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DetectaGene™ Green CMFDG lacZ Gene Expression Kit (D-2920)

For Detecting β-Galactosidase Activity in Living Cells

Quick Facts

Storage upon receipt:

- –20°C
- Protect from light

Abs/Em: 492/517 nm for reaction product

Introduction

The *Escherichia coli* β-D-galactosidase gene (*lacZ*) is an important reporter gene for detecting the expression of recombinant genes in animal cells. Once reporter genes are fused with other genes or genomic regulatory elements, the resulting DNA constructs can be introduced into cells of interest and the reporter gene product assayed. In present analytical techniques, transcription from the transfected promoter is monitored by RNA analysis or by the detection of an encoded protein product. Typically, reporter genes encode enzymes not ordinarily found in the type of cell being studied, and their unique activity is monitored to determine the degree of transcription of the foreign genetic material. The *E. coli lacZ* gene has been extensively studied and utilized for this purpose.

5-Bromo-4-chloro-3-indolyl galactopyranoside (X-gal) is commonly used for detection of genes fused in frame with the *lacZ* gene. When X-gal is cleaved, an intensely blue halogenated indoxyl derivative is formed that is effective for visual identification of transformed cells. However, the cleavage product of X-gal is nonfluorescent and toxic to viable cells and therefore not useful for fluorescence-activated cell sorting analysis. For this reason, the fluorescent β-galactosidase substrate, fluorescein di-β-D-galactopyranoside (FDG), has been used for a highly sensitive flow cytometric β-galactosidase assay.[1,2] Under physiological conditions, however, the fluorescent hydrolysis product (fluorescein) leaks quickly from the *lacZ*-positive cells after enzymatic cleavage. To retard leakage, the cells must be maintained in conditions that reduce cell viability prior to β-galactosidase detection.

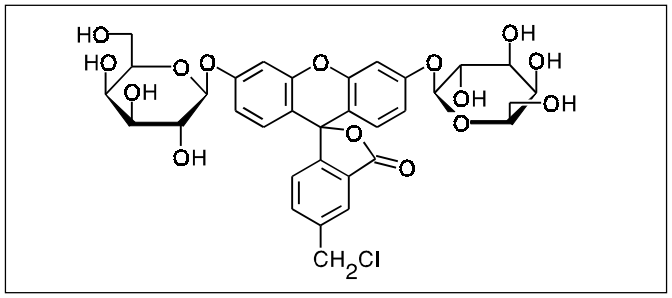

Figure 1. *Structure of CMFDG.*

To overcome the limitations of these substrates, scientists at Molecular Probes have developed the DetectaGene Green CMFDG *lacZ* Gene Expression Kit with a unique β-galactosidase substrate that yields a bright green fluorescent product with greatly improved cellular retention. The fluorogenic substrate in our DetectaGene Green *lacZ* Gene Expression Kit is 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG, Figure 1). This substrate has been designed to react with intracellular glutathione, a ubiquitous tripeptide, through a glutathione *S*-transferase–mediated reaction. In *lacZ*-positive cells, the CMFDG–glutathione adduct is subsequently converted to a bright green fluorescent product. Because peptides do not readily cross cellular membranes, the resulting fluorescein–glutathione adduct is well retained, even in cells that have been incubated for 18 hours in fresh medium at 37°C.

The DetectaGene Green Kit also includes stock solutions of phenylethyl β-D-thiogalactopyranoside (PETG), chloroquine diphosphate, propidium iodide and verapamil. PETG is a competitive inhibitor of β-galactosidase that can be added to terminate reactions prior to analysis. Chloroquine may be used to raise lysosomal pH and thereby inhibit the interfering endogenous lysosomal β-galactosidase activity present in some mammalian cells. Propidium iodide is useful for identifying dead cells in the population; this dye permeates damaged plasma membranes of dead cells and results in a red fluorescent nuclear stain. Verapamil, when added to the medium, can greatly enhance the signals obtained following intracellular hydrolysis of CMFDG by β-galactosidase; verapamil apparently blocks the efflux of fluorescent products produced from the reaction.[3] In addition, the DetectaGene Green Kit includes a vial of our Influx™ pinocytic cell-loading reagent.

The Influx pinocytic cell-loading reagent provides a convenient, rapid and simple procedure for loading CMFDG into live cells. With the Influx reagent, CMFDG 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. Scientists at Molecular Probes have found using the Influx reagent to be the best method for loading CMFDG into live cells.

The Influx cell-loading technique is based on the osmotic lysis of pinocytic vesicles, a technique introduced by Okada and Rechsteiner.[4] 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 (Figure 2). Park and colleagues showed that endosomal compartments containing the hypertonic loading medium do not fuse with lysosomes.[5] Therefore, materials introduced into cells by the Influx cell-loading technique are not exposed to lysosomal enzymes. Furthermore, lysosomal components are not released into the cytosol as a consequence of the procedure.

Materials

Contents

- **DetectaGene Green substrate reagent** (Component A), 100 µL of 10 mM 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG) in 1:1 (v/v) water/dimethylsulfoxide (DMSO)
- **PETG** (Component B), 1 mL of 50 mM phenylethyl β-D-thiogalactopyranoside in water
- **Chloroquine** (Component C), 1 mL of 30 mM chloroquine diphosphate in water
- **Propidium iodide** (Component D), 1 mL of 150 µM propidium iodide in water
- **Verapamil** (Component E), 1 mL of 100 mM verapamil hydrochloride in 1:1 (v/v) water/DMSO
- **Influx pinocytic cell-loading reagent** (Component F), one plastic tube containing an optimized mixture of polyethylene glycol (PEG) and sucrose crystals

Storage and Handling

The stock CMFDG reagent is stable for several months if stored frozen and protected from light. To reduce decomposition of this reagent during freezing and thawing, we recommend that you divide the reagent into several small aliquots and store at -20°C. *Do not keep the CMFDG working solution at elevated temperatures for extended periods, as spontaneous hydrolysis may occur.* **Note:** The presence of a pronounced yellow color in the CMFDG reagent or observation of an unusually high fluorescent background in the cells may indicate deterioration of the reagent.

The other reagents included in this kit are also stable for several months when stored at -20°C. Minimize exposure to light. The Influx cell-loading reagent may be stored at room temperature.

Experimental Protocols

The DetectaGene Green CMFDG *lacZ* Gene Expression Kit can be used for either flow cytometric analysis or fluorescence imaging of β-galactosidase–containing cells. The following protocols are suggested as basic methods for loading CMFDG into live cells and detecting fluorescence in a flow cytometer or fluorescence microscope. Protocol I describes the use of the Influx reagent for loading CMFDG into cells. Protocol II describes the use of hypotonic shock to load cells, and Protocol III describes a method for direct loading of cells. Also described are methods for using the competitive inhibitor, PETG, to slow or completely block β-galactosidase activity, methods for using chloroquine to lower the background from endogenous lysosomal β-galactosidase activity, which is present in some cells, and methods for using verapamil to help prevent the efflux of fluorescent reaction products and thereby boost the signal obtained. These protocols should serve as guidelines and may require modification based on specific experimental requirements.

The DetectaGene Green CMFDG *lacZ* Gene Expression Kit provides sufficient reagents for 50–200 assays when the CMFDG is loaded into cells in suspension, or 10–25 assays when the CMFDG is loaded into cells adhering to coverslips.

Protocol I: Loading Cells Using Influx Reagent

Preparation of Solutions

Hypertonic Loading Medium

1.1 Prewarm 5 mL culture medium, without serum, to 37°C.

1.2 Add 4.7 mL of warm medium to the plastic tube containing the Influx pinocytic cell loading reagent (Component F).

1.3 Place the tube in very hot water (~80°C) for 2 minutes.

1.4 Vortex several times over a 5 minute period to completely dissolve the PEG and sucrose crystals.

1.5 Remove the cap and add 250 µL of serum (the type required for your cell line) and 50 µL of 1 M HEPES buffer, pH 7.4, or other suitable buffer, to the tube.

1.6 Replace the cap and mix by vortexing several times.

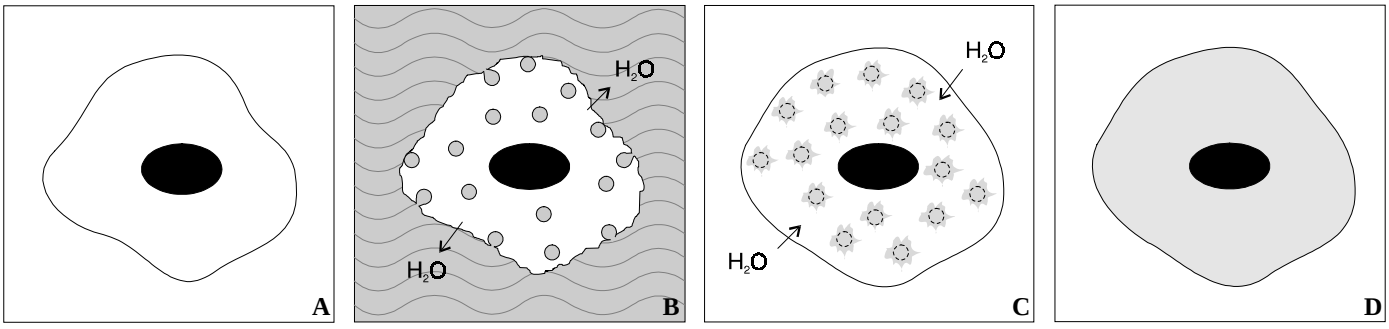

Figure 2. *Principle of the Influx reagent cell-loading method. Cultured cells (A) are exposed to Influx Hypertonic Loading Medium containing the CMFDG, which is carried into the cells via pinocytic vesicles (B). When the cells are placed in Hypotonic Lysis Medium, the pinocytic vesicles burst (C), releasing CMFDG into the cytosol (D).*

1.7 Maintain the Hypertonic Loading Medium at the optimal temperature for your cell line. CMFDG should be added to the Hypertonic Loading Medium immediately prior to use. DO NOT dilute the Hypertonic Loading Medium to less than 70% strength when adding the compound to be loaded into the cells.

Note: The Hypertonic Loading Medium may be filter-sterilized following steps 1.4 or 1.6. We recommend using a 0.8/0.2 µm Supor® Acrodisc® PF syringe sterilization filter (Gelman Sciences #4187). Filter-sterilized Hypertonic Loading Medium may be stored at 4°C for later use.

Hypotonic Lysis Medium

Prepare Hypotonic Lysis Medium by combining culture medium, without serum, and sterile deionized water in a 6:4 ratio. The volume required per loading will vary from 3–10 mL depending upon the method used.

Recovery Medium

Prepare 10 mL of culture medium supplemented with serum (i.e. the growth medium used for your cell line).

Loading Cells in Suspension Using the Influx Reagent

2.1 For each sample, prewarm at least 20 µL of Hypertonic Loading Medium containing 1 mM CMFDG to the ideal growth temperature for your cell type. In addition, prewarm 3 mL of Hypotonic Lysis Medium and ~2 mL of Recovery Medium, as well as all glassware. The following protocol assumes that the ideal temperature is 37°C.

2.2 Use trypsin or EDTA to remove cells from the surface of culture dishes or flasks, or use cells that are naturally in suspension (note **A**).

2.3 Wash the cells to remove the trypsin or EDTA by suspending the cells in medium and then pelleting the cells by centrifugation.

2.4 Resuspend the cells in a 1 mL volume of fresh medium so that the cell density is no higher than 1×10^6 cells per mL. Transfer the cell suspension to a sterile 1.5 mL microfuge tube.

2.5 Pellet the cells by centrifugation in a microfuge for 2 minutes at 2000 rpm.

2.6 Carefully remove the supernatant solution. Make sure to remove as much of the supernatant solution as possible to minimize dilution of the Hypertonic Loading Medium, which will be added next.

2.7 Add 20 µL of prewarmed Hypertonic Loading Medium containing 1 mM CMFDG. Gently resuspend the cells by tapping on the tube. If desired, the suspension medium may contain 200 µM verapamil to inhibit the efflux of the fluorescent product (note **B**).

2.8 Incubate the cells at 37°C for 10 minutes.

2.9 Quickly, but gently, add 1 mL of Hypotonic Lysis Medium to the cell suspension, then transfer the suspension to a separate 5 mL tube containing 2 mL of Hypotonic Lysis Medium.

2.10 Aliquot the cell suspension between two 1.5 mL microfuge tubes, then incubate the cells for 1.5 minutes at 37°C. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

2.11 Pellet the cells by centrifugation in a microfuge for 2 minutes at 2000 rpm.

2.12 Quickly, but carefully, remove the supernatant.

2.13 Add at least 1 mL of Recovery Medium to each microfuge tube and resuspend the cells.

2.14 Allow 30 minutes prior to observing the cells. Alternatively you can plate them immediately onto fresh coverslips, culture dishes or flasks for future examination. If desired, 1.5 µM propidium iodide may be included in the Recovery Medium to facilitate the identification of dead cells (note **C**).

Loading Adherent Cells Using the Influx Reagent

3.1 Prewarm at least 100 µL of Hypertonic Loading Medium containing 1 mM CMFDG to the ideal growth temperature for your cell type. In addition, prewarm ~10 mL of Hypotonic Lysis Medium and 10 mL of Recovery Medium, as well as all glassware. The following protocol assumes that the ideal temperature is 37°C.

3.2 Using sterile forceps, remove a coverslip from the culture dish in which the cells were grown.

3.3 Touch the edge of the coverslip to a sterile Kimwipe® to remove excess media.

3.4 Place the coverslip cell-side up in a staining dish (a 60 or 100 mm–tissue culture dish with a lid). To ensure that the coverslip does not adhere to the dish, we recommend using a "pedestal," e.g. resting the coverslip on the inverted top removed from a 1.5 mL microfuge tube or on a 10 mm–diameter O-ring, sterilized with ethyl alcohol.

3.5 Quickly, but gently, pipet 100 µL of the prewarmed Hypertonic Loading Medium, containing 1 mM CMFDG, onto a *corner* of the coverslip so that the viscous Hypertonic Loading Medium will displace the small amount of residual medium without significantly diluting the loading solution.

3.6 Place the lid on the staining dish and incubate the coverslip at 37°C for 10 minutes.

3.7 Using sterile forceps, quickly, but gently, lift the coverslip and remove the excess Hypertonic Loading Medium by touching an edge of the coverslip to a sterile Kimwipe. As an alternative, gently remove staining solution by tipping coverslip and pipetting off the solution.

3.8 Place the coverslip *vertically* in a coverslip staining jar filled with *at least* 7 mL of prewarmed Hypotonic Lysis Medium, making certain that the coverslip is fully submerged.

3.9 Incubate the coverslip for *only* 2 minutes in the Hypotonic Lysis Medium. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

3.10 Using sterile forceps, quickly, but gently, remove the coverslip from the Hypotonic Lysis Medium. Touch an edge of the coverslip to a sterile Kimwipe to remove excess medium.

3.11 Submerge the coverslip in ~10 mL of prewarmed Recovery Medium in a new coverslip staining jar or staining dish. If desired, 1.5 µM propidium iodide may be included in the Recovery Medium to facilitate the identification of dead cells (note **C**).

3.12 Allow the cells on the coverslip to recover at 37°C for at least 30 minutes before observing in the microscope.

Protocol II: Loading Cells by Hypotonic Shock

Preparation of Solutions

Make up 10 mL of Staining Medium. A typical staining medium is phosphate-buffered saline (PBS), 4% (v/v) fetal calf serum and 10 mM HEPES, pH 7.2.

Loading Cells in Suspension by Hypotonic Shock

4.1 Centrifuge the cells to obtain a cell pellet and aspirate the supernatant (note **A**). Resuspend the cells in Staining Medium (prepared as described above) and draw the sample through a pipet to obtain a single cell suspension. Filter out any cell clumps with a nylon screen. Centrifuge the cells again and remove the supernatant.

4.2 Resuspend the cells in Staining Medium to approximately 10^7 cells/mL (note **D**) and pipet 100 µL into a centrifuge tube. If inhibition of endogenous β-galactosidase is desired, prepare Staining Medium with 1 mM chloroquine diphosphate (freshly diluted from the 30 mM stock solution (Component C); concentrations greater than 1 mM may be deleterious to cells (note **E**). Proceed to step 4.3 immediately, or put the cells on ice.

4.3 Pre-warm the tube containing 100 µL of the cells in a 37°C water bath for 10 minutes, or for 30 minutes when inhibiting endogenous β-galactosidase with chloroquine diphosphate.

4.4 Immediately before use, prepare 100 µL of 200 µM CMFDG substrate working solution in deionized water from the 10 mM stock solution (Component A) (notes **F** and **G**). Warm the solution at 37°C for about 10 minutes.

4.5 Combine 100 µL of the pre-warmed CMFDG substrate working solution with the 100 µL of pre-warmed cells from step 4.3. Mix rapidly and *thoroughly*. Return the sample to the 37°C water bath for 2 minutes. **Note:** The optimal working concentration of the CMFDG substrate must be determined experimentally. The recommended 200 µM working concentration suggested may have to be varied based on the method of loading (note **G**) and the level of β-galactosidase activity in cells.

4.6 Stop the CMFDG loading at the end of 2 minutes by adding 1.8 mL of Staining Medium to the 200 µL volume of CMFDG and cell suspension.

4.7 Wash the cells by centrifugation and resuspend them in 2.0 mL of Staining Medium. If desired, 1.5 µM propidium iodide may be included in the Staining Medium to facilitate the identification of dead cells (note **C**).

4.8 Keep the cells under normal culture conditions for 30 minutes to allow for turnover of the substrate prior to analysis. **Note:** At any point after the termination of loading, you may inhibit further intracellular hydrolysis of the substrate by treatment with PETG (see note **H**).

Loading Adherent Cells by Hypotonic Shock

5.1 Grow cells on coverslips according to normal tissue culture procedures. Use cells at a 40% to 70% confluency for best results (note **A**). If inhibition of endogenous β-galactosidase is desired, prepare Staining Medium with 1 mM chloroquine diphosphate (freshly diluted from the 30 mM stock solution (Component C)); concentrations greater than 1 mM may be deleterious to cells (note **E**).

5.2 Immediately before use, dilute the CMFDG substrate stock reagent (Component A) to 400 µM using a 1:1 mixture of deionized water and Staining Medium. Warm the substrate solution at 37°C for 10 minutes. A 100 µL volume will be used for each coverslip.

5.3 Rinse the cells once with a physiological saline solution, such as Hank's balanced salt solution or PBS.

5.4 Place the coverslip with adherent cells in a petri dish. Apply 100 µL of the substrate solution to the coverslip and incubate the sample at room temperature for 1 minute.

5.5 Stop the CMFDG loading by flooding the petri dish with Staining Medium. If desired, 1.5 µM propidium iodide may be included in the Staining Medium to facilitate the identification of dead cells (note **C**). **Note:** Do not remove the substrate solution before flooding the cells with medium, as this will often wash away many of the cells.

5.6 Return the cells to the 37°C incubator and allow the cells to recover for 1–3 hours. **Note:** At any point after the termination of loading, you may inhibit furthur intracellular hydrolysis of the substrate by treatment with PETG (note **H**).

5.7 Mount the cells in Staining Medium on a slide. Seal and view immediately. For flow cytometric assay, treat adherent cells with trypsin in phosphate buffer until they can be removed from the plate by gentle agitation. Afterwards, remove the trypsin by washing in Staining Medium. Centrifuge the cell suspension, aspirate the supernatant and resuspend the cells in Staining Medium.

Protocol III: Direct Loading of Cells

The following simple procedure has been developed using the mouse fibroblast CRE BAG 2 cell line, an NIH 3T3-derived cell line that stably expresses *lacZ*-encoded β-galactosidase under the control of a murine leukemia virus promoter. The procedure may be generally applicable to other cell lines.

6.1 Culture the cells in suitable growth medium (e.g. Dulbecco's modification of Eagle's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamicin, 300 µg/mL L-glutamine and 10 mM HEPES, pH 7.4), in a humidified atmosphere of 5% CO_2 in air.

Subculture every 2 to 3 days by trypsinization using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) (note **A**).

6.2 Prior to an experiment, trypsinize the cells, collect by centrifugation and resuspend at a density of 10^6 to 10^7 cells per mL (note **D**) in pre-warmed culture medium supplemented with 10% FBS and containing at 50–200 µM CMFDG. A 100 µL volume is usually sufficient for analysis. If desired, the suspension medium may contain 200 µM verapamil to inhibit the efflux of fluorescent product (note **B**).

6.3 Incubate the cell suspension at 37°C for the desired time interval, typically 10–60 minutes. Following incubation, the cells should be placed on ice, or diluted into ice-cold PBS in order to increase the volume, and then analyzed promptly. Alternatively, the turnover of CMFDG can be inhibited by the addition of PETG (note **H**).

6.4 For analysis by flow cytometry, propidium iodide can be diluted into the cell suspension to attain a final concentration of 1.5 µM to facilitate the identification of dead cells (note **C**), so that they may be eliminated electronically from the analysis.

Analysis

Flow Cytometry

Set up and calibrate the flow cytometer to detect fluorescein, propidium iodide and forward scatter according to standard procedures. Use unstained cells of the same type you are analyzing to set the background autofluorescence compensation (note **I**).

Imaging β-Galactosidase Activity

Fluorescence is detected using standard fluorescein or FITC filter sets.

Notes

[A] Keep the cells as healthy as possible. Endogenous lysosomal β-galactosidase activity increases dramatically if the cells are abused or allowed to reach confluency (see note **E** on inhibition of endogenous β-galactosidase activity with chloroquine diphosphate).

[B] Verapamil (Component E), an inhibitor of plasma membrane–resident drug efflux systems is effective in reducing the efflux of fluorescent products produced from the action of β-galactosidase on the CMFDG substrate.[3] Inclusion of verapamil at 100–200 µM in the staining and post-staining media can result in substantially improved detection of β-galactosidase activity. Concentrations of verapamil above 200 µM may be toxic to cells, and the ideal concentration of verapamil for a particular application may need to be determined experimentally.

[C] Propidium iodide (Component D) is impermeant to the plasma membrane and selectively labels the nuclei of dead cells with red fluorescence. Prepare dye solution by diluting the 150 µM propidium iodide stock solution (Component D) 100-fold to obtain a 1.5 µM solution.

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

[E] Some mammalian cells have endogenous lysosomal β-galactosidase that can interfere with accurate measurement of *lacZ* expression. The endogenous activity can be selectively depressed by pre-incubating cells with the weak base, chloroquine (Component C).

[F] For bacterial cells or yeast, the cell wall restricts the swelling induced by osmotic loading, thus preventing CMFDG entry. Brief (1–3 minute) *hypertonic* shrinking of the cell membrane within the wall, followed by a 2-minute hypotonic loading of CMFDG can correct this difficulty with entry.

[G] The loading procedure described in steps 4.4 and 5.2 subjects cells to hypotonic shock in order to facilitate entry of the substrate. This treatment may not be necessary for some cell types. For loading under isotonic conditions, prepare the CMFDG working solution in the staining medium instead of the deionized water and increase the incubation time from 2 to about 30 minutes, or see Protocol III.

[H] Competitive inhibition of β-galactosidase by PETG (Component B) can be used to terminate CMFDG turnover prior to analysis. After terminating CMFDG loading (steps 4.6, 5.5 or 6.3), select a time interval between zero and 60 minutes (zero time for cells with high *lacZ* expression levels, 60 minutes for cells with low *lacZ* expression levels) and add an aliquot of the 50 mM PETG stock reagent to yield a final PETG concentration of 1 mM. Mix thoroughly. PETG is a competitive, reversible inhibitor of *E. coli* β-galactosidase in mammalian cells. It is hydrophobic and can readily cross the cell membrane to inhibit β-galactosidase. Because it has a low K_i (3×10^{-6} M), very little PETG is required to inhibit the reaction. In addition, PETG is not hydrolyzed by the enzyme, which simplifies its influence on the kinetics.

[I] Some endogenous constituents of cells give rise to broad bandwidth autofluorescence when excited by the argon-ion laser. It is essential to compensate for autofluorescence in order to accurately measure low levels of β-galactosidase activity. Correction for the autofluorescence component of the emission signal is typically based on the proportionality of measured *autofluorescence* at one wavelength to that at another wavelength.

References

1. Proc Natl Acad Sci USA 85, 2603 (1988); **2.** Cytometry 12, 291 (1991); **3.** Cytometry 28, 36 (1997); **4.** Cell 29, 33 (1982); **5.** J Cell Physiol 135, 443 (1988).

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

Cat #	Product Name	Unit Size
D-2920	DetectaGene™ Green CMFDG *lacZ* Gene Expression Kit	1 kit
I-14402	Influx™ pinocytic cell-loading reagent *makes 10 x 5 mL*	1 set

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Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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