ToxBLAzer™ DualScreen Protocol

Cat. nos. K1138 and K1139

O-13409-r1 US 1104

1.0 INTRODUCTION

ToxBLAzer™ DualScreen combines the advantages of LiveBLAzer™-FRET B/G with the ability to evaluate cytotoxicity in one simple assay. LiveBLAzer™-FRET B/G features the robust beta-lactamase reporter gene technology in a live-cell format, enabling visual observation of cells and flow cytometry after substrate loading. The FRET (fluorescence resonance energy transfer)-based substrate provides the benefit of ratiometric readout, leading to high Z'-factor values and low coefficients of variation (CVs). With ToxBLAzer™ DualScreen, the advantages of LiveBLAzer™-FRET B/G are multiplexed with a cytoxicity readout that *does not require* any extra reagents or reagent manipulations. This multiplexing strategy not only gives twice the information out of a single assay, but by combining the information from the transcriptional readout and the toxicity readout, it also allows the identification of false positives due to toxic screening compounds.

The ToxBLAzer™ reagent contains both, the LiveBLAzer™-FRET B/G substrate and a proprietary cytotoxic indicator. Both compounds readily enter the cell, where cleavage by endogeneous cytoplasmic esterases rapidly converts them into their negatively charged forms, trapping them inside the cell. The LiveBLAzer™-FRET B/G substrate is green fluorescent; upon beta lactamase hydrolysis of the lactam ring, the substrate undergoes an elimination reaction, resulting in loss of FRET and a product that is highly blue fluorescent. Beta lactamase expression is quantified by measuring the ratio of blue (product) to green (substrate) fluorescence. Cytotoxicity is determined by the level of red fluorescence from viable cells. Both, an intact cell membrane and active esterases are required for the cytotoxic probe to fluoresce.

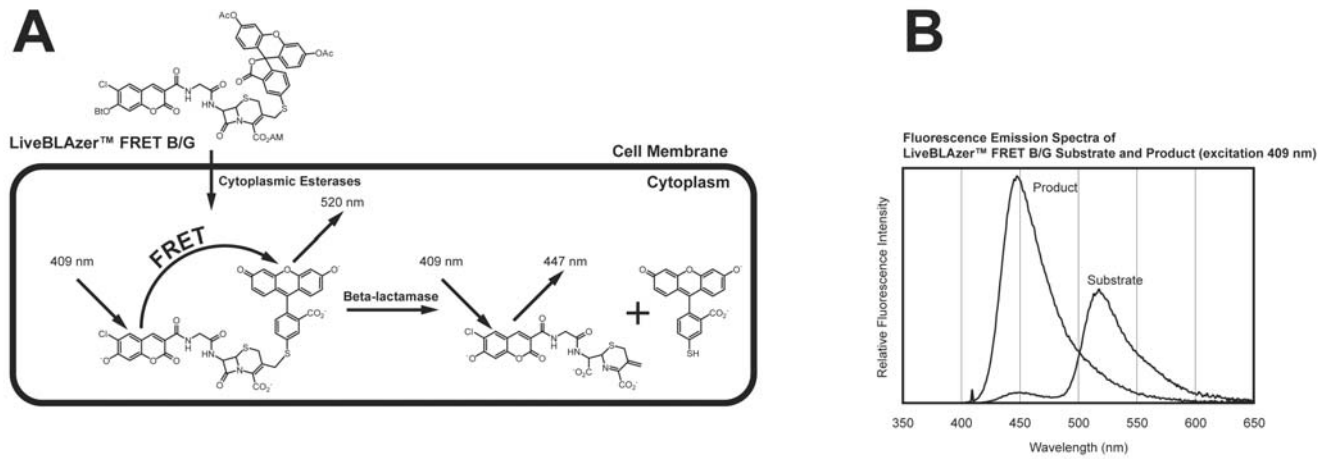


Figure 1—The lipophilic, esterified form of LiveBLAzer™-FRET B/G Substrate readily enters the cell. Cleavage by endogenous cytoplasmic esterases rapidly converts this molecule into the negatively charged substrate, which is retained in the cytosol. In the absence of beta-lactamase activity, excitation of the coumarin (at 409 nm) in the intact molecule, results in FRET to the fluorescein, which emits a green fluorescence signal (at 520 nm) (**Panel A**). In the presence of beta-lactamase, enzymatic cleavage of LiveBLAzer™-FRET B/G Substrate spatially separates the two dyes and disrupts FRET, so that excitation of the coumarin (at 409 nm) now produces a blue fluorescence signal (450 nm) (**Panel A** and **B**).

2.0 MATERIALS SUPPLIED

ToxBLAzer™ DualScreen Kit (200 µg) (K1138)			
Component	**Description**	**Quantity**	**Cat. no.**
ToxBLAzer™ substrate	Dried down under vacuum from acetonitrile	200 µg	K1136
DMSO for Solution A		1 ml	K1040
Solution B	Aids solubility and cell loading of ToxBLAzer™ substrate	5 ml	K1041
Solution C	Required for achieving optimum signal window.	32 ml	K1048

ToxBLAzer™ DualScreen Kit (5 mg) (K1139)			
Component	**Description**	**Quantity**	**Cat. no.**
ToxBLAzer ™ substrate	Dried down under vacuum from acetonitrile	5 mg	K1137
DMSO for Solution A		10 ml	K1035
Solution B	Aids solubility and cell loading of ToxBLAzer ™ substrate	75 ml	K1036
Solution C	Required for achieving optimum signal window.	800 ml	K1037

3.0 MATERIALS AND EQUIPMENT REQUIRED, BUT NOT SUPPLIED

Consumables	Recommended Source
Black-wall, clear-bottom, assay plates (with low fluorescence background)	Costar

Equipment	Recommended Source
Fluorescent plate reader with bottom read mode	Various
Filters (see **Section 7.3.2**)	Chroma Technologies

3.1 Optional Equipment and Materials

- Epifluorescence microscope equipped with appropriate filters (**Section 7.4**)
- Sodium hydroxide (400 mM)
- Sodium phosphate buffer (100 mM, pH 8.0)
- Solution D (Invitrogen, K1156 or K1157)

4.0 STORAGE AND HANDLING

ToxBLAzer™ Dual Screen Substrates and Loading Solutions			
Description	**Storage**	**Handling**	**Notes**
ToxBLAzer™ substrate	–20°C	Desiccate and protect from light	
DMSO for Solution A	22–25°C	Protect from direct light	Used to dissolve ToxBLAzer™ substrate when preparing Solution A.
Solution B	22–25°C	Protect from direct light	If stored at cooler temperatures, a white precipitate may form or the solution may freeze. This change does not affect the quality of the product. Warm and mix (at ~35°C) the solution until the precipitate dissolves, then use as described.
Solution C	22–25°C	Protect from direct light	

5.0 GENERAL PROTOCOL FOR USING TOXBLAZER™ DUAL SCREEN

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 loading cells with ToxBLAzer™ before visual observation or analysis in a fluorescence plate reader. Use tissue culture-treated, black-wall, clear-bottom plates for this assay.

5.1 Controls

Each assay should include the following controls:

5.1.1 Positive Control

Cells should be stimulated with a known stimulant for the particular assay to ensure a detectable signal is obtained. Cells should have a significantly higher blue/green fluorescence ratio than the Negative Control.

5.1.2 Negative Control

This control is used to determine the amount of blue and green fluorescence to expect in an unstimulated sample and is needed when determining the Response Ratio of your assay. The negative control is also used to determine the amount of red fluorescence to expect in a sample of viable cells.

5.1.3 No Cells Background Control

This is a control used to determine the amount of blue and green background fluorescence that exists in a cell-free sample. The amount of background fluorescence seen in the blue and green channels will need to be subtracted from the experimental wells containing cells to obtain accurate data. This control is also used to determine the amount of red fluorescence to expect in a sample where all the cells are dead, such as when exposed to a highly toxic compound.

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 (1 mM stock solution of ToxBLAzer™ in DMSO)

1. Add 912 µl of the provided DMSO per mg of ToxBLAzer™ substrate.
2. 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 three months. Once thawed, Solution A may appear slightly yellow. This color change does not affect the quality or function of the product.

5.4 Choose a Substrate Loading Solution

For most cells, the Standard Loading Solution (**Section 5.4.1**) is recommended. Some cells (such as CHO-K1) may load or retain the substrate poorly. For these cells, the Alternative Loading Solution (**Section 5.4.2**) is recommended.

5.4.1 Standard Substrate Loading Solution

1. Add 6 µl of Solution A to 60 µl of Solution B and vortex.
2. Add 934 µl of Solution C to the combined Solutions A and B and vortex.

Note: Under typical laboratory conditions, the 6X Substrate Loading Solution is stable for up to 12 hours.

5.4.2 Alternative Substrate Loading Solution

1. Add 12 µl of Solution A to 60 µl of Solution B and vortex.
2. Add 925 µl of Solution C to the combined Solutions A and B and vortex.
3. Add 30 to 75 µl of Solution D (a nonspecific anion exchange blocker that can be added to the loading solution to prevent undesired export of the substrate from the cell in cell types with active multidrug transporters) to the 6X Substrate Loading Solution and vortex. The exact volume of the Solution D may need to be optimized for the specific cell line you are using.

5.5 Loading Cells with Substrate

1. Add 6X Substrate Loading Solution to cells to 1X final concentration (*e.g.*, add 20 µl of Substrate Loading Solution to 100 µl of cells in buffer).
2. Add the same volume of 6X Substrate Loading Solution to the No Cells Background Control wells (containing assay medium or buffer) to 1X final concentration.

5.6 Incubation

1. Cover the plate to protect it from light and evaporation.
2. Incubate at room temperature for 60–120 minutes; see **Section 7.2** for more information.

Note: During the incubation, the cells will settle to the bottom of each well. Handle the plate gently as the cells must remain at the bottom of each well for accurate detection to occur.

5.7 Plate Reader Detection

Note: See **Section 7.4** for guidelines on the visual observation of your plate. All measurements are made at room temperature from the bottom of the wells, preferably in a 384-well, black-wall, clear-bottom assay plates with low fluorescence background.

1. Remove dust from the bottom of the plate with compressed air.
2. Be sure the plate reader is set to bottom-read mode.
3. Select the appropriate filters to allow excitation of the coumarin (~410 nm) and detection of the blue coumarin emission (~450 nm) and green fluorescein emission (~520 nm).
4. Read the plate.
5. Select the appropriate filters to allow excitiation (~600 nm) and detection (~650 nm) of the cytotoxic indicator.
6. Read the same plate again.

Note: The correct filters are *essential* for a successful assay. For more information see **Section 7.3.2**.

6.0 DATA ANALYSIS

Note: Using the blue-to-green ratio to analyze samples reduces any well-to-well variations and provides for more consistent results.

Note: Background subtraction for the blue and the green channels (460 nm and 530 nm) is essential to obtain accurate data (Background subtraction is not necessary for the red channel). This can be accomplished either automatically using software connected to the fluorescence plate reader, or manually after each assay plate has been read.

6.1 Beta-Lactamase Data Analysis

1. Calculate the average emission for the No Cells Background Control for both the blue (~450 nm) and green (~ 520 nm) channels. This is your *average blue background* and *average green background*.
2. Subtract the average blue background from all of your controls and sample blue emissions. Subtract the average green background from all of your controls and sample green emissions. This is your *net blue signal* and *net green signal*.
3. Calculate the ratio of blue to green fluorescence by dividing the net blue signal by the net green signal. This is your *blue-to-green ratio*.
4. Determine the average blue-to-green ratio for your Negative Controls. This is your *average negative ratio*.
5. Calculate the Response Ratio for your Positive Control and experimental samples by dividing their blue-to-green ratio by the average negative ratio. Your Positive Control and experimental samples with beta-lactamase activity should have a Response Ratio greater than 1.

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

6.2 Cytotoxicity Data Analysis

Compare the relative fluorescent units (RFUs) (~650 nm) of your sample to the RFUs of both your negative controls and your No Cells Background Controls.

1. If the sample RFUs are similar to your No Cells Background Controls, there is a low number of viable cells in your assay.
2. If the sample RFUs are similar to your Negative Controls, the cells are viable in your assay.
3. If the sample RFUs are between the Negative Controls and No Cells Background Controls RFUs, your sample may be partially cytotoxic under the conditions of the assay.

7.0 ASSAY CONSIDERATIONS

Several variables may 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.
- For the most sensitive cytotoxicity analysis, it is critical to use either buffer or media with low serum (2% or less) with the cells.

7.2 Loading Cells with Substrate

- Suspension cells are typically loaded at 1×10^6–2×10^6 cells/ml.
- Loading efficiency for adherent cells is density-dependent. Loading is most efficient when cells are 60–80% confluent; completely confluent cells may load substrate much less efficiently. Cell density that is too low results in less sensitive cytotoxicity results.
- Different cell types load the substrate at different rates. To optimize substrate loading time, we recommend reading the plate every 30 minutes for 2 hours the first time an assay is run.

  Note: For increased sensitivity, substrate loading time can be extended (depending on the cell type).
- Some cell types exhibit poor substrate retention. For these cell types we recommend using the Alternative Substrate Loading Solution as described in **Section 5.4.2**.
- The loading rate is temperature-sensitive. Increasing the temperature (*e.g.*, to 37°C) will increase the loading rate but actually results in a lower overall steady-state uptake of ToxBLAzer™ substrate, because the export rate of substrate also increases. We recommend loading at room temperature.
- For the most sensitive cytotoxicity analysis, it is critical to use either buffer or media with low serum (2% or less) with the cells.

7.3 Plate Reader Detection

All measurements are made at room temperature from the bottom of the wells, preferably in black-wall, clear-bottom assay plates with low fluorescence background. Before reading the plate, remove dust from the bottom with compressed air.

Note: Some plates/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

Fluorescent plate reader with bottom read capability.

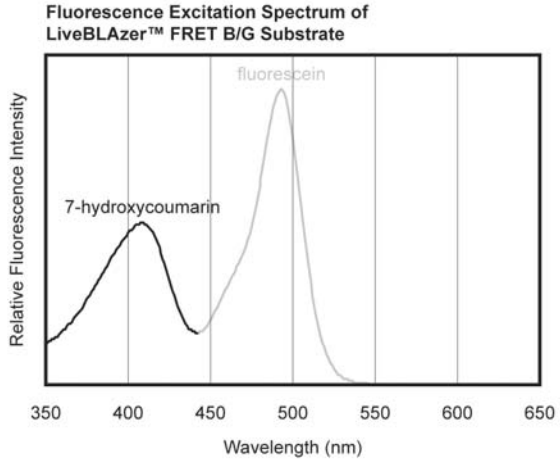
7.3.2 Filter Selection

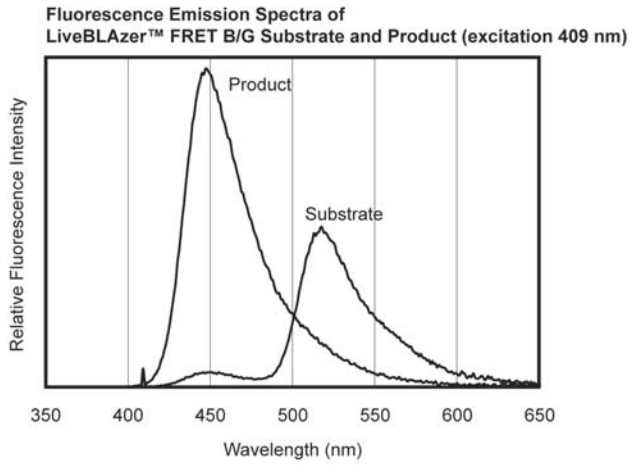
If your fluorescent plate reader does not include the appropriate filters, we recommend using the following filter sets available from Chroma Technologies (800-824-7662; www.chroma.com):

Chroma Set #APR1
Excitation filter: HQ405/20x (405 ± 10 nm)
Emission filter: HQ460/40m (460 ± 20 nm)
Emission filter: HQ530/30m (530 ± 15 nm)

Chroma Set #41013
Excitation filter HQ590/55x (590 ± 27 nm)
Emission filter HQ665/65m (665 ± 32 nm)

You may select alternative filters appropriate for LiveBLAzer™-FRET B/G Substrate by using the spectra shown below. Filter sets vary for specific plate readers and need to be specified at the time of ordering.





Note: To allow for FRET and ensure proper assay performance, the substrate must be excited at the 7-hydroxycourmarin wavelengths and not the fluorescein wavelengths.

7.4 Visual Observation of Intracellular ToxBLAzer™ Substrate Fluorescence (Optional)

Note: Photobleaching may occur during visual observation of the cells with a microscope. Therefore, if you plan to analyze the cells using a fluorescent plate reader, we recommend reading the plate before using the microscope.

1. An inverted microscope equipped for epifluorescence and either a xenon or mercury excitation lamp are typically required to view the substrate fluorescence signal in cells.
2. To inspect the cells, use a long-pass filter passing blue and green fluorescence light, so that you can visually identify whether the cells are fluorescing green or blue.
3. For epifluorescence microscopes, a long-pass dichroic mirror is needed to separate excitation and emission light and should be matched to the excitation filter (to maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light).
4. Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662; www.chroma.com).

Chroma Set # 41031

Excitation filter: HQ405/20x (405 ±10)
Dichroic mirror: 425 DCXR
Emission filter: HQ435LP (435 long-pass)

Chroma Set 41013

Excitation Filter: HQ590/55x
Dichroic Mirror: Q625LP
Emission Filter: HQ665/65m

Filter sizes vary for specific microscopes and need to be specified when the filters are ordered.

8.0 PURCHASER NOTIFICATION

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