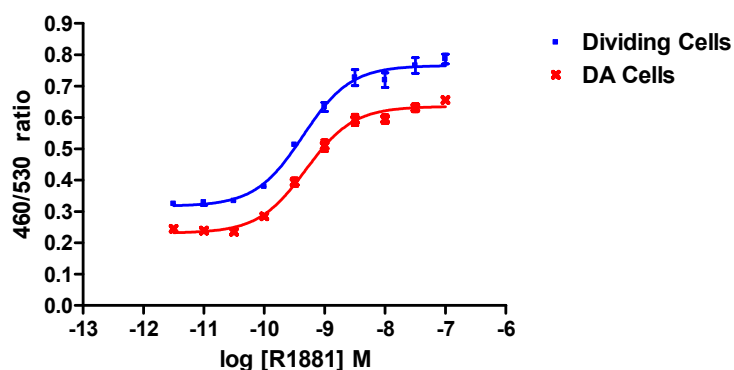


<b>Table of Contents</b>		<b>Page</b>
<b>1.</b>	<b>Description</b> .....	<b>1</b>
<b>2.</b>	<b>Overview of GeneBLazer<sup>®</sup> Beta-Lactamase Reporter Technology</b> .....	<b>2</b>
<b>3.</b>	<b>Materials Supplied</b> .....	<b>2</b>
<b>4.</b>	<b>Materials Required</b> .....	<b>3</b>
<b>5.</b>	<b>Detailed Cell Handling Procedures</b> .....	<b>4</b>
5.1	DA Cells .....	4
5.2	Non-DA Cells.....	4
<b>6.</b>	<b>Media Requirements</b> .....	<b>5</b>
<b>7.</b>	<b>Assay Procedure</b> .....	<b>6</b>
7.1	Quick Assay Reference Guides .....	6
7.2	Detailed Assay Protocol .....	7
7.3	Substrate Preparation, Loading and Incubation .....	8
7.4	Detection .....	8
<b>8.</b>	<b>Data Analysis</b> .....	<b>9</b>
8.1	Background Subtraction and Ratio Calculation .....	9
8.2	Visual Observation of Intracellular Beta-lactamase Activity Using LiveBLazer <sup>™</sup> -FRET B/G Substrate (CCF4-AM).....	9
<b>9.</b>	<b>References</b> .....	<b>10</b>
<b>10.</b>	<b>Purchaser Notification</b> .....	<b>11</b>

## 1. Description

GeneBLazer<sup>®</sup> AR GripTite<sup>™</sup> DA (Division-arrested) cells and AR-UAS-*bla* GripTite<sup>™</sup> cells contain an androgen receptor ligand-binding domain/Gal4 DNA binding domain chimera stably integrated into the UAS-*bla* GripTite<sup>™</sup> cell line. UAS-*bla* GripTite<sup>™</sup> contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into GripTite<sup>™</sup> cells. AR GripTite<sup>™</sup> DA cells and AR-UAS-*bla* GripTite<sup>™</sup> cells have been functionally validated for Z'-Factor and EC<sub>50</sub> concentrations of the synthetic androgen methyltrienolone (R1881).



	Division-arrested Cells	Dividing Cells
EC <sub>50</sub>	0.49 nM	0.44 nM
Z'-factor at EC <sub>100</sub>	0.76	0.68

**Dose response of AR GripTite<sup>™</sup> DA cells and AR-UAS-*bla* GripTite<sup>™</sup> cells to R1881.** AR GripTite<sup>™</sup> DA cells and AR-UAS-*bla* GripTite<sup>™</sup> cells were plated at 10,000 cells/well and stimulated with R1881 in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLazer<sup>™</sup>-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained and the response ratios were plotted against the indicated concentrations of R1881.

## 2. Overview of GeneBLAzer® Beta-Lactamase Reporter Technology

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy-to-use method of monitoring cellular response to drug candidates or other stimuli (1). The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the dual-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, transfection efficiency, substrate concentration, excitation path length, fluorescence detectors, and volume changes. The GeneBLAzer® Beta-lactamase Reporter 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). The utility of division arrest cells in HTS has also been demonstrated (8-11).

## 3. Materials Supplied

Product:	Name	Size	Catalog #
	<b>GeneBLAzer® AR GripTite™ DA Assay Kit</b> Each system contains sufficient division-arrested cells and substrate to assay one 384-well plate. Includes: <ul style="list-style-type: none"> <li>• AR GripTite™ DA cells (K1413A)</li> <li>• LiveBLAzer™-FRET B/G Loading Kit, 70 µg</li> <li>• Protocol</li> <li>• Certificate of Analysis</li> </ul>	1 plate	K1413
	<b>GeneBLAzer® AR-UAS-<i>bla</i> GripTite™ cells</b> Includes: <ul style="list-style-type: none"> <li>• AR-UAS-<i>bla</i> GripTite™ cells (K1698)</li> <li>• Protocol</li> <li>• Certificate of Analysis</li> </ul>	1 tube	K1698
<b>Shipping Condition:</b>	Dry ice		
<b>Storage Condition of Cells:</b>	Immediately upon receipt, store at -80°C or thaw for immediate use. <b>For long term storage, store at -80°C for at least 1 day followed by liquid nitrogen.</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™ (80 µg/mL), Hygromycin (80 µg/mL) <i>Note:</i> GripTite™ cells contain the macrophage scavenger receptor type 1 and are thus Geneticin® resistant. These cells are also Blasticidin resistant.		
<b>Mycoplasma Testing:</b>	Negative		
<b>BioSafety Level:</b>	2		

## 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 #	Required Separately?	
			AR GripTite <sup>™</sup> DA Assay Kit (K1413)	AR-UAS- <i>bla</i> GripTite <sup>™</sup> cells (K1698)
LiveBLAzer <sup>™</sup> -FRET B/G Loading Kit, contains: <ul style="list-style-type: none"> <li>• LiveBLAzer<sup>™</sup>-FRET B/G Substrate (CCF4-AM)</li> <li>• DMSO for Solution A</li> <li>• Solution B</li> <li>• Solution C</li> </ul>	Invitrogen	K1427 (70 µg) K1095 (200 µg) K1096 (1 mg) K1030 (5 mg)	No (included in kit)	Yes
Recovery <sup>™</sup> Cell Culture Freezing Medium	Invitrogen	12648-010	No	Yes
DMEM (high-glucose), with GlutaMAX <sup>™</sup>	Invitrogen	10569-010	No	Yes
DMSO	Fluka	41647	Yes	Yes
OPTI-MEM	Invitrogen	11058-021	Yes	Yes
Fetal bovine serum (FBS), dialyzed, tissue-culture grade ( <b>DO NOT SUBSTITUTE!</b> )	Invitrogen	26400-036	Yes	Yes
Non-essential amino acids (NEAA)	Invitrogen	11140-050	Yes	Yes
Penicillin/Streptomycin (antibiotics)	Invitrogen	15140-122	Yes	Yes
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136	No	Yes
HEPES (1 M, pH 7.3)	Invitrogen	15630-080	Yes	Yes
Sodium Pyruvate	Invitrogen	11360-070	Yes	Yes
Methyltrienolone (R1881)	Perkin-Elmer	NLP005005MG	Yes	Yes
Cyproterone Acetate	Sigma	C3412	Yes	Yes
0.05% Trypsin/EDTA	Invitrogen	25300-054	No	Yes
Hygromycin (antibiotic)	Invitrogen	10687-010	No	Yes
Zeocin <sup>™</sup> (antibiotic)	Invitrogen	R250-01	No	Yes

The following tables list materials 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 <b>Section 7.4.1</b> )	Chroma Technologies
<b>Optional:</b> Epifluorescence- or fluorescence-equipped microscope, with appropriate filters	Various
<b>Optional:</b> Microplate centrifuge	Various

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## 5. Detailed Cell Handling Procedures

**Note:** Division-arrested (DA) cells have different thawing procedures than non-DA cells. Refer to the instructions below for your particular application.

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

### 5.1 DA Cells

#### 5.1.1 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 7, 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 fresh Assay Medium. For the 1 × 384-well plate size of cells, dilute in 1 mL.
6. Count the cells. Adjust the cell density with Assay Medium to the appropriate cell density as specified in Section 7. Proceed to **Section 7, Assay Procedure**, for guidance on using cells in an assay.

### 5.2 Non-DA Cells

#### 5.2.1 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 9 mL of Thawing Medium into a T25 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 T25 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.

#### 5.2.2 Propagation Method

1. Passage or feed cells at least twice a week. Maintain cells between 5% and 95% confluence. **Do not allow cells to reach confluence.**
2. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (1 mL for a T25 flask, 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 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. Centrifuge cells at 200 × *g* for 5 minutes and resuspend in Growth Medium.

#### 5.2.3 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 2 × 10<sup>6</sup> 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

**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 non-DA cells, we recommend that you create and store an aliquot for back up.

Component	Assay Medium (DA and Non-DA cells)	Growth Medium (Non-DA cells only)	Thawing Medium (Non-DA cells only)	Freeze Medium (Non-DA cells only)
DMEM	--	90%	90%	—
OPTI-MEM	90%	—	—	—
Dialyzed FBS (Do not substitute!)	10%	10%	10%	—
Sodium Pyruvate	1 mM	—	—	—
NEAA	0.1 mM	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	--	25 mM	25 mM	—
Penicillin (antibiotic)	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin (antibiotic)	100 µg/mL	100 µg/mL	100 µg/mL	—
Hygromycin (antibiotic)	—	80 µg/mL	—	—
Zeocin <sup>™</sup> (antibiotic)	—	80 µg/mL	—	—
Recovery <sup>™</sup> Cell Culture Freezing Medium	—	—	—	100%

## 7. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of AR using LiveBLAzer<sup>™</sup>-FRET B/G Substrate as the readout. If alternative substrates are used (*e.g.*, ToxBLAzer<sup>™</sup> DualScreen or LyticBLAzer<sup>™</sup> 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
<b>Step 1</b> Plate cells, incubate	32 $\mu$ L cells in Assay Medium (10,000 cells/well)	32 $\mu$ L cells in Assay Medium (10,000 cells/well)	32 $\mu$ L Assay Medium (no cells)	32 $\mu$ L cells in Assay Medium 10,000 cells/well)
No incubation, proceed to Step 2				
<b>Step 2</b> Add Agonist or Test Compounds	8 $\mu$ L Assay Medium with 2.5% DMSO	8 $\mu$ L 5X R1881 in Assay Medium with 2.5% DMSO	8 $\mu$ L Assay Medium with 2.5% DMSO	8 $\mu$ L 5X Test Compounds in 2.5% DMSO
<b>Step 3</b> Incubate cells	Incubate in a humidified 37°C/5% CO <sub>2</sub> incubator for 16 hours			
<b>Step 4</b> Prepare 6X Substrate Mix	6 $\mu$ L of 1 mM LiveBLAzer <sup>™</sup> -FRET B/G (CCF4-AM) Substrate + 60 $\mu$ L of solution B, mix. Add 934 $\mu$ L of Solution C, mix.			
<b>Step 5</b> Add Substrate Mixture	8 $\mu$ L per well			
<b>Step 6</b> Incubate Substrate Mix. + cells	2 hours at room temperature in the dark			
<b>Step 7</b> Detect activity	See <b>Section 7.4</b>			
<b>Step 8</b> Analyze data	See <b>Section 8</b>			

#### Antagonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Antagonist Control Wells	Cell-free Wells	Test Compound Wells
<b>Step 1</b> Plate cells, incubate	32 $\mu$ L cells in Assay Medium (10,000 cells/well)	32 $\mu$ L cells in Assay Medium (10,000 cells/well)	32 $\mu$ L cells in Assay Medium (10,000 cells/well)	32 $\mu$ L Assay Medium (no cells)	32 $\mu$ L cells in Assay Medium (10,000 cells/well)
No incubation, proceed to Step 2					
<b>Step 2</b> Add Antagonist or Test Compounds	4 $\mu$ L Assay Medium with 2.5% DMSO	4 $\mu$ L Assay Medium with 2.5% DMSO	4 $\mu$ L 10X Cyproterone Acetate in Assay Medium with 2.5% DMSO	4 $\mu$ L Assay Medium with 2.5% DMSO	4 $\mu$ L 10X Test Compounds in Assay Medium with 2.5% DMSO
<b>Optional Step:</b>	Incubate plate with Antagonist for 30 minutes before proceeding				
<b>Step 3</b> Add Agonist	4 $\mu$ L Assay Medium with 2.5% DMSO	4 $\mu$ L 10X R1881 in Assay Medium with 2.5% DMSO	4 $\mu$ L 10X R1881 in Assay Medium with 2.5% DMSO	4 $\mu$ L 10X R1881 in Assay Medium with 2.5% DMSO	4 $\mu$ L 10X R1881 in Assay Medium with 2.5% DMSO
<b>Step 4</b> Incubate cells	Incubate in a humidified 37°C/5% CO <sub>2</sub> incubator for 16 hours				
<b>Step 5</b> Prepare 6X Substrate Mix	Add 6 $\mu$ L of 1 mM LiveBLAzer <sup>™</sup> -FRET B/G (CCF4-AM) substrate + 60 $\mu$ L of solution B, mix. Add 934 $\mu$ L of Solution C, mix.				
<b>Step 6</b> Add Substrate Mixture	8 $\mu$ L per well				
<b>Step 7</b> Incubate Mixture	2 hours at room temperature in the dark				
<b>Step 8</b> Detect activity	See <b>Section 7.4</b>				
<b>Step 9</b> Analyze data	See <b>Section 8</b>				

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## 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 0.5% 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  $14 \times g$ ) after additions to ensure all assay components are on the bottom of the wells.

### 7.2.2 Plating Cells

1. Thaw DA cells/harvest non-DA cells and resuspend in Assay Medium to a density of  $3.125 \times 10^5$  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. There is no incubation for the cells prior to adding the agonist/antagonist, 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 2.5% DMSO in Assay Medium.
2. Prepare a 5X stock of Test Compounds in Assay Medium with 2.5% DMSO.
3. Prepare a 5X stock of agonist in Assay Medium with 2.5% 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 2.5% 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 ~16 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 2.5% DMSO in Assay Medium.
2. Prepare a 10X stock of Test Compounds in Assay Medium with 2.5% DMSO.
3. Prepare a 10X stock of agonist in Assay Medium with 2.5% DMSO. We recommend running a dose response curve to determine the optimal agonist concentration. For antagonist assays, we recommend stimulating cells 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 2.5% 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 2.5% DMSO in assay media 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 2.5% DMSO to the Antagonist Control wells.
8. Incubate the cells in a humidified 37°C/5% CO<sub>2</sub> incubator before proceeding. Typically, 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 2.5% DMSO to the Unstimulated Control and Cell-free Control wells.

11. Incubate the Antagonist assay plate in a humidified 37°C/5% CO<sub>2</sub> incubator for ~16 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<sup>™</sup>-FRET B/G Substrate Mixture (CCF4-AM) Substrate Mixture. If you use alternative substrates, follow the loading protocol provided with the substrate.

Prepare LiveBLAzer<sup>™</sup>-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<sup>™</sup>-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<sup>™</sup>-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 934 µL of Solution C 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.

### 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
<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



## 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<sup>™</sup>-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<sup>™</sup>-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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