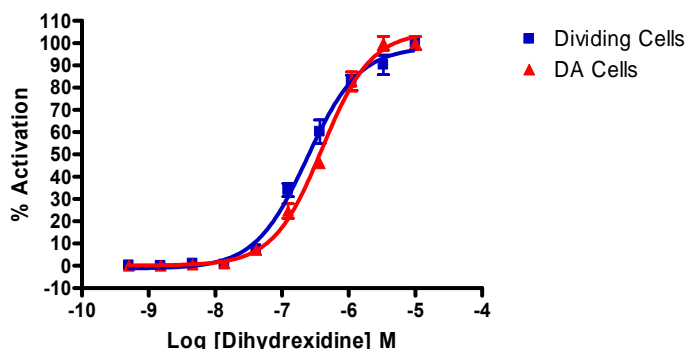


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## 1. Description

Tango™ D1-*bla* U2OS DA (division-arrested) cells and dividing cells contain the human Dopamine Receptor 1 (D1) 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™ D1-*bla* U2OS cells have been functionally validated for a response to Dihydroxidine.



|                                      | Division-arrested cells | Dividing Cells |
|--------------------------------------|-------------------------|----------------|
| <b>EC<sub>50</sub></b>               | <b>400 nM</b>           | <b>240 nM</b>  |
| <b>Z'-factor at EC<sub>100</sub></b> | <b>0.77</b>             | <b>0.76</b>    |

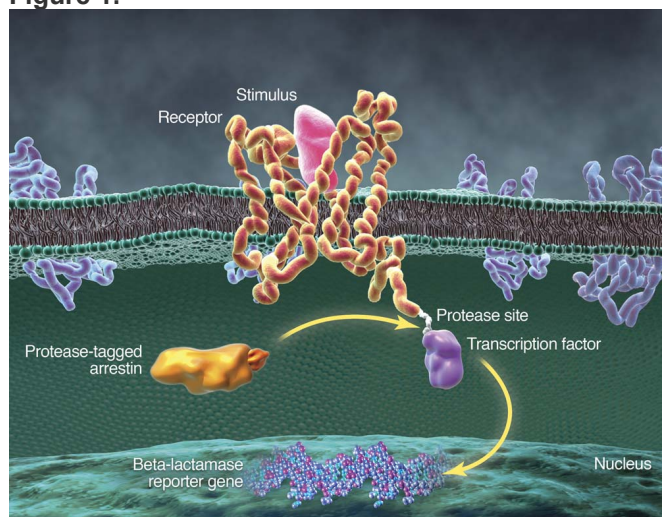
Dose response of Tango™ D1-*bla* U2OS DA cells and dividing cells to Dihydroxidine.

## 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 (*bla*) 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.

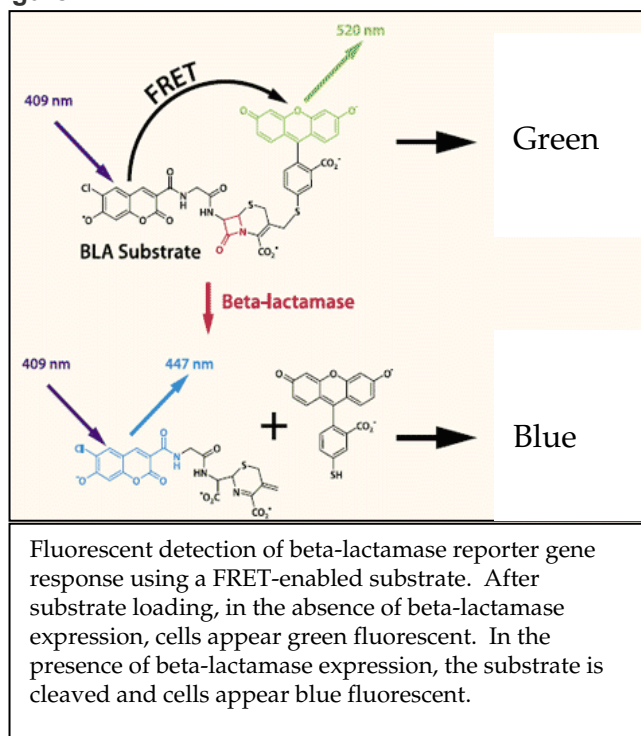
The design of the Tango GPCR assay is shown in Figure 1. The target GPCR is fused at its intracellular C-terminus to an exogenous transcription factor. Interposed between the receptor and the transcription factor is a specific cleavage sequence for a non-native protease. This chimeric receptor protein 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.



Upon ligand binding and receptor activation, a protease-tagged beta-arrestin 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 in turn cleaves the transcription factor from the GPCR, the transcription factor immediately translocates to the nucleus, and beta-lactamase activity is activated.

Figure 2.



Fluorescent detection of beta-lactamase reporter gene response using a FRET-enabled substrate. After substrate loading, in the absence of beta-lactamase expression, cells appear green fluorescent. In the presence of beta-lactamase expression, the substrate is cleaved and cells appear blue fluorescent.

The Tango™ assay technology uses a mammalian-optimized *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™ beta-lactamase reporter technology has been proven in high-throughput screening (HTS) campaigns for a range of target classes, including GPCRs (2, 3), nuclear receptors (4-6) and kinase signaling pathways (7). The utility of division-arrested cells in HTS has also been demonstrated (8-11).

### 3. Overview of Dividing and Division-Arrested Cells

Many of Invitrogen's cell lines are available in two forms: dividing or division arrested. Invitrogen's division-arrest technology allows the use of frozen cells, made from the exact same cell line sold in its dividing form, as ordinary, cost-effective assay reagents for screening. Division-arrested (DA) cells exhibit response profiles similar to those of dividing cells, thus ensuring that you obtain the correct pharmacological profile.

DA cells may be plated and assayed within 24 hours of thawing. Cell numbers for DA cells increase only marginally after plating, thereby removing the variability caused by cell division during the course of an assay and providing more consistent results.

### 4. Materials Supplied

| Product:<br>(One of these)                                  | Name   | Size    | Catalog # |
|---|--|---------|-----------|
|   | <b>Tango™ D1-<i>bla</i> U2OS DA Assay Kit</b><br>Each system contains sufficient division-arrested cells and substrate to assay one 384-well plate. Includes: <ul style="list-style-type: none"> <li>• Tango™ D1-<i>bla</i> U2OS DA cells (K1505A)</li> <li>• LiveBLAzer™ -FRET B/G Loading Kit, 70 µg</li> <li>• Solution D, 1 ml</li> <li>• Protocol</li> <li>• Certificate of Analysis</li> </ul> | 1 plate | K1505     |
|   | <b>Tango™ D1-<i>bla</i> U2OS cells</b><br>Includes: <ul style="list-style-type: none"> <li>• Tango™ D1-<i>bla</i> U2OS cells (K1485A)</li> <li>• Protocol</li> <li>• Certificate of Analysis</li> </ul>  | 1 tube  | K1485     |
| <b>Shipping Condition:</b>                                  | Dry ice  |         |           |
| <b>Storage Condition of Cells:</b>                          | Liquid nitrogen. <b>Immediately upon receipt, cells must be stored in liquid nitrogen or thawed for immediate use. Cells stored at -80°C can quickly lose viability.</b>   |         |           |
| <b>Growth Properties of Non-Division-arrested Cells:</b>    | Adherent   |         |           |
| <b>Cell Phenotype:</b>                                      | Epithelial   |         |           |
| <b>Selection Marker(s) for Non-Division arrested cells:</b> | Zeocin™ 200 µg/ml, Geneticin® 100 µg/ml, and Hygromycin 50 µg/ml   |         |           |
| <b>Mycoplasma Testing:</b>                                  | Negative   |         |           |
| <b>BioSafety Level:</b>                                     | 1  |         |           |

## 5. Materials Required

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

| Media/Reagents   | Recommended Source | Part #  | Required Separately?                            |  |
|--|--------------------|---|---|--|
|  |                    |   | Tango™ D1- <i>bla</i> U2OS DA Assay Kit (K1505) | Tango™ D1- <i>bla</i> U2OS cells (K1485) |
| 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) | No (included in kit)                            | Yes                                      |
| Solution D   | Invitrogen         | K1156 (1 ml)<br>K1157 (25 ml)                                   | No (included in kit)                            | Yes                                      |
| Recovery™ Cell Culture Freezing Medium   | Invitrogen         | 12648-010   | No  | Yes                                      |
| FreeStyle™ Expression Medium   | Invitrogen         | 12338-018   | Yes   | Yes                                      |
| McCoy's 5A Medium (modified) (1X)  | Invitrogen         | 16600-082   | No  | Yes                                      |
| DMSO   | Fluka              | 41647   | Yes   | Yes                                      |
| Fetal bovine serum (FBS), dialyzed, (DO NOT SUBSTITUTE!)   | Invitrogen         | 26400-036   | No  | Yes                                      |
| Non-essential amino acids (NEAA)   | Invitrogen         | 11140-050   | No  | Yes                                      |
| Penicillin/Streptomycin (antibiotics)  | Invitrogen         | 15140-122   | No  | Yes                                      |
| Sodium Pyruvate  | Invitrogen         | 11360-070   | No  | Yes                                      |
| Phosphate-buffered saline without calcium and magnesium [PBS(-)]   | Invitrogen         | 14190-136   | No  | Yes                                      |
| HEPES (1 M, pH 7.3)  | Invitrogen         | 15630-080   | No  | Yes                                      |
| 0.05% Trypsin/EDTA   | Invitrogen         | 25300-054   | No  | Yes                                      |
| Dihydraxidine  | Tocris             | 884   | Yes   | Yes                                      |
| Zeocin™  | Invitrogen         | R250-01   | No  | Yes                                      |
| Hygromycin   | Invitrogen         | 10687-010   | No  | Yes                                      |
| Geneticin®   | Invitrogen         | 10131-027   | No  | Yes                                      |

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 8.4.1)                           | Chroma Technologies |        |

### 5.1 Optional Equipment and Materials

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

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## 6. Detailed Cell Handling Procedures

**Note:** DA cells have different thawing procedures than dividing cells. Refer to the instructions below for your particular application.

**Note:** Refer to **Section 7, Media Requirements** for specific media recipes.

### 6.1 DA Cells Thawing Method

**Note:** Once cells are thawed per the instructions below, cells must be counted and the density adjusted to the appropriate level as specified in **Section 8.2.2, Assay Procedure**, prior to analysis.

1. Rapidly thaw the vial of cells by placing at 37°C in a water bath with gentle agitation for 1–2 minutes. Do not submerge vial in water.
2. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
3. Transfer the vial contents drop-wise into 10 ml of Assay Medium in a sterile 15-ml conical tube.
4. Centrifuge cells at 200 × *g* for 5 minutes.
5. Aspirate supernatant and resuspend the cell pellet in 1 ml fresh Assay Medium. (For the 1 × 384-well plate size of cells, dilute in 1 ml.)
6. Count the cells.
7. Adjust the cell density with Assay Medium to the appropriate cell density as specified in Section 7. Proceed to **Section 8, Assay Procedure**, for guidance on using cells in an assay.

### 6.2 Dividing Cells

#### 6.2.1 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 14 ml of Thawing Medium into a T75 flask.
2. Place the flask in a humidified 37°C/5% CO<sub>2</sub> 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 T75 tissue culture flask containing pre-equilibrated Thawing Medium and place flask in the humidified 37°C/5% CO<sub>2</sub> incubator.
9. At first passage, switch to Growth Medium.

#### 6.2.2 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.

#### 6.2.3 Freezing Method

1. Harvest the cells as described in **Subsection 6.2.2** (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 2E6 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.

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4. Transfer to liquid nitrogen the next day for storage.

## 7. Media Requirements

**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<br>(DA and dividing<br>cells) | Growth Medium<br>(dividing cells only) | Thawing Medium<br>(dividing cells only) | Freeze Medium<br>(dividing cells<br>only) |
|---|--|--|---|---|
| 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<br>Medium | —  | —                                      | —                                       | 100%                                      |
| Zeocin™                                   | —  | 200 µg/ml                              | —                                       | —   |
| Hygromycin                                | —  | 50 µg/ml                               | —                                       | —   |
| Geneticin®                                | —  | 100 µg/ml                              | —                                       | —   |

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## 8. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of D1 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.

### 8.1 Quick Assay Reference Guides

For a more detailed assay protocol, see **Section 8.2**.

#### Agonist Assay Quick Reference Guide

|   | Unstimulated Wells   | Stimulated Wells                                | Cell-free Wells                  | Test Compound Wells                             |
|---|--|---|----------------------------------|---|
| <b>Step 1</b><br>Plate cells, incubate                    | 32 µl cells in Assay Medium (15,000 cells/well)  | 32 µl cells in Assay Medium (15,000 cells/well) | 32 µl Assay Medium (no cells)    | 32 µl cells in Assay Medium (15,000 cells/well) |
| Incubate cells for 16-24 hrs. at 37°C / 5%CO <sub>2</sub> |  |   |                                  |   |
| <b>Step 2</b><br>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             |
| <b>Step 3</b><br>Incubate cells                           | Incubate in a humidified 37°C/5% CO <sub>2</sub> incubator for 5 hours   |   |                                  |   |
| <b>Step 4</b><br>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. |   |                                  |   |
| <b>Step 5</b><br>Add Substrate Mixture                    | 8 µl per well  |   |                                  |   |
| <b>Step 6</b><br>Incubate Substrate Mix. + cells          | 2 hours at room temperature in the dark  |   |                                  |   |
| <b>Step 7</b><br>Detect activity                          | See <b>Section 8.4</b>   |   |                                  |   |
| <b>Step 8</b><br>Analyze data                             | See <b>Section 9</b>   |   |                                  |   |

#### Antagonist Assay Quick Reference Guide

|   | Unstimulated Wells   | Stimulated Wells                                | Antagonist Control Wells                           | Cell-free Wells                                 | Test Compound Wells                                    |
|---|--|---|--|---|--|
| <b>Step 1</b><br>Plate cells, incubate                          | 32 µl cells in Assay Medium (15,000 cells/well)  | 32 µl cells in Assay Medium (15,000 cells/well) | 32 µl cells in Assay Medium (15,000 cells/well)    | 32 µl Assay Medium (no cells)                   | 32 µl cells in Assay Medium (15,000 cells/well)        |
| Incubate cells for 16-24 hrs. at 37°C / 5%CO <sub>2</sub>       |  |   |  |   |  |
| <b>Step 2</b><br>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 |  |   |  |   |  |
| <b>Step 3</b><br>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        |
| <b>Step 4</b><br>Incubate cells                                 | Incubate in a humidified 37°C/5% CO <sub>2</sub> incubator for 5 hours   |   |  |   |  |
| <b>Step 5</b><br>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. |   |  |   |  |
| <b>Step 6</b><br>Add Substrate Mixture                          | 8 µl per well  |   |  |   |  |
| <b>Step 7</b><br>Incubate Mixture                               | 2 hours at room temperature in the dark  |   |  |   |  |
| <b>Step 8</b><br>Detect activity                                | See <b>Section 8.4</b>   |   |  |   |  |
| <b>Step 9</b><br>Analyze data                                   | See <b>Section 9</b>   |   |  |   |  |

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## 8.2 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.

### 8.2.1 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  $14 \times g$ ) after additions to ensure all assay components are on the bottom of the wells.

### 8.2.2 Plating Cells

1. Thaw DA cells/harvest dividing cells and resuspend in Assay Medium to a density of 468,750 cells/ml.
2. Add 32  $\mu$ l per well of the Assay Medium to the Cell-free Control wells. Add 32  $\mu$ l 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<sub>2</sub> for 16-24 hours. Proceed to **Section 8.2.3** for an Agonist assay or **Section 8.2.4** for an Antagonist assay.

### 8.2.3 Agonist Assay Plate Setup

**Note:** This subsection provides directions for performing an Agonist assay. See **Section 8.2.4** 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  $\mu$ l of the stock solution of 0.5% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
5. Add 8  $\mu$ l of the 5X stock solution of agonist to the Stimulated Control wells.
6. Add 8  $\mu$ l 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<sub>2</sub> incubator for 5 hours. Then proceed to **Section 8.3** for Substrate Loading and Incubation.

### 8.2.4 Antagonist Assay Plate Setup

**Note:** This subsection provides directions for performing an Antagonist assay. See **Section 8.2.3** 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<sub>50</sub>-EC<sub>80</sub> 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  $\mu$ l of the 10X stock of Test Compounds to the Test Compound wells.
6. Add 4  $\mu$ l 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  $\mu$ l 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<sub>2</sub> incubator before proceeding. Typically, a 30-minute incubation is sufficient.
9. Add 4  $\mu$ l of the 10X stock solution of agonist to the Test Compound wells, the Stimulated Control wells, and the Antagonist Control wells.
10. Add 4  $\mu$ l of Assay Medium with 0.5% DMSO to the Unstimulated Control and Cell-free Control wells.

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11. Incubate the Antagonist assay plate in a humidified 37°C/5% CO<sub>2</sub> incubator for 5 hours. Then proceed to **Section 8.3** for Substrate Loading and Incubation.

### 8.3 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 -20°C 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<sub>2</sub> 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.

### 8.4 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.

#### 8.4.1 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

#### 8.4.2 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                                   |
|---------------------------|--|--|
| <b>Purpose:</b>           | Measure fluorescence in the Blue channel | Measure FRET signal in the Green channel |
| <b>Excitation filter:</b> | 409/20 nm                                | 409/20 nm                                |
| <b>Emission filter:</b>   | 460/40 nm                                | 530/30 nm                                |

## 9. Data Analysis

---

### 9.1 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.

### 9.2 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](http://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).

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