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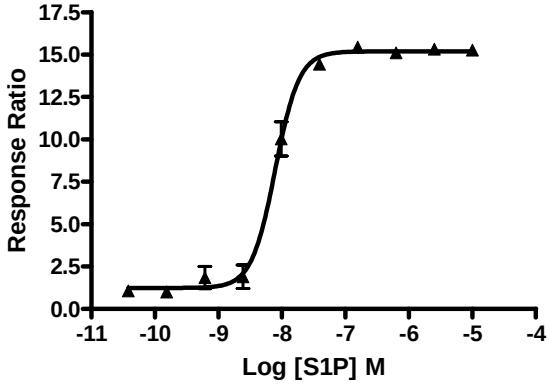
## 1. Description

Tango™ EDG1-*bla* U2OS cells contain the human Endothelial Differentiation Gene 1 (EDG1) linked to a TEV protease site and a Gal4-VP16 transcription factor stably integrated into the Tango™ GPCR-*bla* U2OS parental cell line. This parental cell line stably expresses a beta-arrestin/TEV protease fusion protein and the beta-lactamase reporter gene under the control of a UAS response element. The Tango™ EDG1-*bla* U2OS cells have been functionally validated for a response to Sphingosine-1-phosphate.




| | |
|---|---|
| **$EC_{50}$** | **7.8 nM** |
| **Z′-factor at $EC_{100}$** | **0.85** |

**Dose response of Tango™ EDG1-*bla* U2OS cells to Sphingosine-1-phosphate.**

## 2. Overview of Tango™ GPCR Cell-Based Assays

The Tango™ GPCR Assay technology combines the benefits of the Tango™ assay platform with the highly accurate, sensitive, and easy-to-use GeneBLAzer® beta-lactamase reporter system. The Tango™ assay platform is based upon ligand binding to G-Protein Coupled Receptors (GPCRs) that triggers desensitization, a process mediated by the recruitment of intracellular arrestin proteins to the activated receptor. As a result, the ligand-induced activation of GPCRs may be assayed by monitoring the interaction of arrestin with the test GPCR. A major advantage of this approach is that it does not depend on knowledge of the G-protein signaling specificity of the target receptor.

The design of the Tango™ GPCR assay is shown in Figure 1. The target GPCR is fused at its intracellular C-terminus to an exogenous transcription factor. Interposed between the receptor and the transcription factor is a specific cleavage sequence for a non-native protease. This chimeric receptor protein is expressed in a cell line containing the *bla* reporter gene responsive to the transcription factor. The cell line also expresses an arrestin-protease fusion protein that recognizes and cleaves the site between the receptor and transcription factor. The assay is performed by adding a ligand to the growing cells for a defined period and measuring the activity of the reporter gene. Activation of the reporter gene provides a quantifiable measurement of the degree of interaction between the target receptor and the protease-tagged arrestin partner. Additionally, it is unaffected by other signaling pathways in the cell, thus providing a highly selective readout of target receptor activation.

**Figure 1.**

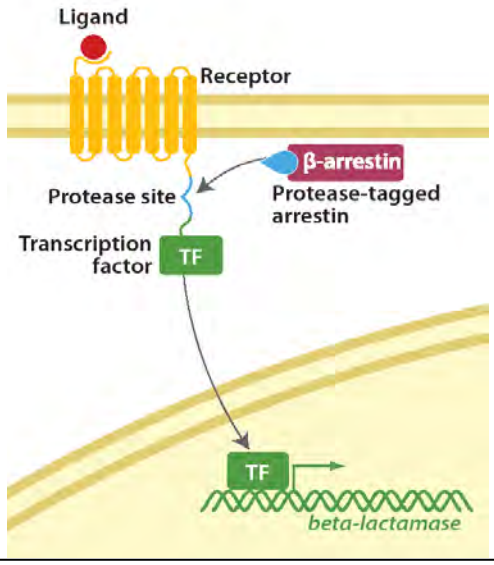



**Figure 1—Tango™ GPCR cell-based beta-arrestin signaling.** Upon ligand binding and receptor activation, a protease-tagged beta-arrestin molecule is recruited to the GPCR that has been modified at the C-terminus to include a transcription factor linked by a protease cleavage site. The protease cleaves the transcription factor from the GPCR, which translocates to the nucleus and activates the expression of beta-lactamase.

**Figure 2.**

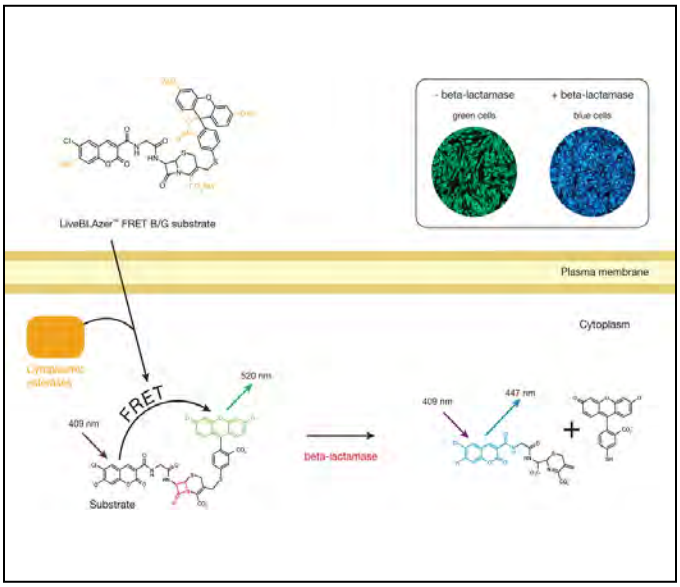



**Figure 2—Sensitive fluorescent detection of beta-lactamase reporter gene response using GeneBLAzer® technology.** After loading the cell permeable LiveBLAzer™ FRET B/G substrate, cellular fluorescence is measured. In the absence of betalactamase expression, cells generate green fluorescence. In the presence of beta-lactamase expression, the substrate is cleaved and the cells generate blue fluorescence.

The Tango™ assay technology uses a mammalian-optimized beta-lactamase (*bla*) reporter gene combined with a FRET-enabled substrate to provide reliable and sensitive detection in cells (1) (Figure 2). Cells are loaded with an engineered fluorescent substrate containing two fluorophores, coumarin and fluorescein. In the absence of *bla* expression, the substrate molecule remains intact. In this state, excitation of the coumarin results in fluorescence resonance energy transfer to the fluorescein moiety and emission of green fluorescent light. However, in the presence of *bla* expression, the substrate is cleaved separating the fluorophores and disrupting energy transfer. Excitation of the coumarin in the presence of *bla* enzyme activity results in a blue fluorescence signal.

The resulting coumarin:fluorescein ratio provides a normalized reporter response that can minimize experimental noise that masks subtle underlying biological responses of interest. The Tango™ assay technology has been proven 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).

## 3. Materials Supplied

| Product: | Name | Size | Catalog # |
|---|---|---|---|
| | **Tango™ EDG1-*bla* U2OS cells**<br>Includes:<br>• Tango™ EDG1-*bla* U2OS cells (K1520B)<br>• Protocol<br>• Certificate of Analysis | 1 tube | K1520 |
| **Shipping Condition:** | Dry ice | | |
| **Storage Condition of Cells:** | 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.** | | |
| **Growth Properties of Cells:** | Adherent | | |
| **Cell Phenotype:** | Epithelial | | |
| **Selection Marker(s) for Cells:** | Zeocin™ 200 µg/ml, Geneticin® 100 µg/ml, and Hygromycin 50 µg/ml | | |
| ***Mycoplasma* Testing:** | Negative | | |
| **BioSafety Level:** | 1 | | |

## 4. Materials Required

Use the table below to determine the additional media and reagents required for use with each kit:

| Media/Reagents | Recommended Source | Part # |
|---|---|---|
| LiveBLAzer™-FRET B/G Loading Kit**:**<br>LiveBLAzer™-FRET B/G Substrate (CCF4-AM)<br>DMSO for Solution A<br>Solution B<br>Solution C | Invitrogen | K1427 (70 µg)<br>K1095 (200 µg)<br>K1096 (1 mg)<br>K1030 (5 mg) |
| Solution D | Invitrogen | K1156 (1 ml)<br>K1157 (25 ml) |
| Recovery™ Cell Culture Freezing Medium | Invitrogen | 12648-010 |
| FreeStyle™ Expression Medium | Invitrogen | 12338-018 |
| McCoy's 5A Medium (modified) (1X) | Invitrogen | 16600-082 |
| DMSO | Fluka | 41647 |
| Fetal bovine serum (FBS), dialyzed, (**DO NOT SUBSTITUTE!**) | Invitrogen | 26400-036 |
| Non-essential amino acids (NEAA) | Invitrogen | 11140-050 |
| Penicillin/Streptomycin (antibiotics) | Invitrogen | 15140-122 |
| Sodium Pyruvate | Invitrogen | 11360-070 |
| Phosphate-buffered saline without calcium and magnesium [PBS(-)] | Invitrogen | 14190-136 |
| HEPES (1 M, pH 7.3) | Invitrogen | 15630-080 |
| 0.05% Trypsin/EDTA | Invitrogen | 25300-054 |
| Sphingosine-1-phosphate | Avanti Polar Lipids | 860492P |
| Zeocin™ | Invitrogen | R250-01 |
| Hygromycin | Invitrogen | 10687-010 |
| Geneticin® | Invitrogen | 10131-027 |

The following table lists additional items required for use with all kits:

| Consumables | Recommended Source | Part # |
|---|---|---|
| Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background) | Corning | 3712 |
| Compressed air | Various | --- |
| **Equipment** | **Recommended Source** | |
| Fluorescence plate reader with bottom-read capabilities | Various | |
| Filters if required for plate reader (see **Section 7.4.1**) | Chroma Technologies | |
| **Optional:** Epifluorescence- or fluorescence-equipped microscope, with appropriate filters | Various | |
| **Optional:** Microplate centrifuge | Various | |

# 5. Detailed Cell Handling Procedures

*Note:* Refer to **Section 6, Media Requirements** for specific media recipes.

## 5.1 Thawing Method

*Note:* Cells are shipped to you on dry ice and as such may require a short period of time prior to full recovery and normal growth.

1. Place 30 ml of Thawing Medium into a T225 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 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 drop-wise into 10 ml of Thawing 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 Thawing Medium.
8. Transfer contents to the T225 tissue culture flask containing pre-equilibrated Thawing Medium and place flask in the humidified 37°C/5% $CO_2$ incubator.
9. At first passage, switch to Growth Medium.

## 5.2 Propagation Method

1. Passage or feed cells at least twice a week. Maintain cells between 25% and 95% confluence. Do not allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once in PBS, add 0.05% Trypsin/EDTA (3 ml for a T75 flask, 5 ml for a T175 flask, and 7 ml for T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2–5 minutes exposure to 0.05% Trypsin/EDTA. Add an equal volume of Growth Medium to inactivate 0.05% Trypsin/EDTA.
3. Verify under a microscope that cells have detached and clumps have completely dispersed.
4. Centrifuge cells at 200 × g for 5 minutes and resuspend in Growth Medium.

## 5.3 Freezing Method

1. Harvest the cells as described in **Subsection 5.2.2** (above), Step 2. After detachment, count the cells, centrifuge cells at 200 × *g* for 5 minutes, and resuspend in 4°C Freeze Medium to a density of 8 x $10^6$ 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.

# 6. Media Requirements

## 6.1 Media Required

*Note:* Unless otherwise stated, have all media and solutions at least at room temperature (we recommend 37°C for optimal performance) before adding to cells.

*Note:* Make **NO MEDIA SUBSTITUTIONS**, as these cell lines have been specifically validated for optimal assay performance with these media. For dividing cells, we recommend that you create and store an aliquot for back up.

| Component | Assay Medium | Growth Medium | Thawing Medium | Freeze Medium |
|---|---|---|---|---|
| **FreeStyle™ Expression Medium** | 100% | — | — | — |
| **McCoy's 5A Medium** | — | 90% | 90% | — |
| **Dialyzed FBS (Do not substitute!)** | — | 10% | 10% | — |
| **NEAA** | — | 0.1 mM | 0.1 mM | — |
| **HEPES (pH 7.3)** | — | 25 mM | 25 mM | — |
| **Sodium Pyruvate** | — | 1 mM | 1 mM | — |
| **Penicillin (antibiotic)** | — | 100 U/ml | 100 U/ml | — |
| **Streptomycin (antibiotic)** | — | 100 µg/ml | 100 µg/ml | — |
| **Recovery™ Cell Culture Freezing Medium** | — | — | — | 100% |
| **Zeocin™** | — | 200 µg/ml | — | — |
| **Hygromycin** | — | 50 µg/ml | — | — |
| **Geneticin®** | — | 100 µg/ml | — | — |

# 7. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of EDG1 using LiveBLAzer™-FRET B/G Substrate as the readout. If alternative substrates are used (*e.g.*, ToxBLAzer™ DualScreen or LyticBLAzer™ Loading kits), follow the loading protocol provided with the product.

## 7.1 Quick Assay Reference Guides

For a more detailed assay protocol, see **Section 7.2**.

**Agonist Assay Quick Reference Guide**

| | Unstimulated Wells | Stimulated Wells | Cell-free Wells | Test Compound Wells |
|---|---|---|---|---|
| **Step 1** Plate cells, incubate | 32 µl cells in Assay Medium (10,000 cells/well) | 32 µl cells in Assay Medium (10,000 cells/well) | 32 µl Assay Medium (no cells) | 32 µl cells in Assay Medium (10,000 cells/well) |
| | Incubate cells for 48 hrs. at 37°C/ 5%$CO_2$ | | | |
| **Step 2** Add Agonist or Test Compounds | 8 µl Assay Medium with 0.5% DMSO | 8 µl 5X agonist in Assay Medium with 0.5% DMSO | 8 µl Assay Medium with 0.5% DMSO | 8 µl 5X Test Compounds in 0.5% DMSO |
| **Step 3** Incubate cells | Incubate in a humidified 37°C/5% $CO_2$ incubator for 5 hours | | | |
| **Step 4** Prepare 6X Substrate Mix | 6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 904 µl of Solution C, mix. Add 30 µl of Solution D, mix. | | | |
| **Step 5** Add Substrate Mixture | 8 µl per well | | | |
| **Step 6** Incubate Substrate Mix + cells | 2 hours at room temperature in the dark | | | |
| **Step 7** Detect activity | See **Section 7.4** | | | |
| **Step 8** Analyze data | See **Section 8** | | | |

**Antagonist Assay Quick Reference Guide**

| | Unstimulated Wells | Stimulated Wells | Antagonist Control Wells | Cell-free Wells | Test Compound Wells |
|---|---|---|---|---|---|
| **Step 1** Plate cells, incubate | 32 µl cells in Assay Medium (10,000 cells/well) | 32 µl cells in Assay Medium (10,000 cells/well) | 32 µl cells in Assay Medium (10,000 cells/well) | 32 µl Assay Medium (no cells) | 32 µl cells in Assay Medium (10,000 cells/well) |
| | Incubate cells for 48 hrs. at 37°C/ 5%$CO_2$ | | | | |
| **Step 2** Add Antagonist or Test Compounds, incubate | 4 µl Assay Medium with 0.5% DMSO | 4 µl Assay Medium with 0.5% DMSO | 4 µl 10X antagonist in Assay Medium with 0.5% DMSO | 4 µl Assay Medium with 0.5% DMSO | 4 µl 10X Test Compounds in Assay Medium with 0.5% DMSO |
| | Incubate plate with Antagonist for 30 minutes before proceeding | | | | |
| **Step 3** Add Agonist | 4 µl Assay Medium with 0.5% DMSO | 4 µl 10X agonist in Assay Medium with 0.5% DMSO | 4 µl 10X agonist in Assay Medium with 0.5% DMSO | 4 µl 10X agonist in Assay Medium with 0.5% DMSO | 4 µl 10X agonist in Assay Medium with 0.5% DMSO |
| **Step 4** Incubate cells | Incubate in a humidified 37°C/5% $CO_2$ incubator for 5 hours | | | | |
| **Step 5** Prepare 6X Substrate Mix | 6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 904 µl of Solution C, mix. Add 30 µl of Solution D, mix. | | | | |
| **Step 6** Add Substrate Mixture | 8 µl per well | | | | |
| **Step 7** Incubate Mixture | 2 hours at room temperature in the dark | | | | |
| **Step 8** Detect activity | See **Section 7.4** | | | | |
| **Step 9** Analyze data | See **Section 8** | | | | |

## 7.2 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, Stimulated Control, and Cell-free Control.

*Note:* Some solvents may affect assay performance. Assess the effects of solvent before screening. The cell stimulation procedure described below is carried out in the presence of 0.1% DMSO to simulate the effect that a Test Compound's solvent might have on the assay. If you use other solvents and/or solvent concentrations, optimize the following assay conditions appropriately.

### *7.2.1 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 (for 1 minute at 14 × *g*) after additions to ensure all assay components are on the bottom of the wells.

### *7.2.2 Plating Cells*

1. Harvest cells and resuspend in Assay Medium to a density of 312,500 cells/ml.
2. Add 32 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. Incubate cells at 37°C/ 5% $CO_2$ for 48 hours. Proceed to **Section 7.2.3** for an Agonist assay or **Section 7.2.4** for an Antagonist assay.

### *7.2.3 Agonist Assay Plate Setup*

*Note:* This subsection provides directions for performing an Agonist assay. See **Section 7.2.4** for directions for performing an Antagonist assay.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 5X stock of Test Compounds in Assay Medium with 0.5% DMSO.
3. Prepare a 5X stock of agonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal concentration of the agonist solution.
4. Add 8 µl of the stock solution of 0.5% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
5. Add 8 µl of the 5X stock solution of agonist to the Stimulated Control wells.
6. Add 8 µl of the 5X stock of Test Compounds to the Test Compound wells.
7. Incubate the Agonist assay plate in a humidified 37°C/5% $CO_2$ incubator for 5 hours. Then proceed to **Section 7.3** for Substrate Loading and Incubation.

### *7.2.4 Antagonist Assay Plate Setup*

*Note:* This subsection provides directions for performing an Antagonist assay. See **Section 7.2.3** for directions for performing an Agonist assay.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 10X stock of Test Compounds in Assay Medium with 0.5% DMSO.
3. Prepare a 10X stock of agonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal agonist concentration. For antagonist assays, we recommend stimulating cells initially with an agonist concentration in the $EC_{50}$-$EC_{80}$ range.
4. Prepare a 10X stock of antagonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal inhibition concentration for the Antagonist solution.
5. Add 4 µl of the 10X stock of Test Compounds to the Test Compound wells.
6. Add 4 µl of the stock solution of 0.5% DMSO to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-free Control wells.
7. Add 4 µl of the 10X stock of antagonist in Assay Medium with 0.5% DMSO to the Antagonist Control wells.
8. If desired, incubate the Test Compounds with the cells humidified 37°C/5% $CO_2$ incubator before proceeding. Typically, a 30-minute incubation is sufficient.
9. Add 4 µl of the 10X stock solution of agonist to the Test Compound wells, the Stimulated Control wells, and the Antagonist Control wells.
10. Add 4 µl of Assay Medium with 0.5% DMSO to the Unstimulated Control and Cell-free Control wells.

11. Incubate the Antagonist assay plate in a humidified 37°C/5% $CO_2$ incubator for 5 hours. Then proceed to **Section 7.3** for Substrate Loading and Incubation.

## 7.3 Substrate Preparation, Loading and Incubation

This protocol is designed for loading cells with LiveBLAzer™-FRET B/G Substrate Mixture (CCF4-AM) Substrate Mixture. If you use alternative substrates, follow the loading protocol provided with the substrate.

Prepare LiveBLAzer™-FRET B/G Substrate Mixture (CCF4-AM) Substrate Mixture and load cells 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) Substrate Mixture in dry DMSO by adding 912 µl of DMSO per mg of dry substrate. Store the aliquots of the stock solution at –20ºC until use. The molecular weight of the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) is 1096 g/mol.
2. Prepare 6X Loading Solution:
   a. Add 6 µl of Solution A to 60 µl of Solution B and vortex.
   b. Add 904 µl of Solution C to the above solution and vortex.
   c. Add 30 µl of Solution D to the above solution and vortex.
3. Remove assay plate from the humidified 37°C/5% $CO_2$ incubator.

   ***Note:*** Handle the plate gently and do not touch the bottom.
4. Add 8 µl of the 6X Substrate Mixture to each well.
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 2 hours.

## 7.4 Detection

Make measurements 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.

### *7.4.1 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

### *7.4.2 Reading an Assay Plate*

1. Set the fluorescence plate reader to bottom-read mode with optimal gain and 5 reads.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before making measurements.
3. Use the following filter selections:

| | Scan 1 | Scan 2 |
|---|---|---|
| **Purpose:** | Measure fluorescence in the Blue channel | Measure FRET signal in the Green channel |
| **Excitation filter:** | 409/20 nm | 409/20 nm |
| **Emission filter:** | 460/40 nm | 530/30 nm |

# 8. Data Analysis

## 8.1 Background Subtraction and 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 Control wells. These Control wells are used for background subtraction.
2. Determine the average emission from the Cell-free Control 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 the Blue/Green Emission Ratio for each well, by dividing the background-subtracted Blue emission values by the background-subtracted Green emission values.

## 8.2 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 with either a xenon or mercury excitation lamp may be used 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) |
| 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).

## 9. 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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7. Whitney M, Rockenstein E, Cantin G, Knapp T, Zlokarnik G, Sanders P, Durick K, Craig FF, Negulescu PA., **A Genome-wide Functional Assay of Signal Transduction in Living Mammalian Cells,** (1998) *Nat. Biotechnol.*;**16**: p1329-1333.

## 10. Purchaser Notification

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## Use of Genetically Modified Organisms (GMO)

**Information for European Customers** The Tango™ EDG1-*bla* U2OS DA and Tango™ EDG1-*bla* U2OS cell lines are genetically modified with the plasmids pTango™ β-Arr/TEV, pLenti-zeo/UAS-bla (note this construct was utilized as a plasmid not as a lentiviral stock) and pTangoEDG1. 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.