GeneBLAzer® ADRA1A CHO-K1 DA and ADRA1A-NFAT-*bla* CHO-K1 Cell-based Assay

invitrogen™

Cat. nos. K1577 and K1470 | **Shipping: Dry Ice** | **Storage: Liquid Nitrogen**

Protocol part no. K1470.pps | Rev. date: 27 October 2010

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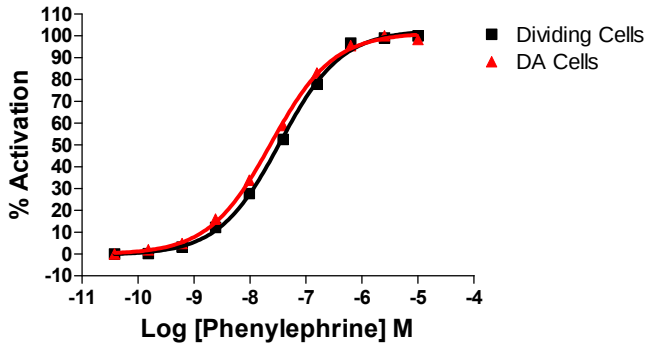
1. Description

GeneBLAzer® ADRA1A CHO-K1 DA (Division-arrested) cells and ADRA1A-NFAT-*bla* CHO-K1 cells contain the human Adrenergic Alpha-1A Receptor (ADRA1A) stably integrated into the CellSensor® NFAT-bla CHO-K1 cell line. CellSensor® NFAT-bla CHO-K1 contains a beta-lactamase reporter gene under control of a NFAT response element stably integrated into CHO-K1 cells. ADRA1A CHO-K1 DA cells and ADRA1A-NFAT-*bla* CHO-K1 cells have been functionally validated for Z'-Factor and EC_{50} concentrations of Phenylephrine.



	Division-arrested cells	Dividing Cells
EC_{50}	23.7 nM	35.3 nM
Z'-factor at EC_{100}	0.84	0.78

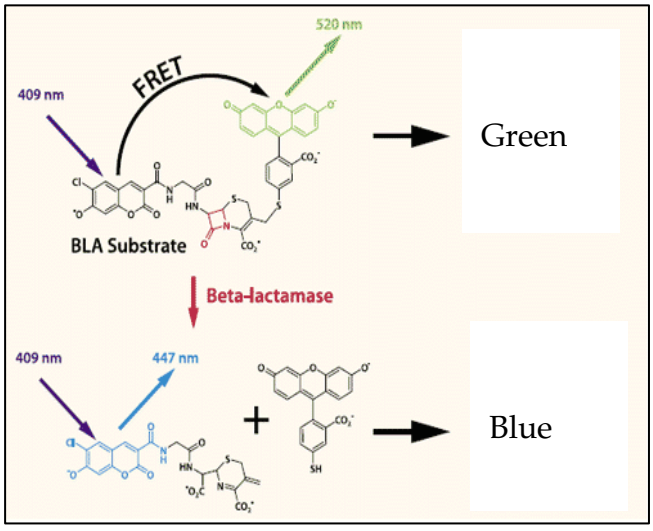
Dose response of ADRA1A CHO-K1 DA cells and ADRA1A-NFAT-*bla* CHO-K1 cells to Phenylephrine.

2. Overview of GeneBLAzer® GPCR Cell-based Assays

The GeneBLAzer® GPCR Cell-based Assay 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® assay technology is a beta-lactamase (*bla*) 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® GPCR 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). The utility of division-arrested cells in HTS has also been demonstrated (8-11).

The GeneBLAzer® 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 1). 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.

Figure 1.



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.

3. Overview of Dividing and Division-Arrested (DA) 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:	Name	Size	Catalog #
	GeneBLAzer® ADRA1A CHO-K1 DA Assay Kit Each system contains sufficient division-arrested cells and substrate to assay one 384-well plate. Includes: • GeneBLAzer® ADRA1A CHO-K1 DA cells (K1577A) • LiveBLAzer™-FRET B/G Loading Kit, 70 µg • Solution D, 1 ml • Protocol • Certificate of Analysis	1 plate	K1577
	GeneBLAzer® ADRA1A-NFAT-*bla* CHO-K1 cells Includes: • GeneBLAzer® ADRA1A-NFAT-*bla* CHO-K1 cells (K1470A) • Protocol • Certificate of Analysis	1 tube	K1470
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 Non-Division-arrested Cells:	Adherent		
Cell Phenotype:	Epithelial		
Selection Marker(s) for Non-Division arrested cells:	Zeocin™ 100 µg/mL; Hygromycin 600 µg/mL;		
***Mycoplasma* Testing:**	Negative		
BioSafety Level:	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?	
			ADRA1A CHO-K1 DA Assay Kit (K1577)	ADRA1A-NFAT-*bla* CHO-K1 cells (K1470)
LiveBLAzer™-FRET B/G Loading Kit: LiveBLAzer™-FRET B/G Substrate (CCF4-AM) DMSO for Solution A Solution B Solution C	Invitrogen	K1427 (70 µg) K1095 (200 µg) K1096 (1 mg) K1030 (5 mg)	No (included in kit)	Yes
Solution D	Invitrogen	K1156 (1 ml) K1157 (25 ml)	No (included in kit)	Yes
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010	No	Yes
DMEM (high-glucose), with GlutaMAX™	Invitrogen	10569-010	Yes	Yes
DMSO	Fluka	41647	Yes	Yes
Fetal bovine serum (FBS), dialyzed, (**DO NOT SUBSTITUTE!**)	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
0.05% Trypsin/EDTA	Invitrogen	25300-054	No	Yes
Phenylephrine	Sigma	P6126	Yes	Yes
Zeocin™	Invitrogen	R250-01	No	Yes
Hygromycin	Invitrogen	10687-010	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

6. Detailed Cell Handling Procedures

Note: Division-arrested (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 8.2.2. 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 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% 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 T75 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.

6.2.2 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 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.
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 (DA and dividing cells)	Growth Medium (dividing cells only)	Thawing Medium (dividing cells only)	Freeze Medium (dividing cells only)
DMEM	90%	90%	90%	—
Dialyzed FBS (Do not substitute!)	10%	10%	10%	—
NEAA	0.1 mM	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	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	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%
Zeocin™	—	100 µg/mL	—	—
Hygromycin	—	600 µg/mL	—	—

8. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of ADRA1A 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
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 16-20 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 8.4**			
Step 8 Analyze data	See **Section 9**			

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 16-20 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 8.4**				
Step 9 Analyze data	See **Section 9**				

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 × *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 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 16-20 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 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 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_{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 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 –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.

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
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

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).

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).

10. References

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The purchaser of this product may not remove or obtain components from cells sold under this label license or use these cells in a manner inconsistent with protocols provided by Life Technologies Corporation for use of division arrested cells. The purchaser of this product may not attempt to expand division arrested cells and may only use the purchased amount of this product.

Use of Genetically Modified Organisms (GMO)

Information for European Customers The ADRA1A CHO-K1 DA and ADRA1A-NFAT-*bla* CHO-K1 cell lines are genetically modified to contain p3XNFATBlaX-Zeo and pcDNA6.2 V5/Hygro ADRA1A As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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