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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:	T-REx™ FOXO3 DBE-*bla* HeLa
Description:	CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa contains a stably integrated FOXO3-response element driving beta-lactamase reporter gene expression along with tetracycline repressor and tetracycline-inducible FOXO3 constructs. This cell line is a clonal population isolated by flow cytometry that has been tested for robust assay performance by assessing a variety of assay parameters. Addition of doxycycline, a tetracycline analog, allows for FOXO3 transcription factor expression and subsequent beta-lactamase expression. Growth hormone (*e.g.,* insulin or IGF1) activation of the PI3K/AKT signaling pathway leads to phosphorylation and inactivation of FOXO3 and concomitant suppression of beta-lactamase expression. Application of pathway inhibitors (*e.g.,* PI-103) restores FOXO3 activity and beta-lactamase expression.
Product Number:	K1468
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:	~2,000,000 (2×10^6 cells/ml)
Application:	Detection of inhibitors for the PI3K/AKT/FOXO3 signaling pathway.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial, cervical adenocarcinoma
Selection Marker:	Blasticidin (5 µg/ml), Hygromycin B (125 µg/ml), Zeocin™ (75 µg/ml)
Vectors Used:	pLenti-*bsd*/DBE-*bla* Vector, pLenti-*hygro*/TR, and pLenti-*zeo*/FOXO3
***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)
DMEM + GlutaMax™-1	Invitrogen	10569-010
Dialyzed Fetal Bovine Serum (dFBS) (**DO NOT SUBSTITUTE!**)	Invitrogen	26400-044
HEPES (pH 7.3)	Invitrogen	15630-080
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin	Invitrogen	15140-122
Insulin solution, Human (10 mg/ml = 1.72 mM) (light sensitive)	Sigma	I9278
Blasticidin	Invitrogen	R210-01
Hygromycin B	Invitrogen	10687-010
Zeocin™	Invitrogen	R250-01
Doxycycline hydrochloride (light sensitive)	MP Biomedicals	195044
0.05% Trypsin/EDTA	Invitrogen	25300-054
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-144
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
DMSO	Fluka	41647

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 5.3**)	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. Cell Culture Conditions

4.1 Media Required

Component	Thaw Medium	Growth Medium	Assay Medium	Freezing Medium
DMEM w/ GlutaMAX™	500 ml bottle	500 ml bottle	500 ml bottle	—
Dialyzed FBS	50 ml	50 ml	0.5 ml	—
HEPES (1 M)	12.5 ml	12.5 ml	12.5 ml	—
NEAA (10 mM)	5 ml	5 ml	5 ml	—
Penicillin/Streptomycin	5 ml	5 ml	5 ml	—
Blasticidin	—	5 µg/ml	—	—
Hygromycin B (50 mg/ml)	—	125 µg/ml	—	—
Zeocin™ (100 mg/ml)	—	75 µg/ml	—	—
Recovery™ Cell Culture Freezing Medium		—	—	100%

Note: We prepare our media by adding the listed components directly to the medium bottle. Blasticidin, Hygromycin B, and Zeocin™ can be added directly to the cell culture flask to reach desired concentrations. 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.

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 Thaw Medium **without selection** and culture them in Growth Medium with selection. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 10% to 90% confluency. Do not allow cells to reach confluence.
2. Freeze cells at 2×10^6 cells/ml in Recovery™ Cell Culture Freezing Medium.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring PI3K/AKT/FOXO3 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.

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 0.1% 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.

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	Insulin-stimulated Wells	Cell-free wells
Step 1 **Doxycycline treatment**	Incubate the Stock Flask supplemented with 5 ng/ml Doxycycline at 37°C/5% CO_2 for at least 5 hours (up to 24 hours) prior to plating the cells.		
Step 2 Plate cells	32 µl cells suspended in Assay Medium plus 6.25 ng/ml Doxycycline (~10,000 cells/well)	32 µl cells suspended in Assay Medium plus 6.25 ng/ml Doxycycline (~10,000 cells/well)	32 µl Assay Medium plus Doxycycline (no cells)
Step 3 Add DMSO	Add 4 µl of 1% DMSO in Assay Medium to each well		
Step 4 Add Insulin	4 µl Assay Medium to each well	4 µl 10X Insulin in Assay Medium to each well	4 µl Assay Medium to each well
Step 5 Incubate cells	Incubate the plate at 37°C/5% CO_2 for 16-20 hours.		
Step 6 Prepare 6X Substrate Mixture	6 µl 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM) in dry DMSO + 60 µl Solution B, mix. Add 934 µl Solution C, mix.		
Step 7 Load Substrate Mixture	8 µl per well		
Step 8 Incubate Substrate + cells	4 hours at room temperature in the dark.		
Step 9 Detect Activity	See **Section 5.3**		
Step 10 Analyze data	See **Section 6.0**		

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, Insulin-stimulated Control, and Cell-free 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 × *g*) after additions to ensure all assay components are on the bottom of the wells.
- For best assay performance, cells should be grown to a confluency of 50 to 80% before plating the assay; higher cell densities may result in more variability and lower Z' values.

5.2.2 Prepare Stock Solutions

- Prepare Assay Medium with 1% DMSO.
- Doxycycline is diluted to 1 mg/ml (2.08 mM) in nanopure water, sterile-filtered, and stored in aliquots at -20°C for long-term storage (several months). Doxycycline is not freeze/thaw sensitive and can also be stored at 4°C for at least 1-2 weeks without any detectable loss of activity. Doxycycline is light-sensitive and should be stored in the dark.
- Prepare 6.25 ng/ml Doxycycline in Assay Medium using the 1 mg/ml stock.
- Prepare 10X Insulin in Assay Medium (10X EC_{80}). We recommend preparing a dose response curve to determine the EC_{80} for your Stimulation Solution. See **Section 6.3** for a representative curve.

5.2.3 Doxycycline Treatment (to induce FOXO3 expression) of Stock Flask

1. In the morning on the day the cells are to be plated for the assay, prepare a 1/1000 dilution of the Doxycycline stock (1 mg/ml) by adding 1 µl Doxycycline stock to 1 ml of Growth Medium to yield a 1 ng/µl Doxycycline solution.
2. Measure the amount of Growth Medium present in the Stock Flask by pipeting, and then add a final of 5 ng/ml of Doxycycline to the Stock Flask.
3. Incubate the Stock Flask supplemented with 5 ng/ml Doxycycline at 37°C/5% CO_2 for at least 5 hours (up to 24 hours) prior to plating the cells.

5.2.4 Plate Cells

1. Harvest cells from culture in Growth Medium and resuspend in Assay Medium plus 6.25 ng/ml Doxycycline to a density of 312,500 cells/ml.
2. Add 32 µl per well of Assay Medium plus 6.25 ng/ml Doxycycline to the Cell-free control wells. Add 32 µl per well of the cell suspension to Unstimulated and Insulin-stimulated wells.

5.2.5 Insulin Stimulation

1. Add 4 µl Assay Medium with 1% DMSO to all the wells.
2. Add 4 µl 10X Insulin in Assay Medium to the Insulin-Stimulated wells, and 4 µl Assay Medium to the Unstimulated and cell free control wells.
3. Incubate the assay plate in a humidified 37°C/5% CO_2 incubator for 16-20 hours.

5.2.6 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 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 to the combined solutions from above step with vortexing.
3. Remove assay plate from the humidified 37°C/5% CO_2 incubator and 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 4 hours.

5.3 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.

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 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. Data Analysis

6.1 Background Subtraction and Calculation of 460/530 nm Emission Ratio

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)

***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.

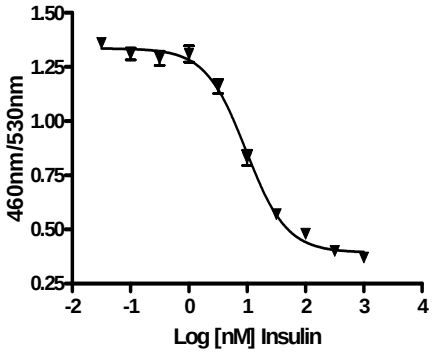
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

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).

6.3 Representative Data



EC_{50}	9.6 nM
EC_{100}	1000 nM
Z' (EC_{100})	0.75

Figure 1. Dose response of T-REx™ FOXO3 DBE-*bla* HeLa cells to insulin. T-REx™ FOXO3 DBE-*bla* HeLa cells pre-treated with Doxycycline were plated in 384-well format (10,000 cells/well) and incubated with a serial dilution series of insulin in the presence of 0.1% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 4 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 nm ratios were plotted for the indicated concentrations of insulin (n=16 for each data point).

7. Detailed Cell Handling Procedures

7.1 Thawing Method

1. Place 14 ml of Thaw Medium (without selection markers) 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 to a sterile 15-ml conical tube.
6. Add 10 ml of Thaw Medium (without selection markers) drop-wise into the cell suspension.
7. Centrifuge cells at 200 × *g* for 5 minutes.
8. Aspirate supernatant and resuspend the cell pellet in 1 ml of fresh Thaw Medium (without selection markers).
9. Transfer contents to the T75 tissue culture flask containing pre-equilibrated Thaw Medium (without selection markers) and place flask in a humidified 37°C/5% CO_2 incubator.
10. At first passage, switch to Growth Medium with selection markers.

7.2 Propagation Method

1. Cells should be passaged or fed at least twice times a week. Cells should be maintained between 10% and 90% confluency. Do not allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once with 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 minutes exposure to Trypsin/EDTA. Add an equal volume of Growth Medium to inactivate Trypsin. 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 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.

8. References

1. Zlokarnik, G., *et al,* **Quantitation of Transcription and Clonal Selection of Single Living Cells with Beta-Lactamase as Reporter,** (1998) *Science*; **279:** p84-88.
2. Kunapuli P., Ransom R., Murphy K., Pettibone D., Kerby J., Grimwood S., Zuck P., Hodder P., Lacson R., Hoffman I., Inglese J., Strulovici B., **Development of an Intact Cell Reporter Gene Beta-lactamase Assay for G Protein-coupled Receptors, (**2003) *Analytical Biochem.;* **314:** p16-29.
3. Xing, H., Pollok, B., *et al*, **A Fluorescent Reporter Assay For The Detection of Ligands Acting Through G1 Protein-coupled Receptors,** (2000) *J. Receptor & Signal Transduction Research*; **20**: p189-210.
4. Qureshi, S., *et al,* **A One-Arm Homologous Recombination Approach for Developing Nuclear Receptor Assays in Somatic Cells,** (2003) *Assay and Drug Dev. Tech;* **1**: p755-766.
5. Peekhaus, N. *et al,* **A Beta-Lactamase-Dependent Gal4-Estrogen Receptor Transactivation Assay for the Ultra-High Throughput Screening of Estrogen Receptor Agonists in a 3,456-Well Format,** (2003) *Assay and Drug Dev Tech*; **1**: p789-800.
6. Chin, J., *et al*, **Miniaturization of Cell-Based Beta-Lactamase-Dependent FRET Assays to Ultra-High Throughput Formats to Identify Agonists of Human Liver X Receptors**, (2003) *Assay and Drug Dev. Tech.*; **1**: p777-787.
7. Whitney M., Rockenstein E., Cantin G., Knapp T., Zlokarnik G., Sanders P., Durick K., Craig F.F., Negulescu P.A., **A Genome-wide Functional Assay of Signal Transduction in Living Mammalian Cells,** (1998) *Nat. Biotechnol.*; **16**: p1329-1333.

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Use of Genetically Modified Organisms (GMO)

Information for European Customers The CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa cell line(s) are genetically modified with the plasmid pLenti-*bsd*/DBE-*bla*, pLenti-*hygro*/TR, and pLenti-*zeo*/FOXO3. 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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