

Tango™ MLNR-*bla* U2OS Cell-Based Assay

Catalog Number K1862

Doc. Part No. K1862.pps Pub. No. MAN0003027 Rev. 2.0

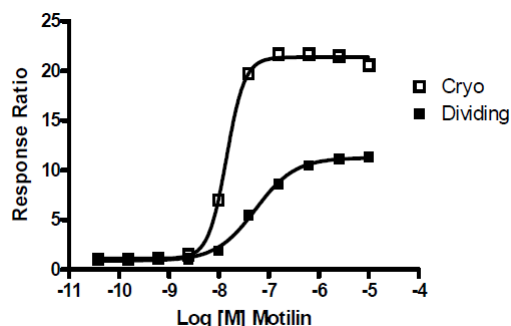
WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

Tango™ MLNR-*bla* U2OS cells contain the human Motilin Receptor linked to a TEV protease site and a Ga14-VP16 transcription factor stably integrated into the Tango™ GPCR-*bla* U2OS 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™ MLNR-*bla* U2OS cells have been functionally validated for a response to Motilin.

Table 1 Dose response of Tango™ MLNR-*bla* U2OS cells to Motilin

Parameter	Response
EC ₅₀	54 nM
Z'-factor at EC ₁₀₀	0.60 0.80



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-protase 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.

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 (Zlokarnik, 1998) (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) (Kunapuli, 2003; Xing, 2000), nuclear receptors (Qureshi, 2003; Peekhaus, 2003; Chin, 2003), and kinase signaling pathways (Whitney, 1998).

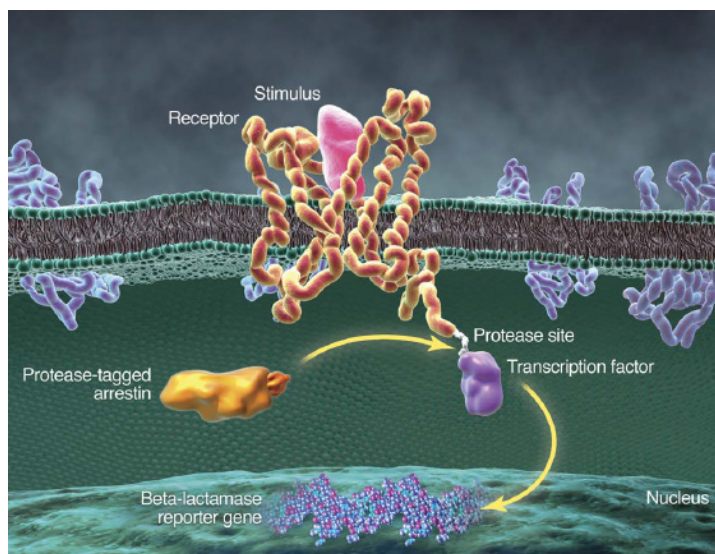


Figure 1 Tango™ GPCR assay design

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.

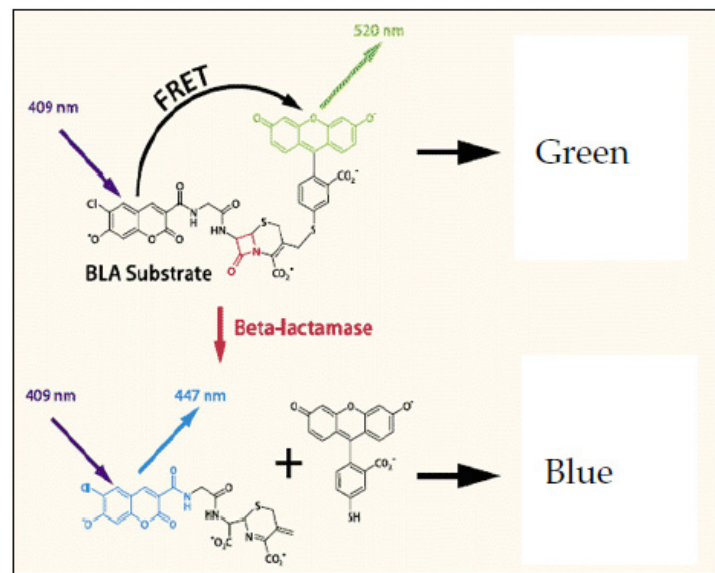


Figure 2 Fluorescent detection of beta-lactamase reporter gene response using a FRET-enabled substrate

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.

Contents and storage

Table 2 Tango™ MLNR-*bla* U2OS cells (Cat. No. K1862)

Contents	Amount	Storage
Tango™ MLNR- <i>bla</i> U2OS cells (Cat. No. K1862A)	1 tube	Liquid nitrogen, vapor phase
Protocol and Certificate of Analysis	—	

IMPORTANT! Cells are shipped overnight on dry ice. Immediately upon receipt, either thaw for immediate use or store in liquid nitrogen, vapor phase. Cells stored for more than 1 day at -80°C quickly lose viability.

Product details

Feature	Details
Shipping condition	Dry ice overnight
Growth properties of cells	Adherent
Cell phenotype	Epithelial
Selection marker(s) for cells	<ul style="list-style-type: none"> • Zeocin™ 200 µg/mL • Geneticin™ 100 µg/mL • Hygromycin-B 50 µg/mL
<i>Mycoplasma</i> testing	Negative
Biosafety level	1

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Item	Source
Equipment	
Fluorescence plate reader with bottom-read capabilities	MLS
Filters (if required for the plate reader)	Chroma Technologies
(Optional) Mr. Frosty™ Freezing Container	5100-0001
(Optional) Epifluorescence- or fluorescence-equipped microscope (with appropriate filters)	MLS
(Optional) Microplate centrifuge	MLS
Consumables	
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Corning 3764
Compressed air	MLS

Item	Source
Reagents	
LiveBLazer™-FRET B/G Loading Kit: <ul style="list-style-type: none"> • LiveBLazer™-FRET B/G Substrate (CCF4-AM) (Cat. No. K1089)^[1] • DMSO for Solution A • Solution B • Solution C 	K1095 ^[2]
Solution D	K1156
Recovery™ Cell Culture Freezing Medium	12648010
FreeStyle™ 293 Expression Medium	12338018
McCoy's 5A (Modified) Medium	16600082
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (Do Not Substitute.)	26400036
MEM Non-Essential Amino Acids Solution (100X)	11140050
DPBS, no calcium, no magnesium	14190136
Sodium Pyruvate (100 mM)	11360070
HEPES (1 M, pH 7.3)	15630080
0.05% Trypsin-EDTA	25300054
Motilin	Phoenix Pharmaceuticals 045-03
Penicillin-Streptomycin (10,000 U/mL) (antibiotics)	15140122
Zeocin™ Selection Reagent	R25001
Geneticin™ Selective Antibiotic (G418 Sulfate) (50 mg/mL)	10131027
Hygromycin-B	10687010

^[1] Cat. No. K1089 can be purchased separately from the kit.

^[2] Additional kit sizes are available.

Cell culture conditions

Media requirements

Unless otherwise indicated, equilibrate all media and solutions to 37°C (recommended) or room temperature before adding to the cells.

IMPORTANT! Do not make media substitutions. The cell lines have been validated for optimal assay performance using the media indicated. For dividing cells, we recommend storing an aliquot of cells for back-up in case of contamination or loss of cell supply.

Component	Assay Medium	Growth Medium	Thawing Medium	Freezing Medium
FreeStyle™ 293 Expression Medium	100%	—	—	—
McCoy's 5A (Modified) 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 (100 mM)	—	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™ Selection Reagent	—	200 µg/mL	—	—
Hygromycin-B	—	50 µg/mL	—	—
Geneticin™ antibiotic	—	100 µg/mL	—	—

Thawing method

Note: The cells are shipped on dry ice and may require a short recovery period before normal growth.

1. Place 14 mL of Thawing Medium into a T75 flask.
2. Place the flask in a humidified 37°C/5% CO₂ incubator for 15 minutes to allow the medium to equilibrate to the proper pH and temperature.
3. Remove the vial of cells to be thawed from liquid nitrogen, then rapidly thaw by placing the vial in a 37°C water bath with gentle agitation for 1–2 minutes. Do not submerge the 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 the cells at 200 × *g* for 5 minutes.
7. Aspirate the supernatant, then resuspend the cell pellet in 1 mL of fresh Thawing Medium.
8. Transfer the contents to the T75 tissue culture flask containing pre-equilibrated Thawing Medium, then place the flask in the humidified 37°C/5% CO₂ incubator.
9. At first passage, switch to Growth Medium.

Propagation method

- Passage or feed cells at least twice a week.
- Maintain cells between 25% and 95% confluence. Do not allow cells to reach confluence.

1. 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), then swirl to coat the cells evenly. The cells usually detach after approximately 2–5 minutes exposure to 0.05% Trypsin-EDTA.
2. 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 the cells at $200 \times g$ for 5 minutes, then resuspend in Growth Medium.

Freezing method

1. Harvest the cells as described (see “Propagation method” on page 5). After detachment, count the cells, centrifuge at $200 \times g$ for 5 minutes, then resuspend the cells in 4°C Freeze Medium at a density of 2×10^6 cells/mL.
2. Transfer 1.0-mL aliquots into cryogenic vials.
3. Place in an insulated container for slow cooling (we recommend Mr. Frosty™ Freezing Container, Cat. No. 5100-0001), then store overnight at -80°C.
4. Transfer to liquid nitrogen the next day for long-term storage.

Assay procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of MLNR using LiveBLazer™-FRET B/G Substrate as the readout. If alternative substrates are used (for example, ToxBLazer™ DualScreen or LyticBLazer™ Loading kits), follow the loading protocol provided with the product.

Procedural guidelines

- Work on a dust-free, clean surface.
- Do not touch the bottom of the 384-well, black-wall, clear-bottom assay plate. Always handle the assay plate by the sides.
- If pipetting manually, briefly centrifuge the plate at room temperature (at $14 \times g$ for 1 minute) after adding all assay components to ensure the reagents are on the bottom of the wells.
- 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.
- Some solvents may affect assay performance. Assess the effects of solvents before screening. The cell stimulation procedure described in the following sections is carried out in the presence of 0.1% DMSO to simulate the effect that a Test Compound's solvent can have on the assay. If other solvents and/or solvent concentrations are used, optimize the assay conditions appropriately.

Quick assay reference guides

For a more detailed assay protocol, see the next section.

Table 3 Agonist Assay Quick Reference Guide

	Unstimulated Control wells	Stimulated Control wells	Cell-Free Control wells	Test Compound wells
Step 1: Plate cells, incubate	32 μ L cells in Assay Medium (10,000 cells/well)	32 μ L cells in Assay Medium (10,000 cells/well)	32 μ L Assay Medium (no cells)	32 μ L cells in Assay Medium (10,000 cells/well)
Incubate cells at 37°C/ 5%CO ₂ for 16 hours.				
Step 2: Add Agonist or Test Compounds	8 μ L Assay Medium with 0.5% DMSO	8 μ L 5X Agonist in Assay Medium with 0.5% DMSO	8 μ L Assay Medium with 0.5% DMSO	8 μ L 5X Test Compounds with 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 Mixture	<ol style="list-style-type: none"> Combine 6 μL of 1 mM LiveBLazer™-FRET B/G (CCF4-AM) (Solution A) + 60 μL of Solution B, then vortex. Add 904 μL of Solution C, then vortex. Add 30 μL of Solution D, then vortex. 			
Step 5: Add Substrate Mixture	Add 8 μ L per well.			
Step 6: Incubate Substrate Mixture with cells	Incubate at room temperature for 2 hours in the dark.			
Step 7: Detect activity	See “Detection” on page 9.			
Step 8: Analyze data	See “Data analysis” on page 10.			

Table 4 Antagonist Assay Quick Reference Guide

	Unstimulated Control wells	Stimulated Control wells	Antagonist Control wells	Cell-Free Control wells	Test Compound wells
Step 1: Plate cells, incubate	32 μ L cells in Assay Medium (10,000 cells/well)	32 μ L cells in Assay Medium (10,000 cells/well)	32 μ L cells in Assay Medium (10,000 cells/well)	32 μ L Assay Medium (no cells)	32 μ L cells in Assay Medium (10,000 cells/well)
Incubate cells at 37°C/ 5%CO ₂ for 16 hours.					
Step 2: Add Antagonist or Test Compounds, incubate	4 μ L Assay Medium with 0.5% DMSO	4 μ L Assay Medium with 0.5% DMSO	4 μ L 10X Antagonist in Assay Medium with 0.5% DMSO	4 μ L Assay Medium with 0.5% DMSO	4 μ L 10X Test Compounds in Assay Medium with 0.5% DMSO
Incubate plate with Antagonist for 30 minutes before proceeding.					
Step 3: Add Agonist	4 μ L Assay Medium with 0.5% DMSO	4 μ L 10X Agonist in Assay Medium with 0.5% DMSO	4 μ L 10X Agonist in Assay Medium with 0.5% DMSO	4 μ L 10X Agonist in Assay Medium with 0.5% DMSO	4 μ L 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 Mixture	<ol style="list-style-type: none"> Combine 6 μL of 1 mM LiveBLazer™-FRET B/G (CCF4-AM) (Solution A) + 60 μL of Solution B, then vortex. Add 904 μL of Solution C, then vortex. Add 30 μL of Solution D, then vortex. 				
Step 6: Add Substrate Mixture	Add 8 μ L per well.				
Step 7: Incubate Substrate Mixture with cells	Incubate at room temperature for 2 hours in the dark.				
Step 8: Detect activity	See “Detection” on page 9.				
Step 9: Analyze data	See “Data analysis” on page 10.				

Detailed assay protocol

Plate cells

1. Harvest dividing cells, then resuspend in Assay Medium to a density of 312,500 cells/mL.
2. Add 32 μ L per well of Assay Medium to the Cell-Free Control wells. Add 32 μ L per well of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells.
3. Incubate cells at 37°C/ 5% CO₂ for 16 hours.
4. Proceed to the appropriate assay procedure.
 - For the Agonist assay—see “Agonist assay plate setup” on page 8.
 - For the Antagonist assay—see “Antagonist assay plate setup” on page 8.

Agonist assay plate setup

This section provides directions for performing an Agonist assay. See the next section 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.
Note: We recommend running a dose response curve to determine the optimal concentration of the Agonist solution.
4. Add 8 μ L of Assay Medium with 0.5% DMSO (prepared in step 1) to the Unstimulated Control and Cell-Free Control wells.
5. Add 8 μ L of the 5X Agonist stock solution to the Stimulated Control wells.
6. Add 8 μ L of the 5X Test Compound stock solution to the Test Compound wells.
7. Incubate the Agonist assay plate in a humidified 37°C/5% CO₂ incubator for 5 hours. Then proceed to “Substrate preparation, loading, and incubation” on page 9.

Antagonist assay plate setup

This section provides directions for performing an Antagonist assay. See the previous section 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.
Note: 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 EC₅₀–EC₈₀ range.
4. Prepare a 10X stock of Antagonist in Assay Medium with 0.5% DMSO.
Note: We recommend running a dose response curve to determine the optimal inhibition concentration for the Antagonist solution.
5. Add 4 μ L of the 10X Test Compound stock solution to the Test Compound wells.
6. Add 4 μ L of the stock solution of 0.5% DMSO in Assay Medium to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-Free Control wells.
7. Add 4 μ L of the 10X Antagonist stock solution in Assay Medium with 0.5% DMSO to the Antagonist Control wells.
8. *(Optional)* If desired, incubate the Test Compounds with the cells in a humidified 37°C/5% CO₂ incubator before proceeding. A 30-minute incubation is typically sufficient.
9. Add 4 μ L of the 10X Agonist stock solution 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 (prepared in step 1) to the Unstimulated Control and Cell-Free Control wells.
11. Incubate the Antagonist assay plate in a humidified 37°C/5% CO₂ incubator for 5 hours. Then proceed to “Substrate preparation, loading, and incubation” on page 9.

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.

IMPORTANT! 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 aliquots of the stock solution at –20°C until use.

Note: The molecular weight of the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) is 1096 g/mol.

2. Prepare 6X Substrate Mixture.
 - a. Add 6 μL of Solution A to 60 μL of Solution B, then vortex.
 - b. Add 904 μL of Solution C to the above solution, then vortex.
 - c. Add 30 μL of Solution D to the above solution, then vortex.
3. Remove the assay plate from the humidified 37°C/5% CO₂ incubator.

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

Detection

Measure fluorescence at room temperature from the bottom of the wells.

Before reading the plate, use compressed air to remove any dust from the bottom of the plate.

Instrumentation, filters, and plates

- Use a fluorescence plate reader with bottom-reading capabilities.
- Use the following recommended filters for the fluorescence plate reader:
 - Excitation filter: 409/20 nm
 - Emission filter: 460/40 nm
 - Emission filter: 530/30 nm

Read 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 ^[1]	Scan 2 ^[2]
Excitation filter	409/20 nm	409/20 nm
Emission filter	460/40 nm	530/30 nm

^[1] Measures fluorescence in the Blue channel.

^[2] Measures FRET signal in the Green channel.

Data analysis

Subtract the background, then calculate the emission ratio

We recommend subtracting 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.

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 can 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.

The following recommended filter set for observing beta-lactamase activity is available from Chroma Technology (www.chroma.com).

Chroma Complete Filter 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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