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1. 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. Materials Supplied

Cell Line Name:	NFκB-*bla* THP-1
Description:	CellSensor® NFκB-*bla* THP-1 cells contain a beta-lactamase reporter gene under control of the Nuclear Factor kappa B (NFκB) response element stably integrated into THP-1 cells. This cell line can be used to detect agonists/antagonists of the NFκB signaling pathway. NFκB-*bla* THP-1 cells have been shown to respond to Tumor Necrosis Factor alpha (TNFα).
Catalog Number:	K1662 (cell line part no. K1176)
Shipping Condition:	Dry Ice
Storage Condition:	Liquid Nitrogen. **Immediately upon receipt, thaw the cells (preferred) or store the cells in liquid nitrogen. Cells CANNOT be stored at –80°C (dry ice) as they will quickly lose viability.**
Quantity:	Refer to COA
Application:	This cell line can be used to detect agonists/antagonists of the NFκB signaling pathway.
Growth Properties:	Suspension
Propagation:	Maintain between 200,000 and 2,000,000 cells/mL (2×10^5 and 2×10^6 cells/mL)
Cell Phenotype:	Monocyte
Selection Marker:	Blasticidin (5 µg/mL)
Vector Used:	pLenti-*bsd*/NFκB-*bla* Vector
***Mycoplasma* Testing:**	Negative
BioSafety Level:	1

3. Materials Required, but Not Supplied

Media/Reagents	Recommended Source	Cat. no.
LiveBLAzer™ Loading Kit containing: LiveBLAzer™ FRET B/G Substrate (CCF4-AM) substrate DMSO for Solution A Solution B Solution C	Invitrogen	K1030 or K1095 Other sizes or CCF2-AM are available
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
RPMI 1640	Invitrogen	72400-047
DMSO	Fluka	41647
Dialyzed Fetal Bovine Serum (dFBS) **(DO NOT SUBSTITUTE)**	Invitrogen	26400-044
Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
Sodium Pyruvate	Invitrogen	11360-070
TNFα	Invitrogen	PHC3015
Blasticidin antibiotic	Invitrogen	R210-01

Consumables	Recommended Source	Cat. no.
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Costar	3712
Compressed Air	Various	—
Conical tubes, 15 mL, sterile	Various	—
1.8 mL Internally threaded cryogenic vials	Various	—
Tissue culture flasks	Various	—

Equipment	Recommended Source
Class II biological safety cabinet	Various
Dual wave-length bottom-reading fluorescence plate reader	Various
Filters (see **Sections 4.5.1** and **5.3**)	Chroma Technologies
Hemacytometer (or another cell counting method)	Various
Humidified 37°C/5% CO_2 incubator	Various
Inverted Microscope (with phase contrast capabilities)	Various
Liquid nitrogen tank	Various
Optional: Epifluorescence or fluorescence-equipped microscope, equipped with appropriate filters	Various
Optional: Microplate centrifuge	Various

4. Cell Culture Conditions

4.1 Media Required

Component	Thaw Medium	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	500 mL bottle	500 mL bottle	500 mL bottle	—
Dialyzed FBS	50 mL	50 mL	50 mL	—
NEAA	5 mL	5 mL	5 mL	—
Sodium Pyruvate	5 mL	5 mL	5 mL	—
Penicillin (10,000 U/mL) / Streptomycin (10,000 µg/mL)	5 mL	5 mL	5 mL	—
Blasticidin antibiotic	—	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Note: Unless otherwise stated, all media and solutions should be at least room temperature (37°C is best) before adding them to the cells.

Note: Thaw medium and assay medium are the same.

Note: We prepare our media by adding the listed components directly to the medium bottle. However, the Blasticidin is added directly to the cell culture flask to reach 5 µg/mL. Similar methods may be suitable.

4.2 Methods Used

Please follow these methods exactly, as they have been validated specifically for optimal performance of this cell line.

Note: Make NO MEDIA SUBSTITUTIONS as this cell line has been specifically validated for viability and optimal assay performance with these media. At first opportunity, create and store an aliquot of cells for back-up.

Note: After shipping, with first usage, cells prefer higher densities than after they have fully recovered.

4.2.1 Thawing Method

1. Place 9 mL of Thaw Medium **without Blasticidin** into a T25 tissue culture flask, standing the flask on its end so that the smallest surface area will be used to grow the cells.
2. Place the flask in a 37°C/5% CO_2 incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
3. Remove vial of cells to be thawed from liquid nitrogen and rapidly thaw by placing in a 37°C 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 dropwise into 10 mL of Thaw Medium 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 Thaw Medium.
8. Transfer contents to the T25 tissue culture flask containing pre-equilibrated Thaw Medium (final volume is ~10 mL, final cell concentration is ~5×10^5 cells/mL) and place flask, standing on its end, in the 37°C/5% CO_2 incubator. Leave the cells for 2 -3 days.
9. Add an equal volume of fresh media to the flask without removing the original media thereby diluting the cells 1:2. Place the flask in the normal or flat orientation for proper gas exchange.
10. When the cells approach or reach 1×10^6 cells/mL, expand to a T75 flask equilibrated with an equal volume of medium, taking care to not let the cell density fall below 5×10^5 cells/mL.
11. Before switching to Growth Medium with Blasticidin, freeze several vials of cells.

4.2.2 Propagation Method

1. When the cells have fully recovered, switch to Growth Medium with Blasticidin.
2. Cells should be maintained at a density between 2×10^5 cells/mL and 2×10^6 cells/mL. Passage and feed the cells as required to maintain this density.
3. Add Growth Medium to reach the desired cell density.

4.2.3 Freezing Method

1. Harvest and count the cells, then spin cells down and resuspend in 4°C Freezing Medium (**Section 4.1**) at 5×10^6 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.

5. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators (agonists or antagonists) of the NFκB signaling pathway using beta-lactamase as the readout.

5.1 Controls

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

Note: Certain solvents may affect assay performance. The effect of a test compound solvent should be assessed prior to screening. The cell stimulation described below is carried out in the presence of 0.5% DMSO to simulate the effect that the test compound solvent may have on the assay. If other solvents and/or solvent concentrations are used, the following assay should be changed accordingly.

Data Point	Agonist Assay	Antagonist Assay	Purpose of Data Point
Unstimulated Control Wells	1. 32 µL cells diluted in Assay Medium. 2. 4 µL 5% DMSO stock solution. 3. 4 µL Assay Medium.	1. 32 µL cells diluted in Assay Medium 2. 4 µL 5% DMSO stock solution. 3. 4 µL Assay Medium.	Provides the Unstimulated Emission Ratio, which is a portion of the Response Ratio. Unstimulated Emission Ratio = (Unstimulated 460 nm/Unstimulated 530 nm). See **Section 6** (Data Analysis).
Stimulated Control Wells	1. 32 µL cells diluted in Assay Medium. 2. 4 µL 5% DMSO stock solution. 3. 4 µL 10X stock solution of TNFα.	1. 32 µL cells diluted in Assay Medium. 2. 4 µL 5% DMSO stock solution. 3. 4 µL 10X stock solution of TNFα.	Provides the Stimulated Emission Ratio, which is a portion of the Response Ratio. Stimulated Emission Ratio = (Stimulated 460 nm/ Stimulated 530 nm). See **Section 6** (Data Analysis).
Cell-free Control Wells	1. 36 µL assay Medium. 2. 4 µL 5% DMSO stock solution.	1. 36 µL Assay Medium. 2. 4 µL 5% DMSO stock solution.	Provides the background blue and green emission values to be subtracted from both Unstimulated and Stimulated blue and green emission values to yield the net Unstimulated and Stimulated blue and green emission values.
Test Compound Wells	1. 32 µL Cells diluted in Assay Medium. 2. 4 µL 10X stock of Test Compounds in 5% DMSO. 3. 4 µL Assay Medium.	1. 32 µL Cells diluted in Assay Medium. 2. 4 µL 10X stock of Test Compounds in 5% DMSO. 3. 4 µL 10X stock solution of TNFα.	Provides the experimental data on whether or not a test compound is active as an agonist or antagonist in the assay.

5.2 Cell Stimulation and Assay Plate Set-up

5.2.1 Precautions

1. 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.
2. If pipetting manually, it may be necessary to centrifuge the plate briefly at room temperature (for 1 min. at 14 × *g*) after additions to ensure all the assay components are on the bottom of the wells.

5.2.2 Plating Cells

1. Harvest cells from culture in Growth Medium as described in **Section 4.2.2,** Step 2 and resuspend in Assay Medium at a density of 6.25×10^5 cells/mL.
2. Add 36 µL per well of the Assay Medium to the cell free control wells. Add 32 µL (20,000 cells) per well of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells.

   Note: Cells were plated on day of assay. Cells can be plated on previous day at half the concentration in Step 1.

5.2.3 Preparation of Stock Solution

1. Prepare a stock solution of 5% DMSO.
2. Prepare a 10X stock of test compounds in 5% DMSO.
3. TNFα is reconstituted to 50 µg/mL with PBS.
4. Prepare a 10X stock of TNFα in assay media. For an antagonist screen, use ~0.294 ng/mL final concentration or EC_{80} (10X = 2.94 ng/mL). For an agonist screen, use ~6 ng/mL final concentration or max stim. (10X = 60 ng/mL). We recommend running a dose response curve to determine the EC_{80} for your 10X TNFα solution.

5.2.4 Agonist Assay Plate Setup

Note: This subsection provides directions for performing an agonist assay. Directions for performing an antagonist assay can be found in **Section 5.2.5**.

1. Add 4 µL of the stock solution of 5% DMSO to the Unstimulated Control wells, the Stimulated Control wells, and to the Cell-free control wells.
2. Add 4 µL of Assay Medium to the Test Compound and Unstimulated Control wells.
3. Add 4 µL of the 10X EC_{80} stock solution of TNFα to Stimulated Control wells.
4. Add 4 µL of the 10X stock of Test Compounds to the Test Compound wells.
5. Incubate the agonist assay plate in a humidified 37°C/5% CO_2 incubator for 5 hours.

5.2.5 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an antagonist assay. Directions for performing an agonist assay are provided in **Section 5.2.4**.

1. Add 4 µL of the 10X stock of Test Compounds to the Test Compound wells.
2. Add 4 µL of the stock solution of 5% DMSO to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-free Control wells.
3. If desired, incubate the test compounds with the cells at 37°C/5% CO_2 before proceeding. Typically, a one-hour incubation is sufficient.
4. Add 4 µL of the 10X EC_{80} stock solution of TNFα to the Test Compound wells and the Stimulated Control wells.
5. Add 4 µL of Assay Medium to the Unstimulated Control wells.
6. Incubate the antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 5 hours.

5.3 Substrate Loading

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

5.3.1 LiveBLAzer™ FRET B/G Substrate (CCF4-AM) or CCF2-AM Loading Protocol

This protocol is designed for loading cells with LiveBLAzer™ FRET B/G Substrate (CCF4-AM) or CCF2-AM, using the following solutions. The following protocol is written for LiveBLAzer™ FRET B/G (CCF4-AM), however, CCF2-AM can be substituted.

Reagents	Storage and Handling
LiveBLAzer™ FRET B/G Substrate (CCF4-AM)	The product is supplied as a dried powder. The molecular weight of CCF2-AM is 1082 g/mole; the molecular weight of LiveBLAzer™ FRET B/G Substrate (CCF4-AM) is 1096 g/mole. Store the product at –20°C, desiccated and protected from light.
Dry DMSO	Used to dissolve LiveBLAzer™ FRET B/G Substrate (CCF4-AM) for preparation of Solution A.
Solution A	LiveBLAzer™ FRET B/G Substrate (CCF4-AM) stock solution; 1 mM LiveBLAzer™ FRET B/G Substrate in dry DMSO. Prepare a 1 mM LiveBLAzer™ FRET B/G Substrate stock solution in dry DMSO. Store the LiveBLAzer™ FRET B/G Substrate stock solution at –20°C, desiccated, protected from light. Before each use, let frozen stock solution warm to room temperature and remove desired amount of reagent. Immediately recap the vial after each use to reduce moisture uptake and return to –20°C storage. Stored under these conditions, Solution A is stable for approximately three months. Once thawed, Solution A may appear slightly yellow. This is normal.
Solution B	Store the reagent at room temperature (18–22°C) protected from direct light. Under cold lab conditions [colder than 18°C (65°F)], the solution may freeze or a white precipitate may form. In this case, warm and mix the solution (~35°C) until thawed and the precipitate dissolves. Mix thoroughly before use.
Solution C	Keep tightly closed and store in a cool, dry place. Store the reagent at room temperature (18–22°C) protected from direct light.

5.3.2 Preparation of 6X LiveBLAzer™ FRET B/G Substrate (CCF4-AM) Loading Solution

1. Add 6 µL of Solution A to 60 µL of Solution B and vortex.
2. Add 934 µL Solution C to the combined solutions from Step 1 with vortexing.

5.3.3 Cell Loading

1. Remove assay plate from incubator and allow to equilibrate to room temperature prior to loading.
2. Add 8 µL of the 6X Loading Buffer to each well [C_f of LiveBLAzer™ FRET B/G (CCF4-AM) = 1 µM].

5.4 Incubation

1. Cover the plate to protect it from light and evaporation.
2. Incubate at room temperature for 2 hours.

   Note: Handle the plate gently and do not touch the bottom.

5.5 Detection

All measurements are made at room temperature from the bottom of the wells, preferably in 384-well 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.

5.5.1 Instrumentation, Filters, and Plates

- Almost all dual wavelength, bottom read fluorescence plate readers can be used to detect beta-lactamase using LiveBLAzer™ FRET B/G (CCF4-AM) or CCF2-AM.

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

- Filters for fluorescence plate reader:

  For ratiometric readout using a fluorescence plate reader:

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

5.5.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode and for eight scans per cycle.
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:

	Scans 1-4	Scans 5-8
Purpose:	Measure fluorescence in the blue channel	Measure fluorescence in the green channel
Excitation Filter:	409/20 nm	409/20 nm
Emission Filter:	460/40 nm (using the gain determined during calibration)	530/30 nm (using the gain determined during calibration)

Set the fluorescence plate reader to 5 reads/well.

6. Data Analysis

6.1 Background Subtraction

Background subtraction for both channels (460 nm and 530 nm) is essential to obtain meaningful data. This can be accomplished either automatically using software connected to the fluorescence plate reader, or manually after each assay plate has been read.

Use the assay plate layout to identify the location of the Cell-free Control wells. These control wells are used for background subtraction.

6.1.1 Automatic Background Subtraction

Use the assay plate layout to designate appropriate Cell-free Control wells and enable background subtraction for both sets of emission scans.

6.1.2 Manual Background Subtraction

1. Determine the average emission from the Cell-free Control wells at both 460 nm (Average Em_{460} Background) and 530 nm (Average Em_{530} Background).
2. Calculate the Net Stimulated Em_{460} and Em_{530} values and the Net Unstimulated Em_{460} and Em_{530} values, as shown below:

Stimulated Cells

Net Stimulated Em_{460} = Em_{460} of stimulated sample – Average Em_{460} Background

Net Stimulated Em_{530} = Em_{530} of stimulated sample – Average Em_{530} Background

Unstimulated Cells

Net Unstimulated Em_{460} = Em_{460} of unstimulated sample – Average Em_{460} Background
Net Unstimulated Em_{530} = Em_{530} of unstimulated sample – Average Em_{530} Background

6.2 Response Ratio Calculations

1. Calculate the Stimulated Emission Ratio for each well, as shown below.

$$\textbf{Stimulated Emission Ratio} = \frac{\textbf{Net Stimulated } Em_{460}}{\textbf{Net Stimulated } Em_{530}}$$

2. Calculate the Unstimulated Emission Ratio for each well, as shown below.

$$\textbf{Unstimulated Emission Ratio} = \frac{\textbf{Net Unstimulated } Em_{460}}{\textbf{Net Unstimulated } Em_{530}}$$

3. Calculate the Mean Unstimulated Emission Ratio for each assay plate, as shown below.

$$\text{Mean Unstimulated Emission Ratio} = \frac{\text{Sum of Unstimulated Emission Ratios}}{\text{Total number of wells containing unstimulated cells}}$$

4. Calculate the Response Ratio for each well of interest. The Response Ratio, as shown below.

$$\text{Response Ratio} = \frac{\text{Stimulated Emission Ratio } (Em_{460}/Em_{530})}{\text{Mean Unstimulated Emission Ratio } (Em_{460}/Em_{530})}$$

Note: All Response Ratios are calculated using the Mean Unstimulated Emission Ratio. The Response Ratio determined with 6 ng/mL TNFα and LiveBLAzer™ FRET B/G Substrate (CCF4-AM) was ~20.

6.3 Visual Observation of Intracellular 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, a long-pass filter passing blue and green fluorescence light is needed 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).

Chroma Set No. 41031

Excitation filter: HQ405/20x (405 ±10)
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).

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Use of Genetically Modified Organisms (GMO)

Information for European Customers The NFκB-*bla* THP-1 cell line(s) are genetically modified with the plasmid pLenti-*bsd*/NFκB-*bla* Vector. 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.