FluoReporter® lacZ *Flow Cytometry Kits*

For Detection of β-Galactosidase Activity in Single Cells

F-1930 FluoReporter *lacZ* Flow Cytometry Kit *50 assays*
F-1931 FluoReporter *lacZ* Flow Cytometry Kit *250 assays*

Quick Facts

Storage upon receipt:

- ≤–20°C
- Protect from light

Abs/Em of reaction product: 491/514 nm

Note: Do not keep the FDG solutions at elevated temperatures for extended periods, as spontaneous hydrolysis will occur. A pronounced yellow color in the FDG solution indicates substrate hydrolysis.

Introduction

Introducing recombinant DNA constructs into organisms and cultured cells has become a standard method for defining the mechanisms that regulate gene transcription. Typically, transcription from the transfected promoter is monitored by detecting the RNA transcript or the encoded protein product. A reporter gene, which encodes an enzyme not ordinarily found in the type of cell being studied, is often incorporated into the recombinant DNA construct. The unique activity of this gene product is then assayed to determine the degree of transcription from the foreign promoter. The *Escherichia coli lacZ* gene, which encodes β-galactosidase, is a popular reporter gene for transformation and transfection experiments with bacteria, yeast, plants and mammalian cells.

Chromogenic β-galactosidase substrates such as *o*-nitrophenyl β-D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, B-1690, B-22015) have been widely used to monitor β-galactosidase activity resulting from *lacZ* gene expression. However, these colorimetric assays are relatively insensitive and often require the use of bulk cell extracts to obtain sufficient signal. More recently, the fluorogenic β-galactosidase substrate fluorescein di β-D-galactopyranoside (FDG, F-1179) has been employed in conjunction with fluorescence-activated cell sorting to develop an extremely sensitive fluorescence-based assay of β-galactosidase activity.[1,2] Nonfluorescent FDG is sequentially hydrolyzed by β-galactosidase, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein.[3-6] Low levels of β-galactosidase activity are readily detectable with FDG due to the superior spectral characteristics of fluorescein (maximum extinction coefficient 90,000 $cm^{-1}M^{-1}$, fluorescence quantum yield 0.92 at pH >8). Moreover, because this flow cytometric technique is carried out on a cell-by-cell basis, it allows researchers to detect heterogeneous expression patterns and to sort and clone individual cells expressing known quantities of β-galactosidase.

The FluoReporter® *lacZ* Flow Cytometry Kits provide materials and protocols for quantitating β-galactosidase activity with FDG in single cells using flow cytometry. A single-cell suspension is prepared from exponentially growing cells, and the substrate FDG is loaded into the cells at 37°C by hypotonic shock. The loading process is terminated by diluting the cells into ice-cold isotonic medium. At 4°C, the enzymatic turnover rate is reduced 10-fold and the membrane permeability of fluorescein is inhibited more than 200-fold relative to that at 37°C.[1] This property allows hydrolysis of the substrate to proceed without leakage of the fluorescent product from the cell. The β-galactosidase reaction can be stopped by adding the competitive inhibitor, phenylethyl β-D-thiogalactopyranoside (PETG, P-1692). Fluorescence is then measured with a flow cytometer, and individual cell populations expressing β-galactosidase can be selectively sorted and cloned. These FluoReporter Kits also contain the nucleic acid counterstain propidium iodide to detect cells that have compromised cell membranes (dead cells), and chloroquine to raise lysosomal pH levels and prevent hydrolysis of the substrate by endogenous lysosomal β-galactosidase.

Materials

Contents

- **FDG** (Component A), 0.5 mL (F-1930) or 2.5 mL (F-1931) of 20 mM fluorescein di-β-D-galactopyranoside in H_2O/DMSO/ethanol, 8:1:1 (v/v).
- **PETG** (Component B), 2.0 mL (F-1930) or 10 mL (F-1931) of 50 mM phenylethyl β-D-thiogalactopyranoside in water.
- **Chloroquine diphosphate** (Component C), 1.0 mL (F-1930) or 5.0 mL (F-1931) of 30 mM chloroquine diphosphate in water.
- **Propidium iodide** (Component D), 1.0 mL (F-1930) or 5.0 mL (F-1931) of 150 μM propidium iodide in water.

Sufficient reagents are provided for approximately 50 assays (F-1930) or 250 assays (F-1931) using the provided protocol.

Storage

The stock FDG reagent and the 2 mM working solution (see step 2.1) should be stored frozen at ≤–20°C, protected from light. *Do not keep the FDG solution at elevated temperatures for extended periods, as spontaneous hydrolysis will occur.* All other reagents may be stored, protected from light, either at 4°C or at ≤–20°C. When stored properly, these reagents are stable for several months. A pronounced yellow color in the FDG reagent or a high fluorescence background (as compared to previously obtained results) may indicate that the reagent has spontaneously hydrolyzed and no longer meets appropriate standards for use.

Materials Required but Not Provided

- Staining medium: phosphate-buffered saline, 4% (v/v) fetal calf serum, 10 mM HEPES, pH 7.2

Protocol

Overview

The following protocol describes a method for fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-galactosidase activity. Procedures are given for preparing the cells and staining them with FDG using transient permeabilization by hypotonic shock. Methods are also described for using the competitive inhibitor PETG to stop substrate turnover by β-galactosidase and chloroquine to lower the endogenous lysosomal β-galactosidase activity present in some cell types. Cells grown to confluency may contain higher levels of endogenous β-galactosidase activity.

Preparing Cell Suspension

1.1 Prepare cells using one of the following two methods:
For adherent cells (note **A**): Treat exponentially growing cells with trypsin in phosphate buffer until they can be removed from the plate by gentle agitation. Inactivate trypsin by washing in staining medium (phosphate-buffered saline, 4% (v/v) fetal calf serum, 10 mM HEPES, pH 7.2) or tissue culture growth medium. Centrifuge the cell suspension and aspirate off the supernatant.
For suspension cells: Centrifuge the suspension to obtain a cell pellet and aspirate off the supernatant. Resuspend cells in staining medium and pipet up and down to obtain a suspension of single cells. If cell clumps are present, they must be filtered out with a nylon screen. Centrifuge cells again and remove the supernatant.

1.2 Resuspend cells in staining medium to ~ 10^7 cells/mL (note **B**) and pipet 100 µL into an appropriate flow cytometer tube. Place cells on ice.

Loading Cells with FDG by Hypotonic Shock (see note C)

2.1 Prepare a 2 mM working solution: Thaw the FDG reagent (Component A). It is normal for the frozen reagent to appear to have precipitated from solution. To return the reagent to a homogeneous mixture, warm briefly at 37°C and vortex or shake to dissolve any crystals. *Do not keep the FDG solutions at elevated temperatures for extended periods, as spontaneous hydrolysis will occur.* Prepare the 2 mM FDG working solution by diluting the FDG reagent 10-fold in deionized water. Prewarm the FDG working solution to 37°C for 10 minutes prior to use. This solution can be stored at ≤–20°C, protected from light, for future use.

2.2 Prepare staining medium with 1.5 µM (1 µg/mL) propidium iodide by diluting the propidium iodide reagent 100-fold in staining medium. Chill on ice.

2.3 Prewarm the flow cytometer tube containing 100 µL of cells in a 37°C water bath for 10 minutes.

2.4 Start FDG loading by adding 100 µL of prewarmed (37°C) 2 mM FDG working solution (notes **D**, **E**). Mix rapidly and *thoroughly*. Return to the 37°C water bath for exactly one minute.

2.5 Stop the FDG loading at the end of one minute by adding 1.8 mL *ice-cold* staining medium containing 1.5 µM propidium iodide (note **F**). Use *ice-cold* pipets to aliquot the staining medium into the cells. Keep the cells *on ice* prior to flow cytometry analysis (note **G**).

Flow Cytometer Set-Up and Calibration

3.1 Set up and calibrate the flow cytometer to detect fluorescein, propidium iodide and forward scatter, according to standard procedures.[7]

3.2 Using unstained cells of the same type you are analyzing, set the background autofluorescence compensation[8] (note **H**).

3.3 If the cells are to be sorted, take great care to keep the suspension *ice-cold,* because at room temperature fluorescein will leak out of the cells and into β-galactosidase–negative cells making the measurement inaccurate. Use chilled water or ice-water in the jacket of the flow cytometer to ensure that the cells remain at 4°C prior to sorting.

Inhibition of Lysosomal β-Galactosidase Using Chloroquine (see note I)

4.1 Prepare staining medium containing 300 µM chloroquine by diluting the chloroquine diphosphate stock reagent 100-fold in staining medium. Prepare staining medium containing both 300 µM chloroquine and 1.5 µM propidium iodide by diluting both chloroquine diphosphate stock reagent and propidium iodide stock reagent 100-fold into a common volume of staining medium; chill on ice.

4.2 Prepare exponentially growing cells as described above. After filtering to remove cell clumps, resuspend the cells at ~10^7 cells/mL in staining medium containing 300 µM chloroquine. Aliquot 100 µL into an appropriate flow cytometer tube and place the cells on ice.

4.3 Place the cells in a 37°C water bath for 20 minutes and load with FDG as previously described in steps 2.1–2.5.

4.4 After one minute, stop FDG loading by adding 1.8 mL *ice-cold* staining medium containing 1.5 µM propidium iodide and 300 µM chloroquine. Place the cells *on ice* and analyze by flow cytometry as described in steps 3.1–3.3.

Inhibition of β-Galactosidase with PETG (see note J)

5.1 For cells with high β-galactosidase activity, prepare staining medium with 1.5 μM propidium iodide and 1 mM PETG by diluting propidium iodide stock reagent 100-fold and PETG stock reagent 50-fold into a common volume of staining medium; chill on ice. For cells with medium or low β-galactosidase activity, prepare staining medium with 1.5 μM propidium iodide (no PETG) by diluting propidium iodide stock reagent 100-fold into staining medium; chill on ice.

5.2 Prepare cells and stain them with FDG as described in steps 2.1–2.5. Depending on the degree of β-galactosidase activity, perform one of the following protocols:

For cells with high β-galactosidase activity: Stop FDG loading at the end of one minute by adding 1.8 mL *ice-cold* staining medium containing 1.5 μM propidium iodide and 1 mM PETG.

For cells with medium or low β-galactosidase activity: Stop FDG loading at the end of one minute by adding 1.8 mL *ice-cold* staining medium containing 1.5 μM propidium iodide. Place the cells *on ice*. At a predetermined time (usually 10, 30 or 60 minutes, depending on activity), add 40 μL of the 50 mM PETG stock reagent and mix thoroughly.

5.3 Place the cells back *on ice*. The β-galactosidase activity is now strongly inhibited, and the flow cytometric analysis can be done at a convenient time.

Calculation of Results

Data analysis will depend on the type of flow cytometer used and the data reduction programs that have been developed or adopted by individual laboratories. For detailed information, consult the manufacturer's applications manual or a reference manual on flow cytometry. A typical histogram generated on a Becton-Dickinson FACS® system is shown in Figure 1. The following points should be noted regarding quantitative interpretation of fluorescence resulting from FDG hydrolysis in terms of β-galactosidase activity:

6.1 Fluorescence intensity per cell has a linear dependence on the intracellular substrate concentration. The intracellular concentration of FDG produced by the above protocol has been estimated to be approximately 5 μM.[2] This concentration may be varied by adjusting either the external FDG concentration (nominally 1 mM), the duration of the FDG incubation or the hypotonicity of the loading medium (note that increasing hypotonicity will compromise cell viability). However, the loading of FDG by hypotonic permeabilization is sufficiently uniform for a given cell type that fluorescence intensity variations from cell to cell reflect β-galactosidase activity and not variations in intracellular substrate concentration.[2]

6.2 Because each cell contains only a finite amount of FDG, fluorescence readings must be taken before the substrate is exhausted in order to be quantitatively related to β-galactosidase activity. In practice, this can be accomplished either by taking readings at precisely controlled time points after substrate loading (which may be difficult in cells with very high levels of β-galactosidase activity), or by use of the competitive β-galacto-sidase inhibitor PETG to stop or slow the hydrolysis reaction. PETG is effective at low concentrations (K_i = 2.5 μM) and is cell-permeant, even at 4°C (notes **G, J**).

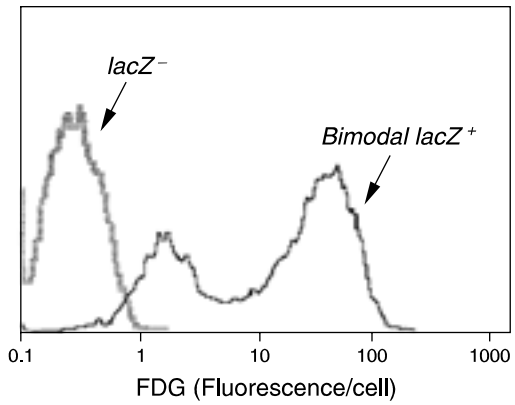

Figure 1. Bimodal expression of β-galactosidase in a T cell lymphoma. BW5147 cells were infected with the MMULV-SVnlslacZ retrovirus. After two days, cells expressing the *lacZ* gene were detected using the FluoReporter *lacZ* Flow Cytometry Kit and then cloned. After two weeks of growth, one clone was analyzed for β-galactosidase activity, again using the FluoReporter *lacZ* Flow Cytometry Kit. Cells were stained with FDG, stopped at 1 minute with PETG and analyzed by flow cytometry. The cells derived from the clone showed a bimodal distribution of *lacZ* expression (solid line), while *lacZ*-negative cells exhibited background autofluorescence (dashed line).

6.3 The relationship between fluorescence intensity and intracellular β-galactosidase activity is nonlinear.[2,9]

Notes

[A] Keep cells as healthy as possible. Certain adherent cell types, such as 293 or NIH 3T3 cells, will have higher endogenous β-galactosidase activity if they are abused or allowed to grow to confluency (see *Inhibition of Lysosomal β-Galactosidase Using Chloroquine* for further information on inhibition of endogenous β-galactosidase activity and note **I** below).

[B] The staining results are not critically dependent on cell concentration. Staining patterns are essentially the same using cell concentrations ranging from 10^5 cells/mL to 5×10^7 cells/mL.

[C] Alternatively, the Influx™ pinocytic cell-loading reagent provides a convenient, rapid and simple procedure for loading FDG into live cells. With the Influx reagent, FDG can be introduced into many cells simultaneously without significantly altering normal cell function. In general, the Influx reagent provides a more gentle cell-loading method than the typical cell-loading techniques of microinjection, electroporation, hypotonic shock or scrape loading, which are all physically disruptive to cells.

The Influx cell-loading technique is based on the osmotic lysis of pionocytic vesicles, a technique introduced by Okada and Rechsteiner.[11] Briefly, compounds to be loaded are mixed at high concentration with a hyperosmotic medium, allowing the material to be carried into the cells via endocytosis. The cells are then transferred to a hypotonic medium, which results in the release of trapped material from the pinocytic vesicles within the cells, filling the cytosol with the compound. Park and colleagues showed that endosomal compartments containing the hypertonic loading medium do not fuse with lysosomes.[12] Therefore, materials introduced into cells by the Influx cell-loading techniques are not exposed to lysosomal enzymes. Furthermore, lysosomal components are not released into the cytosol as a consequence of

the procedure. Optical filters designed for fluorescein or FITC are required for imaging â-galactosidase activity using FDG.

[D] For bacteria or yeast, the cell wall restricts the swelling induced by osmotic loading, thus preventing FDG entry. Methods for flow cytometric sorting of viable bacteria and yeast using FDG have been described by Nir and co-workers.[10]

[E] This staining procedure can be scaled up as long as equal volumes of cells and 2 mM FDG are used and the cells are thoroughly mixed with FDG.

[F] This staining procedure relies on osmotic shock of the cells. For one minute at 37°C, FDG enters the cells by passive osmotic loading. The uptake is then stopped by rapid dilution into cold isotonic staining medium, thereby "freezing" the membrane and locking the substrate and product inside the cells. The nucleic acid counterstain propidium iodide can be used to selectively stain cells with compromised membranes.

[G] Even though the cells are on ice, the conversion of FDG to fluorescein is proceeding. Therefore, all of the substrate will eventually be hydrolyzed, giving a distribution that reflects the amount of FDG initially loaded into each cell. This situation is acceptable if simple discrimination of *lacZ*-positive cells from *lacZ*-negative cells is desired. However, for other purposes, such as comparing promoter strengths of different cell types, the cells must be analyzed at the same times after loading and before the substrate is exhausted. This can be done either by accurate timing and analysis of the reactions or by use of a competitive inhibitor of β-galactosidase (see *Inhibition of* β-*Galactosidase with PETG* and note **J** below).

[H] Many types of cells contain endogenous substances that, when excited at 488 nm with an argon-ion laser, exhibit a broad bandwidth autofluorescence. This background autofluorescence can interfere with the accurate measurement of β-galactosidase activity. Therefore, it is necessary to use a compensation technique that removes the autofluorescence component of the emission signal. Typically, a mathematical correction based on the proportionality of the measured autofluorescence at one wavelength to that at another wavelength is used.[8] It is essential to accurately compensate for autofluorescence to measure low *lacZ* enzyme activities.

[I] Some mammalian cell types have endogenous β-galactosidase activities in their lysosomes that can interfere with accurate measurement of *lacZ* expression. The activity of these endogenous enzymes can be selectively depressed by preincubating the cells with the weak base, chloroquine. Chloroquine treatment can be used in concert with the competitive inhibitor PETG described in *Inhibition of β-Galactoside with PETG.*

[J] PETG is a competitive inhibitor of *E. coli* β-galactosidase in mammalian cells. It has a low K_i (2.5×10^{-6} M) and is not hydrolyzed by the enzyme, which simplifies its influence on the kinetics. PETG is hydrophobic and readily crosses the cell membrane to inhibit β-galactosidase, even at 4°C.

References

1. Proc Natl Acad Sci USA 85, 2603 (1988); **2.** Cytometry 12, 291 (1991); **3.** Proc Natl Acad Sci USA 50, 1 (1963); **4.** Anal Biochem 131, 180 (1983); **5.** Biochemistry 30, 8535 (1991); **6.** Eur J Biochem 222, 75 (1994); **7.** Parks, D.R. *et al.* in *Handbook of Experimental Immunology, Fourth Edition*, D.M. Wier *et al.*, Eds., Blackwell, Edinburgh, pp. 29.1–29.21 (1986); **8.** Cytometry 8, 114 (1988); **9.** Methods 2, 248 (1991); **10.** Appl Environ Microbiol 56, 3861 (1990); **11.** Cell 29, 33 (1982); **12.** J Cell Physiol 135, 443 (1998).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
F-1930	FluoReporter® *lacZ* Flow Cytometry Kit *50 assays*	1 kit
F-1931	FluoReporter® *lacZ* Flow Cytometry Kit *250 assays*	1 kit
I-14402	Influx™ pinocytic cell-loading reagent *makes 10 x 5 mL*	1 set

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