Part # K1129 **Shipping Condition: Dry Ice** **Storage: Liquid Nitrogen**

O-13341-r1 US 1010

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1.0 MATERIALS SUPPLIED

Description: This cell line utilizes the GeneBLAzer® Beta-lactamase technology. CellSensor™ CRE-*bla* CHO-K1 contains a beta-lactamase reporter gene under control of a cAMP response element (CRE) stably integrated into CHO-K1 cells. This cell line can be used as a parental cell line to build specific G-protein coupled receptor (GPCR) assays after transfection of additional genes of interest or to detect changes in intracellular cAMP levels. CRE-*bla* CHO-K1 cells have been shown to be responsive to forskolin stimulation.

Product Number: K1129

Shipping Condition: Dry Ice

Storage Condition: Liquid nitrogen

Quantity: ~2,000,000 cells (2 x 10^6 cells/ml)

Cell Line Name: CRE-*bla* CHO-K1

Application: Parental cell line for constructing specific GPCR assays and to detect changes in intracellular cAMP levels.

Growth Properties: Adherent

Cell Phenotype: Epithelial

Selection Marker: YFP

Vector Used: pLenti-*bsd*/CRE-*bla* Vector

***Mycoplasma* Testing:** Negative

Biosafety Level: 1

2.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Reagents	Recommended Source	Cat. no.
LiveBLAzer™ FRET B/G loading kit CCF2-AM or LiveBLAzer™ FRET B/G (CCF4-AM) substrate (5 mg) DMSO for Solution A Solution B Solution C	Invitrogen	K1025 (CCF2-AM) K1030 LiveBLAzer™ FRET B/G (CCF4-AM)
Cell Culture Freezing Medium	Invitrogen	11101-011
DMEM, high-glucose	Invitrogen	11965-092
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade	Invitrogen	26400-044
Forskolin	Sigma	F6886
HEPES (pH 7.3)	Invitrogen	15630-080
Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
Solution D	Invitrogen	K1156 or K1157
Sodium pyruvate	Invitrogen	11360-070
Trypsin/EDTA	Invitrogen	25300-054

Consumables	Recommended Source	Cat. no.
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Costar	3712
Compressed Air	Various	—
Conical tubes, 15-ml, sterile	Various	—
1.8-ml Internally threaded cryogenic vials	Various	—
Tissue culture flasks	Various	—

Equipment	Recommended Source
Class II biological safety cabinet	Various
CytoFluor® 4000 fluorescence plate reader (or equivalent bottom-reading fluorescence plate reader)	PerSeptive Biosystems
Filters (see **Sections 4.6.1** and **5.3**)	Chroma Technologies
Hemacytometer (or another cell counting method).	Various
Humidified 37°C/5% CO_2 incubator	Various
Insulated Container	Various
Liquid nitrogen tank	Various

2.1 Optional Equipment and Materials

- Epifluorescence or fluorescence-equipped microscope, equipped with appropriate filters
- Microplate centrifuge

3.0 CELL CULTURE CONDITIONS

3.1 Media Required

Component	Growth/Assay Medium
DMEM, high glucose	90%
FBS, dialyzed, tissue-culture grade	10%
HEPES (pH 7.3)	25 mM
NEAA	0.1 mM
Penicillin	100 U/ml
Sodium pyruvate	1 mM
Streptomycin	100 µg/ml

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

3.2 Methods Used

3.2.1 Thawing Method

1. Prepare at least 25 ml of fresh Growth Medium. (See note, above and apply throughout)
2. Place 15 ml of fresh Growth Medium into a sterile T75 flask.
3. Place T75 flask in a humidified 37°C/5% CO_2 incubator to equilibrate to the proper pH and temperature.
4. Rapidly thaw cells by placing frozen vial in a water bath at 37°C for 1–2 min. with gentle agitation.
5. Swab the vial with 70% EtOH before opening in hood.
6. Transfer the contents to a sterile 15-ml conical tube.
7. Slowly add 10 ml Growth Medium dropwise to the cell suspension.
8. Centrifuge for 5 minutes at 200 x *g*.
9. Discard the supernatant, and resuspend the cells in the 15-ml conical tube containing 15 ml of pre-equilibrated Growth Medium (prepared in the T75 flask from Step 4 in this Section).
10. Return the cells to the T75 flask prepared in Step 1 in this Section for culturing.

3.2.2 Propagation Method

1. Cells should be fed or passaged 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 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 ~3-5 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. If necessary, gently pipette up and down to disperse clumps.
4. Spin down cells and resuspend in Growth Medium.

3.2.3 Freezing Method

1. Harvest the cells as described in **Section 3.2.2, Step 2**.
2. Count the cells.
3. Spin cells down (200 x *g*, 5 min.) and resuspend in 4°C Cell Culture Freezing Medium at 2 x 10^6 cells/ml.
4. Dispense 1.0 ml aliquots into cryogenic vials.
5. Place vials in an insulated container (for slow cooling) and store overnight at -80°C.
6. Transfer to liquid nitrogen the next day for storage.

4.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for determining activity of compounds as modulators (agonists or antagonists) of the cAMP signaling pathway in a 384-well plate.

4.1 Controls

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 5.0** (Data Analysis).
Stimulated Control Wells	1. 32 µl cells diluted in Assay Medium. 2. 4 µl 5% DMSO stock solution. 3. 4 µl 10X Forskolin stock solution.	1. 32 µl cells diluted in Assay Medium. 2. 4 µl 5% DMSO stock solution. 3. 4 µl 10X Forskolin stock solution.	Provides the Stimulated Emission Ratio, which is a portion of the Response Ratio. Stimulated Emission Ratio = (Stimulated 460 nm/ Stimulated 530 nm). See **Section 5.0** (Data Analysis).
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 Forskolin stock solution.	Provides the experimental data on whether or not a test compound is active as an agonist or antagonist in the assay.

4.2 Cell Stimulation and Assay Plate Set-up

4.2.1 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 min. at 14 x *g*) after additions to ensure all the assay components are on the bottom of the wells.

4.2.2 Plating Cells

- Harvest cells from culture in Growth Medium as described in **Section 3.2.2,** Step 2 and resuspend in Assay Medium at a density of 6.25 x 10^5cells/ml.
- 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 day of assay. Cells can be plated on previous day at half the concentration in Step #1.

4.2.3 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 forskolin as a control in the agonist or antagonist assay. For an antagonist assay, use ~0.8 µM final concentration or EC_{80} (10X = 8 µM). For an agonist assay, use ~5 µM final concentration, or max stim (10X = 50 µM). We recommend running a dose response curve to determine the EC_{80} for your 10X forskolin solution.

4.2.4 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 4.2.5**.

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 forskolin 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.

4.2.5 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an antagonist assay. Directions for performing an agonist assay are provided in **Section 4.2.4**.

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. Add 4 µl of the 10X stock solution of an appropriate agonist to the Test Compound wells and the Stimulated Control wells.
4. Add 4 µl of Assay Medium to the Unstimulated Control wells.
5. Incubate the antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 5 hours.

4.3 Substrate Loading

Preparation of 6X CCF2-AM or LiveBLAzer™ FRET B/G Substrate (CCF4-AM) Loading Solution and cell loading should be done in the absence of direct strong lighting. Turn off the light in the hood.

4.3.1 LiveBLAzer™ FRET B/G Loading Protocol

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

Reagents	Storage and Handling
LiveBLAzer™ FRET B/G	The product is supplied as a dried powder. The molecular weight of CCF2-AM is 1082 g/mole; the molecular weight of LiveBLAzer™ FRET B/G Substrate (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 (CCF4-AM) for preparation of Solution A.
Solution A	LiveBLAzer™ FRET B/G Substrate (CCF4-AM) 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.
Solution D	Optional: Solution D is a non-specific anion exchange blocker that can be added to the loading solution to prevent undesired export of the substrate from the cell in cell types with active multi-drug transporters. Solution D requires neutralization with base before it will dissolve in aqueous solutions.

4.3.2 Preparation of 6X LiveBLAzer™ FRET B/G Substrate (CCF4-AM) Loading Solution

1. Add 12 µl of Solution A to 60 µl of Solution B and vortex.
2. Add 898 µl Solution C to the combined solutions from Step 1 and vortex.
3. Add 30 µl of Solution D, pH 8.0 to the combined solutions from Step 2 and vortex.

4.3.3 Cell Loading

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

4.4 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.

4.5 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: Some plates/fluorescence plate readers experience edge effects which may affect data. If edge effects are noticed, plate layout should be considered when setting up the assay.

Note: For CytoFluor® 4000 fluorescence plate reader gain setting calibration, see the Section 6.0, Technical Appendix.

4.5.1 Instrumentation, Filters, and Plates

- Fluorescence plate reader: CytoFluor® 4000 or equivalent fluorescence plate reader.

  Note: If you are using an instrument other than the CytoFluor® 4000, please contact Technical Support before you begin.

- Filters for fluorescence plate reader:

  For ratiometric readout using a fluorescence plate reader:

  Excitation filter: 409/20 nm
  Emission filter: 460/40 nm
  Emission filter: 530/30 nm

4.5.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode and for eight scans per cycle.
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:

	Scans 1-4	**Scans 5-8**
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 (using the gain determined during calibration)	530/30 nm (using the gain determined during calibration)

Typically, set the fluorescence plate reader to 5 reads/well. See **Section 6.0, Technical Appendix** for more information.

5.0 DATA ANALYSIS

5.1 Background Subtraction

Background subtraction for both channels (460 nm and 530 nm) is essential to obtain meaningful data. This can be accomplished either automatically using software connected to the fluorescence plate reader, or manually after each assay plate has been read.

Use the assay plate layout to identify the location of the Cell-free Control wells. These control wells are used for background subtraction.

5.1.1 Automatic background subtraction

Use the assay plate layout to designate appropriate Cell-free Control wells and enable background subtraction for both sets of emission scans.

5.1.2 Manual background subtraction

1. Determine the average emission from the Cell-free Control wells at both 460 nm (Average Em_{460} Background) and 530 nm (Average Em_{530} Background).
2. Calculate the Net Stimulated Em_{460} and Em_{530} values and the Net Unstimulated Em_{460} and Em_{530} values, as shown below:

Stimulated Cells

Net Stimulated Em_{460} = Em_{460} of stimulated sample – Average Em_{460} Background

Net Stimulated Em_{530} = Em_{530} of stimulated sample – Average Em_{530} Background

Unstimulated Cells

Net Unstimulated Em_{460} = Em_{460} of unstimulated sample – Average Em_{460} Background

Net Unstimulated Em_{530} = Em_{530} of unstimulated sample – Average Em_{530} Background

5.2 Response Ratio Calculations

1. Calculate the Stimulated Emission Ratio for each well, as shown below.

$$\text{Stimulated Emission Ratio} = \frac{\text{Net Stimulated } Em_{460}}{\text{Net Stimulated } Em_{530}}$$

2. Calculate the Unstimulated Emission Ratio for each well, as shown below.

$$\text{Unstimulated Emission Ratio} = \frac{\text{Net Unstimulated } Em_{460}}{\text{Net Unstimulated } Em_{530}}$$

3. Calculate the Mean Unstimulated Emission Ratio for each assay plate, as shown below.

$$\text{Mean Unstimulated Emission Ratio} = \frac{\text{Sum of Unstimulated Emission Ratios}}{\text{Total number of wells containing unstimulated cells}}$$

4. Calculate the Response Ratio for each well of interest. The Response Ratio, as shown below.

$$\text{Response Ratio} = \frac{\text{Stimulated Emission Ratio } (Em_{460}/Em_{530})}{\text{Mean Unstimulated Emission Ratio } (Em_{460}/Em_{530})}$$

Note: All Response Ratios are calculated using the Mean Unstimulated Emission Ratio. The Response Ratio determined with 4 µM Forskolin and LiveBLAzer™ FRET B/G Substrate (CCF4-AM) was 10-15.

5.3 Visual Observation of Intracellular LiveBLAzer™ FRET B/G Substrate

***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 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).

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).

6.0 TECHNICAL APPENDIX

This Technical Appendix describes the calibration methods we use routinely. Other calibration methods may yield different results.

6.1 Materials Required

LiveBLAzer™ FRET B/G Substrate (CCF4-AM) or CCF2-AM Loading Kit (Cat. no. K1025 or K1032)

- LiveBLAzer™ FRET B/G Substrate (CCF4-AM) or CCF2-AM
- DMSO for Solution A
- Solution B
- Solution C

Materials Required, but not supplied

Reagent	Recommended Source	Cat. no.
Hank's Balanced Salt Solution (pH 7.3)	Invitrogen	14175-095
2.5 µM 5,6-carboxyfluorescein	Molecular Probes	C194
125 nM 7-hydroxycoumarin-3-carboxylate	Molecular Probes	H185
10 mM sodium borate buffer (pH 9.2)	Various sources	—
96-well, black-wall, clear bottom assay plate	Costar	3603

6.2 CytoFluor® 4000 fluorescence plate reader Gain Setting Calibration

6.2.1 Standards and Controls

Note: The calibration plate does not contain cells.

Prepare the following set of controls and standards (ten replicates per control or standard, 100 µl per well) in a 96-well, black-wall, clear-bottom assay plate (Costar Cat. No. 3603), as follows:

- **LiveBLAzer™ FRET B/G Control wells**. Dilute 6X LiveBLAzer™ FRET B/G Substrate Loading Solution (see **Section 4.6)** to 1X with Hank's Balanced Salt Solution (pH 7.3).
- **2.5 µM 5,6-Carboxyfluorescein** in 10 mM sodium borate buffer (pH 9.2). This is a 1:400 dilution from a 1 mM stock solution.
- **125 nM 7-Hydroxycoumarin-3-carboxylate** in 10 mM sodium borate buffer (pH 9.2). This is a 1:8000 dilution from a 1 mM stock solution.

6.2.2 Using a Calibration Plate

1. Set the fluorescence plate reader to bottom-read mode and for two scans per cycle (one scan at 460 nm and one scan at 530 nm).
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 min. before making measurements.
3. Excite the plate at 409 nm. Read the calibration plate at 10 reads/well, with an initial gain of 70 for the 460-nm read and a gain of 80 for the 530-nm read. The readings should fall within the following ranges:

Expected Ranges of Fluorescence Signal Intensities on CytoFluor® 4000 Fluorescence Plate Reader				
Solution	**LiveBLAzer™ FRET B/G Control Wells**	**125 nM 7-Hydroxycoumarin-3-carboxylate**	**LiveBLAzer™ FRET B/G Control Wells**	**2.5 µM 5,6-Carboxyfluorescein**
Detection Wavelength	460 nm	460 nm	530 nm	530 nm
Range	250–350	2200–3000	150–250	1700–3200

4. If necessary, adjust the gain settings so that the intensities of the calibration plate fall in the expected range. Once the appropriate gain settings are determined, these settings can be used routinely. Recalibration frequency depends on instrument usage.

7.0 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 CRE-*bla* CHO-K1 cell line(s) are genetically modified and the plasmid pLenti-bsd/ CRE-*bla*. 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.

CellSensor™ is a trademark of Invitrogen Corporation.
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