Invitrogen™
life technologies

Instruction Manual

GeneBLAzer™ Detection Kits

For *in vivo* or *in vitro* fluorescent detection of β-lactamase reporter activity in mammalian cells

Catalog nos. 12578-126 and 12578-134

Version B
26 January 2004
25-0661

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below, as well as with various GeneBLAzer™ vector kits. For more information about the GeneBLAzer™ vector kits, see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Product	Catalog no.
GeneBLAzer™ *In Vitro* Detection Kit	12578-126
GeneBLAzer™ *In Vivo* Detection Kit	12578-134

Shipping/Storage

The GeneBLAzer™ Detection Kits are shipped as detailed below Upon receipt, store as indicated.

Kit	Shipping	Storage
GeneBLAzer™ *In Vitro* Detection Kit	Dry ice	**CCF2-FA:** -20°C, dessicated and protected from light
GeneBLAzer™ *In Vivo* Detection Kit	Room Temperature	**CCF2-AM:** -20°C, dessicated and protected from light **Solutions:** Room temperature, protected from light

GeneBLAzer™ *In Vitro* Detection Kit Component

The GeneBLAzer™ *In Vitro* Detection Kit includes 100 µg of CCF2-FA Substrate and is supplied as an orange powder. **Store CCF2-FA at –20°C, dessicated and protected from light.** The product is stable for at least 6 months under these conditions.

GeneBLAzer™ *In Vivo* Detection Kit Components

The following reagents are included with the GeneBLAzer™ *In Vivo* Detection Kit. **Store CCF2-AM at –20°C, dessicated and protected from light. Store the other reagents at room temperature, protected from light.** All components are stable for at least 6 months under these conditions.

Note: The amount of CCF2-AM supplied is sufficient to perform 375 detection reactions with cells plated in a 96-well format.

Reagent	Composition	Amount
CCF2-AM Substrate	Dry powder	50 µg
Anhydrous DMSO	Liquid	1 ml
Solution B	100 mg/ml Pluronic®-F127 surfactant in DMSO and 0.1% acetic acid	500 µl
Solution C	24% (w/v) PEG 400 18% (v/v) TR40 in water	7.5 ml

continued on next page

Pluronic® is a registered trademark of BASF Corporation.

Kit Contents and Storage, continued

Substrate Molecular Weights

The table below lists the molecular weight and nmol supplied for each CCF2 substrate.

Substrate	Molecular Weight	nmol Supplied
CCF2-FA	856.23 g/mol	117 nmol
CCF2-AM	1082 g/mol	46 nmol

Accessory Products

The table below provides ordering information for larger sizes of the CCF2-FA and CCF2-AM substrates as well other products available from Invitrogen that may be used with the GeneBLAzer™ Detection Kits. For more information about these products, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Item	Amount	Catalog no.
CCF2-FA Substrate	1 mg	K1027
CCF2-AM Loading Kit	200 µg	K1032
	5 mg	K1025
CCF2-AM Substrate	5 mg	K1023
	20 mg	K1024
Hank's Balanced Salt Solution (HBSS)	500 ml	14175-095
	1 L	14175-079
HEPES Buffer Solution (1 M)	20 ml	15630-106
	100 ml	15630-080

Introduction

Overview

Introduction

The GeneBLAzer™ Detection Kits use the GeneBLAzer™ Technology to facilitate *in vivo* or *in vitro* detection of β-lactamase reporter activity in mammalian cells using a unique fluorescent substrate. Use of the GeneBLAzer™ Technology provides a highly sensitive and accurate method to quantitate gene expression in mammalian cells.

Advantages of the GeneBLAzer™ Detection System

Using the GeneBLAzer™ Technology and the GeneBLAzer™ Detection System as a reporter of gene expression in mammalian cells provides the following advantages:

- Suitable for use as a sensitive reporter of gene expression in living mammalian cells using fluorescence microscopy.
- Provides a ratiometric readout to minimize differences due to variability in cell number, substrate concentration, fluorescence intensity, and emission sensitivity.
- Compatible with a wide variety of *in vivo* and *in vitro* applications including microplate-based transcriptional assays and flow cytometry.
- Provides a flexible and simple assay development platform for gene expression in mammalian cells.
- Using a non-toxic substrate allows continued cell culturing after quantitative analysis.

Purpose of this Manual

This manual provides the following information:

- An overview of the GeneBLAzer™ Technology including the CCF2 fluorescent substrate used in the GeneBLAzer™ Detection System.
- Guidelines and instructions to use the GeneBLAzer™ *In Vitro* Detection Kit to quantitate β-lactamase reporter activity in mammalian cell lysates.
- Guidelines and instructions to use the GeneBLAzer™ *In Vivo* Detection Kit to qualitatively or quantitatively assess β-lactamase reporter activity in live mammalian cells using a variety of fluorescence measurement techniques.

The GeneBLAzer™ Technology

Components of the GeneBLAzer™ System

The GeneBLAzer™ System facilitates fluorescent detection of β-lactamase reporter activity in mammalian cells, and consists of two major components:

- The β-lactamase reporter gene, *bla*(M), a truncated form of the *E. coli bla* gene. When fused to promoter sequences or to a gene of interest (in the context of a GeneBLAzer™ vector), the *bla*(M) gene can be used as a reporter of promoter activity or gene expression in mammalian cells, respectively. For more information about the *bla*(M) gene, see below.
- A fluorescence resonance energy transfer (FRET)-enabled substrate, CCF2 to facilitate fluorescent detection of β-lactamase reporter activity. In the absence or presence of β-lactamase reporter activity, cells loaded with the CCF2 substrate fluoresce green or blue, respectively. Comparing the ratio of blue to green fluorescence in a population of live cells or in a cell extract of your sample to a negative control provides a means to quantitate gene expression. For more information about the CCF2 substrate and how FRET works, see the next page.

β-Lactamase (*bla*) Gene

β-lactamase is the product encoded by the ampicillin resistance gene (*bla*) and is the bacterial enzyme that hydrolyzes penicillins and cephalosporins. The *bla* gene is present in many cloning vectors and allows ampicillin selection in *E. coli*. β-lactamase enzyme activity is not found in mammalian cells.

bla(M) Gene

The GeneBLAzer™ Technology uses a modified *bla* gene as a reporter in mammalian cells. This *bla* gene is derived from the *E. coli TEM-1* gene present in many cloning vectors (Zlokarnik *et al.*, 1998), and has been modified in the following ways:

- 72 nucleotides encoding the first 24 amino acids of β-lactamase were deleted from the N-terminal region of the gene. These 24 amino acids comprise the bacterial periplasmic signal sequence, and deleting this region allows cytoplasmic expression of β-lactamase in mammalian cells.
- The amino acid at position 24 was mutated from His to Asp to create an optimal Kozak sequence for optimal translation initiation.

This modified reporter gene is named *bla*(M).

Note: The *TEM-1* gene also contains 2 mutations (at nucleotide positions 452 and 753) that distinguish it from the *bla* gene in pBR322 (Sutcliffe, 1978).

continued on next page

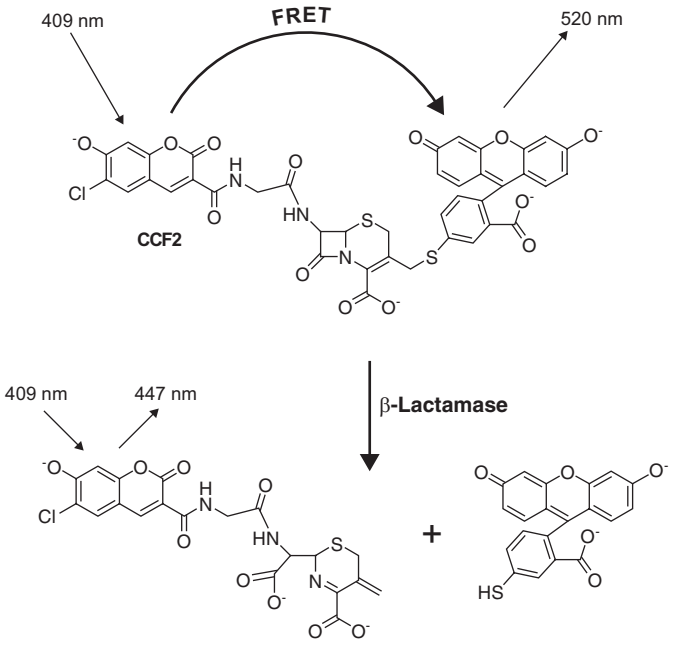
The GeneBLAzer™ Technology, continued

CCF2 Fluorescent Substrate and How FRET Works

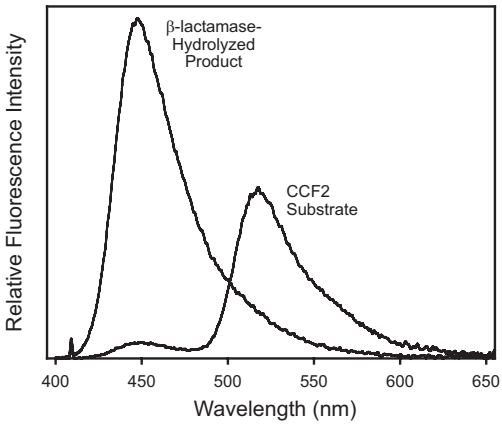
The second component of the GeneBLAzer™ Technology is the fluorescent CCF2 substrate for β-lactamase. CCF2 consists of a cephalosporin core linked to two fluorophores, 7-hydroxycoumarin and fluorescein. In the absence of β-lactamase reporter activity, the substrate molecule remains intact. Excitation of the coumarin at 409 nm results in fluorescence resonance energy transfer (FRET) to the fluorescein moiety. This energy transfer causes the fluorescein to emit a green fluorescence signal with an emission peak of 520 nm. In the presence of β–lactamase reporter activity, the CCF2 substrate is cleaved, disrupting FRET. In this case, excitation of the coumarin at 409 nm results in emission of a blue fluorescence signal with an emission peak of 447 nm. In a population of cells loaded with CCF2 substrate, those that fluoresce blue contain β-lactamase reporter activity while those that fluoresce green do not.

In the figure below, panel A illustrates how CCF2 is hydrolyzed by β-lactamase and how CCF2 FRET works. Panel B depicts the fluorescence emission spectra of the CCF2 substrate and its hydrolyzed product after excitation at 409 nm.

A



B



Adapted with permission from *Science*, Vol. 276, p. 85 (January 2, 1998). ©1998.

CCF2 Derivatives

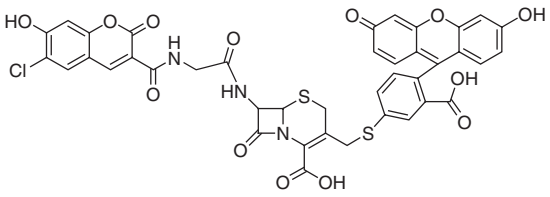
Two derivatives of CCF2 have been developed to enable use of the fluorescent substrate for *in vivo* or *in vitro* applications. CCF2-FA is supplied in the GeneBLAzer™ *In Vitro* Detection Kit while CCF2-AM is supplied in the GeneBLAzer™ *In Vivo* Detection Kit. These substrates are described further on the next page.

continued on next page

The GeneBLAzer™ Technology, continued

CCF2-FA

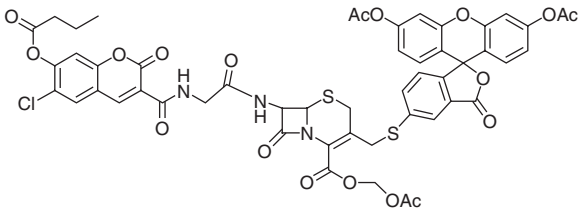
CCF2-FA is the free acid form of the CCF2 substrate and is supplied in the GeneBLAzer™ *In Vitro* Detection Kit (see figure below for the structure of CCF2-FA). This free acid form is water soluble, making it suitable for direct addition to cell lysates.



Formula: $C_{40}H_{26}ClN_3O_{13}S_2$
Exact Mass: 855.06
Molecular Weight: 856.23

CCF2-AM

CCF2-AM is a hydrophobic, membrane-permeable, esterified form of the CCF2 substrate and is supplied in the GeneBLAzer™ *In Vivo* Detection Kit (see figure below for the structure of CCF2-AM). This esterified form is non-toxic, lipophilic and readily enters the cell. Once inside the cell, the CCF2-AM is converted into CCF2. For more information, see below.

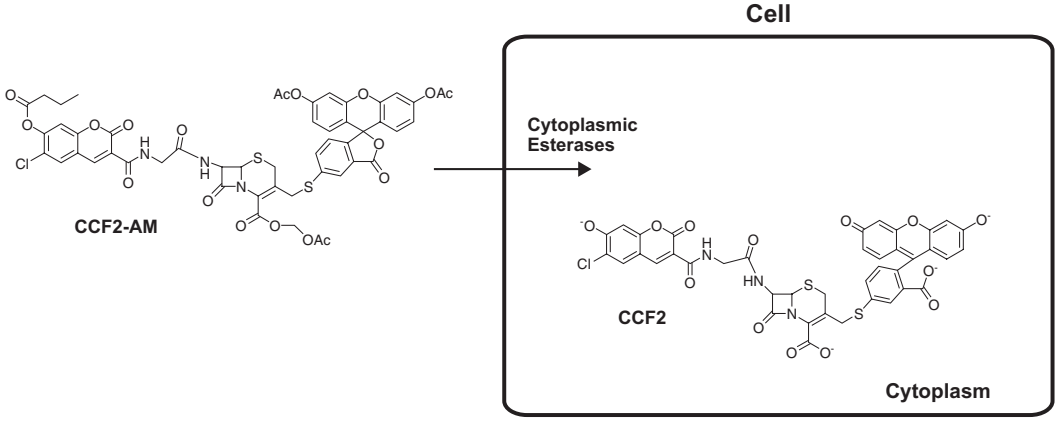


Formula: $C_{51}H_{40}ClN_3O_{18}S_2$
Exact Mass: 1081.14
Molecular Weight: 1082.46

Ac = acetyl

How CCF2-AM is Converted to CCF2

When added to mammalian cells, the lipophilic, esterified CCF2-AM substrate enters the cell via diffusion, where it is cleaved by endogenous cytoplasmic esterases and rapidly converted into its negatively charged form, CCF2 (see figure below). The hydrophilic, charged CCF2 substrate is trapped inside the cell. Over time, this results in cells "loading" with more substrate, thereby increasing the intracellular substrate concentration. This increases the sensitivity of the detection assay without the need for addition of higher concentrations of substrate.



Experimental Outline

In vitro Detection

The table below outlines the steps required to use the GeneBLAzer™ *In Vitro* Detection Kit to assay for β-lactamase reporter activity in your mammalian cell lysates.

Step	Action	Page
1	Prepare a cell lysate from your mammalian cells of interest using a method that preserves the enzyme activity of β-lactamase.	8-9
2	Prepare a 100 μM CCF2-FA Stock Solution (10X stock) and add the appropriate amount of CCF2-FA solution to the mammalian cell lysate.	10
3	Detect CCF2-FA fluorescence signal using a fluorescence plate reader or fluorometer.	25-27

In vivo Detection

The table below outlines the steps required to use the GeneBLAzer™ *In Vivo* Detection Kit to assay for β-lactamase reporter activity in your live mammalian cells.

Step	Action	Page
1	Prepare 1 mM CCF2-AM Stock Solution (Solution A).	14
2a	Prepare 6X CCF2-AM Loading Solution and use the General Loading Protocol to load your mammalian target cells.	15-18
2b	Alternatively, prepare 6X CCF2-AM Enhanced Loading Solution and use the Enhanced Loading Protocol to load your mammalian target cells.	19-22
3	Detect CCF2-AM fluorescence signal using the method of choice (*i.e.* fluorescence microscopy, ratiometric imaging, fluorescence plate reader, FACS).	23-29

Methods

Using β-Lactamase as a Reporter of Gene Expression

Introduction

Before you can use one of the GeneBLAzer™ Detection Kits to assay for gene expression in mammalian cells, you must generate an expression construct containing your gene or promoter of interest fused to the *bla*(M) reporter gene. Depending on your application of choice, a number of GeneBLAzer™ vectors are available from Invitrogen (see below).

Expression Vectors

The table below and on the next page lists the GeneBLAzer™ vectors available from Invitrogen and their features. Most vectors are supplied with a choice of the GeneBLAzer™ *In Vivo* or *In Vitro* Detection Kit. For more information about each vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Vector	Features
pGeneBLAzer-TOPO®	Allows 5-minute, TOPO® Cloning of your DNA fragment of interest upstream of β-lactamase for promoter analysis
pcDNA™6.2/nGeneBLAzer™-DEST	• Allows N-terminal fusion of β-lactamase to the gene of interest • High-level expression from the CMV promoter • Contains *att*R sites for efficient recombination with a Gateway® entry clone
pcDNA™6.2/cGeneBLAzer™-DEST	• Allows C-terminal fusion of β-lactamase to the gene of interest • High-level expression from the CMV promoter • Contains *att*R sites for efficient recombination with a Gateway® entry clone
pcDNA™6.2/nGeneBLAzer™-GW/D-TOPO®	• Allows N-terminal fusion of β-lactamase to the gene of interest • High-level expression from the CMV promoter • 5-minute, TOPO® Cloning of your PCR product directly into an expression vector • Designed for streamlined downstream analysis in multiple systems using Gateway® recombinational cloning

continued on next page

Using β-Lactamase as a Reporter of Gene Expression, continued

Expression Vectors, continued

Vector	Features
pcDNA™6.2/cGeneBLAzer™-GW/D-TOPO®	• Allows C-terminal fusion of β-lactamase to the gene of interest • High-level expression from the CMV promoter • 5-minute, TOPO® Cloning of your PCR product directly into an expression vector • Designed for streamlined downstream analysis in multiple systems using Gateway® recombinational cloning
pENTR™/GeneBLAzer™	Gateway® entry clone containing the *bla*(M) gene for generating a β-lactamase expression control in any Gateway® destination vector

Transfection

Once you have generated an expression construct containing the *bla*(M) reporter gene, you may assay for β-lactamase reporter activity in a transient assay or from a stable cell line. For transient assays, perform transient transfection using your transfection method of choice. To generate a stable cell line, transfect cells and select for stable cell lines using the appropriate selection reagent.

For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-019) available from Invitrogen. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Using the GeneBLAzer™ *In Vitro* Detection Kit

Introduction

This section provides guidelines to use the GeneBLAzer™ *In Vitro* Detection Kit to measure β-lactamase reporter activity in mammalian cell lysates. Using this kit allows you to quantitate the amount of intracellular β-lactamase in cells based on the β-lactamase activity in lysates from transfected cells as compared to an appropriate negative control. To detect β-lactamase activity in cell lysates, you will use the CCF2-FA substrate. CCF2-FA is the non-esterified, free acid form of CCF2, and is recommended for *in vitro* use because it is readily soluble in aqueous solution and may be added directly to pre-made cell lysates. Once added to cell lysates, you may quantitate the fluorescent CCF2-FA signal using a fluorescence plate reader or a fluorometer.

Note

If you want to visualize CCF2 fluorescence signal in your cells before making cell lysates, you will need to use the GeneBLAzer™ *In Vivo* Detection Kit and load cells with the CCF2-AM substrate provided in the kit.

Materials Needed

To prepare a cell lysate, have the following materials on hand before beginning.

- Adherent or suspension cell line of interest expressing β-lactamase
- Versene (for adherent cell lines only; Catalog no. 15040-066)
- Reagents and supplies to count cells
- Hank's Balanced Salt Solution (HBSS, Catalog no. 14175-095) or HEPES Buffer Saline (HBS, Catalog no. 15630-080)
- Liquid nitrogen or dry ice/ethanol bath
- 30°C water bath

continued on next page

Preparing Cell Lysates

To prepare lysates from mammalian cells containing the *bla*(M) reporter gene, you **must** use a method that will preserve the activity of the β-lactamase enzyme. A procedure to prepare cell lysates using three cycles of freezing and thawing is provided below. Other protocols are suitable (see below for alternatives). For high-throughput applications, we suggest using one of the alternative methods below.

1. Harvest cells as follows.
   - For adherent cells, dissociate cells with an EDTA-containing buffer using standard methods (*e.g.* Versene). Count cells using a cell counter or a hemacytometer. Centrifuge cells to pellet. Wash the cell pellet twice with HBSS or HBS. Resuspend the cell pellet in HBSS or HBS to a density of 1×10^7 cells/ml in a microcentrifuge tube.

     Note: Do not dissociate cells using trypsin-EDTA. Over trypsinizing cells may reduce β-lactamase activity by causing cell lysis and proteolysis.
   - For suspension cells, remove an aliquot and count cells using a cell counter or a hemacytometer. Harvest cells by centrifugation. Resuspend the cell pellet in HBSS or HBS to a density of 1×10^7 cells/ml in a microcentrifuge tube.
2. Freeze cells by placing the tube in liquid nitrogen or a dry ice/ethanol bath.
3. Transfer the tube to a 30°C water bath until cells are thawed. To prevent degradation, avoid excessive incubation at 30°C. Performing Steps 2 and 3 constitutes 1 freeze/thaw cycle.
4. Repeat Steps 2 and 3 twice more for a total of 3 freeze/thaw cycles.
5. Centrifuge the sample in a microcentrifuge at +4°C at maximum speed to pellet cell debris.
6. Transfer the supernatant to a sterile microcentrifuge tube. Store the cell lysate at -20°C or at -80°C.

Alternative Lysis Protocols

You may also prepare cell lysates using sonication or a gentle detergent such as 1% NP-40, 1% IGEPAL CA-630 (Sigma, Catalog no. I-3021) or 0.5% CHAPS, if desired.

For high-throughput applications, we recommend preparing cell lysates using one of the detergents suggested above. Lyse cells directly in the tissue culture well.

continued on next page

Preparing the CCF2-FA Stock Solution

To use CCF2-FA, prepare a 100 µM stock solution of CCF2-FA in Hank's Balanced Salt Solution (HBSS) or HEPES Buffer Saline (HBS).

Note: Other phosphate-based buffers such as Phosphate-Buffered Saline (PBS) are also suitable.

1. Add 1.17 ml of HBS to the vial containing CCF2-FA powder to obtain a 100 µM stock solution. Vortex to dissolve.
2. Aliquot desired volumes into cryovials and freeze quickly by placing the vials on dry ice or in liquid nitrogen.

   Note: This minimizes freeze/thaw cycles during use.
3. Once the solutions are frozen, transfer the cryovials to a -20°C freezer. Store the solutions protected from light. **When stored under these conditions, the aqueous CCF2-FA stock solution is stable for at least one month.**

Preparing the Samples

Follow the guidelines below to add the CCF2-FA substrate to cell lysates.

Materials Needed

- Mammalian cell lysate(s) of interest (thaw on ice before use)
- 100 µM CCF2-FA stock solution (thaw to room temperature immediately before use, remove the desired amount and return the stock solution to -20°C storage)

Procedure

1. For each sample, add the desired amount of cell lysate to a 96-well microtiter plate. Do not exceed a volume of 90 µl.
2. To each well containing sample, add the CCF2-FA stock solution to obtain a final concentration of 10 µM (10-fold dilution). For example, add 10 µl of CCF2-FA to 90 µl of cell lysate (total volume = 100 µl).
3. Proceed to read the fluorescence signal in a fluorescence plate reader (see **Detecting CCF2 Signal Using a Fluorescence Plate Reader**, page 25) or fluorometer.

   Recommendation: Note that although β-lactamase cleaves the CCF2 substrate rapidly, longer incubation times may be required to optimize the fluorescence signal when low levels of the enzyme are present in the cell lysate. We recommend reading the fluorescence signal every 15 minutes for 1 hour.

General Guidelines to Use the GeneBLAzer™ *In Vivo* Detection Kit

Introduction

Use the GeneBLAzer™ *In Vivo* Detection Kit to measure β-lactamase reporter activity in live mammalian cells. Using this kit allows you to monitor cellular gene expression under real-time, physiological conditions. Once β-lactamase reporter activity has been measured, cells may be cultured further for use in additional assays or other downstream applications.

To detect β-lactamase reporter activity in live mammalian cells, you will use the CCF2-AM substrate. CCF2-AM is the membrane-permeable, esterified form of CCF2, and is recommended for *in vivo* use because it is lipophilic and readily enters the cell. Once cells are "loaded" with CCF2-AM, you may quantitate the CCF2 fluorescence signal using a variety of methods.

Important: Do not use CCF2-FA for *in vivo* detection. The CCF2-FA substrate will not enter the cells.

Factors Affecting Cell Loading

A number of factors can influence the degree of cell loading, and consequently, the success of your detection experiment. These factors include:

- The cell type or cell line you are using
- The density of your cells at the time of loading
- The temperature at which you perform cell loading
- The degree to which your cell line retains the CCF2-AM substrate
- The loading protocol used

Each of these factors is discussed further in this section.

Selecting a Cell Line

You may use any mammalian cell line or cell type of choice to express your β-lactamase reporter construct for detection using the GeneBLAzer™ System. This includes cell lines that grow in suspension or as adherent monolayers. Note however, that cell lines may vary significantly in their rate and ability to load and retain the CCF2-AM substrate. Depending on the cell line that you choose for your experiments, we recommend using the guidelines below regarding cell density, loading temperature, loading time, and loading protocol as a starting point. Once you have evaluated the blue and green fluorescence signal, you may vary conditions and loading protocols, as desired to optimize your detection experiment.

continued on next page

General Guidelines to Use the GeneBLAzer™ *In Vivo* Detection Kit, continued

Cell Density

Suspension cells: These cells typically load most efficiently at a density of 1-2 x 10^6 cells/ml.

Adherent Cells: For these cells, the efficiency of loading is dependent on the cell density. Cells load with CCF2-AM substrate most efficiently when they are 60-80% confluent at the time of loading. In contrast, confluent cells load poorly.

- For analysis of gene expression from a stable cell line, we recommend plating cells such that they will be 60-80% confluent at the time of loading.
- For transient analysis of gene expression, we recommend the following:
  a. Use Lipofectamine™ 2000 Reagent available from Invitrogen (Catalog no. 11668-027) for transfection.
  b. Transfect cells as recommended in the Lipofectamine™ 2000 manual (*i.e.* 90% confluence for 4-6 hours). Incubate cells at 37°C overnight, then trypsinize and re-plate the transfected cells such that they are 50-60% confluent. Incubate the cells overnight at 37°C, and load cells the next day.

Loading Temperature

We recommend loading cells at room temperature. The rate at which cells load with CCF2-AM substrate is affected by temperature. Generally, increasing the temperature (*e.g.* from room temperature to 37°C) will increase the loading rate. However, increasing the temperature also increases the rate at which the substrate is exported from the cell, resulting in lower overall steady-state uptake of CCF2-AM.

Loading Time

As a starting point, we recommend loading cells for one hour. Cell lines vary in their ability to load and retain the CCF2-AM substrate. For example, lymphoma cells tend to load in 15-30 minutes, while most adherent cells load well in 30 minutes to 1 hour at room temperature. Generally, fluorescence signal is detectable by 15 minutes after loading and increases steadily for about 60 minutes. Longer incubation times may further increase the intensity of the fluorescence signal, but the increase in intensity is smaller than that observed in the first hour. Depending on your cell line and the application, you may vary the CCF2-AM loading time to optimize the fluorescence signal.

If you are performing *in vivo* detection for the first time, we recommend verifying adequate substrate loading for your cell line and application. You may accomplish this by visualizing cell loading (*e.g.* take a reading every 15 minutes for up to 2 hours) using a fluorescence microscope (see **Detecting CCF2 Signal Using Fluorescence Microscopy**, page 23) and determining how quickly the cells fluoresce green. Alternatively, you may monitor loading using a bottom-read fluorescence plate reader (*e.g.* Gemini-EM Fluorescence Microtiter Plate Reader, Molecular Devices or CytoFluor® 4000 Fluorescence Plate Reader, PerSeptive Biosystems).

continued on next page

General Guidelines to Use the GeneBLAzer™ *In Vivo* Detection Kit, continued

Selecting a Loading Protocol

Two loading protocols are provided in this manual to facilitate cell loading of CCF2-AM, a **General Loading Protocol** and an **Enhanced Loading Protocol**. For most cell lines, the General Loading Protocol is recommended and results in efficient cell loading and a highly detectable CCF2-AM fluorescence signal. In some cell lines, using the General Loading Protocol results in a weak fluorescence signal. These cell lines are generally those that possess active anion transport, resulting in export of the substrate (see examples below). For these cell lines, we recommend loading cells using the Enhanced Loading Protocol. Depending on the nature of your cell line, choose the loading protocol that best suits your needs.

Examples

The table below lists a number of mammalian cell lines and the recommended protocol to use for loading.

Recommended Loading Protocol	Cell Line
General Loading Protocol	HEK293 COS-7 Jurkat
Enhanced Loading Protocol	CHO-K1 CV-1 ME-180 HepG2

Method of Detection

Once cells have been loaded with the CCF2-AM substrate, you may use a variety of methods to analyze the fluorescence signal including:

- Visual inspection of fluorescent cells using fluorescence microscopy
- Quantitative analysis of blue and green fluorescence by ratiometric imaging using a fluorescence microscope
- Quantitative analysis of blue and green fluorescence using a fluorescence plate reader
- Fluorescence-activated cell sorting (FACS) to isolate cells expressing β-lactamase

These methods are discussed in greater detail later in this manual.

Preparing CCF2-AM Stock Solution (Solution A)

Introduction

This section provides instructions to prepare the CCF2-AM stock solution.

Note

For optimal results, use the anhydrous DMSO supplied with the kit to solubilize CCF2-AM. The anhydrous DMSO does not contain inhibitory contaminants and is assay grade.

Preparing and Using the CCF2-AM Stock Solution (Solution A)

Follow the instructions below to prepare a 1 mM stock solution of CCF2-AM in anhydrous DMSO (supplied with the kit).

1. Add 46 µl of anhydrous DMSO to the vial containing 50 µg of CCF2-AM powder. Vortex to dissolve. This 1 mM stock solution is called **Solution A**.
2. Store Solution A at -20°C, dessicated and protected from light. **When stored under these conditions, Solution A is stable for at least one month.**

When to Use Solution A

When you are ready to load cells with CCF2-AM, you will combine Solution A with Solutions B and C to prepare the 6X CCF2-AM Loading Solution. The 6X CCF2-AM Loading Solution differs slightly depending on whether you plan to load cells using the **General Loading Protocol** or the **Enhanced Loading Protocol**. To determine which protocol to use, refer to **Selecting a Loading Protocol**, page 13. For instructions to prepare the 6X CCF2-AM Loading Solution for use in the General Loading Protocol or the Enhanced Loading Protocol, see page 15 or page 19, respectively.

Note

Before each use, let the frozen Solution A warm to room temperature and remove the desired amount of reagent. Immediately recap the vial to reduce moisture uptake and return to -20°C storage.

Note: Once thawed, Solution A may appear slightly yellow. This color change is normal and does not affect the performance of the reagent.

Using the General Loading Protocol for *in vivo* Detection

Introduction

To assay for β-lactamase reporter activity in your mammalian cell line of interest, you will load cells with the fluorescent CCF2-AM substrate and evaluate the difference in blue and green signal intensity compared to a negative control (cells with no β-lactamase reporter activity). Guidelines and instructions are provided in this section to prepare and load cells with a 6X CCF2-AM Loading Solution using the General Loading Protocol. Two protocols are provided; one to load adherent cells and one to load suspension cells. Choose the protocol that best fits your needs.

Important

If you plan to culture cells further after measuring β-lactamase reporter activity, be sure to maintain sterility throughout the experiment.

- Perform all manipulations within a tissue-culture hood
- Prepare solutions using sterile reagents

Preparing 6X CCF2-AM Loading Solution

Follow this protocol to prepare 1 ml of 6X CCF2-AM Loading Solution.

Materials Needed

- Solution A (see previous page; warm to room temperature and vortex briefly)
- Solution B (supplied with the GeneBLAzer™ *In Vivo* Detection Kit; keep at room temperature)

  Note: If Solution B is stored at cooler temperatures, a white precipitate may form or the solution may freeze. Warm and mix the solution at 37°C until the precipitate dissolves. Use as directed below.
- Solution C (optional; supplied with the GeneBLAzer™ *In Vivo* Detection Kit; keep at room temperature, protected from light). See **Note** on the next page for additional information.
- HBSS (Catalog no. 14175-095; if reading fluorescence signal in a top-read fluorescence plate reader or performing FACS)

Procedure

1. Add 6 µl of Solution A to 54 µl of Solution B and vortex to mix thoroughly.
2. Add 940 µl of Solution C or 940 µl of HBSS (see **Note** on the next page) to the combined Solutions A and B (60 µl volume) to obtain a final volume of 1 ml. Vortex to mix thoroughly.
3. Proceed directly to load cells. To load adherent cells, see **General Loading Protocol for Adherent Cells**, page 17. To load suspension cells, see **General Loading Protocol for Suspension Cells**, page 18.

Important

Use the 6X CCF2-AM Loading Solution within two hours of preparation as the substrate degrades over time in aqueous solution. Discard any unused solution.

continued on next page

Using the General Loading Protocol for *in vivo* Detection, continued

Note

Solution C is added to the 6X CCF2-AM Loading Solution to reduce non-specific fluorescence due to substrate that has not entered the cell. If you plan to read fluorescence signal from your cells using a top-read fluorescence plate reader **or** perform FACS, note that the presence of Solution C will interfere with the fluorescence signal. In either case,

1. Prepare 6X CCF2-AM Loading Solution as directed on the previous page, except substitute 940 µl of HBSS or PBS (Ca^{2+}- and Mg^{2+}-free) for Solution C. Load cells using the General Loading Protocol.
2. After loading, remove the loading solution and wash the cells with HBSS.
3. Replace with an equal volume of HBSS before taking a reading (if using a fluorescence plate reader) or prepare cells for flow cytometry (if performing FACS).

Tip: Reading fluorescence signal from a bottom-read fluorescence plate reader provides the best sensitivity.

Recommended Loading Conditions

Use the following recommended conditions to load your cells using the 6X CCF2-AM Loading Solution. For more information, see the section entitled **General Guidelines to Use the GeneBLAzer™ *In Vivo* Detection Kit**, page 11.

Condition	Recommendation
Tissue culture format	• You may plate cells in any size tissue culture plate of choice (*e.g.* 96-well format). • Make sure that your tissue culture plate is compatible with your detection instrument. • If you plan to perform detection in 96-well plates, plate cells in black-walled, clear bottom 96-well plates (see page 26 for more information).
Cell density	For optimal loading efficiency: • Plate adherent cells such that they will be 60-80% confluent at the time of loading. • Load suspension cells at a density of 1-2 x 10^6 cells/ml.
Loading temperature	Load cells at room temperature.
Loading time	For most cell lines, load cells for one hour.
Loading buffer	For optimal efficiency, load cells in HBSS or HBS. **Note:** You may use serum-containing media, however, CCF2-AM may hydrolyze during prolonged exposure to serum. This may affect the rate of CCF2-AM loading.

continued on next page

Using the General Loading Protocol for *in vivo* Detection, continued

Materials Needed

Be sure to have the following materials on hand before beginning cell loading:

- 6X CCF2-AM Loading Solution (prepare immediately before use, see page 15)
- Hank's Balanced Salt Solution (HBSS; Catalog no. 14175-095)
- Mammalian cell line of interest (plated in the tissue culture format of choice or in suspension, as appropriate)

General Loading Protocol for Adherent Cells

Follow this protocol to load adherent cells with the CCF2-AM substrate. You may plate your cells in any tissue culture format of choice. Refer to the table in **Recommended Reagent Volumes**, below for the appropriate reagent amounts to add. We recommend including a negative control (no cells) and an untransfected or uninduced control in your experiment so that you can determine the background blue and green fluorescence.

1. Remove the growth medium from the cells and wash cells once with HBSS. Add the appropriate amount of HBSS to each well (see **Recommended Reagent Volumes**, below). For example, add 100 µl of HBSS to each well of a 96-well plate.
2. For each sample, add the appropriate amount of 6X CCF2-AM Loading Solution to the well (6-fold dilution) to obtain a final concentration of 1X (see **Recommended Reagent Volumes**, below). This is 1 µM CCF2-AM. For example, add 20 µl of 6X CCF2-AM Loading Solution to 100 µl of cells in HBSS in a 96-well plate (total volume = 120 µl).
3. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 1 hour, protected from light.

   Note: Extending the incubation time may increase the fluorescence signal, but may also increase the background.
5. Proceed to detect fluorescence signal using the method of choice (see **What to Do Next**, page 18). Alternatively, you may remove the CCF2-AM Loading Solution and replace with fresh, growth medium or HBSS, then proceed to detection.

Recommended Reagent Volumes

The table below lists the recommended volumes of 6X CCF2-AM Loading Solution and HBSS to use for loading cells cultured in various tissue culture formats.

Culture Vessel	6X CCF2-AM Loading Solution	HBSS
96-well	20 µl	100 µl
48-well	40 µl	200 µl
24-well	100 µl	500 µl
12-well	150 µl	750 µl
6-well	250 µl	1250 µl

continued on next page

Using the General Loading Protocol for *in vivo* Detection, continued

General Loading Protocol for Suspension Cells

Follow this protocol to load suspension cells with the CCF2-AM substrate. We recommend including a negative control (no cells) and an untransfected or uninduced control in your experiment so that you can determine the background blue and green fluorescence.

1. For each sample, pellet 1-2 x 10^5 cells by centrifugation. Wash the cell pellet once with HBSS, then resuspend in 100 µl of HBSS.
2. To each sample, add 20 µl of the 6X CCF2-AM Loading Solution to 100 µl of cells in buffer to obtain a final concentration of 1X. This is 1 µM CCF2-AM.
3. Transfer the cells and CCF2-AM Loading Solution to a black-walled, clear bottom 96-well tissue culture plate. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 1 hour, protected from light.
5. Proceed to detect fluorescence signal using the method of choice (see **What to Do Next**, below).

   Note: During the incubation, cells will settle to the bottom of the well. If you are using a bottom-read fluorescence plate reader, handle the plate gently as the cells must remain at the bottom of each well for accurate detection to occur. Be careful not to touch the bottom of the plate.

What to Do Next

After loading cells with the CCF2-AM substrate, we recommend inspecting the cells visually in a fluorescence microscope first to qualitatively assess the fluorescence signal. If the blue and green CCF2-AM fluorescence signal is detectable, you may:

- Quantitate β-lactamase reporter activity in live cells using a fluorescence plate reader (see page 25) or ratiometric imaging with a fluorescence microscope (see page 24).

  Recommendation: If you are using a fluorescence plate reader to detect fluorescence signal in whole cells, note that optimal sensitivity is obtained with a bottom-read fluorescence plate reader.
- Prepare cell lysates and measure β-lactamase reporter activity using a fluorescence plate reader. See **Preparing Cell Lysates**, page 9 for a procedure to prepare cell lysates.
- Perform FACS to select cells based on their β-lactamase reporter activity. See page 28 for guidelines to prepare samples for cell sorting.

If your fluorescence signal appears weak after using the general loading protocol, you may want to repeat the experiment and load cells using the **Enhanced Loading Protocol**, page 19.

Using the Enhanced Loading Protocol for *in vivo* Detection

Introduction

For cells that display weak fluorescence signal (*i.e.* poor substrate retention) by visual inspection on a fluorescence microscope after being loaded with CCF2-AM using the General Loading Protocol, we recommend repeating CCF2-AM loading using a 6X CCF2-AM Enhanced Loading Solution. Cell lines that typically exhibit an increased fluorescence signal after being loaded with the 6X CCF2-AM Enhanced Loading Solution are those that possess active ion transport mechanisms including CHO-K1, CV-1, ME-180, and HepG2. Guidelines and instructions are provided in this section to prepare and load cells with a 6X CCF2-AM Enhanced Loading Solution.

Choosing Which 6X CCF2-AM Enhanced Loading Solution to Use

The 6X CCF2-AM Enhanced Loading Solution contains a higher concentration of CCF2-AM and probenecid, a non-specific inhibitor of anion transport (DiVirgilio *et al.*, 1988). Although the presence of probenecid can increase the amount of substrate retained in the cell, it may be toxic to some cell types. If you observe cell toxicity upon using the 6X CCF2-AM Enhanced Loading Solution containing probenecid, we recommend preparing the 6X CCF2-AM Enhanced Loading Solution as directed, but omit the probenecid.

Preparing Probenecid

Probenecid (p-[Dipropylsulfamoyl]benzoic acid) is available from Sigma (Catalog no. P-8761). To use probenecid, prepare a 250 mM stock solution (100X) as directed below.

Materials Needed

- Probenecid
- 500 mM NaOH
- 100 mM sodium phosphate buffer, pH 8.0
- 1 M HCl
- 1 M NaOH

Procedure

1. Prepare a 500 mM stock solution of probenecid in 500 mM NaOH.
2. Add an equal volume of 100 mM sodium phosphate buffer, pH 8.0.
3. Adjust the pH of the resulting 250 mM solution to pH 8.0 with 1 M HCl or 1 M NaOH.
4. Aliquot the 250 mM probenecid stock solution (100X) in 1 ml aliquots into microcentrifuge tubes. Store at -20°C. The solution is stable for at least 4 months.

continued on next page

Using the Enhanced Loading Protocol for *in vivo* Detection, continued

Preparing 6X CCF2-AM Enhanced Loading Solution

Follow the instructions below to prepare 6X CCF2-AM Enhanced Loading Solution.

Materials Needed

- Solution A (see page 14; warm to room temperature)
- Solution B (supplied with the GeneBLAzer™ *In Vivo* Detection Kit; keep at room temperature)

  Note: If Solution B is stored at cooler temperatures, a white precipitate may form or the solution may freeze. Warm and mix the solution at 37°C until the precipitate dissolves. Use as directed below.
- Solution C (supplied with the GeneBLAzer™ *In Vivo* Detection Kit; keep at room temperature, protected from light)
- 250 mM (100X) Probenecid Stock Solution (see previous page; warm to room temperature)

Procedure

1. Add 12 µl of Solution A to 48 µl of Solution B and vortex.
2. Add 60 µl of probenecid stock solution (optional) to the combined Solutions A and B (total volume = 120 µl).
3. Add 880 µl of Solution C (940 µl if probenecid is omitted) to the loading buffer to obtain a final volume of 1 ml. Vortex to mix.
4. Proceed directly to load cells. To load adherent or suspension cells, see **Enhanced Loading Protocol for Adherent Cells** or **Enhanced Loading Protocol for Suspension Cells**, respectively, on pages 21-22.

Important

Use the 6X CCF2-AM Enhanced Loading Solution within two hours of preparation as the substrate degrades over time in aqueous solution. Discard any unused solution.

Materials Needed

Be sure to have the following materials on hand before beginning cell loading:

- 6X CCF2-AM Enhanced Loading Solution (prepare immediately before use, see the previous page)
- Hank's Balanced Salt Solution (HBSS; Catalog no. 14175-095)
- Mammalian cell line of interest (plated in the tissue culture format of choice or in suspension, as appropriate)

continued on next page

Using the Enhanced Loading Protocol for *in vivo* Detection, continued

Enhanced Loading Protocol for Adherent Cells

Follow this protocol to load adherent cells with the CCF2-AM substrate. You may plate your cells in any tissue culture format of choice. Refer to the table in **Recommended Reagent Volumes**, below for the appropriate reagent amounts to add. We recommend including a negative control (no cells) and an untransfected control in your experiment so that you can determine the background blue and green fluorescence.

1. Remove the growth medium from the cells and wash cells once with HBSS. Add the appropriate amount of HBSS to each well (see **Recommended Reagent Volumes**, below). For example, add 100 µl of HBSS to each well of a 96-well plate.
2. For each sample, add the appropriate amount of 6X CCF2-AM Enhanced Loading Solution to the well (6-fold dilution) to obtain a final concentration of 1X (see **Recommended Reagent Volumes**, below). This is 2 µM CCF2-AM. For example, add 20 µl of 6X CCF2-AM Loading Solution to 100 µl of cells in HBSS in a 96-well plate (total volume = 120 µl).
3. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 1 hour, protected from light.

   Note: Extending the incubation time may increase the fluorescence signal, but may also increase the background.
5. Proceed to detect fluorescence signal using the method of choice (see **What to Do Next**, page 18). Alternatively, you may remove the CCF2-AM Enhanced Loading Solution and replace with fresh, growth medium containing 1% probenecid stock or HBSS containing 1% probenecid stock, then proceed to detection.

Recommended Reagent Volumes

The table below lists the recommended volumes of 6X CCF2-AM Enhanced Loading Solution and HBSS to use for loading cells cultured in various tissue culture formats.

Culture Vessel	6X CCF2-AM Enhanced Loading Solution	HBSS
96-well	20 µl	100 µl
48-well	40 µl	200 µl
24-well	100 µl	500 µl
12-well	150 µl	750 µl
6-well	250 µl	1250 µl

continued on next page

Using the Enhanced Loading Protocol for *in vivo* Detection, continued

Enhanced Loading Protocol for Suspension Cells

Follow this protocol to load suspension cells with the CCF2-AM substrate. We recommend including a negative control (no cells) and an untransfected control in your experiment so that you can determine the background blue and green fluorescence.

1. For each sample, pellet 1-2 x 10^5 cells by centrifugation. Wash the cell pellet once with HBSS, then resuspend in 100 µl of HBSS.
2. To each sample, add 20 µl of the 6X CCF2-AM Enhanced Loading Solution to 100 µl of cells in buffer to obtain a final concentration of 1X. This is 2 µM CCF2-AM.
3. Transfer the cells and CCF2-AM Enhanced Loading Solution to a black-walled, clear bottom 96-well tissue culture plate. Cover the plate to prevent the solution from evaporating.
4. Incubate the cells at room temperature for 1 hour, protected from light.
5. Proceed to detect fluorescence signal using the method of choice.

   Note: During the incubation, cells will settle to the bottom of the well. If you are using a bottom-read fluorescence plate reader, handle the plate gently as the cells must remain at the bottom of each well for accurate detection to occur.

Detecting CCF2 Signal Using Fluorescence Microscopy

Introduction

Once you have loaded your mammalian cells with the CCF2 substrate, you may qualitatively or quantitatively assess the amount of β-lactamase reporter activity in live cells by visually observing the intracellular CCF2 fluorescence or performing ratiometric analysis, respectively. General guidelines are provided below to select the type of fluorescence microscope and filter sets to optimally visualize or analyze the fluorescence signal of CCF2 or its β-lactamase-catalyzed hydrolysis product.

Fluorescence Microscope

You may view the fluorescence signal of CCF2 and its β-lactamase-catalyzed hydrolysis product in cells using any type of fluorescence microscope with a long-pass dichroic mirror to separate excitation and emission light. Make sure that the dichroic mirror is matched to the excitation filter to maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light.

Recommended Filter Sets for Visualization

Use of the best filter sets will ensure that the optimal regions of the β-lactamase spectra are excited and passed (emitted). To visually inspect the cells, use a long-pass filter passing blue and green fluorescence light so that your eye can visually identify whether the cells are fluorescing blue or green. For best results, we recommend using one of the following filter sets available from Chroma Technologies (www.chroma.com) or Omega Optical (www.omegafilters.com) as specified below.

Important: Do not use FITC filters. Most FITC filters block emission of blue light so all cells (even those that contain β-lactamase) will appear green.

	Chroma Filter Set #41031	**Omega Optical Filter Set #XF106-2**
Excitation filter:	HQ405/20x (405 ± 10)	400AF30
Dichroic mirror:	425 DCXR	435DRLP
Emission filter:	HQ435LP (435 long-pass)	435ALP

Color Camera

If desired, you may use a color camera that is compatible with the microscope to photograph the cells. We recommend using a digital camera or high sensitivity film, such as 400 ASA or greater.

What You Should See

Reminder: Wild-type cells that do not contain the *bla*(M) reporter gene and possess no β-lactamase activity will emit a green fluorescence signal, while those that contain the *bla*(M) reporter gene and are expressing β-lactamase will emit a blue fluorescence signal.

continued on next page

Detecting CCF2 Signal Using Fluorescence Microscopy, continued

Performing Ratiometric Analysis

To monitor β-lactamase expression in single cells over time, you may perform microscopic imaging and ratiometric analysis. For microscope-based ratiometric analysis, the blue and green fluorescence emissions are analyzed separately by filtering the emitted light through two emission filters, passing either blue or green fluorescence (analogous to using a fluorescence plate reader). By calculating the ratio of blue to green fluorescence intensities, it is possible to numerically analyze β-lactamase activity. For recommended filter sets to perform ratiometric analysis, see below.

Note: This technique is labor-intensive and time-consuming. For an easier method to quantitate β-lactamase activity over time, we recommend using a fluorescence plate reader (see the next section).

Recommended Filter Sets for Ratiometric Analysis

To perform ratiometric analysis, you will need to obtain a filter set containing separate blue and green emission filters. We recommend using one of the filter sets available from Chroma Technologies (www.chroma.com) or Omega Optical (www.omegafilters.com) as specified below. Note that filter sizes vary for specific microscopes and need to be specified at the time of ordering.

	Chroma Filter Set #71008	**Omega Optical Filter Set #XF124**
Excitation filter:	HQ405/20x (405 ± 10)	400DF15
Dichroic mirror:	425 DCXR	415DRLP
Emission filter (blue):	HQ460/40m (460 ± 20 nm)	450DF65
Emission filter (green):	HQ530/30m (530 ± 15 nm)	535DF35

As with other fluorescent dyes, avoid photo-bleaching the dye-loaded cells. The CCF2 substrate is particularly sensitive to continuous illumination through a high magnification, high numerical aperture objective with UV or any other wavelength of light that can excite the dye. Continuous excitation of the dye can cause the acceptor fluorophore to be bleached (destroyed) with loss of FRET and appearance of donor fluorescence. This effect is progressive and nonreversible.

To reduce photo-bleaching, limit exposure of cells to excitation light by analyzing fluorescence signal for a few seconds at a time. Alternatively, use a lower magnification objective to reduce exposure of the substrate to light.

Detecting CCF2 Signal Using a Fluorescence Plate Reader

Introduction

To quantitatively determine the β-lactamase activity in your cells, you may assay for CCF2 fluorescence signal using a fluorescence plate reader. Three options exist to assay for CCF2 fluorescence signal. You may:

- Measure the fluorescence intensity in cell lysates containing CCF2-FA.
- Measure the fluorescence intensity in live CCF2-AM-loaded cells.
- Lyse the CCF2-AM-loaded cells and measure fluorescence intensity in cell lysates. This method may provide better sensitivity if using a top-read fluorescence plate reader.

Fluorescence Plate Readers and Filter Sets

You may use any fluorescence plate reader to detect CCF2 fluorescence signal in your cells. Keep the following in mind:

- For optimal sensitivity, we recommend using a bottom-read fluorescence plate reader (*e.g.* Gemini-EM Fluorescence Microtiter Plate Reader, Molecular Devices, CytoFluor® 4000 Fluorescence Plate Reader, PerSeptive Biosystems, or Safire Microplate Reader, Tecan). Top-read fluorescence plate readers (*e.g.* Gemini-XS Fluorescence Microtiter Plate Reader, Molecular Devices) can be used, however, lower sensitivity may be observed and extra manipulation steps are required before fluorescence signal can be measured in live cells (see **Reading Fluorescence Signal in a Top-Read Machine**, next page).
- Use the optimal filter set to detect ratiometric blue and green readout. Filter sets are included with some fluorescence plate readers, while others require that filters be obtained separately. If you need to obtain filters separately, we recommend using the filters available from either Chroma Technologies (www.chroma.com) or Omega Optical (www.omegafilters.com) as specified below.

	Chroma Filter Set #APR1	**Omega Optical Filters**
Excitation filter:	HQ405/20x (405 ± 10)	400AF30 (Part no. XF1076)
Emission filter (blue):	HQ460/40m (460 ± 20 nm)	450DF65 (Part no. XF3002)
Emission filter (green):	HQ530/30m (530 ± 15 nm)	535DF35 (Part no. XF3007)

continued on next page

Detecting CCF2 Signal Using a Fluorescence Plate Reader, continued

General Recommendations

Follow the general recommendations below when using a fluorescence plate reader to quantitate your CCF2 signal.

- You may plate your cells in any size tissue culture format of your choice; however, make sure that your fluorescence plate reader can accommodate your plate format.
- If you are assaying for β-lactamase activity in a 96-well format, plate cells in a black-walled, clear-bottom microplate with low autofluorescence (Costar, Catalog no. #3603). Using a black-walled microplate blocks any signal from adjoining wells during reading. For larger-sized tissue culture formats, use of clear tissue culture plates is acceptable.
- Some plates/plate readers exhibit edge effects that may affect data. If edge effects are noticed, consider the plate layout when setting up the assay.
- Do not touch the bottom of the microtiter plate or allow dust to cover the tissue culture surface. Fingerprints and dust can autofluoresce, introducing well-to-well variability in replicate wells.
- Include negative controls (loading buffer with no cells and cells with no β-lactamase activity) in your experiment so that you can determine the background blue and green fluorescence.

Reading Fluorescence Signal in a Top-Read Machine

If you are quantitating CCF2-AM fluorescence signal in live cells using a top-read fluorescence plate reader, note that the dyes from Solution C in the 6X CCF2-AM Loading Solution will interfere with the fluorescence signal. In addition, some components of cell culture media may also interfere with the fluorescence signal. **Before reading the fluorescence signal, you must remove the 6X CCF2-AM Loading Solution and any cell culture media from the cells.** Follow the protocol below.

1. Load cells with 6X CCF2-AM Loading Solution containing Solution C using the General Loading Protocol on page 15.
2. Before reading the fluorescence signal, remove the CCF2-AM Loading Solution from the well. Wash the cells once with HBSS.
3. Add an appropriate amount of HBSS to the well and read the fluorescence signal using the fluorescence plate reader.

   Alternative: If you do not plan to culture the cells further, you may lyse the cells and then read the fluorescence signal in the cell lysate (see **Preparing Cell Lysates**, page 9).
4. After reading the fluorescence signal, remove the HBSS and replace with an appropriate amount of fresh, complete growth media. Incubate the cells at 37°C to culture cells further.

continued on next page

Detecting CCF2 Signal Using a Fluorescence Plate Reader, continued

Analyzing Data

Calculate the ratio of blue and green fluorescence signal by dividing the 460 nm emission (blue channel) reading by the 530 nm emission (green channel) reading. Remember to subtract the background fluorescence obtained at each wavelength. This is determined by reading the fluorescence signal from the negative (no cells) control.

$$\text{Ratio} = \frac{(\text{signal at 460 nm - background at 460 nm})}{(\text{signal at 530 nm - background at 530 nm})}$$

Compare the ratio obtained from your experimental sample to the ratio obtained from the appropriate negative controls.

Note: Determine background values **for each read** since these reads are highly dependent on instrument-specific factors and on the length of time the lamp in the instrument has been lit.

Performing Fluorescence-Activated Cell Sorting of CCF2-AM-Loaded Cells

Introduction

You may use fluorescence-activated cell sorting (FACS) to rapidly screen and select cells based on their pattern of β-lactamase gene expression. General guidelines are provided below to configure the flow cytometer and to load and prepare cells for sorting using flow cytometry.

Flow Cytometry Instrumentation

You may use any flow cytometer to detect CCF2-AM-loaded cells by flow cytometry. For optimal results, we recommend the following general guidelines:

- Use a Krypton laser with violet excitation (407 nm, 413 nm, or multiline violet 407-415 nm) at 60 mW.
- Make sure that the flow cytometer is equipped with the proper optical filters to detect the CCF2 fluorescence signal. We suggest using HQ460/50m (blue) and HQ535/40m (green) bandpass filters separated by a 490 nm dichroic mirror or the following filters available from Omega Optical (www.omegafilters.com).

	Omega Optical Filters
Dichroic mirror:	495DRLP (Part no. XF2026)
Emission filter (blue):	450DF65 (Part no. XF3002)
Emission filter (green):	535DF35 (Part no. XF3007)

- After the instrument has been optically aligned and optimized, run a negative control sample (untransfected or uninduced cells) and a positive control sample (cells expressing β-lactamase) loaded with CCF2-AM to adjust PMT levels and compensation values for optimal separation of the blue and green fluorescence signals.

For more help to configure your flow cytometer for detection of CCF2 fluorescence signal, we suggest consulting the cell sorting facility at your institution.

Loading Cells with CCF2-AM

To load adherent or suspension cells with CCF2-AM for FACS, prepare 6X CCF2-AM Loading Solution as directed on page 15, but use HBSS in place of Solution C as the presence of Solution C can interfere with the detection of fluorescence signal. Follow the General Loading Protocol on pages 17 or 18, respectively to load adherent or suspension cells.

continued on next page

Performing Fluorescence-Activated Cell Sorting of CCF2-AM-Loaded Cells, continued

General Guidelines to Prepare Cells for FACS Sorting

Follow the guidelines below to prepare cells for FACS:

- **Sorting Buffer:** Use calcium- and magnesium-free HBSS (Catalog no. 14175-095) containing 25 mM HEPES (pH 7.3) and 0.1% BSA. If necessary to preserve cell viability, use serum-free medium buffered with 25 mM HEPES (pH 7.3) and 0.1% BSA.

  Note: Do not sort cells in tissue culture medium as the buffering capacity is weak and can cause the sample pH to increase in air.
- **Adherent Cells:** After loading, remove cells from the tissue culture surface and wash cells once with calcium- and magnesium-free HBSS. Resuspend the cell pellet in Sorting Buffer at a density of 3-5 x 10^6 cells/ml. Make sure that the cells are in a single cell suspension (see **Important Note** below for more information).
- **Suspension Cells:** After loading, wash cells once with calcium- and magnesium-free HBSS, and resuspend in Sorting Buffer at a density of 5-10 x 10^6 cells/ml.

Important

For optimal results, make sure that cells are in a single cell suspension during sorting. Formation of aggregates (a major problem with adherent cells) can result in subobtimal sorting due to clogging of the flow cytometer and potential contamination of the sample with unwanted cells. To prevent aggregation, do the following:

- Perform all washes with Ca^{2+}- and Mg^{2+}-free solutions.
- Resuspend cells in Ca^{2+}- and Mg^{2+}-free buffers.
- If adding serum to the cell suspension to preserve cell viability, dialyze the serum before use to remove Ca^{2+} and other divalent cations.

Troubleshooting

Introduction

Use the information in this section to troubleshoot your GeneBLAzer™ detection experiments.

In vitro Detection

The table below lists solutions to some potential problems you may encounter when using the GeneBLAzer™ *In Vitro* Detection Kit.

Problem	Reason	Solution
Weak fluorescence signal	Low β-lactamase expression	• Increase the incubation time of the cell lysate with CCF2-FA. • Re-assess transfection conditions. • Use Lipofectamine™ 2000 for transfection.
	When preparing the cell lysate, adherent cells dissociated using trypsin-EDTA	Over-trypsinizing cells may affect fluorescence signal by causing cell lysis and proteolysis. Use Versene to dissociate cells.
	Loaded cells with CCF2-AM substrate	For in vitro detection, load cells with CCF2-FA substrate.
No fluorescence signal	CCF2-FA substrate or stock solution exposed to light during storage	Store CCF2-FA protected from light.
	CCF2-FA stock solution not stored at -20°C	Store the CCF2-FA stock solution at -20°C.
	Cell lysate prepared using a method that destroys the activity of the β-lactamase enzyme	Prepare cell lysates using a method that preserves the activity of the β-lactamase enzyme (see page 9).
Observe well-to-well variability in replicate wells (most notable when using top-read fluorescence plate readers)	Bubbles are present in the cell lysates	Carefully transfer cell lysates to a new tissue culture plate, taking care not to introduce bubbles. Read fluorescence signal.
	Touched the bottom of the microtiter plate	Do not touch the bottom of the microtiter plate as fingerprints can autofluoresce.
	Microtiter plate covered with dust or lint	Dust can autofluoresce. Keep the bottom and top surface of the microtiter plate free of dust.

continued on next page

Troubleshooting, continued

In vivo Detection

The table below lists solutions to some potential problems you may encounter when using the GeneBLAzer™ *In Vivo* Detection Kit.

Problem	Reason	Solution
All cells fluoresce green	Poor transfection efficiency	• Re-assess transfection conditions. • Use Lipofectamine™ 2000 for transfection.
	Used a FITC filter set or other improper filter set	Use a filter set that allows both blue (460 nm) and green (520 nm) visualization (see page 23 for recommendations).
Weak fluorescence signal	Poor substrate retention	Use the Enhanced Loading Protocol, page 19.
	Cells too dense	Plate cells such that they will be 60-80% confluent at the time of loading.
	Low β-lactamase expression	• Increase cell loading time. • Use the Enhanced Loading Protocol, page 19. • Re-assess transfection conditions.
	Cells loaded at 37°C	Load cells at room temperature.
	Cells loaded in serum-containing media	For optimal efficiency, load cells in HBSS or HBS.
	Used a top-read fluorescence plate reader in the presence of media or Solution C	Remove the media or 6X CCF2-AM Loading Solution containing Solution C, wash the cells with HBSS, and replace with HBSS before reading fluorescence signal.
Hazy background or difficulty visualizing fluorescing cells under the microscope	Cells loaded in the absence of Solution C	Add Solution C to the 6X CCF2-AM Loading Solution.
No fluorescence signal	6X CCF2-AM Loading Solution degraded	Use 6X CCF2-AM Loading Solution within two hours of preparation.
	Solution A exposed to light during storage	Store Solution A at -20°C, dessicated and protected from light.
Cells detach (in sheets) from the surface of the well	Cells not adherent	Plate cells on Matrigel-treated wells
	Cells sensitive to the surfactant (from Solution B) in the 6X CCF2-AM Loading Solution	Load cells for less time (*e.g.* 30 to 45 minutes).
Cells exhibit toxicity when loaded using the enhanced loading protocol	Probenecid is present in the 6X CCF2-AM Enhanced Loading Solution	• Prepare the 6X CCF2-AM Enhanced Loading Solution, but omit the probenecid. • Load cells for less time (*e.g.* 30 to 45 minutes).

Appendix

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
USA

Tel: 1 760 603 7200

Tel (Toll Free): 1 800 955 6288

Fax: 1 760 602 6500

E-mail: tech_service@invitrogen.com

Japanese Headquarters:

Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park
Bldg. 4F
2-35-4, Hama-Cho,
Nihonbashi

Tel: 81 3 3663 7972

Fax: 81 3 3663 8242

E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK

Tel: +44 (0) 141 814 6100

Tech Fax: +44 (0) 141 814 6117

E-mail: eurotech@invitrogen.com

MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

continued on next page

Technical Service, continued

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

Use of the GeneBLAzer™ Detection Kits is covered under the licenses detailed below.

Limited Use Label License No. 150: GeneBLAzer™ Technology

This product and/or its use is the subject of one or more of U.S. Patent Nos. 5,741,657, 5,955,604, 6,291,162, and 6,472,205 and foreign equivalents 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.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 152: TR40

This product and/or its use is the subject of one or more of U.S. Patent Nos. 6,200,762, 6,214,563, and 6,221,612 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 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 a scientific 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.

Product Qualification

Introduction	The components of the GeneBLAzer™ *In Vitro* and *In Vivo* Detection Kits are qualified as described below.
CCF2-FA	CCF2-FA is dried under vacuum from methanol, and is qualified as follows: **Purity:** Determined by HPLC **Mass:** Determined by mass spectroscopy **Structure:** Verified by NMR **Functionality:** Tested using purified β-lactamase enzyme
CCF2-AM	CCF2-AM is dried under vacuum from acetonitrile, and is qualified as follows: **Purity:** Determined by HPLC **Mass:** Determined by mass spectroscopy **Structure:** Verified by NMR **Functionality:** Tested using the Jurkat CMV-β-lactamase host cell line to ensure performance.
Anhydrous DMSO	Anhydrous DMSO is qualified to be ≥ 99.5% pure.
Solutions B and C	Solutions B and C are functionally tested using the Jurkat CMV-β-lactamase host cell line to ensure performance.

References

DiVirgilio, F., Steinberg, T. H., Swanson, J. A., and Silverstein, S. C. (1988). Fura-2 Secretion and Sequestration in Macrophages: A Blocker of Organic Anion Transport Reveals that these Processes Occur Via a Membrane Transport System for Organic Anions. J. Immunol. *140*, 915-920.

Sutcliffe, J. G. (1978). Nucleotide Sequence of the Ampicillin Resistance Gene of *Escherichia coli* Plasmid pBR322. Proc. Nat. Acad. Sci. USA *75*, 3737-3741.

Zlokarnik, G., Negulescu, P. A., Knapp, T. E., Mere, L., Burres, N., Feng, L., Whitney, M., Roemer, K., and Tsien, R. Y. (1998). Quantitation of Transcription and Clonal Selection of Single Living Cells with b-Lactamase as Reporter. Science *279*, 84-88.

Notes

Notes

Notes

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 603 7229
Email: tech_service@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
Email: eurotech@invitrogen.com

International Offices:
Argentina 5411 4556 0844
Australia 1 800 331 627
Austria 0800 20 1087
Belgium 0800 14894
Brazil 0800 11 0575
Canada 800 263 6236
China 10 6849 2578
Denmark 80 30 17 40

France 0800 23 20 79
Germany 0800 083 0902
Hong Kong 2407 8450
India 11 577 3282
Italy 02 98 22 201
Japan 03 3663 7974
The Netherlands 0800 099 3310
New Zealand 0800 600 200
Norway 00800 5456 5456

Spain & Portugal 900 181 461
Sweden 020 26 34 52
Switzerland 0800 848 800
Taiwan 2 2651 6156
UK 0800 838 380
For other countries see our website

www.invitrogen.com