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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

Cell Line Name:	p53RE-*bla* HCT-116
Description:	CellSensor™ p53RE-*bla* HCT-116 cells contain a beta-lactamase reporter gene under control of the p53 response elements stably integrated into HCT-116 cells. This cell line can be used to detect agonists/antagonists of the p53 signaling pathway. p53RE-*bla* HCT-116 cells have been shown to respond to Mitomycin and Etoposide.
Product Number:	K1202
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:	~2×10^6 cells/ml
Application:	This cell line can be used to detect agonists/antagonists of the p53 signaling pathway.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin
Vector Used:	pLenti-*bsd*/p53RE-*bla* Vector
***Mycoplasma* Testing:**	Negative
Biosafety Level:	1

3.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Media/Reagents	Recommended Source	Cat. no.
LiveBLAzer™ Loading Kit LiveBLAzer™ -FRET B/G Substrate (CCF4-AM), 5 mg DMSO for Solution A Solution B Solution C	Invitrogen	K1030 Other sizes and Loading Kits are available
McCoy's 5A Medium	Invitrogen	16600-082
DMSO	Fluka	41647
Opti-MEM® Reduced Serum Medium	Invitrogen	11058-021
Fetal bovine serum, (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)	Invitrogen	26400-036
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
Mitomycin	Calbiochem	475820
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin antibiotic	Invitrogen	R210-01
Solution D	Invitrogen	K1157

Consumables	Recommended Source	Catalog #
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Corning®	3712
Compressed Air	Various	—

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

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
McCoy's 5A Medium	90%	-	85%
Opti- MEM®	-	99.5%	-
Dialyzed FBS	10%	0.5%	10%
NEAA	-	0.1 mM	0.1 mM
Sodium pyruvate	-	1 mM	1 mM
Penicillin	100 U/ml	100 U/ml	100 U/ml
Streptomycin	100 µg/ml	100 µg/ml	100 µg/ml
Blasticidin antibiotic	5 µg/ml	-	-
DMSO	-	-	5%

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 (See Section 7.0 for detailed protocol)

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.
2. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells at a cell density between 5% and 60% confluence. Do not allow cells to reach confluence.
3. Freeze cells at 2×10^6 cells/ml in Freezing Medium.
4. For detailed growth and maintenance directions, see **Section 7.0**

5.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for monitoring Mitomycin- induced p53 signaling 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.

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.

Note: 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.

	Unstimulated Wells	Stimulated Wells	Cell-Free Wells
Step 1 **Plating Cells**	32 µl cells in Assay Medium (30,000 cells/well).	32 µl cells in Assay Medium (30,000 cells/well).	32 µl Assay Medium (no cells)
Step 2 **Incubation**	Incubate the plate in the 37ºC/5% CO_2 incubator for 6 hours.		
Step 3 **Adding DMSO**	4 µl Assay Medium with 5% DMSO.	4 l Assay Medium with 5% DMSO.	4 µl Assay Medium with 5% DMSO.
Step 4 **Adding Agonist**	4 l Assay Medium	4 l 10X Mitomycin in Assay Medium	4 l Assay Medium
Step 5 **Incubation**	Incubate the plate in the 37ºC/5% CO_2 incubator for 16 hours.		
Step 6 **Prepare 6X Substrate Mixture**	12 µl of 1 mM LiveBLAzer™ -FRET B/G substrate (CCF4-AM) + 120 µl of solution B, mix 1848 µl of Solution C and 20 µl Solution D, mix		
Step 7 **Substrate Loading**	8 µl per well		
Step 8 **Substrate Incubation**	2.5 hrs at room temperature in the dark		
Step 9 **Detection**	See **Section 5.3**		
Step 10 **Data Analysis**	See **Section 6.0**		

5.2 Detailed Assay Protocol

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 pipeting manually, you may need to centrifuge the plate briefly at room temperature (30 seconds at 14 × *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 9.38×10^5 cells/ml. ***Note:*** Plate cells on the day of the assay.
2. Add 32 µl per well of Assay Medium to the cell-free control wells. Add 32 µl per well of the cell suspension to Unstimulated and Stimulated wells.
3. After plating, incubate the plates in a 37°C/5% CO_2 incubator for 6 hours.

5.2.3 Prepare Stimulation Stock Solution

1. Prepare Assay Medium with 5% DMSO.
2. Prepare 10X Mitomycin solution in Assay Medium. We recommend preparing a dose response curve to determine the EC_{80} for your Stimulation Solution.

5.2.4 Stimulate Cells

1. Add 4 µl of Assay Medium with 5% DMSO to Stimulated, Unstimulated and Cell-Free wells.
2. Add 4 µl of Assay Medium without DMSO to Unstimulated wells and Cell-Free wells.
3. Vortex 10X Mitomycin solution and add 4 µl to Stimulated wells.
4. Incubate the assay plate in a humidified 37°C/5% CO_2 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 -20ºC until use.
2. Prepare 6X LiveBLAzer™ -FRET B/G (CCF4-AM) Substrate Mixture.

   Add 12 µl of Solution A to 120 µl of Solution B and vortex.

   Add 1848 µl Solution C and 20 µl of Solution D to the combined solutions from above step with vortexing.
3. Remove assay plate from the incubator.
4. Add 8 µl 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 2.5 hours.

5.3 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: 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.

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

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

***Important*:** 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.

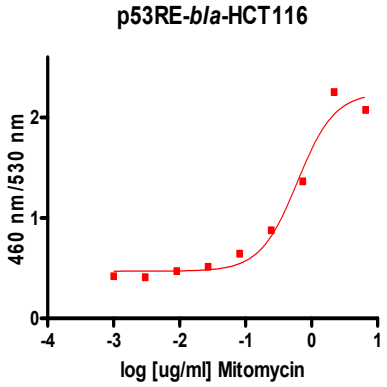
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

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).

6.3 Representative Data



EC_{50}	0.81 µg/ml
EC_{100}	2.2 µg/ml
Z′ at EC_{100}	0.74

Dose response of p53RE-*bla* HCT-116 cells to TNFα. p53RE-*bla* HCT-116 cells were treated with agonist Mitomycin over the indicated concentration range in a 384-well format. Cells were incubated for 16 hours with agonist and 0.5% DMSO and then combined with LiveBLAzer™ -FRET B/G Substrate (CCF4-AM) for 2.5 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted against the concentration of the agonist.

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_2 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 x *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_2 incubator.
9. At first passage, switch to Growth Medium with Blasticidin.

7.2 Propagation Method

Note: Cells should be passaged or fed at least twice a week. Cells should be maintained at a cell density between 5% and 60% confluency.

1. 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.
2. Verify under a microscope that cells have detached and clumps have completely dispersed.
3. 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 at 2 x 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.

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9.0 PURCHASER NOTIFICATION

Limited Use Label License No. 150: GeneBLAzer® Technology

This product and/or its use is the subject of one or more of U.S. Patent Nos. 5,741,657, 5,955,604, 6,291,162, and 6,472,205 and foreign equivalents licensed to Life Technologies Corporation. The right to use this product for internal research specifically excludes the right to use this product to identify, discover, and profile compounds that act as a flavor, fragrance or taste-enhancers and modify a target identified in taste, olfaction, or pheromone detection, which compound does not require FDA approval of an NDA for claims of safety and efficacy. The right to use methods claimed in the foregoing patents with this product for research purposes can only be acquired by the use of GeneBLAzer® substrates purchased from Life Technologies Corporation or its authorized distributors.

Use of Genetically Modified Organisms (GMO)

Information for European Customers The CellSensor™ p53RE-*bla* HCT-116 cell line is genetically modified with the plasmid pLenti-*bsd*/ p53RE-*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.