LyticBLAzer™-FRET B/G Homogeneous Kit Protocol

Cat. nos. K1148 and K1149

O-13392-r1 US 0305

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1.0 INTRODUCTION

GeneBLAzer® beta-lactamase reporter gene technology is a robust method of monitoring cellular events. Beta-lactamase is a small 29 kDa enzyme from bacteria that is utilized as a reporter gene. Since mammalian cells have no homologous proteins to beta-lactamase, there is no endogenous background. Furthermore, beta-lactamase has no cofactors or metal requirements for activity, making it an ideal enzyme-amplified reporter gene. The GeneBLAzer® technology is a tool available for drug discovery researchers interested in a variety of targets and cellular processes including GPCRs, nuclear receptors, kinases, proteases, drug metabolism, and apoptosis.

Fluorescent substrates have been developed for detecting beta-lactamase expression levels. LyticBLAzer™_h-FRET B/G (Substrate for the homogenous assay with FRET readout using Blue and Green fluorophores) is a Förster resonance energy transfer (FRET)-based substrate for the ratiometric detection of beta-lactamase. LyticBLAzer™_h-FRET B/G is green fluorescent and upon enzyme cleavage the product becomes highly blue fluorescent.

Specialized fluorescent substrates have distinct advantages. LyticBLAzer™_h-FRET B/G substrate has the advantage of ratiometric data processing, similar to Invitrogen's widely used and proven LiveBLAzer™ FRET B/G (CCF4-AM) Substrate. Ratiometric methods eliminate the well-to-well differences caused by normal variations in hand pipetting or liquid handling instrumentation. Ratiometric methods also reduce or eliminate variations caused by excitation pathlength, fluorescence detectors, or volume changes. The ratiometric advantage of LyticBLAzer™_h-FRET B/G leads to high Z'-factors.

Monitoring reporter gene expression levels in cell lysates has many benefits. First, the addition of a Lysis & Detection solution directly to cells in cell culture media with serum is a simple one-step addition resulting in a completely homogeneous assay (Figure 1). This assay technology eliminates the need to aspirate medium from cells prior to conducting the assay. Moreover, the addition of a lysis solution has the ability to "freeze" the cellular system at a defined point in time and therefore yield long "read windows," or timeframes for reading results of an assay. Lysis of cells also eliminates aberrant effects of non-uniform plating of cells and the requirement for instrumentation capable of reading from the bottom of the assay well. Taken together, the fluorescence and lysis advantages of LyticBLAzer™ homogeneous kits make them ideal beta-lactamase detection technologies for many HTS applications.

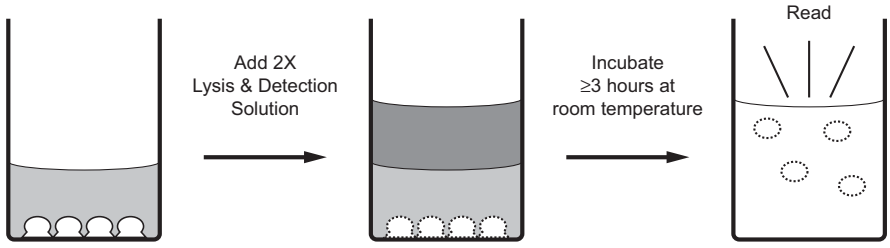

Figure 1—Schematic diagram illustrating the ease of use of LyticBLAzer™_h-FRET B/G homogeneous kits. Starting with cells in culture media (with up to 10% serum), assay media, or buffer, simply add 2X Lysis & Detection Solution directly to cells in culture medium. There is no requirement for plate shaking or mixing by pipetting. Following an incubation step, read the results of the assay on a fluorescence plate reader. Results are stable and can be read up to 72 hours after addition of Lysis & Detection Solution.

2.0 MATERIALS SUPPLIED

LyticBLAzer™-FRET B/G Homogeneous Kit (150 ml) (K1148) Sufficient for ~3,750 standard volume (80 µl final volume) wells			
Component	**Description**	**Quantity**	**Cat. no.**
LyticBLAzer™_h-FRET B/G	Proprietary Blue/Green FRET-based beta-lactamase substrate	1 unit	K1140
DMSO for Solution A	Dimethylsulfoxide for resuspension of substrate	1 ml	K1040
Homogeneous lysis buffer	Optimized buffer solution for detection of beta-lactamase in cell culture media	150 ml	K1158

LyticBLAzer™-FRET B/G Homogeneous Kit (3.75 L) (K1149) Sufficient for ~93,750 standard volume (80 µl final volume) wells			
Component	**Description**	**Quantity**	**Cat. no.**
LyticBLAzer™_h-FRET B/G	Proprietary Blue/Green FRET-based beta-lactamase substrate	25 units	K1141
DMSO for Solution A	Dimethylsulfoxide for resuspension of substrate	2 x 10 ml	K1035
Homogeneous lysis buffer	Optimized buffer solution for detection of beta-lactamase in cell culture media	4 x 950 ml	K1159

Note: GeneBLAzer® substrates should not be substituted between different product protocols (*i.e.* LyticBLAzer™_h-FRET B/G is **NOT** interchangeable with LiveBLAzer™ FRET B/G)

3.0 MATERIALS AND EQUIPMENT REQUIRED, BUT NOT SUPPLIED

Consumables	Recommended Source
Black-wall, clear or black-bottom, assay plates	Costar

Equipment	Recommended Source
Fluorescence plate reader (see **Section 7.3.1**)	Multiple vendors
Optical Filters for non-monochromator-based plate readers (see **Section 7.3.2**)	Chroma Technologies

4.0 STORAGE AND HANDLING

Beta-Lactamase Substrates and Lysis Solutions			
Description	**Storage**	**Handling**	**Notes**
LyticBLAzer™_h-FRET B/G	–20°C	Desiccate and protect from light.	Store dry substrate at -20°C, and keep protected from light. Once the substrate has been resuspended in DMSO, store the substrate at -20°C and keep protected from light. Stored under these conditions, LyticBLAzer™_h-FRET B/G Substrate in DMSO is stable for at least 6 months.
DMSO for Solution A	20–25°C	Protect from direct light.	Use to dissolve LyticBLAzer™_h-FRET B/G when preparing Solution A.
Homogeneous lysis buffer	20–25°C	Protect from direct light.	

5.0 GENERAL PROTOCOL FOR USING LYTICBLAZER™_H-FRET B/G

Note: Section 7.0, Assay Considerations provides critical information necessary for a successful assay. Before proceeding with the assay for the first time, read this information carefully.

This general protocol is designed for the detection of beta-lactamase in cells with LyticBLAzer™_h-FRET B/G using a fluorescence plate reader or other fluorescence detection instrumentation.

5.1 Controls

Each assay should include the following controls:

Positive Control (stimulated cells)

Cells should be stimulated with a known stimulant for the particular assay to ensure that a detectable signal is obtained. Cells should have a significantly higher blue/green fluorescence ratio when assayed using LyticBLAzer™_h-FRET B/G than the Negative Control.

Negative Control (unstimulated cells)

This control is used to determine the amount of blue and/or green fluorescence to expect in an unstimulated sample. This value will also be used when determining the Response Ratio of your assay.

5.2 Plate Incubation

1. After preparing your plate of cells, incubate at 37°C/5% CO_2 for an amount of time sufficient for stimulation.
2. At the end of the incubation, allow the plate to equilibrate to room temperature before proceeding to **Section 5.5**.

5.3 Preparation of Solution A (1X stock solution of LyticBLAzer™_h-FRET B/G in DMSO)

Add 750 µl of DMSO per unit of LyticBLAzer™_h-FRET B/G.

Mix well.

Note: Store the solution at –20°C, desiccated and protected from light. Before each use, allow the frozen stock solution to thaw at room temperature and remove the desired amount of reagent. To reduce moisture uptake, recap the vial immediately after each use and return it to the desiccator in the –20°C freezer. Stored under these conditions, Solution A is stable for at least six months. Once thawed, Solution A will appear green fluorescent; this color is normal and does not affect the quality or function of the product.

5.4 Prepare 2X Lysis & Detection Solution

1. Add 5 µl of Solution A to 995 µl of homogeneous lysis buffer.
2. Mix well, but avoid rigorous shaking and/or vortexing as foaming will occur.

   Note: Under typical laboratory conditions, the 2X Lysis & Detection Solution is stable for at least 12 hours.

5.5 Addition of 2X Lysis & Detection Solution to Cells

1. Add 2X Lysis & Detection Solution to all wells to 1X final concentration (*e.g.*, 40 µl of 2X Lysis & Detection Solution to 40 µl of cells in cell culture media with serum, assay media, or buffer).

   Note: Effects of serum: The 2X Lysis & Detection Solution has been optimized to lyse cells and detect beta-lactamase expression in typical cell culture media (RPMI and DMEM) supplemented with up to 10% serum.

5.6 Incubation

1. Cover the plate to protect it from light and evaporation.
2. Incubate at room temperature for >3 hours. Incubation at 37ºC will speed the reaction time.

5.7 Plate Reader Detection

1. A top-read plate reader works well. Alternatively, if using clear-bottom plates, a bottom-read plate reader can be used.

   Note: If you are uncertain of whether your instrument can be used to read LyticBLAzer™-FRET B/G, please contact Technical Support for assistance.

2. Select appropriate excitation and emission wavelengths:

   Note: For LyticBLAzer™_h-FRET B/G, excitation should be centered at 405 nm, with a 20 nm (or narrower) bandpass. Emission of the blue signal should be measured with a filter or monochromator centered at 460 nm, with a 40 nm (or narrower) bandbass, and emission of the green signal should be measured with a filter or monochromator centered at 530 nm, with a 30 nm (or narrower) bandpass. Bandpasses larger than those recommended will contribute to increased background and lowered measured response ratios. Smaller bandpasses may be used, but the overall signal strength will be lower, which may result in increased error in the resulting measurements, and therefore, lower Z'-factors.

   Note: The correct filters or instrument settings are *essential* for a successful assay. For more information on filter selection see **Section 7.3.2**.

3. Read the plate.

6.0 DATA ANALYSIS

6.1 LyticBLAzer™_h-FRET B/G

Note: Using the blue/green ratio to analyze samples reduces well-to-well variations and provides for more consistent results by reducing the contribution of normal assay noise.

Note: Background subtraction is not required.

1. Calculate the ratio of blue/green fluorescence by dividing the blue signal (530 nm) by the green signal (460 nm). This is your *blue/green ratio* (*B/G*).
2. Determine the average blue/green ratio for your Negative Controls. This is your *average negative ratio*.
3. Calculate the Response Ratio for your Positive Control and experimental samples by dividing each of their blue/green ratios by the average negative ratio. Your Positive Control and experimental samples with beta-lactamase activity should have a Response Ratio greater than 1.

   Response Ratio = $(B_s/G_s)/(B_n/G_n)$, where B_s and G_s are the blue and green signals of the sample (or Positive Control) and B_n and G_n are the blue and green signals of the negative control (unstimulated) samples.

   Note: Due to the error-correcting nature of ratiometric readouts, very subtle differences in response ratios with beta-lactamase are more likely to be statistically significant compared to other reporter technologies.

7.0 ASSAY CONSIDERATIONS

Several variables can affect assay performance and will need to be empirically determined. The following suggestions are only starting points; further evaluation may be necessary to optimize individual assay performance.

7.1 Cell Stimulation

- Factors such as the type of cell being tested and the necessary conditions for induction can affect the optimal stimulation conditions for cells.
- Better results may be obtained with certain adherent cell types if the cells are adhered before stimulation.
- Certain assays may require the cells to be incubated in serum-free media for up to 24 hours before stimulation (serum starvation).
- Alternative assay media formulations can help improve the response of some assays. Using serum that has been stripped of some components (including charcoal-dextran-treated and delipidated serum) is helpful in certain assays.

7.2 Detection of Beta-Lactamase

- Adherent cell lines may take longer to lyse than suspension cell lines; therefore fluorescence signals may stabilize more slowly than suspension cell lines
- Incubation of cells with Lysis & Detection solution at 37°C can shorten the incubation time (the time from adding the Lysis & Detection solution to reading the results).
- Quicker incubation times may be obtained with gentle plate shaking or mixing of Lysis & Detection solution after addition to cells in media.

7.3 Plate Reader Detection

All measurements are made at room temperature, preferably in black-wall, clear-bottom (for bottom reading instruments) or black-bottom bottom (for top-reading instruments) assay plates with low fluorescence background.

Note: Some plates and/or fluorescence plate readers experience edge effects, which may affect data. If edge effects are noticed, plate layout should be considered when setting up the assay.

7.3.1 Instrumentation

Almost all standard and advanced fluorescence plate readers and single tube fluorescence spectrophotometers can be used to detect beta-lactamase with LyticBLAzer™_h-FRET B/G kits.

Note: If you are uncertain of whether your instrument can be used for this assay, please contact Technical Support for assistance.

7.3.2 Filter Selection for non-monochromator-based instruments

LyticBLAzer™_h-FRET B/G

If your fluorescent plate reader does not include the appropriate filters, we recommend using the filter set available from Chroma Technologies (800-824-7662; www.chroma.com).

Chroma Set #71008a
Excitation filter: HQ405/20x (405 ± 10 nm)
Dichroic mirror: 425DCXR
Emission filter: HQ460/40m (460 ± 20 nm)
Emission filter: HQ530/30m (530 ± 15 nm)

Alternatively, you can select appropriate filters for LyticBLAzer™_h-FRET B/G using the spectra shown below.

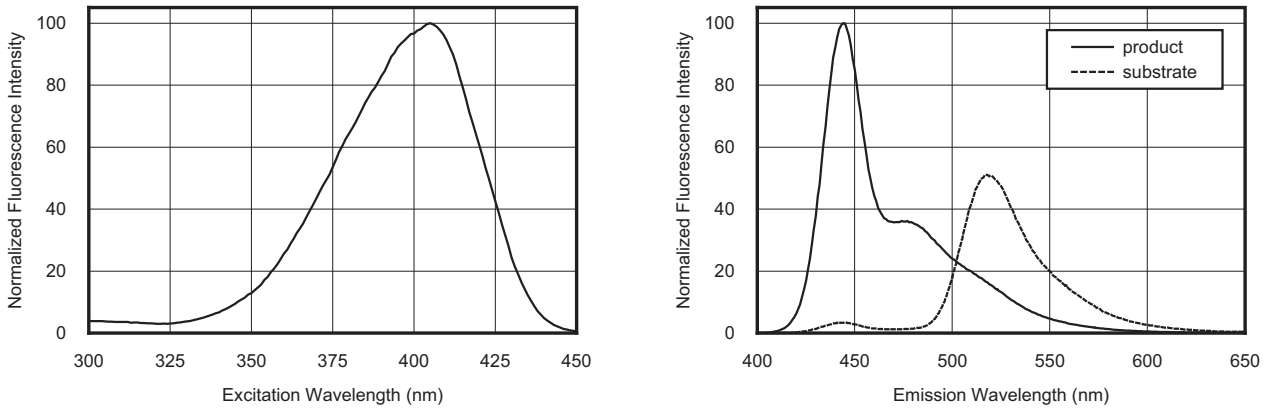

Figure 2—Fluorescence excitation and emission spectra of LyticBLAzer™_h-FRET B/G. The peak excitation wavelength for LyticBLAzer™_h-FRET B/G is 405 nm and the peak emission wavelengths are 445 nm and 520 nm.

8.0 PURCHASER NOTIFICATION

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