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GeneBLAzer™ VDR HEK 293T DA and VDR-UAS-*bla* HEK 293T Cell-based Assay

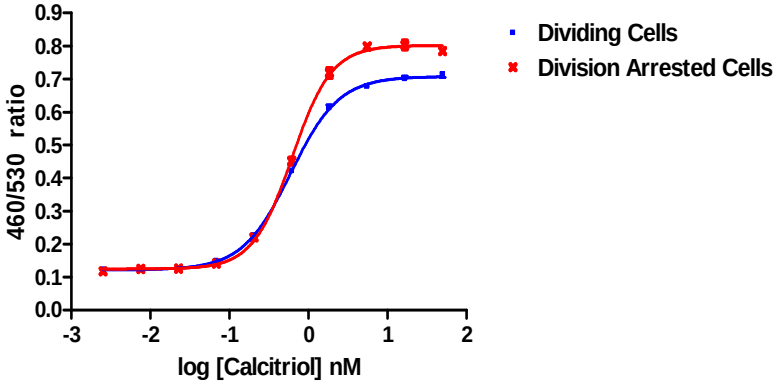
Catalog Numbers K1417, K1700

Pub. No. MAN0002785

Rev. A.0

Product description

GeneBLAzer™ VDR HEK 293T DA (Division-arrested) cells and VDR-UAS-*bla* HEK 293T cells contain a human vitamin D receptor ligand-binding domain/Gal4 DNA binding domain chimera stably integrated into the CellSensor™ UAS-*bla* HEK 293T cell line. CellSensor™ UAS-*bla* HEK 293T contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into HEK 293T cells. VDR HEK 293T DA cells and VDR-UAS-*bla* HEK 293T cells have been functionally validated for Z′-Factor and EC_{50} concentrations of 1α,25-dihydroxyvitamin D_3 (calcitriol).



	Division-arrested	Non-Division-arrested
EC_{50}	0.62 nM	0.60 nM
Z′-factor at EC_{100}	0.86	0.70

Dose response of VDR HEK 293T DA cells and VDR-UAS-*bla* HEK 293T cells to 1α,25-dihydroxyvitamin D_3.

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).

Contents and storage

Note: Cat. Nos. K1417 and K1700 are sold as separate products.

Contents	Part Number	Amount	Storage
GeneBLAzer™ VDR HEK 293T DA Assay Kit **(Cat. No. K1417)** (Each system contains sufficient division-arrested cells and substrate to assay one 384-well plate)			
• VDR HEK 293T DA cells	K1417A[1]	1 vial	Liquid nitrogen
• LiveBLAzer™-FRET B/G Loading Kit:		Kit	
o LiveBLAzer™-FRET B/G Substrate (CCF4-AM) (70 µg)	K1426		–20°C
o DMSO for Solution A	K1040		Room temperature
o Solution B	K1041		Room temperature
o Solution C	K1049		Room temperature
GeneBLAzer™ VDR-UAS-*bla* HEK 293T cells **(Cat. No. K1700)**			
• VDR-UAS-*bla* HEK 293T cells	K1099[1]	1 vial	Liquid nitrogen

[1]**Storage and Handling**: After shipment either thaw for immediate use or store in liquid nitrogen. Cells stored for more than 1 day at –80°C quickly lose viability.

Product details

Condition	Details
Shipping condition:	Dry ice
Storage condition of cells:	Thaw for immediate use of store in liquid nitrogen of dry vapor liquid nitrogen.
Growth properties of non-division-arrested cells:	Adherent
Cell phenotype:	Epithelial
Selection marker(s) for non-division arrested cells:	Zeocin™ (80 µg/mL), Hygromycin B (80 µg/mL) **Note:** HEK 293T cells contain the large T antigen and are thus Geneticin™ resistant. These cells are also Blasticidin resistant.
***Mycoplasma* testing:**	Negative
BioSafety level:	2

Product names and abbreviations

Product name	Part number	Type	Abbreviation
VDR HEK 293T DA cells	K1417A	Division–arrested cells	DA Cells
VDR-UAS-*bla* HEK 293T cells	K1099	Non-division arrested cells Dividing cells	Non-DA Cells

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com.**

Required for Non-DA Cells (Cat. No. K1700) workflow

Item	Cat. No.
LiveBLAzer™-FRET B/G Loading Kit: • LiveBLAzer™-FRET B/G Substrate (CCF4-AM) (200 µg) (K1089) • DMSO for Solution A (K1040)[2] • Solution B (K1041)[2] • Solution C (K1049)[2]	K1095[1]
Recovery™ Cell Culture Freezing Medium	12648-010
DMEM, high-glucose, GlutaMAX™ Supplement	10569-010
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)	26400-036
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	14190-136
HEPES (1 M, pH 7.3)	15630-080
Trypsin-EDTA (0.05%), phenol red	25300-054
Hygromycin B (antibiotic)	10687-010
Zeocin™ Selection Reagent (antibiotic)	R250-01
Matrigel™ matrix, growth factor reduced	BD; 354230

[1]Additional sizes of Cat. No. K1095 are available.
[2] Sold only as part of Cat. No. K1085. K1085 is available separately.

Required for Non-DA Cells (Cat. No. K1700) and DA Cells (Cat. No. K1417) workflows

Item	Cat. No.
Reagents	
DMEM, high glucose, HEPES, no phenol red	21063-029
Fetal bovine serum (FBS), charcoal-stripped	12676-029
Non-essential amino acids (NEAA)	11140-050
Sodium Pyruvate	11360-070
Penicillin/Streptomycin (antibiotics)	15140-122
DMSO (for compound preparation)	MP Biomedical; 196055
1,25-dihydroxyvitamin D_3	Calbiochem; 679101
Consumables	
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Corning; 3712
Compressed air	Various
Equipment	
Fluorescence plate reader with bottom-read capabilities	Various
Filters if required for plate reader (see **Detection**)	Chroma Technologies
Optional: Epifluorescence- or fluorescence-equipped microscope, with appropriate filters	Various
Optional: Microplate centrifuge	Various

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. (Matrigel™ should be kept cold prior to coating flasks.)

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.

Note: All media components can be added directly to the 500 mL bottle of base media (DMEM).

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)	1X Matrigel™ Matrix (Non-DA cells only)
DMEM, high-glucose, GlutaMAX™ Supplement	--	90% (500 mL)	90% (500 mL)	—	—
DMEM, high glucose, HEPES, no phenol red	98% (500 mL)	—	—	—	99.75%
Dialyzed FBS (Do not substitute!)	—	10% (50 mL)	10% (50 mL)	—	—
Charcoal-stripped FBS	2% (10 mL)	—	—	—	—
Sodium Pyruvate	1 mM (5 mL)	—	—	—	—
NEAA	0.1 mM (5 mL)	0.1 mM (5 mL)	0.1 mM (5 mL)	—	—
HEPES (pH 7.3)	—	25 mM (12.5 mL)	25 mM (12.5 mL)	—	—
Penicillin/Streptomycin (antibiotics)	100 U/mL and 100 µg/mL (5 mL)	100 U/mL and 100 µg/mL (5 mL)	100 U/mL and 100 µg/mL (5 mL)	—	—
Hygromycin B (antibiotic)	—	80 µg/mL	—	—	—
Zeocin™ Selection Reagent (antibiotic)	—	80 µg/mL	—	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%	—
Matrigel™ matrix	—	—	—	—	0.25%

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. Refer to **Media Requirements** for specific media recipes.

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 **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.
6. Count the cells.

7. Proceed to **Assay Procedure**. Adjust the cell density with Assay Medium to the appropriate cell density as specified.

Non-DA Cells

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. Precoat a T25 flask with 3 mL 1X Matrigel™ matrix. Incubate 15 min. at 37ºC. Aspirate the Matrigel™ matrix.
2. Place 9 mL of Thawing Medium into the precoated T25 flask. 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. Count Cells.
9. Transfer ~1x10^6 cells to the T25 tissue culture flask (~40,000 cells/cm^2) containing pre-equilibrated Thawing Medium and place flask in the humidified 37°C/5% CO_2 incubator.
10. At first passage, switch to Growth Medium.

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. All flasks must be coated with 1X Matrigel™ matrix (3 mL for a T75 flask, 5 mL for a T175 flask, and 7 mL for T225 flask) and incubated 15 min. in a humidified 37°C/5% CO_2 incubator before plating. After coating and incubation, aspirate the Matrigel™ matrix and plate cells.
3. To passage cells, aspirate medium, rinse once in PBS, add 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 Trypsin/EDTA. Add an equal volume of Growth Medium to inactivate Trypsin.
4. Verify under a microscope that cells have detached and clumps have completely dispersed.
5. Centrifuge cells at 200 × g for 5 minutes and resuspend in Growth Medium.

Freezing Method

1. Harvest the cells as described in **Propagation**. 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 × 10^6 cells/mL.
2. Dispense 1.0-mL aliquots into cryogenic vials.
3. Place in an insulated container for slow cooling, we recommend a Mr. Frosty™ container. Store at –80°C.
4. Transfer to liquid nitrogen the next day for storage.

Assay procedure

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

Quick Assay Reference Guides

For a more detailed assay protocol, see section following Quick Reference Guides.

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 (20,000 cells/well)	32 µL cells in Assay Medium (20,000 cells/well)	32 µL Assay Medium (no cells)	32 µL cells in Assay Medium (20,000 cells/well)
Step 2 Add Agonist or Test Compounds	8 µL Assay Medium with 0.5% DMSO	8 µL 5X 1α,25-dihydroxyvitamin D_3 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 934 µL of Solution C, 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 **Detection**			
Step 8 Analyze data	See **Data analysis**			

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 (20,000 cells/well)	32 µL cells in Assay Medium (20,000 cells/well)	32 µL cells in Assay Medium (20,000 cells/well)	32 µL Assay Medium (no cells)	32 µL cells in Assay Medium (20,000 cells/well)
Step 2 Add Antagonist or Test Compounds	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
Optional Step:	Incubate plate with Antagonist for 30 minutes before proceeding				
Step 3 Add Agonist	4 µL Assay Medium with 0.5% DMSO	4 µL 10X 1α,25-dihydroxyvitamin D_3 in Assay Medium with 0.5% DMSO	4 µL 10X 1α,25-dihydroxyvitamin D_3 in Assay Medium with 0.5% DMSO	4 µL Assay Medium with 0.5% DMSO	4 µL 10X 1α,25-dihydroxyvitamin D_3 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	Add 6 µL of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) substrate + 60 µL of solution B, mix. Add 934 µL of Solution C, 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 **Detection**
Step 9 Analyze data	See **Data analysis**

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.

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.

Plating Cells

DA Cells

1. Thaw DA cells into Assay Medium and count (as described in **Thawing Method**). Dilute cells to a density of 6.2×10^5 cells/mL in Assay Medium.
2. Add 32 µL per well of the Assay Medium to the Cell-free Control wells. Add 32 µL per well (20,000 cells/well) of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells. Proceed to **Plate setup** for an Agonist assay or for an Antagonist assay.

Non-DA Cells

1. Harvest non-DA cells from culture at **70–90%** confluency. Spin down cells and suspend cells in Assay Medium and count. Dilute cells to a density of 6.2×10^5 cells/mL in Assay Medium.
2. Add 32 µL per well of the Assay Medium to the Cell-free Control wells. Add 32 µL per well (20,000 cells/well) of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells. Proceed to **Plate setup** for an Agonist assay or for an Antagonist assay.

Agonist Assay Plate Setup

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 (or if test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%)
3. Prepare a 5X stock of 1α,25-dihydroxyvitamin D_3 in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal concentration for the 1α,25-dihydroxyvitamin D_3 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 1α,25-dihydroxyvitamin D_3 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 **Substrate Preparation, Loading and Incubation** section.

Antagonist Assay Plate Setup

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 (or if test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%).
3. Prepare a 10X stock of 1α,25-dihydroxyvitamin D_3 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 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 in Assay Medium 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 in a 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 1α,25-dihydroxyvitamin D_3 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 Then proceed to **Substrate Preparation, Loading and Incubation** section.

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 934 µL of Solution C to the above solution and vortex.

   Note: If more than 1 mL 6X Substrate Mixture is needed, scale up the amount of each solution proportionally
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.

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.

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

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

Data analysis

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.

Note: You may also calculate response ratio to know your assay window. The response ratio is calculated as the Blue/Green Emission Ratio of the 1α,25-dihydroxyvitamin D_3 –Stimulated wells divided by the Blue/Green Emission Ratio of the unstimulated wells. Generally, a response ratio of >3 has been shown to yield a $Z' \geq 0.6$.

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).

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