GeneBLAzer® CMV-*bla* Jurkat Cell-based Assay Protocol

Cat. no. K1045 **Shipping Condition: Frozen** **Storage: Liquid Nitrogen**

O-12776-r1 1210

TABLE OF CONTENTS

1.0 MATERIALS SUPPLIED

Description:	The CMV-*bla* Jurkat cell line has been engineered to express the beta-lactamase (*bla*) reporter gene under the control of the CMV promoter. A stable population was obtained using 1 mg/mL Geneticin (G418) selection followed by isolation of a clone by fluorescence-activated cell sorting (FACS). This cell line constitutively expresses Beta-Lactamase.
Cat. no.:	K1045
Shipping Condition:	Frozen
Storage Condition:	Liquid Nitrogen
Quantity:	~5,000,000 cells (5×10^6 cells/mL)
Cell Line Name:	CMV-*bla* Jurkat
Function:	Used as a positive control for beta-lactamase expression
Growth Properties:	Suspension
Cell Phenotype:	Lymphoblast
Antibiotic Selection:	Geneticin (G418)
***Mycoplasma* Testing:**	Negative

2.0 ADDITIONAL MATERIALS

2.1 Required, but Not Supplied

Media/Reagents	Recommended Source	Cat. no.
Loading Kit with CCF2-AM, 5 mg	Invitrogen	K1025
Loading Kit with CCF4-AM, 5 mg	Invitrogen	K1030
Loading Kit with CCF2-AM, 200 µg	Invitrogen	K1032
Jurkat, Clone E6-1	ATCC	TIB-152
RPMI 1640	Invitrogen	11875-093
Hank's Balanced Salt Solution (HBSS)	Invitrogen	14025-092
Fetal Bovine Serum (FBS), dialyzed, tissue culture grade	Invitrogen	26400-044
l-glutamine	Invitrogen	25030-081
Non-essential Amino Acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
HEPES (pH 7.3)	Invitrogen	15630-080
Penicillin/Streptomycin	Invitrogen	15140-122
Geneticin (G418)	Invitrogen	10131-027
Dimethyl sulfoxide (DMSO)	Fluka	41647
5,6-carboxyfluorescein	Molecular Probes	C194
7-hydroxycoumarin-3-carboxylate	Molecular Probes	H185
Sodium Borate buffer (pH 9.2)	Various	

Consumables	Recommended Source	Cat. no.
Black-wall, clear-bottom, 96-well assay plates (with low fluorescence background)	Costar	3603
Compressed Air	Various	—
Conical tubes, 15 mL, sterile	Various	—
1.8-mL Internally threaded cryogenic vials	Various	—
Tissue culture flasks	Various	—

Equipment	Recommended Source
37°C/5% CO_2 Incubator	Various
Class II Biological Safety Cabinet	Various
Liquid Nitrogen Tank	Various
Hemacytometer (or another cell counting method)	Various
Inverted Microscope	Various
CytoFluor® 4000 Fluorescence Plate Reader, or equivalent bottom-reading instrument	PerSeptive Biosystems
Filters (see **Sections 5.2.1**, **5.2.6** and **5.2.7**)	Chroma Technologies

2.2 Optional Equipment and Materials

- Epifluorescence or fluorescence-equipped microscope

3.0 CELL CULTURE CONDITIONS

3.1 Passage Conditions

Pass cells at least twice weekly. Maintain cells between 1×10^5 and 2×10^6 cells/mL.

3.2 Media Required

	Growth Medium (+)	Growth Medium (-)	Assay Medium*	Freezing Medium
RPMI 1640	90%	90%	100%	80%
Dialyzed FBS	10%	10%	0.1%	10%
L-glutamine	2 mM	2 mM	--	2 mM
NEAA	0.1 mM	0.1 mM	--	0.1 mM
Sodium Pyruvate	1 mM	1 mM	--	1 mM
HEPES (pH 7.3)	25 mM	25 mM	25 mM	25 mM
Penicillin	100 U/mL	--	--	--
Streptomycin	100 µg/mL	--	--	--
G418	500 µg/mL	--	--	--
DMSO	--	--	--	10%

*You may use Hank's Balanced Salt Solution (HBSS) supplemented with 25 mM HEPES (pH 7.3) as an alternative Assay Medium.

3.3 Methods Used

3.3.1 Freezing Method

1. Harvest the cells, and resuspend them in fresh freezing medium.
2. Dispense aliquots into vials (5×10^6 cells/vial).
3. Place in an insulated carrier and store overnight at -80°C.
4. Transfer to liquid nitrogen storage the next day.

3.3.2 Thawing Method

1. Place 15 mL of fresh Growth Medium (-) ***with*** 100 U/mL penicillin and 100 µg/mL streptomycin in a T75 flask.
2. Pre-equilibrate media by placing T75 flask in a 37°C/5% CO_2 incubator.
3. Rapidly thaw cells by placing in a water bath at 37°C for 1–2 minutes with gentle agitation.
4. Swab the vial with 70% ethanol before opening.
5. Transfer the contents to a 15 mL conical tube.
6. Slowly add 10 mL Growth Medium (-) dropwise to the cell suspension.
7. Centrifuge for 5 minutes at $200 \times g$, discard the supernatant, and resuspend the cells in the 15 mL of pre-equilibrated Growth Medium (-) ***with*** 100 U/mL penicillin and 100 µg/mL streptomycin from Step 2 (**Section 3.3.2**).
8. Return the cells to the T75 flask from Step 2 (**Section 3.3.2**) for culturing.
9. At the first passage, change the cells to Growth Medium (+).

4.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for determining beta-lactamase activity in a 96-well Plate. We recommend at least 5 wells per experimental condition.

4.1 Cells to Wells

1. Harvest cells from culture in Growth Medium (+) at a density of no greater than 2×10^6 cells/mL.
2. Resuspend the cells in Assay Medium at a density of 1×10^6 cells/mL.
3. Add 100 µL of cell suspension per well into a 96-well tissue culture treated black-wall, clear-bottom plate (Costar #3603).
4. In a separate set of wells on the same plate, add 100 µL per well of wild-type Jurkat cell suspension in Assay Medium.
5. In a separate set of wells on the same plate, add 100 µL per well of Assay Medium.

4.2 Loading Cells with Substrate

This protocol is designed for loading cells with CCF4-AM or CCF-2AM. Please see **Section 5.1** for more details.

4.2.1 Solutions Required

Solution A: 1 mM CCF4-AM or CCF-2AM in dry DMSO.

Solution B: 100 mg/mL Pluronic®-F127 surfactant in DMSO containing 0.1% acetic acid.

Solution C: 24% w/w PEG 400 and 18% TR40 by volume in water.

4.2.2 Preparation of 1 mL 6X Substrate Loading Buffer

1. Add 6 µL of Solution A to 60 µL of Solution B and vortex.
2. Add the combined solution with vigorous agitation (vortexing) to 934 µL Solution C.

4.2.3 Cell Loading (with 1 µM Substrate)

1. Add 6X loading buffer to the cells to a final concentration of 1X (this is 1 µM Substrate). For example, add 20 µL of 6X loading solution to 100 µL of cells in buffer.
2. Add the same volume of 6X Loading Buffer to wells containing Assay Medium (Step 5, **Section 4.1**)

4.2.4 Incubation

1. Cover the plate to protect it from light and evaporation.
2. Incubate at room temperature for 1.5 hours.

Note: During the incubation, the cells will settle to the bottom of each well. Handle the plate gently as the cells must remain at the bottom of each well for accurate detection to occur.

4.4 Detection

Please note that all measurements are made at room temperature from the bottom of the wells, preferably in black-walled, 96-well plates with low fluorescence background. See **Section 5.2** for more details.

5.0 DATA ANALYSIS

5.1 Background Subtraction

Background subtraction for both channels (460 nm and 530 nm) is essential to obtain meaningful data. This can be accomplished either automatically using software connected to the fluorescence plate reader, or manually after each assay plate has been read.

Use the assay plate layout to identify the location of the Cell-free Control wells. These control wells are used for background subtraction.

5.1.1 Automatic background subtraction

Use the assay plate layout to designate appropriate Cell-free Control wells and enable background subtraction for both sets of emission scans.

5.1.2 Manual background subtraction

1. Determine the average emission from the Cell-free Control wells at both 460 nm (Average Em_{460} Background) and 530 nm (Average Em_{530} Background).
2. Calculate the Net CMV-*bla* Jurkat Em_{460} and Em_{530} values and the Net Jurkat Em_{460} and Em_{530} values, as shown below:

CMV-bla Jurkat Cells

Net CMV-*bla* Jurkat Em_{460} = Em_{460} of CMV-*bla* Jurkat sample – Average Em_{460} Background

Net CMV-*bla* Jurkat Em_{530} = Em_{530} of CMV-*bla* Jurkat sample – Average Em_{530} Background

Jurkat Cells

Net Jurkat Em_{460} = Em_{460} of Jurkat sample – Average Em_{460} Background
Net Jurkat Em_{530} = Em_{530} of Jurkat sample – Average Em_{530} Background

5.2 Response Ratio Calculations

1. Calculate the CMV-*bla* Jurkat Emission Ratio for each well, as shown below:

$$\textbf{CMV-}\textit{bla}\textbf{ Jurkat Emission Ratio} = \frac{\textbf{Net CMV-}\textit{bla}\textbf{ Jurkat Em}_{460}}{\textbf{Net CMV-}\textit{bla}\textbf{ Jurkat Em}_{530}}$$

2. Calculate the Jurkat Emission Ratio for each well, as shown below:

$$\textbf{Jurkat Emission Ratio} = \frac{\textbf{Net Jurkat Em}_{460}}{\textbf{Net Jurkat Em}_{530}}$$

3. Calculate the Mean Jurkat Emission Ratio for each assay plate, as shown below:

$$\textbf{Mean Jurkat Emission Ratio} = \frac{\textbf{Sum of Jurkat Emission Ratios}}{\textbf{Total number of wells containing Jurkat cells}}$$

4. Calculate the Response Ratio for each well of interest, as shown below:

$$\textbf{Response Ratio} = \frac{\textbf{CMV-}\textit{bla}\textbf{ Jurkat Emission Ratio (Em}_{460}\textbf{/Em}_{530}\textbf{)}}{\textbf{Mean Jurkat Emission Ratio (Em}_{460}\textbf{/Em}_{530}\textbf{)}}$$

5.3 Visual Observation of Intracellular CCF2/4-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 either a xenon or mercury excitation lamp is typically required to view the CCF2/4-AM signal in cells. To visually inspect the cells, a long-pass filter passing blue and green fluorescence light is needed 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).

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).

6.0 TECHNICAL APPENDIX

This Technical Appendix describes the calibration methods we use routinely. Other calibration methods may yield different results.

6.1 Materials Required

GeneBLAzer® Loading Kit (Cat. no. K1025 or K1032)

- CCF2-AM
- DMSO for Solution A
- Solution B
- Solution C

Materials Required, but not supplied

Reagent	Recommended Source	Cat. no.
Hank's Balanced Salt Solution (pH 7.3)	Invitrogen	14175-095
2.5 µM 5,6-carboxyfluorescein	Molecular Probes	C194
125 nM 7-hydroxycoumarin-3-carboxylate	Molecular Probes	H185
10 mM sodium borate buffer (pH 9.2)	Various sources	—
96-well, black-wall, clear bottom assay plate	Costar	3603

6.2 CytoFluor® 4000 fluorescence plate reader Gain Setting Calibration

6.2.1 Standards and Controls

Note: The calibration plate does not contain cells.

Prepare the following set of controls and standards (ten replicates per control or standard, 100 µL per well) in a 96-well, black-wall, clear-bottom assay plate (Costar Cat. No. 3603), as follows:

- **CCF2-AM Control wells**. Dilute 6X CCF2-AM Substrate Loading Solution (see **Section 4.6)** to 1X with Hank's Balanced Salt Solution (pH 7.3).
- **2.5 µM 5,6-Carboxyfluorescein** in 10 mM sodium borate buffer (pH 9.2). This is a 1:400 dilution from a 1 mM stock solution.
- **125 nM 7-Hydroxycoumarin-3-carboxylate** in 10 mM sodium borate buffer (pH 9.2). This is a 1:8000 dilution from a 1 mM stock solution.

6.2.2 Using a Calibration Plate

1. Set the fluorescence plate reader to bottom-read mode and for two scans per cycle (one scan at 460 nm and one scan at 530 nm).
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 min. before making measurements.
3. Excite the plate at 409 nm. Read the calibration plate at 10 reads/well, with an initial gain of 70 for the 460-nm read and a gain of 80 for the 530-nm read. The readings should fall within the following ranges:

Expected Ranges of Fluorescence Signal Intensities on CytoFluor® 4000 Fluorescence Plate Reader				
Solution	**CCF2-AM Control Wells**	**125 nM 7-Hydroxycoumarin-3-carboxylate**	**CCF2-AM Control Wells**	**2.5 µM 5,6-Carboxyfluorescein**
Detection Wavelength	460 nm	460 nm	530 nm	530 nm
Range	250–350	2200–3000	150–250	1700–3200

4. If necessary, adjust the gain settings so that the intensities of the calibration plate fall in the expected range. Once the appropriate gain settings are determined, these settings can be used routinely. Recalibration frequency depends on instrument usage.

7.0 PURCHASER NOTIFICATION

Limited Use Label License No. 150: GeneBLAzer® Technology

This product and/or its use is the subject of U.S. and foreign patents owned by and/or licensed to Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The right to use this product for internal research specifically excludes the right to use this product to identify, discover, and profile compounds that act as a flavor, fragrance or taste-enhancers and modify a target identified in taste, olfaction, or pheromone detection, provided the compound does not require FDA approval of an NDA for claims of safety and efficacy. The right to use methods claimed in the foregoing patents with this product for research purposes can only be acquired by the use of GeneBLAzer® substrates purchased from Invitrogen Corporation or its authorized distributors. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to an academic collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Use of Genetically Modified Organisms (GMO)

Information for European Customers The CMV-*bla* Jurkat cell line(s) are genetically modified with the plasmid pCMV-blaX-neo. 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.