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**1.0 OVERVIEW OF GENEBLAZER® TECHNOLOGY**

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy to use method of monitoring cellular responses to drug candidates or other stimuli (1). The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the two-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, transfection efficiency, substrate concentration, excitation path length, fluorescence detectors, and volume changes. The GeneBLAzer® Beta-lactamase Reporter Technology has been proven effective in high-throughput screening (HTS) campaigns for a range of target classes, including G-protein coupled receptors (GPCRs) (2, 3), nuclear receptors (4-6), and kinase signaling pathways (7).

**2.0 MATERIALS SUPPLIED**

<b>Cell Line Name:</b>	dhfr(E2F)-bla NIH3T3
<b>Description:</b>	CellSensor® dhfr(E2F)-bla NIH3T3 cells contain a beta-lactamase reporter gene under control of E2F/DP1 response element present in the DHFR gene promoter that has been stably integrated into NIH3T3 cells. dhfr(E2F)-bla NIH3T3 cells respond to serum treatment.
<b>Product Number:</b>	K1240
<b>Shipping Condition:</b>	Dry Ice
<b>Storage Condition:</b>	Liquid nitrogen. <b>Immediately upon receipt, cells must be stored in liquid nitrogen or thawed for immediate use. Cells stored at -80°C can quickly lose viability.</b>
<b>Quantity:</b>	~2,000,000 (2 × 10 <sup>6</sup> cells/mL)
<b>Application:</b>	Detection of agonists/antagonists of the pRB-E2F cell cycle signaling pathway.
<b>Growth Properties:</b>	Adherent
<b>Cell Phenotype:</b>	Fibroblast
<b>Selection Marker:</b>	Blasticidin (5 µg/mL)
<b>Vector Used:</b>	pLenti-bsd/dhfr(E2F)-bla Vector
<b>Mycoplasma Testing:</b>	Negative
<b>BioSafety Level:</b>	1

### 3.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Media/Reagents	Recommended Source	Part #
LiveBLAzer™ -FRET B/G Loading Kit, containing: LiveBLAzer™ -FRET B/G Substrate (CCF4-AM), DMSO, Solution B, and Solution C	Invitrogen	K1095 (0.2 mg) K1096 (1 mg) K1030 (5 mg)
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
DMEM with GlutaMAX™	Invitrogen	10569-010
DMSO	Fluka	41647
Newborn Calf Serum ( <b>DO NOT SUBSTITUTE!</b> )	Invitrogen	16010-159
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
HEPES (1 M, pH 7.3)	Invitrogen	15630-080
DMEM (without phenol-red)	Invitrogen	21063-029
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin (antibiotic)	Invitrogen	R210-01
Solution D	Invitrogen	K1157

Consumables	Recommended Source	Part #
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Corning Life Sciences	3712
Compressed air	Various	—

Equipment	Recommended Source
Fluorescence plate reader with bottom-read capability	Various
Filters, if required for plate reader (see <b>Section 5.3</b> )	Chroma Technology Corp.

**Note:** If you do not have access to a fluorescence plate reader with bottom-read capabilities, contact our Technical Support for options of other beta-lactamase substrates that can be read with top-reading instruments.

#### 3.1 Optional Equipment and Materials

- Epifluorescence- or fluorescence-equipped microscope with appropriate filters
- Microplate centrifuge

## 4.0 CELL CULTURE CONDITIONS

### 4.1 Media Required

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	500 mL	—	—
DMEM without phenol red	—	500 mL	—
Newborn Calf Serum (NCS)	50 mL	2.5 mL	—
NEAA (10 mM)	5 mL	5 mL	—
HEPES (pH 7.3, 1M)	12.5 mL	12.5 mL	—
Sodium Pyruvate (100 mM)	—	5 mL	—
Penicillin (antibiotic) (10,000 U/ml) and Streptomycin (antibiotic) (10,000 µg/ml)	5 mL	—	—
Blasticidin (antibiotic)	—	—	—
Recovery™ Cell Culture Freezing Medium	—	—	50 mL

**Note:** Unless otherwise stated, have all media and solutions at least at room temperature (we recommend 37°C for optimal performance) before adding them to the cells.

### 4.2 Growth Conditions

For detailed cell growth and maintenance directions, see **Section 7.0**.

**Note:** We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay.

1. Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO<sub>2</sub> incubator. Maintain cells between 20% and 75% confluency. Do not allow cells to reach confluence.
2. Freeze cells at  $2 \times 10^6$  cells/mL in Recovery™ Cell Culture Freezing Medium.

## 5.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for monitoring CDKs/pRB/E2F cell cycle pathway using LiveBLAzer™-FRET B/G Substrate as the readout. If you use alternative substrates (e.g., ToxBLAzer™ DualScreen, or LyticBLAzer™ Loading kits), follow the loading protocol provided with the product.

### Note:

- We recommend using 384-well, black-wall, clear-bottom assay plates with low fluorescence background.
- Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening. The cell stimulation described below is carried out in the presence of 0.5% DMSO to simulate the effect that a test compound solvent might have on the assay. If you use other solvents and/or solvent concentrations, change the following assay conditions and optimize appropriately.
- Due to serum interference, we recommend setting up two types of Cell-free wells for background subtraction: with serum (NCS) and without. The Cell-free Plus Serum wells will be used to subtract background the Stimulated wells, while the Cell-free Minus Serum wells will be used to subtract background from the Unstimulated wells.

### 5.1 Quick Reference Guide

For more detailed protocol information, see [Section 5.2](#).

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each condition: Unstimulated, Stimulated, and Cell-free.

	Unstimulated Wells	Stimulated Wells	Cell-free Plus Serum wells	Cell-free Minus Serum wells
<b>Step 1</b> Plate cells	32 µL cells suspended in Assay Medium (3,750 cells/well)	32 µL cells suspended in Assay Medium (3,750 cells/well)	32 µL Assay Medium (no cells)	32 µL Assay Medium (no cells)
<b>Step 2</b> Incubate cells	Incubate at 37°C/5% CO <sub>2</sub> for 5–6 hours			
<b>Step 3</b> Add agonist	8 µL Assay Medium with 2.5% DMSO	8 µL Assay Medium with 2.5% DMSO plus 50% NCS	8 µL Assay Medium with 2.5% DMSO plus 50% NCS	8 µL Assay Medium with 2.5% DMSO
<b>Step 4</b> Incubate cells	Incubate the plate at 37°C/5% CO <sub>2</sub> for 16 hours.			
<b>Step 5</b> Prepare 6X Substrate Mixture	6 µL 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM) in dry DMSO + 60 µL Solution B, mix. Add 900 µL Solution C and 60 µL Solution D, mix.			
<b>Step 6</b> Load Substrate Mixture	8 µL per well			
<b>Step 7</b> Incubate Substrate + cells	3 hours at room temperature in the dark.			
<b>Step 8</b> Detect Activity	See <a href="#">Section 5.3</a>			
<b>Step 9</b> Analyze data	See <a href="#">Section 6.0</a>			

### 5.2 Detailed Assay Protocol

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each control: Unstimulated Control, Stimulated Control, Cell-free Minus Serum Control, and Cell-free Plus Serum Control.

#### 5.2.1 Precautions

- Work on a dust-free, clean surface. Always handle the 384-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate.

- If pipetting manually, you may need to centrifuge the plate briefly at room temperature (30 seconds at  $14 \times g$ ) after additions to ensure all assay components are on the bottom of the wells.

### 5.2.2 Plate Cells

1. Harvest cells from culture in Growth Medium and resuspend in Assay Medium at a density of  $1.2 \times 10^5$  cells/mL.
2. Add 32  $\mu$ L per well of Assay Medium to the Cell-free Minus Serum and Cell-free Plus Serum wells. Add 32  $\mu$ L per well of the cell suspension to Unstimulated and Stimulated wells.
3. After plating, incubate the plates in a 37°C/5% CO<sub>2</sub> incubator for 5–6 hours.

### 5.2.3 Prepare Stock Solutions

1. Prepare Assay Medium with 2.5% DMSO.
2. Prepare 50% NCS in Assay Medium with 2.5% DMSO.

### 5.2.4 Stimulate Cells

1. Add 8  $\mu$ L Assay Medium with 2.5% DMSO to the Unstimulated wells and Cell-free Minus Serum wells.
2. Add 8  $\mu$ L 50% NCS in Assay Medium with 2.5% DMSO to the Stimulated wells and Cell-free Plus Serum wells.
3. Incubate the assay plate in a humidified 37°C/5% CO<sub>2</sub> incubator for 16 hours.

### 5.2.5 Substrate Loading and Incubation

This protocol is designed for loading cells with LiveBLAzer™-FRET B/G Substrate (CCF4-AM) or CCF2-AM. If alternative substrates are used, please follow the loading protocol provided with the substrate.

Preparation of 6X LiveBLAzer™-FRET B/G Substrate (CCF4-AM) or CCF2-AM Mixture and cell loading should be done in the absence of direct strong lighting. Turn off the light in the hood.

1. Prepare Solution A: 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM, MW = 1096) stock solution in dry DMSO. Store the aliquots of the stock solution at -20°C until use.
2. Prepare the 6X LiveBLAzer™-FRET B/G (CCF4-AM) Substrate Mixture as follows:
  - 2.1 Add 6  $\mu$ L of Solution A to 60  $\mu$ L of Solution B and vortex.
  - 2.2 Add 874  $\mu$ L Solution C and 60  $\mu$ L of Solution D to the combined solutions from above step with vortexing.
3. Remove the assay plate from the humidified 37°C/5% CO<sub>2</sub> incubator.
4. Add 8  $\mu$ L of 6X Substrate Mixture from **Step 2** to each well.
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 3 hours.

## 5.3 Detection

All measurements using LiveBLAzer™-FRET B/G Substrate should be made at room temperature from the bottom of the wells. Before reading the plate, remove dust from the bottom with compressed air.

### 5.3.1 Instrumentation, Filters, and Plates

- Fluorescence plate reader with bottom reading capabilities.
- Recommended filters for fluorescence plate reader:
 

Excitation filter:	409/20 nm
Emission filter:	460/40 nm
Emission filter:	530/30 nm

### 5.3.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before making measurements.
3. Use the following filter selections:

	Scan 1	Scan 2
<b>Purpose:</b>	Measure fluorescence in the blue channel	Measure fluorescence in the green channel
<b>Excitation filter:</b>	409/20 nm	409/20 nm
<b>Emission filter:</b>	460/40 nm	530/30 nm

## 6.0 DATA ANALYSIS

### 6.1 Background Subtraction

We recommend that you subtract the background for both emission channels (460 nm and 530 nm).

1. Use the assay plate layout to identify the location of the Cell-free Minus Serum and Cell-free Plus Serum wells. These control wells are used for background subtraction.
2. Determine the average emission for the Cell-free Minus Serum wells and average emission for the Cell-free Plus Serum wells at both 460 nm (Average Blue Background) and 530 nm (Average Green Background).
3. Subtract the Average Blue Background for the Cell-free Minus Serum wells from the blue emission data for the Unstimulated wells.
4. Subtract the Average Blue Background for the Cell-free Plus Serum wells from the blue emission data for the Stimulated wells.
5. Subtract the Average Green Background for the Cell-free Minus Serum wells from the green emission data for the Unstimulated wells.
6. Subtract the Average Green Background for the Cell-free Plus Serum wells from the green emission data for the Stimulated wells.
7. Calculate the Blue/Green Emission Ratio for each well, by dividing the background-subtracted blue emission values by the background-subtracted green emission values.

### 6.2 Visual Observation of Intracellular Beta-lactamase Activity Using LiveBLAzer™-FRET B/G Substrate (CCF4-AM)

**Note:** Microscopic visualization of cells will cause photobleaching. Always read the assay plate in the fluorescence plate reader before performing microscopic visualization.

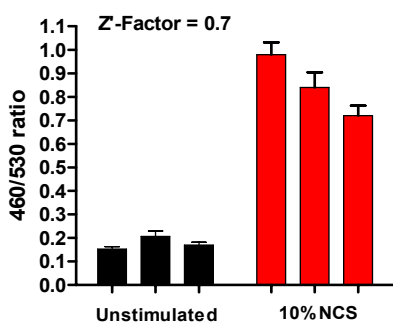
An inverted microscope equipped for epifluorescence and either a xenon or mercury excitation lamp is typically required to view the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) signal in cells. To visually inspect the cells, you will need a long-pass filter passing blue and green fluorescence light so that your eye can visually identify whether the cells are fluorescing green or blue. Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, [www.chroma.com](http://www.chroma.com)).

#### Chroma Set # 41031

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

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

### 6.3 Representative Data



**Figure 1.** Dhfr(E2)-bla NIH3T3 cells (3750 cells/well) were assayed on three separate days represented by the three different columns in each set shown on the graph. Cells were plated in a 384-well format in assay medium and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 emission ratios plotted for unstimulated and serum stimulated samples (n=16 for each data point).

## 7.0 DETAILED CELL HANDLING PROCEDURES

### 7.1 Thawing Method

1. Place 14 mL of Growth Medium without Blasticidin into a T75 flask.
2. Place the flask in a humidified 37°C/5% CO<sub>2</sub> incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
3. Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
4. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
5. Transfer the vial contents drop-wise into 10 mL of Growth Medium without Blasticidin in a sterile 15-mL conical tube.
6. Centrifuge cells at 200 × g for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 1 mL of fresh Growth Medium without Blasticidin.
8. Transfer contents to the T75 tissue culture flask containing pre-equilibrated Growth Medium without Blasticidin and place flask in a humidified 37°C/5% CO<sub>2</sub> incubator.
9. At first passage, switch to Growth Medium with Blasticidin.

### 7.2 Propagation Method

1. Cells should be passaged or fed at least twice a week. Cells should be maintained between 20% and 75% confluence. Do not allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (3 mL for a T75 flask and 5 mL for a T175 flask and 8 mL for T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2–5 minutes exposure to Trypsin/EDTA. Add an equal volume of Growth Medium to inactivate Trypsin.
3. Verify under a microscope that cells have detached and clumps have completely dispersed.
4. Spin down cells and resuspend in Growth Medium.

### 7.3 Freezing Method

1. Harvest the cells as described in **Section 7.2**. After detachment, count the cells, then spin cells down and resuspend in 4°C Freezing Medium to 2 × 10<sup>6</sup> cells/mL.
2. Dispense 1.0-mL aliquots into cryogenic vials.
3. Place in an insulated container for slow cooling and store overnight at –80°C.
4. Transfer to liquid nitrogen the next day for storage.

## 8.0 REFERENCES

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### Use of Genetically Modified Organisms (GMO)

**Information for European Customers** The CellSensor® dhfr(E2F)-bla NIH3T3 cell line is genetically modified with the plasmid pLenti-*bsd*/dhfr(E2F)-*bla*. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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