CellSensor® *NFκB-bla* HEK 293T Cell-based Assay Protocol

Catalog no. K1165 **Shipping: Dry Ice** **Storage: Liquid Nitrogen**

Protocol part no. O-13582-r1 Rev. date: 17 December 2010

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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® NFkB-bla HEK 293T Cells
Description:	This cell line utilizes GeneBLAzer® beta-lactamase technology. CellSensor™ NFkB-*bla* HEK 293T cells contain a beta-lactamase reporter gene under control of the NFkB response element (NFkB) stably integrated into HEK 293T cells. This cell line can be used to detect agonists/antagonists of the NFkB signaling pathway. NFkB-*bla* HEK 293T cells have been shown to respond to Tumor Necrosis Factor Alpha (TNF alpha).
Cat. no.	K1165
Shipping:	Dry Ice
Storage:	Liquid Nitrogen
Quantity:	~2,000,000 cells (2×10^6 cells/mL)
Application:	This cell line can be used to detect agonists/antagonists of the NFkB signaling pathway.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/mL)
Vector Used:	pLenti-bsd/NFkB-bla Vector
***Mycoplasma* Testing:**	Negative
BioSafety Level:	2

3. Materials Required, but Not Supplied

Media/Reagents	Recommended Source	Catalog no.
LiveBLAzer™ FRET B/G Loading Kit LiveBLAzer™ FRET B/G Substrate (CCF4-AM) or CCF2-AM substrate (5 mg) DMSO for Solution A Solution B Solution C	Invitrogen	K1025 (CCF2-AM) K1030 (CCF4-AM) Other sizes are available
Cell Culture Freezing Medium	Invitrogen	12648-010
DMEM with GlutaMAX™	Invitrogen	10569-010
DMSO	Fluka	41647
Fetal bovine serum, (FBS), dialyzed, tissue-culture grade	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
HEPES (1 M, pH 7.3)	Invitrogen	15630-080
Human TNF Alpha	BD Bioscienes	354066
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin antibiotic	Invitrogen	R210-01

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 5.5)	Chroma Technology Corp.
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
DMEM with GlutaMAX™	90% (500 mL)	90% (500 mL)	90% (500 mL)
Dialyzed FBS	10% (50 mL)	10% (50 mL)	10% (50 mL)
NEAA	0.1 mM (5 mL)	0.1 mM (5 mL)	0.1 mM (5 mL)
HEPES (pH 7.3)	25 mM (12.5 mL)	25 mM (12.5 mL)	25 mM (12.5 mL)
Penicillin and Streptomycin	100 U/mL and 100 µg/mL (5 mL)	100 U/mL and 100 µg/mL (5 mL)	100 U/mL and 100 µg/mL (5 mL)
Blasticidin antibiotic	—	5 µg/mL	—

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: Components can be added directly to the 500 mL bottle of DMEM media.

4.2 Methods Used

4.2.1 Thawing Method

1. Place 9 mL of Thaw Medium into a T25 flask
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 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 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. Count cells.
9. Transfer approximately 1–1.5 × 10^6 cells into the T25 tissue culture flask containing pre-equilibrated Thaw Medium (~40,000–60,000 cells/cm^2) and place flask in the 37°C/5% CO_2 incubator.
10. At first passage switch to Growth Medium.

4.2.2 Propagation Method

1. Cells should be passaged or fed at least twice a week. Cells should be maintained between 10% and 90% confluence. Do not allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (1 mL for a T25 flask, 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 ~2–5 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.

4.2.3 Freezing Method

1. Harvest the cells as described in **Section 4.2.2**, Step 2. After detachment, count the cells, then spin cells down and resuspend in 4°C Cell Culture Freezing Medium (see ordering information on page 2) at the desired cell density.
2. Dispense 1-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 Smad 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** for details.
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 TNF alpha	1. 32 µL cells diluted in Assay Medium 2. 4 µL 5% DMSO stock solution 3. 4 µL 10X stock solution of TNF alpha	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** for details.
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 TNF alpha	Provides 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

- 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, it may be necessary to centrifuge the plate briefly at room temperature (for 1 minute 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 6.25×10^5cells/mL.
2. Add 36 µL per well of the Assay Medium to the cell-free control wells. Add 32 µL per well of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells.

Note: Cells were plated on the day of the assay. Cells can be plated on the 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. Prepare a 10X stock of TNF alpha in assay media. For an antagonist screen, use ~2.7 ng/mL final concentration or EC_{80} (10X = 27 ng/mL). For an agonist screen, use ~50 ng/mL final concentration or max stim (10X = 500 ng/mL). We recommend running a dose response curve to determine the EC_{80} for your 10X TNF alpha 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 stock solution of TNF alpha 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 1-hour incubation is sufficient.
4. Add 4 µL of the 10X stock solution of an appropriate agonist 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 CCF2-AM or CCF4-AM solution (from the LiveBLAzer™ FRET B/G Loading Kit) 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 Loading Protocol

This protocol is designed for loading cells with LiveBLAzer™ FRET B/G Substrate (CCF2-AM or CCF4-AM) using the following solutions.

Reagents	Storage and Handling
LiveBLAzer™ FRET B/G Substrate	The product is supplied as a dried powder. The molecular weight of CCF2-AM is 1082 g/mole; the molecular weight of 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 for preparation of Solution A.
Solution A	LiveBLAzer™ FRET B/G Substrate 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 one month. 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 vigorous agitation (vortexing).

5.3.3 Cell Loading

1. Remove the assay plate from the incubator and allow it to equilibrate to room temperature before loading.
2. Add 8 µL of the 6X Loading Buffer to each well.

5.4 Incubation

1. Cover the plate to protect it from light and evaporation.
2. Incubate at room temperature for 120 minutes.

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

5.5 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.

5.5.1 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 or visit www.invitrogen.com/instrumentsetup

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.

Refer to the ToxBLAzer™ protocol for specific instrumentation and filter recommendations.

5.5.2 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 at 405 nm excitation and 460 nm emission; measurement 2 at 405 nm excitation and 530 nm emission).
2. Some instrument settings may also require optimization (e.g., gain, plate height, flash number, integration time, etc.).
3. For specific microplate reader setup information, contact Drug Discovery Technical Support at 1-800-955-6280, select option 3 and enter extension 40266 or visit www.invitrogen.com/instrumentsetup.

6. Data Analysis

6.1 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 TNF alpha –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 $Z' \geq 0.5$.

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).

7. References

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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.

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