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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 (4,5), and kinase signaling pathways (6).

2. Materials Supplied

Cell Line Name:	CellSensor [®] HSE- <i>bla</i> HeLa Cells
Description:	The CellSensor [®] HSE- <i>bla</i> HeLa Cell Line contains a beta-lactamase reporter gene under control of a Heat Shock Response Element (HSE) that has been stably integrated into HeLa cells. This cell line is a stable pool enriched for responsiveness by flow cytometry. Application of inducers of heat shock response (e.g. Hsp90 inhibitor 17-AAG) to these cells activates heat shock factor (HSF) family members leading to increased beta-lactamase expression.
Product Number:	K1813
Shipping Condition:	Dry Ice
Storage Condition:	Liquid nitrogen. Immediately upon receipt, cells must be stored in liquid nitrogen or thawed for immediate use. Cells stored at -80°C can quickly lose viability.
Quantity:	6,400,000 (6.4 × 10 ⁶ cells/ml)
Application:	Detection of activators or inhibitors of heat shock/unfolded protein response
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/ml)
Vector Used:	pLenti- <i>bsd</i> /DBE- <i>bla</i>
Mycoplasma Testing:	Negative
BioSafety Level:	2

3. 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 + GlutaMax™ -1	Invitrogen	10569-010
DMSO	Fluka	41647
Dialyzed Fetal Bovine Serum (dFBS) (DO NOT SUBSTITUTE!)**	Invitrogen	26400-044
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
HEPES (1M, pH 7.3)	Invitrogen	15630-080
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-144
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin (antibiotic)	Invitrogen	R210-01
17-AAG	LC Laboratories	A-6880
Quercetin	Sigma	337951

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 Section 6.3)	Chroma Technology Corp.
Optional: Epifluorescence- or fluorescence-equipped microscope with appropriate filters	Various
Optional: Microplate centrifuge	Various

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. The assay conditions of this cell line have been fully validated with LiveBLAzer™ FRET B/G substrate and bottom-reading instruments. Other beta-lactamase substrates and top-reading method have not been tested with this cell line.

* Some part numbers differ outside of the continental US. Please check with your local Invitrogen Technical Support.

** The cell line has been grown in the presence of dialyzed FBS. Other forms of FBS may kill the cells especially upon first thaw.

*** Alternative assay plates (such as poly-D-lysine coated plates) may or may not work for this assay.

4. Media Requirements

Component	Thaw Medium	Growth Medium	Assay Medium*	Freezing Medium
DMEM + GlutaMax™-1	500 ml bottle	500 ml bottle	500 ml bottle	—
Dialyzed Fetal Bovine Serum (dFBS)	50 ml	50 ml	0.5 mL (~0.1 % final)	—
NEAA (10 mM)	5 ml	5 ml	5 ml	—
HEPES (pH 7.3, 1 M)	12.5 ml	12.5 ml	12.5 ml	—
Penicillin (10,000 U/ml) / Streptomycin (10,000 µg/ml)	5 ml	5 ml	5 ml	—
Blasticidin	—	5 µg/ml	—	—
Recovery™ Cell Culture Freezing Medium		—	—	100%

Note: We prepare our media by adding the listed components directly to the medium bottle. Blasticidin can be added directly to the cell culture flask to reach 5 µg/ml and 200 µg/ml, respectively. Similar methods are suitable.

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.

5. Detailed Cell Handling Procedures

5.1 Thawing Method

- Place 29 ml of Thaw Medium (without Blasticidin) into a T225 flask.
- Place the flask in a humidified 37°C/5% CO₂ incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
- 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.
- Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
- Transfer the vial contents to a sterile 15-ml conical tube.
- Add 10 ml of Thaw Medium (without Blasticidin) drop-wise into the cell suspension.
- Centrifuge cells at 200 × *g* for 5 minutes.
- Aspirate supernatant and resuspend the cell pellet in 1 ml of fresh Thaw Medium (without Blasticidin).
Note: This step is important to fully remove the DMSO present from the Recovery™ Cell Culture Freezing Medium.
- Transfer contents to the T225 tissue culture flask containing pre-equilibrated Thaw Medium (without Blasticidin) and place flask in a humidified 37°C/5% CO₂ incubator.
- Switch to passaging cells in Growth Medium with Blasticidin, once cells appear to be growing at consistent rates for the given cellular background. See **Section 5.4** for special considerations.

5.2 Propagation Method

1. Cells should be passaged or fed at least two times a week. Cells should be maintained between 20% and 90% confluency. Do not allow cells to reach confluence. Cells which have grown to confluence may not show expected agonist response in the assay.
2. To passage cells, aspirate medium, rinse once with PBS, add Trypsin/EDTA (3 ml for a T75 flask, 5 ml for a T175 flask and 8 ml for T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2 minutes exposure to Trypsin/EDTA. Add Growth Medium (7 ml for a T75 flask, 10 ml for T175 and T225 flasks) to inactivate Trypsin and mix. Verify under a microscope that cells have detached and clumps have completely dispersed.
3. Transfer required amount to a new flask containing pre-warmed Growth Medium.

5.3 Freezing Method

1. Harvest and count the cells, then spin cells down and resuspend in 4°C Recovery™ Cell Culture Freezing Medium at a density of 2×10^6 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.

5.4 Special Considerations

- This cell line is a stable pool enriched by Fluorescence Activated Cell Sorting (FACs) based on functional response. Assay performance can be expected to depend upon use of the specified media as responsive cells have been chosen based on these formulations.
- This cell line is Blasticidin.
- This cell line has shown optimal assay performance when cells are harvested for assay at 50% to 70% confluent

6. Assay Procedure

The following instructions outline the recommended procedure for monitoring NGF-induced response using LiveBLazer™-FRET B/G Substrate as the readout.

Note:

- We recommend using black-wall, clear-bottom assay plates with low fluorescence background.
- We recommend including cell-free control wells on the same plate as test wells for background subtraction. See **Section 7** Data Analysis.
- Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening. This cell line has been qualified for DMSO tolerance up to 0.25 %. The example below uses a final concentration of 0.1% DMSO in the Agonist Assay and 0.2% DMSO in the Antagonist Assay. If you use other solvents and/or solvent concentrations, change the following assay conditions and optimize appropriately.

6.1 Quick Reference Guide

For more detailed protocol information, see [Section 6.2](#).

Agonist Assay Quick Reference Guide

	Unstimulated Wells	Agonist Control Wells	Cell-free Wells	Test Compound Wells
Step 1 Plate cells, incubate	36 µl cells in Assay Medium (8,000 cells/well)	36 µl cells in Assay Medium (8,000 cells/well)	36 µl Assay Medium (no cells)	36 µl cells in Assay Medium (8,000 cells/well)
Incubate overnight in a humidified 37°C/5% CO ₂ incubator				
Step 2 Add Agonist or Test Compounds	4 µl Assay Medium with 1% DMSO	4 µl 10X 17-AAG diluted in Assay Medium to final 1.0% DMSO	4 µl Assay Medium with 1% DMSO	4 µl 10X Test Compounds diluted in Assay Medium to final 1% DMSO
Step 3 Incubate cells	Incubate for 5 hours in a humidified 37°C/5% CO ₂			
Step 4 Prepare 6X Substrate Mix	6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 934 µl of Solution C, mix			
Step 5 Add Substrate Mixture	8 µl per well			
Step 6 Incubate	2 hours at room temperature in the dark			
Step 7 Detect activity	See Section 6.3			
Step 8 Analyze data	See Section 7			

Antagonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Cell-free Wells	Antagonist Control Wells	Test Compound Wells
Step 1 Plate cells	32 µl cells in Assay Medium (8,000 cells/well)	32 µl cells in Assay Medium (8,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (8,000 cells/well)	32 µl cells in Assay Medium (8,000 cells/well)
Step 2 Incubate cells	Incubate overnight in a humidified 37°C/5% CO ₂ incubator				
Step 3 Add Antagonist or Test Compounds	4 µl Assay Medium with 1.0% DMSO	4 µl Assay Medium with 1.0% DMSO	4 µl Assay Medium with 1.0% DMSO	4 µl 10X Quercetin diluted in Assay Medium to final 1.0% DMSO	4 µl 10X Test Compounds in Assay Medium with 1% DMSO
Optional Step:	Incubate plate with Antagonist for 30 minutes before proceeding				
Step 4 Add Agonist	4 µl Assay Medium with 1.0% DMSO	4 µl 10X 17-AAG in Assay Medium with 1.0% DMSO	4 µl Assay Medium with 1.0% DMSO	4 µl 10X 17-AAG in Assay Medium with 1.0% DMSO	4 µl 10X 17-AAG in Assay Medium with 1.0% DMSO
Step 5 Incubate	Incubate in a humidified 37°C/5% CO ₂ incubator for 5 hours				
Step 6 Prepare 6X Substrate Mix	6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 934 µl of Solution C, mix				
Step 7 Add Substrate Mixture	8 µl per well				
Step 8 Incubate	2 hours at room temperature in the dark				
Step 9 Detect activity	See Section 5.3				
Step 10 Analyze data	See Section 6.0				

6.2 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, and Cell-free Control.

6.2.1 Precautions and Special Considerations for this Assay

- 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.
- Cells should be grown to a confluency between 40% to 80% before harvesting and plating for the assay.

6.2.2 Plate Cells

1. Harvest cells from culture in Growth Medium and resuspend in Assay Medium to a density of $\sim 222,000$ cells/ml.
2. Add 36 μ L per well of Assay Medium to the Cell-free control wells. Add 36 μ L per well of the cell suspension to the Test Compound, Control Compound, and Unstimulated Control wells.
3. Incubate the plates in a humidified 37°C/5% CO₂ incubator for 16–18 hours
4. Proceed to **Section 6.2.3** for an Agonist assay or **Section 6.2.4** for an Antagonist assay.

6.2.3 Agonist Assay Plate Setup

Note: This subsection provides directions for performing an Agonist assay. See Section 5.2.4 for directions for performing an Antagonist assay.

1. Prepare a stock solution of 1% DMSO in Assay Medium.
2. Prepare a 10X stock of Agonist Control 17-AAG in Assay Medium with 1% DMSO. (17-AAG is reconstituted in DMSO to 10mM and further diluted in assay media to a 10X solution with a DMSO concentration of 1.0%). For maximum stimulation we use 125 nM final concentration (10X = 1.25 μ M). We recommend running a dose response curve to determine the optimal concentration for your Agonist solution. See **Section 7.3** for a representative curve.
3. Prepare a 10X stock of Test Compounds in Assay Medium with 1% DMSO. (Or if test compound is dissolved in DMSO, prepare a 10X stock of Test Compounds in Assay Medium and make sure the DMSO concentration for the 10X solution is 1%.
4. Add 4 μ L of the 10X stock of Agonist Control to the Agonist Control wells.
5. Add 4 μ L of the 10X stock of Test Compounds to the Test Compound wells.
6. Add 4 μ L of the stock solution of 1.0% DMSO in Assay Medium to the Unstimulated Control wells and the Cell-free Control wells.
7. Incubate the assay plate in a humidified 37°C/5% CO₂ incubator for 5 hours. Then proceed to **Section 6.2.5** for Substrate Loading and Incubation.

6.2.4 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an Antagonist assay. See Section 5.2.3 for directions for performing an Agonist assay.

1. Prepare a stock solution of 1% DMSO in Assay Medium.
2. Prepare a 10X stock of Test Compounds in Assay Medium with 1% DMSO. (Or if test compound is dissolved in DMSO, prepare a 10X stock of Test Compounds in Assay Medium and make sure the DMSO concentration for the 10X solution is 1%.
3. Prepare a 10X stock of Antagonist Control Quercetin in Assay Medium with 1.0% DMSO. For maximum inhibition we use 31.6 μ M final concentration (10X = 316 μ M). We recommend running a dose response curve to determine the optimal inhibition concentration for your Antagonist solution.
4. Prepare a 10X stock of Agonist 17-AAG in Assay Medium at an EC₈₀ concentration. We recommend running a dose response curve to determine the EC₈₀ for your 17-AAG solution. See **Section 7.3** for a representative curve. In this example the final EC₈₀ concentration, was ~ 63 nM (10X = 630 nM).
5. Add 4 μ L of the stock solution of 1.0% DMSO in Assay Medium to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-free Control wells.
6. Add 4 μ L of the 10X stock of Test Compounds to the Test Compound wells.
7. Add 4 μ L of the 10X stock of Antagonist Control Quercetin to the Antagonist Control wells.

8. If desired, incubate the Test Compounds with the cells in a humidified 37°C/5% CO₂ incubator before proceeding. Typically, a 30-minute incubation is sufficient.
9. Add 4 µl of the 10X EC₈₀ stock solution of Agonist 17-AAG prepared in step 4 to the Test Compound wells, the Stimulated Control wells, and the Antagonist Control wells.
10. Add 4 µl of the stock solution of 1.0% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
11. Incubate the Antagonist assay plate in a humidified 37°C/5% CO₂ incubator for 5 hours. Then proceed to **Section 6.2.5** for Substrate Loading and Incubation

6.2.4 Substrate Loading and Incubation

This protocol is designed for loading cells with LiveBLAzer™-FRET B/G Substrate (CCF4-AM). If using ToxBLAzer™ substrate, prepare and utilize as you would LiveBLAzer.

Preparation of 6X LiveBLAzer™-FRET B/G Substrate (CCF4-AM) mixture and cell loading should be done quickly in the absence of direct strong light

1. Prepare Solution A: 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM, MW = 1096) stock solution:
 - 1.1 Add anhydrous DMSO directly to the vial of lyophilized CCF4-AM, using 182 µl of DMSO for every 200 µg of CCF4-AM.
 - 1.2 Mix well.
 - 1.3 Store the aliquots of the stock solution at -20°C until use.
2. Prepare 6X LiveBLAzer™-FRET B/G (CCF4-AM) Substrate Mixture:
 - 2.1 Add 6 µl of Solution A to 60 µl of Solution B and vortex.
 - 2.2 Add 934 µl Solution C and 60 µl Solution D to the combined solutions from above step with vortexing.

Note: If more than 1 ml 6X Substrate Mixture is needed, scale up the amount of each solution proportionally.
3. Remove assay plate from the humidified 37°C/5% CO₂ incubator and allow plate to equilibrate to room temperature.
4. Add 8 µl 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 2 hours.

6.3 Detection

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

6.3.1 Microplate Readers and Optical Requirements

- Most fluorescence microplate readers (filter or monochromator) with bottom reading capabilities are suitable for detection. For a current list of compatible microplate readers, contact Drug Discovery Technical Support at 1-800-955-6280, select option 3 and enter extension 40266.
- Recommended filters (or those with similar spectral specifications) for fluorescence microplate readers are listed below, and are also available from Chroma Technologies (800-824-7662, www.chroma.com)

Excitation filter:	405/20 nm (Chroma part# HQ405/20x)
Emission filter:	460/40 nm (Chroma part# HQ460/40m)
Emission filter:	530/30 nm (Chroma part# HQ530/30m)
- Recommended dichroic mirrors: 380 nm, 400 nm, and 425 nm cutoff mirrors have been successfully used, and general 50/50 mirrors may also be suitable.
- If using ToxBLAzer™, refer to that protocol for specific instrumentation and filter recommendations.

6.3.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode, and establish a dual emission wavelength measurement protocol within the instrument software (i.e. measurement 1: 405 nm excitation & 460 nm emission; measurement 2: 405 nm excitation & 530 nm emission). Some instrument settings may also require optimization (e.g. gain, plate height, flash number, integration time, etc.).

Note: For specific microplate reader setup information, contact Drug Discovery Technical Support at 1-800-955-6280, select option 3 and enter extension 40266.

7. Data Analysis

7.1 Background Subtraction and Blue/Green Ratio Calculation

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 wells. These control wells are used for background subtraction.
2. Determine the average emission from the Cell-free wells at both 460 nm (Average Blue Background) and 530 nm (Average Green Background).

3. Subtract the Average Blue Background (data collected at 460 nm) from all of the blue emission data.

Note: Background corrected values should not be near zero.

4. Subtract the Average Green background (data collected at 530 nm) from all of the green emission data.

Note: Background corrected values should not be near zero.

5. Calculate the Blue/Green Emission Ratio for each well, by dividing the background-subtracted blue emission values by the background-subtracted green emission values.

Note: You may also calculate response ratio to know your assay window. The response ratio is calculated as the Blue/Green Emission Ratio of the NGF –Stimulated wells divided by the Blue/Green Emission Ratio of the unstimulated wells. Generally, a response ratio of >3 has been shown to yield a $Z' \geq 0.5$.

7.2 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.

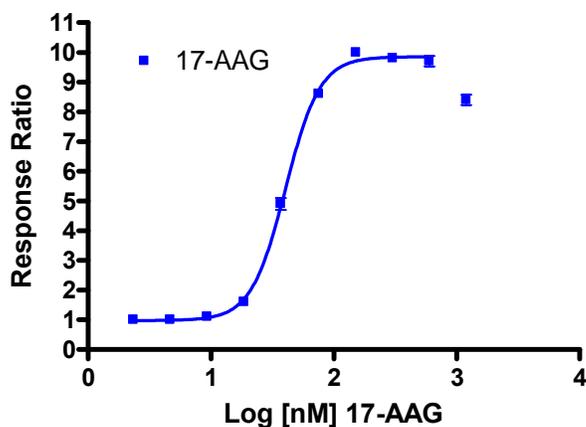
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

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).

7.3 Representative Data



EC₅₀	41 nM
RR_{max}	10.0
Z' at EC₁₀₀	0.81

Figure 1. Dose response of TrkA-NFAT-*bla* CHO-K1 cells to NGF 2.5s. HSE-*bla* HeLa cells were plated in Assay Medium at 8,000 cells/well in 384-well assay format. Following overnight incubation, serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells (0.1 % final DMSO) for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios (RR) were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 16 for each data point).

8. References

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9. Purchaser Notification

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Use of Genetically Modified Organisms (GMO)

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