star from Tropix, were tested with purified enzyme to evaluate the performance of Analyst in luminescence mode. The general protocols of the two assays consist of diluting the enzyme to the appropriate concentrations in a compatible buffer, diluting the substrate to a working concentration with a buffer provided by the substrate vendor, combining the enzyme with the substrate, incubating for a specified period of time at room temperature, and dark adapting the plate before finally reading in Analyst.

Table 1: Analyst Software Settings

<table>
<thead>
<tr>
<th>Parameter</th>
<th>96-well plate</th>
<th>384-well plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate format</td>
<td>choose correct one</td>
<td>choose correct one</td>
</tr>
<tr>
<td>Luminescence Height</td>
<td>1 mm</td>
<td>1 mm</td>
</tr>
<tr>
<td>Readings per well</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Integration time:</td>
<td>500,000 µsec</td>
<td>1,000,000 µsec</td>
</tr>
<tr>
<td>Units</td>
<td>counts/sec</td>
<td>counts/sec</td>
</tr>
<tr>
<td>Attenuator Mode</td>
<td>out</td>
<td>out</td>
</tr>
<tr>
<td>Plate agitation</td>
<td>choose</td>
<td>choose</td>
</tr>
<tr>
<td>Plate settle time</td>
<td>50 msec</td>
<td>0 msec</td>
</tr>
</tbody>
</table>

Assay Development Recommendations

Using purified enzyme and substrate, evaluate reagents in a luminescence assay according to the following steps:

1. Optimize the initial assay and instrument parameters using a cell-free system.
2. Establish the lower detection limit in plates by varying
   - incubation times
   - instrument integration times
3. Extend the assay to include cells once optimal ranges for the parameters are identified.
4. Compare non-expressing cells, un-induced but expressing cells, and fully induced expressing cells.

**Use White Plates and Dark Adapt**

To maximize counts, use white plates for luminescence applications. Only use a black plate if the signals are expected to be over 2 million counts/sec in a white plate. Because most white plates autoluminesce after exposure to room light, work in subdued light when pipetting luminescence plates and ‘dark adapt’ the plates inside Analyst for 5–30 minutes before reading the plate. Dark adapting is accomplished in either of two ways, depending on the software. For CriterionHost version 1.5, perform the following procedure:

1. Set the plate in the gripper.
2. Choose **Command Line** view in the Analyst CriterionHost software.
3. Type the word— load.

For CriterionHost version 2.0, perform the following procedure:

1. Click **Advanced** on the Luminescence Edit Method Screen.
2. Set the **delay before first read** to 5–30 minutes.
3. Read plate.

The plate will remain in the instrument until after the read command has been carried out. Figure 1 shows the improved performance of white plates compared to black plates.

![Figure 1. Luminescence in 96-well white vs. black plates.](image)

Horseradish peroxidase was added to both white and black plates in levels ranging from 24 pg to 46 fg/well. The enzyme was reacted in a total volume of 100 µL/well of SuperSignal LBA. Each plate was dark adapted for at least 15 minutes before reading. The lower limit of detection (LLD) is defined by the intersection of the fitted curve with the horizontal line on the graph signifying 3 standard deviations above background signal. The non-fitted signals are plotted in the figure. For the white plate the LLD was 76 fg (1.1 x 10^6 molecules) and for the black plate it was 684 fg (1.3 x 10^7 molecules). The 10–30 fold reduction in signal between white and black plates is mostly due to the black color of the plastic absorbing much of the emitted light.
A similar comparison was done between 96-well and 384-well white plates (data not shown). For the 96-well plate the LLD was 46 fg (7.0 x 10^5 molecules) in a 100 µL volume and for the 384-well plate it was 149 fg (2.2 x 10^6 molecules) in an 80 µL volume. The signal was greatly reduced in the 384-well plate because of the decreased area (16% of the area of the 96-well aperture) used to collect the light from the wells on a 384-well plate. The decreased signal resulted in a three-fold loss in sensitivity in the 384-well plates.

Optimize Assay Incubation Time

Even though the luminescence reagent manufacturers suggest times for glow signal stability, for the best results empirically determine the optimum incubation time for each assay. The data in Figure 2 demonstrate that lower detection limits change with different plate incubation times.

**Figure 2. Lower limit of detection with varying incubation times.**

Dilutions of β-Galactosidase (β-Gal) from 317 pg to 16 fg/well were added to a white 96-well plate. The enzyme was reacted in a total volume of 100 µL/well. The plate was incubated inside Analyst (dark adapted at least 5 minutes) and read at different time intervals. This figure shows the average of 2 separate experiments. The lowest LLDs (36–40 fg/well) were obtained if the plate was read after 15–60 minutes of incubation. This is consistent with optimal signal production between 30 and 60 minutes of incubation.

Minimize the Distance between the Read Head and Plate

Luminescence height is the distance from the read head to the top of the well. Place the read head as close to the top of the well as possible to capture all of the light emitted from the well and to minimize crosstalk. Usually, a luminescence height of 1 mm achieves minimum cross-talk and maximum signal. The data in Figures 3 and 4 illustrate the effect of increasing luminescence height on well-to-well cross-talk and signal magnitude.
Figure 3. Effect of luminescence height on cross-talk.

Five hundred picograms of β-Gal were added to select wells of a 96-well white plate. The enzyme was reacted with Galacto-Star substrate in a total volume of 100 µL/well. The plate was dark adapted for 5 min before reading each time. The luminescence heights were randomly varied from 0.75–3.0 mm. Signal from background wells, far from the 'hot' wells, was subtracted from both the hot well signal and the surrounding well signal. The % cross-talk was calculated by dividing the average background-subtracted signal of the 8 wells surrounding a hot well by the background-subtracted signal of the hot well and multiplying by 100. The percent cross-talk ranged from 0.004% (at 0.75 mm) to 0.056% (at 3.0 mm).

Figure 4. Effect of luminescence height on signal.

This is the same experiment as described in Figure 3 but shows the effect of signal detected as a function of luminescence height. About 22% of the signal was lost by increasing the luminescence height from 1 mm to 2 mm. A luminescence height of 1 mm is ideal for detecting the maximum signal from each well while minimizing cross-talk.

Determine Necessary Sample Volume

The volume of sample in each well may affect signal magnitude and lower the limit of detection. The volume of sample in the well did not significantly affect cross-talk in either 96- or 384-well plates when using a luminescence height of 1 mm (data not shown). Table 2 shows that for a fixed amount of enzyme, increasing assay volume improves lower limit of detection in a standard plate. For the highest sensitivity, position the sample surface as close to the luminescence aperture as possible.
Dilutions of β-Gal were added to a white 96-well plate at levels from 11.8 pg to 16 fg/well. The enzyme was reacted in Galacto-Star substrate at a total volume of 100, 200, or 300 µL/well. The actual concentration of the enzyme (pg/mL) was higher in the lower volume wells. The plate was dark adapted at least 10 minutes. The LLD was reduced by 27% (from 29 to 21 fg) by increasing the well volume from 100 to 200 µL and by 71% (from 21 to 6 fg) by increasing the well volume from 200 to 300 µL.

If it is imperative to use a low assay volume, then use white 96-well Molecular Devices High Efficiency (HE) plates. Figure 5 shows the results comparing a 10 µL assay in an Molecular Devices HE plate and a 100 µL assay in a Packard Optiplate. Using a lower volume in the HE plate did not diminish the LLD: In both cases it was ~30 fg/well.

Table 2: Effect of well volume on LLD in a 96-well Optiplate

<table>
<thead>
<tr>
<th>Well Volume (µL)</th>
<th>LLD (fg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>200</td>
<td>21</td>
</tr>
<tr>
<td>300</td>
<td>6</td>
</tr>
</tbody>
</table>

If it is imperative to use a low assay volume, then use white 96-well Molecular Devices High Efficiency (HE) plates. Figure 5 shows the results comparing a 10 µL assay in an Molecular Devices HE plate and a 100 µL assay in a Packard Optiplate. Using a lower volume in the HE plate did not diminish the LLD: In both cases it was ~30 fg/well.

**Figure 5. Luminescence in HE plates vs. Optiplates**

Dilutions of the β-Gal were added to a white 96-well Optiplate and a white 96 HE plate at concentrations from 8 ng to 100 fg/mL. The enzyme was reacted for 35 min and dark adapted for 10 min. Wells containing the same concentration of enzyme but in one-tenth the volume showed one-tenth the signal; however, the background signal of the HE plate was only 25% that of the Optiplate. The LLD is defined by the intersection of the fitted curve with the horizontal line on the graph which represents three standard deviations above the background signal.

**Optimize Integration Time**

The integration time for luminescence is set in the Analyst software, with values up to 1.6 sec/well. Longer integration times are selected using the Command Line view.

In these studies, the magnitude of signal rate from each well is not significantly affected by varying integration time (data not shown). Figure 6 shows that integration times between 250,000 µsec and 9.6 sec give similar lower limits of detection.
A 500,000 µsec integration time is optimal for 96-well plates since a 9.6 sec integration time with a 50 msec settle time takes over 15 minutes to read and does not improve LLD.

![Graph showing detection limit with different integration times.](image)

**Figure 6. Detection limit with different integration times.**

Beta-Galactosidase was added to a white 96-well plate at levels from 5 fg–317 pg/well. The enzyme was reacted with Tropix Galacto-Star substrate in a total volume of 100 µL/well. The plate was read at 12 different integration times (10,000 µsec–9.6 sec/well) after dark adapting inside the instrument for 25 minutes. Total incubation of the plate at different read times ranged from 30–70 minutes.

Table 3 shows total plate read times for 0.5 and 1 second integration times in 96- and 384-well plates.

### Table 3: Total elapsed read time for a plate

<table>
<thead>
<tr>
<th>Integration Time (µsec)</th>
<th>96-Well Plate</th>
<th>384-Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>500,000</td>
<td>1 min 20 sec</td>
<td>5 min 15 sec</td>
</tr>
<tr>
<td>1,000,000</td>
<td>2 min 18 sec</td>
<td>8 min 25 sec</td>
</tr>
</tbody>
</table>

Reading plates by rows with no plate agitation and a 50 msec plate settle time for the 96-well plate and 0 msec plate settle time for the 384-well plate.

The following factors affect reproducibility of the luminescence assay:

- Stability of the enzyme and substrate under different storage conditions
- Environmental conditions in the laboratory
- Robustness of the assay protocol, such as pipetting extremely low volumes or using an extreme range of incubation times.

To assess reproducibility, run a standard curve each time the assay is performed, and collect a history of absolute signal magnitudes, curve linearity, and LLD.

Figure 7 shows day-to-day reproducibility of the Galacto-Star luminescence assay under different enzyme storage conditions.
Figure 7. Reproducibility of β-Galactosidase/Galacto-Star Assay.

A dilution series of β-Gal from 317 pg–48 fg/well was tested on 3 separate days. On day 1 the β-Gal enzyme was diluted from stock stored at -20°C in glycerol; day 6 the enzyme was diluted from a stock stored at 4°C in phosphate buffer; day 35 the enzyme was diluted from a stock stored at -20°C in buffer. The enzyme was reacted with Galacto-Star substrate in a total volume of 100 µL/well. The plates were incubated for 45 min and dark adapted inside Analyst for 15 min before reading. LLDs were 22 fg (2.8 x 10^4 molecules), 29 fg (3.8 x 10^4 molecules), and 20 fg (2.6 x 10^4 molecules) on the three days. Raw signals varied by as much as 23%.

Summary

Any new assay system requires optimization of instrument and reagent conditions.

The suggested guidelines for developing a glow-type luminescence assay on the Analyst HTS platform are as follows:

• Determine luminescence assay parameters with purified enzyme
• Use white plates and dark adapt
• Optimize assay incubation time
• Choose a low luminescence height
• Determine necessary sample volume
• Optimize integration time
• Determine day-to-day assay reproducibility

Conclusions

The conclusions from the data evaluating two luminescence reagent kits are as follows:

• Maximize counts by using white plates and dark adapt them.
• Incubate samples between 30 and 60 min.
• Place read head 1 mm from the top of the well.
• Use the largest assay volume possible.
• Use an integration time of 500,000 µsec for a 96-well plate.

Note  The recommendations presented are meant to be used as general guidelines. Each glow-type enzyme and substrate system must be optimized separately.