# CellSensor® NFAT-*bla* HEK 293T Cell-Based Assay Protocol

**Catalog no.** K1179 **Shipping:** Dry Ice **Storage:** Liquid Nitrogen
**Doc. Part no.** O-13786-r1 **Pub. no.** MAN0003091 **Revision** 2.0

## TABLE OF CONTENTS



## 1.0 MATERIALS SUPPLIED

| | |
|---|---|
| **Description:** | This cell line utilizes GeneBLAzer® beta-lactamase technology. CellSensor™ NFAT-*bla* HEK 293T cells contain a beta-lactamase reporter gene under control of the nuclear factor of activated T cells (NFAT) response element stably integrated into HEK 293T cells. This cell line can be used to detect agonists and/or antagonists of the NFAT signaling pathway, or as a parental cell line to build specific G-protein coupled receptor (GPCR) assays after transfection of additional genes of interest. NFAT-*bla* HEK 293T cells have been shown to respond to PMA + thapsigargin. |
| **Cat. no.:** | K1179 |
| **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 will quickly lose viability.** |
| **Quantity:** | ~2,000,000 cells ($2 \times 10^6$ cells/mL) |
| **Cell Line Name:** | NFAT-*bla* HEK 293T |
| **Application:** | This cell line can be used to detect agonists/antagonists of the NFAT signaling pathway or as a parental cell line to build specific G-protein coupled receptor (GPCR) assays after transfection of additional genes of interest. |
| **Growth Properties:** | Adherent |
| **Cell Phenotype:** | Epithelial |
| **Selection Marker:** | Zeocin™ (200 µg/mL) |
| **Vector Used:** | pcDNA 3XNFAT-bla-zeo vector |
| **Response Element Sequence:** | GGAGGAAAAACTGTTTCATACAGAAGGCGT 1 forward, 2 reverse |
| ***Mycoplasma* Testing:** | Negative |
| **Biosafety Level:** | 2 |

## 2.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

| Media/Reagents | Recommended Source | Cat. no. |
|---|---|---|
| LiveBLAzer™ Loading Kit containing:<br>LiveBLAzer™ FRET B/G Substrate (CCF4-AM) substrate (5 mg)<br>DMSO for Solution A<br>Solution B<br>Solution C | Life Technologies | K1030* |
| Cell Culture Freezing Medium | Life Technologies | 11101-011 |
| DMEM (high-glucose) | Life Technologies | 11965-092 |
| DMSO | Fluka | 41647 |
| Fetal bovine serum, (FBS), dialyzed, tissue-culture grade **(DO NOT SUBSTITUTE)** | Life Technologies | 26400-044 |
| Non-essential amino acids (NEAA) | Life Technologies | 11140-050 |
| Penicillin/Streptomycin | Life Technologies | 15140-122 |
| Phosphate-buffered saline without calcium and magnesium [PBS] | Life Technologies | 14190-136 |
| HEPES (1 M, pH 7.3) | Life Technologies | 15630-080 |
| Sodium pyruvate | Life Technologies | 11360-070 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma | P8139 |
| Thapsigargin | Sigma | T9033 |
| 0.05% Trypsin/EDTA | Life Technologies | 25300-054 |
| Zeocin™ antibiotic | Life Technologies | R250-01 |

* Other sizes or Loading Kits containing CCF2-AM are available.

| 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 | — |
| Internally threaded cryogenic vials, 1.8 mL | Various | — |
| Tissue culture flasks | Various | — |

| Equipment | Recommended Source |
|---|---|
| Class II biological safety cabinet | Various |
| Dual wave-length bottom-reading fluorescence plate reader | Various |
| Filters (see **Sections 4.5.1** and **5.3**) | Chroma Technologies |
| Hemacytometer (or another cell counting method) | Various |
| Humidified 37°C/5% $CO_2$ incubator | Various |
| Inverted microscope (with phase contrast capabilities) | 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 Medium (+) | Growth Medium (-)/Assay Medium |
|---|---|---|
| **DMEM** | 90% | 90% |
| **Dialyzed FBS** | 10% | 10% |
| **NEAA** | 0.1 mM | 0.1 mM |
| **Sodium pyruvate** | 1 mM | 1 mM |
| **HEPES (pH 7.3)** | 25 mM | 25 mM |
| **Penicillin** | 100 U/mL | 100 U/mL |
| **Streptomycin** | 100 µg/mL | 100 µg/mL |
| **Zeocin™** | 200 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.

## 3.2 Methods Used

Follow these methods exactly, as they have been validated specifically for optimal performance of this cell line. At first opportunity, create and store an aliquot of cells for back-up.

### *3.2.1 Thawing Method*

1. Place 14 mL of Growth Medium (-) into a T75 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 in a 37°C 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 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 (-).
8. Transfer contents to the T75 tissue culture flask containing pre-equilibrated Growth Medium (-) and place flask in the 37°C/5% $CO_2$ incubator.
9. At first passage switch to Growth Medium (+).

### *3.2.2 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 (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.

### *3.2.3 Freezing Method*

1. Harvest the cells as described in **Section 3.2.2**, Step 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.

## 4.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for determining activity of compounds as modulators (agonists or antagonists) of the NFAT signaling pathway using beta-lactamase as the readout.

### 4.1 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<br>2. 4 µL 5% DMSO stock solution<br>3. 4 µL Assay Medium | 1. 32 µL cells diluted in Assay Medium<br>2. 4 µL 5% DMSO stock solution<br>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<br>2. 4 µL 5% DMSO stock solution<br>3. 4 µL 10X stock solution of PMA + Thapsigargin | 1. 32 µL cells diluted in Assay Medium<br>2. 4 µL 5% DMSO stock solution<br>3. 4 µL 10X stock solution of PMA + Thapsigargin | 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<br>2. 4 µL 5% DMSO stock solution | 1. 36 µL Assay Medium<br>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<br>2. 4 µL 10X stock of test compounds in 5% DMSO<br>3. 4 µL Assay Medium | 1. 32 µL cells diluted in Assay Medium<br>2. 4 µL 10X stock of test compounds in 5% DMSO<br>3. 4 µL 10X stock solution of PMA + Thapsigargin | 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 Setup

### *4.2.1 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 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.

### *4.2.2 Plating Cells (Day 1)*

1. 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 156,250 cells/mL.
2. Add 36 µL per well of the Assay Medium to the Cell-Free Control wells. Add 32 µL (5,000 cells) per well of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells.

   ***Note:*** Cells should be plated one day prior to cell stimulation.

### *4.2.3 Preparation of Stock Solution (Day 2)*

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 PMA + Thapsigargin in assay media. For an antagonist screen, use ~5 nM PMA + 2 nM Thapsigargin final concentration or $EC_{80}$ (10X = 50 nM PMA, 20 nM Thapsigargin). For an agonist screen, use ~5 nM PMA + 3 nM Thapsigargin final concentration or max stim (10X = 50 nM PMA, 30 nM Thapsigargin). We recommend running a dose response curve to determine the $EC_{80}$ for your 10X PMA + Thapsigargin 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 PMA + Thapsigargin 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. If desired, incubate the test compounds with the cells at 37°C/5% $CO_2$ before proceeding. Typically, a one-hour incubation is sufficient.
4. Add 4 µL of the 10X stock solution of PMA + Thapsigargin 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.

## 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 Substrate (CCF4-AM) or CCF2-AM Loading Protocol*

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

| Reagents | Storage and Handling |
|---|---|
| **LiveBLAzer™ FRET B/G Substrate (CCF4-AM)** | The product is supplied as a dried powder. The molecular weight of CCF2-AM is 1082 g/mol; the molecular weight of LiveBLAzer™ FRET B/G Substrate (CCF4-AM) is 1096 g/mol. 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 and 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 three months. 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. |

### *4.3.2 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 vortexing.

### *4.3.3 Cell Loading*

1. Remove assay plate from incubator and allow it to equilibrate to room temperature prior to loading.
2. Add 8 µL of the 6X Loading Buffer to each well ($C_f$ of LiveBLAzer™ FRET B/G (CCF4-AM) = 1 µM).

## 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.

### *4.5.1 Instrumentation, Filters, and Plates*

- Almost all dual wavelength, bottom read fluorescence plate readers can be used to detect beta-lactamase using LiveBLAzer™ FRET B/G (CCF4-AM) or CCF2-AM.

  *Note:* If you are uncertain of whether your instrument can be used for this assay, please contact Technical Support for assistance.

- 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.
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:

| | Read 1 | Read 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 (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.

# 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.

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

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

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

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

$$\textbf{Mean Unstimulated Emission Ratio} = \frac{\textbf{Sum of Unstimulated Emission Ratios}}{\textbf{Total Number of Wells Containing Unstimulated Cells}}$$

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

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

*Note*: All Response Ratios are calculated using the Mean Unstimulated Emission Ratio. The Response Ratio determined with 5nM PMA + 3nM Thapsigargin and LiveBLAzer™ FRET B/G Substrate (CCF4-AM) was 6–8.

## 5.3 Visual Observation of Intracellular 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 are typically required to view the LiveBLAzer™ FRET B/G Substrate (CCF4-AM) signal in cells. To inspect the cells, a long-pass filter passing blue and green fluorescence light is needed so that you 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).

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### Use of Genetically Modified Organisms (GMO)

**Information for European Customers.** The NFAT-*bla* HEK 293T cell line(s) are genetically modified with the plasmid pcDNA 3XNFAT-bla-zeo 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.

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