CellSensor® LEF/TCF-*bla* FreeStyle 293F Cell-based Assay

Cat. nos. K1192 **Shipping: Dry Ice** **Storage: Liquid Nitrogen**

Protocol part no. K1192.pps Rev. date: 28 May 2008

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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 response to drug candidates or other stimuli (1). The core of the GeneBLAzer® Technology is a Förster Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors which 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 in high-throughput screening campaigns for a range of target classes, including G-protein coupled receptors (2, 3), nuclear receptors (4-6) and kinase signaling pathways (7).

2. Materials Supplied

Cell Line Name:	LEF/TCF-*bla* FreeStyle™ 293F
Description:	CellSensor® LEF/TCF-*bla* FreeStyle™ 293F cells contain a beta-lactamase reporter gene under control of the β-catenin/LEF/TCF binding elements stably integrated into FreeStyle™ 293F cells. This cell line can be used to detect agonists/antagonists of the Wnt/β-catenin signaling pathway. LEF/TCF-*bla* FreeStyle™ 293F cells have been shown to respond to mouse Wnt3a stimulation.
Product Number:	K1192
Shipping Condition:	Dry Ice
Storage Condition:	Liquid nitrogen. **Immediately upon receipt, cells must be stored in liquid nitrogen. Cells cannot be stored at –80°C, as they can quickly lose viability.**
Quantity:	~8,000,000 (8×10^6 cells/ml)
Application:	This cell line can be used to detect agonists/antagonists of the Wnt/β-catenin signaling pathway.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/ml)
Vector Used:	pLenti-*bsd*/LEF/TCF-*bla* Vector
***Mycoplasma* Testing:**	Negative
BioSafety Level:	2

3. 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
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
DMEM (high-glucose)	Invitrogen	10569-010
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
HEPES (1M, pH 7.3)	Invitrogen	15630-080
Sodium pyruvate	Invitrogen	11360-070
Mouse Wnt3a	R&D Systems	1324-WN
Lithium Chloride	Sigma	L4408
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin antibiotic	Invitrogen	R210-01

Consumables	Recommended Source	Catalog #
BIOCOAT Poly-D-Lysine CellWare™	BD Biosciences	354663
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. Cell Culture Conditions

4.1 Media Required

Component	Growth Medium	Thaw Medium	Assay Medium	Freezing Medium
DMEM	90%	90%	-	-
Opti- MEM®	-	-	96.5%	-
Dialyzed FBS	10%	10%	0.5%	-
NEAA	0.1 mM	0.1 mM	0.1 mM	-
Sodium pyruvate	-	-	1 mM	-
HEPES (pH 7.3)	25 mM	25 mM	10 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	-	-	-
Cell Culture Freezing Medium	-	-	-	100%

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

4.2 Growth Conditions

See **Section 7** for detailed growth and maintenance conditions.

1. Cells should be thawed in Thaw Medium without Blasticidin and grown in Growth Medium with Blasticidin. Cells should be passed or fed at least twice a week and maintained in a 37ºC/5% CO_2 incubator. Cells should be maintained between 10% and 90% confluency. Do not allow cells to reach confluence.

   Note: We recommend passaging cells three times after thawing before using them in the beta-lactamase assay.

2. Cells should be frozen at 2×10^6 cells/ml in Freezing Medium.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring the Wnt/β-catenin signaling pathway using beta-lactamase as the readout.

5.1 Quick Reference Guide

For more detailed protocol, see **Section 5.2**.

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least 3 wells for each condition: Unstimulated, Stimulated, and Cell-free.

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.

	Unstimulated Wells	Stimulated Wells	Cell-free wells
Step 1 **Plating Cells**	20 µl cells in Assay Medium (10,000 cells/well).	20 µl cells in Assay Medium (10,000 cells/well).	20 µl Assay Medium (no cells)
Step 2 **Incubation**	Incubate the plate in the 37ºC incubator for 2 to 3 hours.		
Step 3 **Adding LiCl**	10 µl Assay Medium with 30 mM LiCl.	10 µl Assay Medium with 30 mM LiCl.	10 µl Assay Medium with 30 mM LiCl.
Step 4 **Incubation**	Incubate the plate in the 37ºC incubator for 15 hours.		
Step 4 **Adding Agonist**	10 µl Assay Medium with 2% DMSO.	10 µl 4 x mWnt3a in Assay Medium with 2% DMSO.	10 µl Assay Medium with 2% DMSO.
Step 5 **Incubation**	Incubate the plate in the 37ºC incubator for 5 hours.		
Step 6 **Prepare 6X Substrate Mixture**	6 µl of 1 mM LiveBLAzer™ -FRET B/G substrate (CCF4-AM) + 60 µl of solution B, mix 934 µl of Solution C, mix		
Step 7 **Substrate Loading**	8 µl per well		
Step 8 **Substrate Incubation**	3 hrs at Room Temperature in the dark		
Step 9 **Detection**	See **Section 5.3**		
Step 10 **Data Analysis**	See **Section 6**		

5.2 Detailed Assay Protocol

5.2.1 Precautions

1. Work on a dust-free, clean surface. Always handle the poly-D-Lysine 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 30 seconds 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 and resuspend in Assay Medium at a density of 5×10^5cells/ml.
2. Add 20 µl per well of the Assay Medium to the cell free control wells. Add 20 µl per well of the cell suspension to the Unstimulated and Stimulated wells.
3. After plating, put the plate in 37ºC incubator and incubate for 2–3 hours.

4. Prepare 30 mM LiCl solution in the Assay Medium.
5. Add 10 µl per well of the 30 mM LiCl in Assay Medium to each well.
6. Incubate the plate in the 37ºC incubator for 15 hours.

5.2.3 Preparation of Stock Solution

1. Prepare Assay Medium with 2% DMSO.
2. Prepare a 4X mWnt3a in Assay Medium with 2% DMSO. We recommend running a dose response curve to determine the EC_{80} for your Stimulation Solution.

5.2.4 Cell Stimulation

1. Add 10 µl of Assay Medium with 2% DMSO to the Unstimulated wells and to the Cell-free wells.
2. Add 10 µl of 4X mWnt3a to Stimulated wells.
3. Incubate the assay plate in a humidified 37°C/5% CO_2 incubator for 5 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 Loading Solution 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 6 µl of Solution A to 60 µl of Solution B and vortex.

   Add 934 µl of Solution C to the combined solutions from above step with vortexing.
3. Remove assay plate from the incubator.
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 3 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 the fluorescence plate reader with bottom read capabilities is not available, please call our tech 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 min. 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. Data Analysis

6.1 Background Subtraction

Background subtraction for both emission channels (460 nm and 530 nm) is recommended.

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 from all of the Blue emission data.
4. Subtract the Average Green background from all of the Green emission data.
5. Calculate 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

***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.

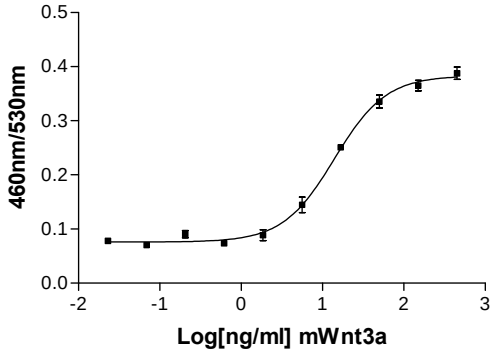
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

Chroma Set # 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).

6.3 Representative Data



EC_{50}	15 ng/ml
EC_{80}	46 ng/ml
EC_{100}	450 ng/ml
Z′ at EC_{100}	0.67

Dose Response of LEF/TCF-*bla* FreeStyle™ 293F cells to mWnt3a. LEF/TCF-*bla* FreeStyle™ 293F cells treated overnight with 10 mM LiCl were stimulated with indicated amount of mWnt3a for 5 hours before β-Lactamase assay was performed.

7. Detailed Growth Methods

7.1 Thawing Method

1. Place 29 ml of Growth Medium without Blasticidin into a T225 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 Growth Medium without Blasticidin 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 Growth Medium without Blasticidin.
8. Transfer contents to the T225 tissue culture flask containing pre-equilibrated Growth Medium without Blasticidin and place flask in the 37°C/5% CO_2 incubator.
9. At first passage switch to Growth Medium with Blasticidin.

7.2 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 (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.
3. Verify under a microscope that cells have detached and clumps have completely dispersed.
4. 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 Cell Culture Freezing Medium at 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.
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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.
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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