Revised: 22–March–2001

ImaGene Green™ C_{12}FDG lacZ Gene Expression Kit (I-2904)

Quick Facts

Storage upon receipt:

- –20°C
- Avoid freeze-thaw cycles
- Desiccate
- Protect from light

Abs/Em for reaction product: 497/518 nm

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; B-1690, B-22015) 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 is 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] However, FDG does not readily permeate cells, and special cell-loading procedures must be used.

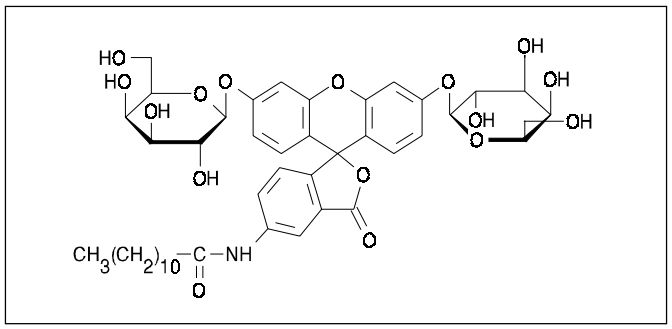

Figure 1. *Structure of C_{12}FDG*

To overcome the limitations of these substrates, Molecular Probes has introduced the ImaGene Green™ C_{12}FDG *lacZ* Gene Expression Kit (I-2904). The β-galactosidase substrate included in the kit, C_{12}FDG, is a variant of FDG that has been covalently modified to include a 12-carbon lipophilic moiety. Unlike FDG, this lipophilic fluorescein can be loaded simply by adding the substrate to the aqueous medium in which the cells or organisms are growing, either at ambient temperatures or at 37°C. Once inside the cell, the substrate is cleaved by β-galactosidase, producing a fluorescent product that is retained by the cells, probably by incorporation of its lipophilic tail within the cellular membranes. Mammalian NIH 3T3 *lacZ*-positive cells grown for several days in medium containing 60 µM C_{12}FDG appear morphologically normal, continue to undergo cell division and remain fluorescent for up to three cell divisions after they are returned to substrate-free medium.[3,4]

The C_{12}FDG substrate has been shown to be superior to FDG for flow cytometric detection of β-galactosidase activity in live mammalian cells.[5] Using C_{12}FDG with flow cytometric methods, researchers have:

- Assessed levels of *lacZ* gene expression in recombinant Chinese hamster ovary (CHO) cells throughout the cell cycle, which was monitored with Hoechst 33342[6]
- Identified endocrine cell precursors in dissociated fetal pancreatic tissue based on their high levels of endogenous acid β-galactosidase[7]
- Measured β-galactosidase activity in single recombinant *E. coli* bacteria[8]
- Sorted β-galactosidase–expressing mouse sperm cells[9] and insect cells that harbor recombinant baculovirus[10,11]

The C_{12}FDG substrate has also proven useful for a fluorescence microscopy study of the expression of a *lacZ* reporter gene under the control of a mammalian homeobox gene promoter in mosaic transgenic zebrafish.[12] In addition, lipophilic β-galactosidase substrates have been employed to diagnose the deficiency in β-galactocerebrosidase activity that typifies Krabbe disease in human patients.[13,14] In some cell types, C_{12}FDG has been reported to produce high levels of background fluorescence that may prohibit its use in assaying low β-galactosidase expression.[15]

In addition to the substrate C_{12}FDG, the ImaGene Green C_{12}FDG *lacZ* Gene Expression Kit also includes stock solutions of phenylethyl β-D-thiogalactopyranoside (PETG) and chloroquine. 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.

Materials

Contents

- **ImaGene Green C_{12}FDG substrate reagent** (Component A), 0.35 mL of 20 mM C_{12}FDG substrate in DMSO.
- **PETG reagent** (Component B), 1.0 mL of 50 mM phenylethyl β-D-thiogalactopyranoside in water.
- **Chloroquine reagent** (Component C), 1.0 mL of 30 mM chloroquine diphosphate in water.

The material provided is sufficient for about 100–200 assays, depending on the volume used for each assay.

Storage

Upon receipt, store the ImaGene Green Kit at -20°C, desiccated and protected from light. To avoid freeze–thaw cycles, divide the C_{12}FDG substrate reagent into several small aliquots. This will minimize potential decomposition of the substrate. Presence of a pronounced yellow color in the ImaGene Green C_{12}FDG β-galactosidase substrate reagent or a high fluorescent background in the cells, as compared to previously obtained results, may indicate that the substrate no longer meets appropriate standards for use. The reagents are stable for at least six months, provided the recommended storage conditions are observed.

Protocols

Overview

The following protocols provide guidelines for introducing the ImaGene Green C_{12}FDG β-galactosidase substrate into adherent or suspension cells and for preparing the cells for imaging. Our suggested initial conditions may require modifications based on the expression level of the cells, the permeability of the substrate into the cells and the background from intrinsic cellular β-galactosidase. Methods are also given for using a selective inhibitor to lower the background endogenous lysosomal β-D-galactosidase activity that is present in some cell types and for using a competitive inhibitor to terminate the β-galactosidase reaction, if desired.

Reagent Preparation

1.1 Prepare the required amount of a 33 µM working solution of the C_{12}FDG by diluting the stock solution (Component A) 600-fold with an appropriate sterile culture medium (notes **A**, **B**).

1.2 Warm the required amount of the diluted reagent to 37°C for about 10 minutes. *Do not keep the substrate working solution at 37°C for extended periods, as spontaneous hydrolysis may occur.*

1.3 Sterilize the substrate-containing media by filtering it through a 0.2 µm Acrodisc® filter (HT Tuffryn® polysulfone membrane). Some researchers have indicated that some other brands of sterilization filters adsorb the substrate from dilute solutions.

Cell Staining

Adherent cells:

2.1 Grow the cells on coverslips inside a Petri dish in an appropriate culture medium to the desired confluency (note **C**).

2.2 Remove the medium from the dish and cover the cells with pre-warmed (37°C) 33 µM substrate–containing culture medium from the working solution prepared above (note **D**).

2.3 Incubate the cell cultures for 20–60 minutes under desired conditions. The coverslip with cells can then be removed and examined for fluorescence intensity (note **E**).

Suspension cells:

3.1 Centrifuge to obtain a cell pellet and aspirate the supernatant.

3.2 Resuspend the cells gently in pre-warmed (37°C) 33 µM substrate–containing culture medium (note **F**).

3.3 Incubate the cell suspension for 15–30 minutes under the desired conditions. Centrifuge and resuspend the pellet in fresh culture medium. Apply the cells to a slide, cover with a coverslip, seal and examine the fluorescence.

Inhibition of Endogenous β-D-Galactosidase Using Chloroquine Reagent (optional, see note G)

4.1 If desired, pre-incubate the cells in the presence of chloroquine *before* staining: Dilute the 30 mM chloroquine stock solution (Component C) 100-fold with culture medium to obtain a 300 µM solution and prepare cell cultures as described above (step 2.1 or 3.1). Incubate the cells for 30 minutes at 37°C.

4.2 Remove the chloroquine-containing culture medium (centrifuge in the case of suspension cells) (note **H**) and add the substrate-containing culture medium. Incubate and examine the cells as described above.

Inhibition of β-D-Galactosidase by PETG Reagent (optional, see note I)

5.1 Prepare and stain cell cultures as described above. To stop β-galactosidase activity at any time, add PETG reagent to the culture medium to a final concentration of 1 mM. The β-galactosidase activity will be strongly inhibited, and the samples can be examined at a convenient time.

Fluorescence Microscopy

The ImaGene Green C_{12}FDG Kit is designed to be used on a wide range of epifluorescence microscopes, with both standard optics and video enhancement. The fluorescent product can be detected with filter sets equipped with excitation and emission filters designed for FITC or fluorescein.

Uniform staining of β-galactosidase–positive cells (e.g. CRE BAG 2, which are NIH 3T3 cells that have been transfected with a retrovirus containing the *lacZ* gene) is confined to the cytosol and is visible during the first 20–60 minutes after incubation with the substrate.[4] After longer incubation periods, bright granules of accumulated fluorescent product begin to appear throughout the cytoplasm. Granular structures corresponding to these products can also be seen in the Nomarski differential interference contrast (DIC) image of these stained cells. Poorly stained cells with numerous large yellow crystals around the cells or attached nonspecifically to the cell surface may indicate that the substrate is not sufficiently soluble in the medium being used.

When cells are incubated for extended periods of time, the substrate may be nonenzymatically hydrolyzed by cell compartments having extremely acidic or basic pH or that have endoge-

nous β-galactosidase activity. Therefore, it may be necessary to further optimize the experimental conditions suggested in these instructions.

Notes

[**A**] The type of culture medium chosen will depend on the cells being examined. Most commonly used culture media should be acceptable; for example, D-MEM containing 10% calf serum has been successfully used. In any case, it is preferable, when possible, to prepare the ImaGene Green C_{12}FDG substrate reagent working solution in the same medium that will be used to grow the cells. The substrate is compatible with most typical additives; however, phenol red should be omitted.

[**B**] The ImaGene Green C_{12}FDG substrate is a very sensitive indicator of β-galactosidase. If the reagent is being used to simply discriminate strong *lacZ*-positive from *lacZ*-negative cells, it may be necessary to reduce the substrate concentration from the recommended concentration of 33 µM to 5–15 µM, and to reduce the incubation time. Examine the cells immediately for fluorescence.

[**C**] Keep cells as healthy as possible. Certain adherent cells such as NIH 3T3 will have higher endogenous β-galactosidase activity if they are abused or allowed to become confluent (see notes **G** and **H** below).

[**D**] Alternatively, a prewarmed 10X working solution of C_{12}FDG in medium can be added directly to the original medium containing the cells. This may serve to minimize potential damage to the cells. The 10X concentrated (300 µM) working solution can be filter sterilized and stored for up to 48 hours at 4°C, or longer at -20°C.

[**E**] After 30 minutes, fluorescence can be easily detected in most cells expressing the *lacZ* gene product. Cells that have very low levels of expression may require longer incubation.

[**F**] The amount of substrate-containing culture medium required will depend on the number and type of cells being stained. It may be necessary to optimize the volume of staining solution.

[**G**] Some mammalian cell types have endogenous β-galactosidase activity in their lysosomes and this can interfere with measurement of the reporter gene activity. If negative controls are appreciably fluorescent, then treatment with chloroquine diphosphate may inhibit endogenous β-galactosidase activity.

[**H**] If the cells have a high level of β-galactosidase activity and need only a short incubation with the substrate reagent (20–120 minutes), then the washing step is optional. Leaving the chloroquine diphosphate in the medium may result in better inhibition of endogenous β-galactosidase activity.

[**I**] PETG is a competitive, reversible inhibitor of *E. coli* β-D-galactosidase. The inhibitor has a low K_i (3×10^{-6} M), and thus very little is required to inhibit the reaction. It is not hydrolyzed by the enzyme, which simplifies its influence on the kinetics. It is hydrophobic and can readily cross the cell membrane to inhibit β-galactosidase.

References

1. Proc Natl Acad Sci USA 85, 2603 (1988); **2.** Cytometry 12, 291 (1991); **3.** J Fluorescence 3, 119 (1993); **4.** FASEB J 5, 3108 (1991); **5.** Appl Environ Microbiol 60, 4638 (1994); **6.** Biotech Bioeng 42, 1113 (1993); **7.** J Clin Endocrinol Metab 78, 1232 (1994); **8.** Biotech Bioeng 42, 708 (1993); **9.** Proc Natl Acad Sci USA 89, 10681 (1992); **10.** Methods Cell Biol 42, 563 (1994); **11.** Biotechniques 14, 274 (1993); **12.** Genes Dev 6, 591 (1992); **13.** Clin Chim Acta 205, 87 (1992); **14.** J Lab Clin Med 110, 740 (1987); **15.** J Neurosci 13, 1418 (1993).

Product List

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

Cat #	Product Name	Unit Size
I-2904	ImaGene Green™ C_{12}FDG *lacZ* Gene Expression Kit	1 kit

Contact Information

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.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

Molecular Probes, Inc.
PO Box 22010, Eugene, OR 97402-0469
Phone: (541) 465-8300 • Fax: (541) 344-6504

Customer Service: 7:00 am to 5:00 pm (Pacific Time)
Phone: (541) 465-8338 • Fax: (541) 344-6504 • order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353 • Fax: (541) 465-4593 • tech@probes.com

Molecular Probes Europe BV
PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.