

Tips for Improved Luminescence Performance

Getting the most out of your Agilent BioTek reader

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Introduction

To achieve the best possible luminescence results from your Agilent BioTek microplate reader, several different parameters must be addressed. While there are an infinite number of possible combinations, addressing just a few key issues will greatly increase the likelihood of experimental success. This technical overview describes some of those issues and parameters.

Agilent offers two reader series capable of measuring fluorescence: the Agilent BioTek Synergy HT multimode reader and Agilent BioTek FLx800 fluorescence reader. Depending on the model and type, the user will be able to perform various luminescent reactions. For example, only those readers configured with injectors have the capability to perform flash luminescence assays, while virtually all the luminescence-capable readers can perform glow assays. Listed in the next section are several different parameters, which may or may not apply to any one specific reader.

Getting started

Plate type

The type of plate used matters. Opaque white plates have been shown to provide the best performance for luminescence assays, as they maximize the signal. Other plates can be used, such as white-sided plates with a clear bottom, if necessary. Solid black plates will also work, but the signal will be reduced significantly. Quality plates should not allow any light leakage through the walls of the microplate.

Filter wheel settings

To prevent any stray light from entering the optical path, an opaque plug is located in the excitation wheel in lieu of an excitation filter. Generally, an open aperture on the emission filter wheel is used when performing luminescence measurements. However, there are occasions where wavelength specificity is required for the luminescent emission. Filters can be used to eliminate much of the autofluorescence generated by white plates exposed to ambient light. The use of a filter instead of an empty position will result in an approximately 20% loss of signal.

PMT sensitivity setting

The correct sensitivity setting is critical in obtaining the best luminescence results. The sensitivity setting must be high enough to obtain a signal, yet stay within the linear range of the reader. There are several ways to arrive at the correct sensitivity setting. With glow assays, the best setting can be determined using multiple filter sets, each with differing sensitivity settings. Alternatively, for many glow-type luminescence assays, scaling to low well, with a value of approximately 500 on a blank well, will ensure that wells are above the X-axis. If this results in high wells returning an over-range message, then scale to high well (using 70,000 to 80,000 as the value) can then be used to optimize the photomultiplier (PMT) sensitivity setting. With flash-type assays, one does not have the luxury of time, and multiple readings are not possible. Trial runs with known samples with expected high levels of luminescence can be performed to determine a sensitivity setting that is appropriate. The PMT performs best with a setting of approximately 150, so this is a good value to start with.

Read and dispense parameters

Most luminescence kit manufacturers recommend any reading and dispensing parameters their kit requires. For example, Promega recommends a 2-second delay after dispensing 100 μL of luciferin/luciferase reagent, then a 10-second integration on each well when measuring ATP with the ENLITEN kit. Most glow-type assays can be performed using a standard endpoint determination. However, detection of low levels can be enhanced with kinetic integration if the reader has that capability (e.g., Agilent BioTek Synergy HT with injectors). Other luminescent compounds with a faster reaction time, such as aequorin, may require reading immediately after dispensing the trigger reagent. When using readers that do not have injectors for glow-type luminescent reactions with low signal levels, the signal-to-noise ratio may be improved by increasing the number of reads per well from the default of 10 to 50 to 100. The increase in read number provides better statistical averaging, resulting in tighter CVs.

Maintaining the reader

Flash-type luminescence assays require the use of reagent injectors to provide reagents that initiate the reaction while the plate is in the reader. Use of these reagent delivery devices requires that the fluid path be maintained and free of contamination. Reader models that do not have reagent injectors generally do not need much maintenance beyond the guidelines outlined in the user manual.

Cleaning the system

To achieve the best results, the fluid path must be maintained. If the reagent manufacturer specifies a cleaning procedure specific to their reagent, please follow it before running the daily cleaning procedure. The daily cleaning of the reagent injectors can be accomplished using deionized or distilled water. Using the "purge" feature to pump the solution back into the reagent bottle can save reagents. Immediately after purging of the reagents, use the prime function in the software to pump deionized water through the syringe and tubing. Using the "prime" function, select 3,000 μL and prime the system to several times. When switching between different assays, particularly when switching between a luciferase assay and an ATP assay, it is recommended that an acid/base cleaning procedure be used (see the following). First, rinse with deionized water using the "prime" function. Next, prime the system with 0.1 N NaOH several times. Let the NaOH remain in the system for 10 to 15 minutes. Next, prime with 0.1 N HCl and leave this solution in the system for 10 to 15 minutes. Finally, wash the syringe with deionized water using the priming routine.

ATP and luciferase assays

The quantitation of luciferase activity and ATP concentration are two of the most commonly performed luminescence assays. These two moieties are often measured using the same light-emitting chemical reaction. Luciferase enzyme catalyzes the reaction shown in Figure 1.

Depending on the limiting component, the intensity of light produced is proportional to its concentration. When luciferase enzyme is being quantitated, a mixture of ATP and luciferin substrate is injected to initiate the assay, while ATP assays require luciferase enzyme and luciferin. Therefore, when switching between these two assays, it is critical to ensure that the reagent injectors and supply tubing are not contaminated with components from the other assay type. Cross-contamination will lead to markedly higher background levels as a result of the luminescent reaction taking place in the tubing.

Sterility

The measurement of ATP in solution is prone to problems associated with contamination. The presence of ATP is often used as a means to detect cellular contamination. To prevent exogenous ATP, an aseptic technique should be used as much as possible. The use of gloves, sterile pipette tips, and a HEPA-filtered biosafety cabinet (if possible) when preparing the samples and performing the ATP assay is highly recommended. While the procurement of sterile white plates may be difficult or impossible, the plate used should be kept covered and free of contamination as much as possible. In addition, any buffers or solutions should be sterile and free of ATP. It is also recommended that the reader fluid path be chemically sterilized with 70% ethanol, followed by rinsing with sterile deionized water immediately before running the assay.

Decontaminating the reagent injection system

The fluid path of the reagent injection system needs to be decontaminated periodically. The fluid path should be treated with 0.5% sodium hypochlorite (bleach) for decontamination. Common household bleach is 5.25% sodium hypochlorite (NaClO) and requires a 1:10 dilution. After priming the reagent injector, keep the fluid lines filled for 15 to 20 minutes. Following decontamination, rinse the fluid lines several times with sterile deionized water to remove any residual amounts of sodium hypochlorite. Residual sodium hypochlorite can affect luminescence results.

Promega Dual Luciferase Assay reagents

One of the components of the Promega Dual Luciferase Assay kit, the Stop and Glo reagent, has an affinity to plastic materials. To avoid cross-contamination when changing reagents, rinse the fluid lines with deionized or distilled water using the priming feature. Next, prime the fluid lines with 70% ethanol and keep them filled for 30 minutes. The Stop and Glo reagent will partition to the 70% ethanol solution. After soaking, remove the 70% ethanol by priming several times with deionized or distilled water.

Running the assays

Plate background luminescence

The white plates commonly used for luminescence experiments will often fluoresce. These plates absorb energy from the ambient light in the room and then emit the energy as light during the luminescence measurement. This phenomenon can be a problem if the plates have been exposed to bright light for a particularly long period of time. Store unused plates in a dark environment and, when pipetting samples into the plate, do so in a reduced light in order to minimize plate autofluorescence. One can also "dark adapt" the plate by incubating it in the dark for approximately 10 minutes. The easiest way to accomplish this is to maintain it in the reader's reading chamber before the initiation of reading.



Figure 1. The reaction of ATP with d-luciferin, catalyzed by luciferase.

Follow the manufacturer's recommendations

Do not alter the amount of sample or decrease the amount of reagent. While using less reagent than suggested can provide adequate results for many applications, it may result in a decrease in the assay's dynamic range or sensitivity.

Allow reagents to equilibrate to temperature

Many reagents are stored frozen and it is important to allow these reagents to equilibrate to room temperature before running the assay. Because many luminescence assays are integrated over a period of 10 seconds per well, it can take tens of minutes to run a plate. If the reagent has not properly equilibrated, it will continue to do so while the plate is being read. The resultant variation in temperature can cause poor precision between replicates, and an overall skewing of the data.

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